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Surface and enzymatic properties of marine and terrestrial bacteria involved in biodegradation of petrochemical hydrocarbons: a comparative study towards improving bioremediation strategies for treating hydrocarbon-polluted sites

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ABSTRACT

A continuous demand for petroleum products leads to large amounts of oil being annually released to the natural environment, causing the threat to all living organisms. Therefore, the clean-up methods are constantly developed towards their efficiency, safety and minimised secondary pollutions. Biodegradation, being considered as the natural clean-up process has recently gained a lot of interest. This method involves the use of microorganisms such as bacteria and fungi, which by expressing the appropriate enzymes, are able to metabolise petroleum contaminants, using them as sole carbon and energy sources.

The Deepwater Horizon oil spill was a great example of the use and development of the methods helpful in an identification of the hydrocarbon-degraders from the sites contaminated by the spill. The research based on the obtained findings is helpful in the analyses of the biodegradation mechanisms, now mainly focused on enzymatic and surface properties of the microbiota involved in the process. What is more, a comprehensive and detailed knowledge on the type and properties of hydrocarbon-degraders is an invaluable contribution to the research focused on exploring and perfecting the bioremediation strategies of the polluted sites, thereby presented in this Thesis.

A very limited or total lack of solubility of hydrocarbons in water considerably reduces their bioavailability and biodegradation. Nonetheless, despite this limitation, microorganisms possess some adhesive properties, which enable them to attach to different surfaces – including hydrophobic organic compounds. Those properties depend on a few main factors such as cell surface charge (that is connected with the electrostatic interactions between a bacterial cell and the substrate surface) and the cell surface hydrophobicity.

Another important aspect of biodegradation is the activity of the enzymes involved in the metabolism of hydrocarbons. The first and the most important step in this process is catalysed by oxygenases (mono- and dioxygenases) and is based on the introduction of the oxygen atoms to the hydrophobic organic compound, which is a substrate for the further degradation steps. This thesis presents the analysis of the enzymatic activity of long chain alkane monooxygenases and catechol 1,2- and 2,3-dioxygenases, which were produced by the selected bacterial strains.

There were eight bacterial strains chosen for this study, all of them isolated from the crude oil contaminated reservoirs. The marine bacterial strains *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 are PAH-

degrading bacteria that were isolated from sea surface oil slick samples collected during the Deepwater Horizon oil spill. *Polycyclovorans algicola* TG408 is an obligate PAH-degrading bacterium that was isolated from a laboratory culture of the cosmopolitan marine diatom *Skeletonema costatum.* The terrestrial strains used in this study *Achromobacter* sp. 4(2010), *Pseudomonas stutzeri* strain 9, *Rahnella* sp. EK12, *Stenotrophomonas maltophilia* strain 6 are hydrocarbon degrading strains isolated from soil contaminated with petroleum hydrocarbons in Poland.

There were three commercially available non-ionic surfactants used in the studies: Glucopon 215 and Lutensol GD 70 (natural surfactants) and Triton X-100 (synthetic). The biodegradation of neither diesel oil nor naphthalene by all tested bacterial strains was inhibited by the abovementioned surface active agents.

The obtained results also indicate that the marine strains are characterised by high catechol (1,2- and 2,3-) dioxygenase activity and proved great ability to degrade naphthalene, which was used as model polycyclic aromatic hydrocarbon. The optimal pH and temperature conditions were also determined for the abovementioned enzymes. Further analyses revealed that the strong relationship between the cell surface hydrophobicity and biodegradation of hydrophobic organic compounds was found in the case of terrestrial strains, which were also characterised with higher long chain alkane monooxygenase activity in comparison with marine strains.

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ABBREVIATIONS

ABLM – activated bleomycin
APGs – alkyl polyglucosides
BATH – bacterial adhesion to hydrocarbons
BLM – bleomycin
BVMO – Baeyer-Villiger monooxygenase
CAM – contact angle measurement
CHDH – cyclohexanol dehydrogenase
CHMO – cyclohexane monooxygenase
CHMO1 – cyclohexanone monooxygenase
CLH – caprolactone hydrolase
CMC – critical micelle concentration
CSH – cell surface hydrophobicity
DCM – dichloromethane
DWH – Deepwater Horizon
DβM – dopamine β-monooxygenase
EPS – extracellular polymeric substances
FAD – flavin adenine dinucleotide
FMO – flavin-containing monooxygenase
HCADH – 6-hydroxyhexanoic acid dehydrogenase
HIC – hydrophobic interaction chromatography
HOC – hydrophobic organic compounds
HWM PAH – high molecular weight polycyclic aromatic hydrocarbon
ITOPF – The International Tanker Owners Pollution Federation Limited
IUPAC – International Union of Pure and Applied Chemistry
LMW – low molecular weight
LO – lipoxygenases
LPS – lipopolysaccharides

MATH – microbial adhesion to hydrocarbons

MATS – microbial adhesion to solvents

NAD(P)H – nicotinamide adenine dinucleotide phosphate (reduced form)

NADH – nicotinamide adenine dinucleotide (reduced form)

NAPLs – non-aqueous phase liquids

OHDH – 6-oxohexanoic acid dehydrogenase

ONR7a – artificial seawater medium ONR7a

pAH – particulate alkane hydroxylases

PAH – polycyclic aromatic hydrocarbons

PCBs – polychlorinated biphenyls

PCP – pentachlorophenol

PHM – peptidylglycine R-hydroxylating monooxygenase

pMMO – particulate methane monooxygenase

PUM – phosphate urea magnesium sulphate buffer

RO – Rieske non-heme iron oxygenases

SCs – shoreline cleaners

sMMO – soluble methane monooxygenase

SWAs – Surface-washing agents

TOPA – 2,4,5-trihydroxylphenylalanine

USEPA – United States Environmental Protection Agency

ZM-10 – Zobell Marine medium diluted 10 times

α-KG – α-ketoglutarate

1. INTRODUCTION AND PURPOSE OF WORK

The global uncontrolled and high release of the petroleum hydrocarbons to the natural environment causes its severe contamination and jeopardizes the nature by their toxic properties. The question of whether there is a possibility to enhance the clean-up strategies based on the natural processes has been preoccupying the experts for some time. The special interest has been put to the bioremediation (especially biodegradation) processes, however, the success can be achieved when a closer look is taken at the molecular basics of the process. This natural process involving microorganisms with the capability to metabolize many different carbon sources, inter alia hydrocarbons, would not be possible without their special cell properties (allowing for the attachment to the hydrophobic compounds) and production of enzymes, which enable the metabolism of the hydrophobic organic compounds. Therefore, a detailed analysis of the properties of the microorganisms involved in the biodegradation of hydrocarbons merits further investigation.

The research performed within this PhD project investigated the similarities and differences between marine and terrestrial bacterial strains regarding their enzymatic and cell surface properties in relation to the biodegradation of petroleum hydrocarbons.

This work increases our understanding on the differences between marine and terrestrial strains in their capacities to interact (via surface and enzymatic properties) and degrade petrochemical pollutants.

There were four marine and four terrestrial strains chosen for this study, all of them isolated from the crude oil contaminated reservoirs. The marine bacterial strains *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 are PAH-degrading bacteria that were isolated from sea surface oil slick samples collected during the Deepwater Horizon oil spill. *Polycyclovorans algicola* TG408 is an obligate PAH-degrading bacterium that was isolated from a laboratory culture of the cosmopolitan marine diatom *Skeletonema costatum.* The terrestrial strains *Achromobacter denitrificans* sp. 4(2010), *Pseudomonas stutzeri* strain 9, *Rahnella* sp. EK12,and *Stenotrophomonas maltophilia* strain 6 were isolated from the soil contaminated with the petroleum hydrocarbons in Poland.

1.1. Specific Objectives

i. *Determine the enzymatic properties of the marine and terrestrial bacterial strains supplemented with diesel oil used as sole carbon and energy source.*

The tests covered the analyses of the activity of long chain alkane monooxygenases and 1,2- or 2,3-catechol dioxygenases for the strains incubated with diesel oil.

ii. *Analyse the surface properties of the above-mentioned bacterial strains in the presence and/or absence of diesel oil.*

The microbial adhesion to hydrocarbons (MATH) test and zeta potential measurements were performed in order to determine bacterial cell surface properties (CSH) in the presence and absence of diesel oil.

iii. *Investigate the biodegradation of diesel oil by the tested bacterial strains.*

A gravimetric method was used to determine the ability of the bacterial strains to degrade diesel oil.

iv. *Establish the biodegradation of naphthalene by terrestrial and marine strains.*

A spectrophotometric method was applied in order to establish the biodegradation of a model polycyclic aromatic hydrocarbon, i.e. naphthalene by the tested organisms in the presence and absence of surfactants and over a specific time.

v. *Evaluate the influence of three non-ionic surfactants (Triton X-100, Lutensol GD 70 and Glucopon 215) on the enzymatic activity, surface properties and biodegradation ability of the bacterial strains.*

The use of surfactants in order to enhance the bioremediation of hydrocarbon-polluted sites has been thoroughly studied for the past two decades (Bury and Miller, 1993; Christofi and Ivshina, 2002; Kim et al., 2001). However, due to the enormous number of different surface-active agents and varied microbial properties, the effect of surfactants on bioremediation might be very individual, depending on the bacterial strain properties, pH, temperature, enzymatic activity and other environmental conditions.. In this study the influence of three non-ionic surfactants on the selected marine and terrestrial bacteria has been investigated.

vi. *Demonstrate the similarities and differences between marine and terrestrial strains regarding the aspects listed above.*

The comparative study was supported with statistical analyses as well as mathematical correlations between the selected parameters.

1.2. Dissertation Organization

This dissertation has been divided into three main chapters that comprise a Literature Overview (Chapter 2), which provides the background for the work presented in the dissertation. The Materials and Methods (Chapter 3) provides a detailed description of the experimental work conducted in the laboratory. Lastly, the Results and Discussion (Chapter 4) provides a description of the results obtained and their discussion and interpretation with relevance and in consideration to previous published work.

2. LITERATURE OVERVIEW

2.1. Petroleum Hydrocarbons in the Environment

In spite of the high attention that alternative sources of energy have gained in recent years, the continuous development of all of the world's industry branches (such as mechanical and civil engineering, transport etc.) still raises the demand for petroleum products. As useful as it is, petroleum commerce also brings some negative consequences, especially to the environment, therefore, to human health. As a matter of fact, an increase in production and consumption of oil consequently raises the threat of the oil pollution. Because of the varied demands of oil around the world, it is transferred via different means of transport, such as tankers, pipelines, railcars and tank trucks (Fingas, 2011a). As such, the oil spills are often a consequence of the storage or transportation accidents.

Although, when speaking about the oils spills, we mostly picture large and well known accidents such as grounding of the oil tankers *Exxon Valdes* (1989) and *Prestige* (2002) or the explosion of the oil platform *Deepwater Horizon* (2010), it should be noted that most of the oil spills happen every day on a smaller scale. Figure 1 presents the average annual amounts of oil spills in the United States between 1998 and 2007, where the pipeline spills constitute almost 80% of the total spills.

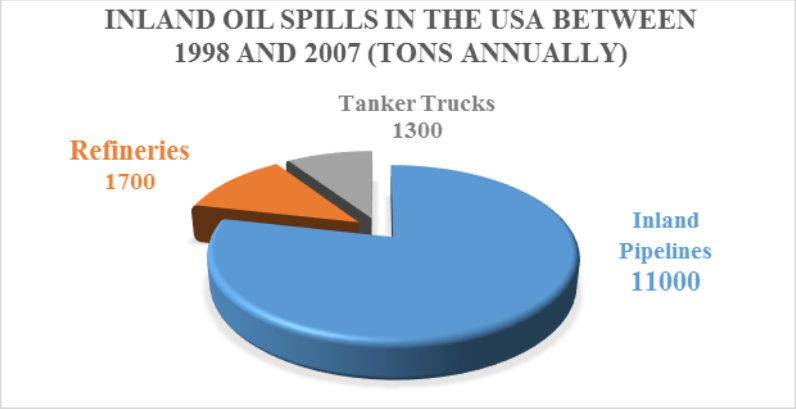


Figure 1: Inland oil spills in the United States between 1998 and 2007.
Graph prepared according to Schmidt-Etkin, (2011) .

The consideration of the oil spills should not only cover the accidental incidents, but also the leaks of oil from the natural sources, called "natural seeps". There are both inland and submarine oil reservoirs, which are nowadays useful for locating rich sources of oil. The oil releases from the natural seeps are estimated to be as high as 600 000 tonnes per year, and often such releases can reach sizes comparable with tanker spills (GESAMP, 2007).

According to Kvenvolden (2003) the annual global oil seepage ranges between 0.2 and 2 Mt, with a "best estimate" of 0.6 Mt. Based on those estimates and a study from National Research Council (2003), it has been assessed that nearly 47% of the annual oil contamination come from the natural seepage, while the other 53% is a result of human actions.

2.1.1. The composition and properties of crude oil

Although crude oil has been known about for more than a thousand years, its first industrial extraction dates back to 17th August 1859, when the first well for a large scale production was opened in Oil Creek, Pennsylvania (Simanzhenkov and Idem, 2003). From this well an expeditious use and application of crude oil started and it continues until now.

Element-wise, crude oil contains mostly carbon (84-87%) and hydrogen (12-14%), but also heteroatoms (1 – 1.5 %), such as oxygen, nitrogen and sulphur (Simanzhenkov and Idem, 2003).

Crude oil consists of thousands of compounds, the major classes of which are listed in Table 1.

Table 1: Classification of the crude oil components.
Based on Fingas (2011b).

Grouping	Chemical Class	Example
SATURATES	alkanes (straight chain and branched)	hexadecane
	cycloalkanes	decalin
	waxes (long chain alkanes)	octadecane
AROMATICS	benzenes	benzene
	BTEX (benzene, toluene, ethylbenzene, xylenes)	ortho-xylene
	PAH (polycyclic aromatic hydrocarbons)	naphthalene
	naphthenoaromatics	tetralin
RESINS	hydrocarbons of different structures, sometimes containing oxygen, nitrogen or sulphur	carbazole
ASPHALTENES	Hydrocarbons of different structures, large molecules, sometimes containing oxygen, nitrogen or sulphur	structures unknown

It is worth noting that the composition of the oil may vary depending on its source of extraction. There are hardly any oils with the same characteristics, as the geographical sources differ between each other with regard to the compounds and their quantities, which make up the oil's composition (Jones, 2008). As an example, crude oil that is extracted in Nigeria is characterised by high amounts of cyclic alkanes and low specific gravity, whereas the oil produced in Venezuela has a high specific gravity and little aliphatic contents (Jones, 2008). Some other examples of such differences are listed in Table 2.

Table 2: Differences in crude oil composition from various locations. Adapted from Jones (2008).

Source of oil / % of total contents	Kuwait	Libyan (Brega)	North Sea (Ekofisk)	South American (Bachequero)
% vol. Boiling below 350°c	49.0	64.0	61.2	30.0
Sulphur % wt.	2.5	0.21	0.21	2.4
Paraffins	67.9	53.0	56.5	27.6
Olefins	-	20 ppm	-	-
Naphthalenes	22.1	39.3	29.5	58.5
Aromatics	10	7.7	14.0	13.9

The distillation of crude oil is one of the processes carried out in refinery plants in order to obtain specific fractions and petroleum products, which can be then further used for transport, chemical technologies, heat transfer and other uses. Table 3 summarises some of the products of a crude oil distillation.

Table 3: Crude oil distillation products.
Adapted from Fingas (2011b).

PRODUCT	DISTILLATION TEMPERATURE RANGE (°C)	APPROXIMATE NUMBER OF CARBON ATOMS
Gasoline	30 – 200	5 – 12
Naphtha	100 – 200	8 – 12
Jet fuel & kerosene	150 – 250	11 – 13
Diesel fuel	160 – 400	13 – 17
Gas-oil	220 – 350	wide range
Heavy fuel oils	315 – 540	20 – 45
Atmospheric residue	>450	30+
Vacuum residue	>600	60+

2.1.2. Sources of oil spills

The marine environment, in particular, is exposed to all sorts of oil tanker spills and some accidents such as explosions during oil's outputs or off-shore drillings. According to The International Tanker Owners Pollution Federation Limited (ITOPF)(2015), the causes of the oil spills are varied, however, they may have an important influence on the final amounts of the spill. In its annual report, ITOPF indicates that 40% of small-sized oil spills (<7 tonnes) took place during loading and discharging operations around ports and oil terminals, while for medium oil spills (7-700 tonnes) it was 20 % of total causes. Approximately 45 % of medium oil spills are reported to be caused by collisions, allisions and groundings. Among large-scale spills (>700 tonnes) nearly 59 % of the incidents were caused by allisions, groundings and collisions as well as by a number of hull and equipment failures as well as fire and/or explosions.

The spillage incidents on the ground are related to the oil transport, storage and refineries, gas stations, pipelines and wells. The deposits of natural gas and oil are located thousands of meters below the surface, under many layers of rock and soil. That means that those precious energy sources are pressurised by the heavy weight of the abovementioned layers, which makes the drilling process difficult especially with regard to the pressure control.

Moreover, drilling also requires a good protection of the other soil layers from the accumulating hydrocarbons from the well. According to the American Petroleum Institute ("Oil Spill Prevention," 2015), the isolation of the environment from this threat can be achieved by a combination of steel casing and cement, which work as a physical barrier.

Another potentially dangerous sources of spills are under- and above-ground pipelines, transporting great amounts of crude oil, gasoline and some other refined fuels such as diesel oil, and heavy fuel. As seen in Figure 1, around 11,000 tons of oil is annually spilled from pipelines (Schmidt-Etkin, 2011) around the world.

Other transport-linked spillages cover mostly railroads (approx. 200 tons per year) and tanker trucks (around 1300 tons annually) (Schmidt-Etkin, 2011).

Oil Refineries, where the crude oil is converted to fuels, diesel and a large number of hydrocarbons, constitute another source of oil spills, where approximately 200 tones oil get accidentally released into the environment (Schmidt-Etkin, 2011). However, it should not be forgotten that the negative impact of refineries on the environment often stems from the large amounts of toxic hydrocarbons being released to the air. Benzene, toluene, ethyl benzene, xylenes and short chain alkanes are only a small example of abovementioned toxic air pollutants (Rourke and Connolly, 2003).

2.1.3. Examples of historic oil spill incidents

Torrey Canyon

When on 18th March 1967 *Torrey Canyon* hit the Seven Stones rocks between Land's End and the Scilly Isles, it quickly became clear that it was the largest oil spill on record at that time. Approximately 118,000 tons of Kuwait crude oil was released into the sea within just a few days. According to Holme (1969), 30,000 tons reached the English Channel, fouling also the French north coast. Another 20,000 tons reached the west coast of Cornwall, and after the ship's sinking approximately 50,000 tons drifted to Biscay. By the end of March, the ship's wreck was finally bombed with an intention to burn the rest of the oil. According to the reports from that time, the broken tanker was actually exposed to a "rain of bombs" (BBC News, 1967) between 28th and 30th of March. Final statistics from that period presented in the news reported that: "the RAF and the Royal Navy have dropped 62,000 lbs of bombs, 5,200 gallons of petrol, 11 rockets and large quantities of napalm onto the ship" (BBC News, 1967). This was, however, not the end of the problems that the local

environment had to face. Just 12 hours after the spill the Navy had used so called "detergents" to deal with the spill. Those substances, although named like domestic cleaning agents, were in fact very toxic chemicals, produced mostly by British Petroleum at that time (The Guardian 2010). Dating back, the Guardian reported that "British Petroleum, which has the Torrey Canyon on charter but does not own her (and therefore disclaims any responsibility for the oil pollution) has sent all the detergent it can lay hands on".

The French idea of the clean-up process, however, was considerably different. They used "Craie de Champagne", a French blackboard chalk consisting of calcium carbonate treated with 1% sodium stearate (Cooper, 1968), which turned out to be more effective than the expensive and toxic British detergents (The Guardian 2010). Cooper (1968) reported her observations of the oil patches becoming pink after using "Craie de Champagne", stating that the good efficiency of the chalk was also enhanced by the microorganisms, flagellate *Noctiluca miliaris*. Yet, Almeda et al. (2014) indicate that, although interesting, this statement has never been scientifically proven and the oil ingestion by dinoflagellates has not been studied so far.

Although there is not much recent information on the state of the places covered with oil due to this large accident, according to Hawkins el al. (2002) the recovery took around 10 years. However, on shores that were badly affected by dispersants it took longer – up to 15 years. The authors indicated that in the areas where there were no dispersants used, the recovery took around 2-3 years.

Exxon Valdez

In the early morning on 24th March 1989 the oil tanker Exxon Valdez grounded on a reef in Prince William Sound in Alaska. Within only a few days, between 42,000 and 120,000 metric tonnes of the Alaskan North Slope crude oil got into the Sound, contaminating around 1990 km of the shoreline (Anderson et al., 2014; Peterson et al., 2003). Although Prince William Sound was said to be most seriously polluted, the oil drifted approximately 750 km further south, passing Kenai Peninsula, Kodiak archipelago, and the Alaska Peninsula (Peterson et al., 2003). Such an enormous incident took its toll soon after the spill, causing death to thousands of marine birds and mammals, fishes, invertebrates and kelps (Bodkin et al., 2012).

In the late 1990s and early 2000s a fair number of research papers concentrated on an evaluation of the recovery of harmed ecosystems and species. Some of the unexpected findings concerned the delays in recovery for several species occupying the nearshore habitats (Bodkin et al., 2012). Golet et al. (2002) presented their findings on population recovery of pigeon guillemots *Cepphus columba*. Apart from the general conclusions on reduced animal survival due to the continued oil exposure and their reduced mass and size, the authors also indicated the presence of hepatic cytochrome P4501A, aspartate aminotransferase, and lactate dehydrogenase. Based on the enzymes' presence and activities, the authors suggested that the continuous closeness of petroleum hydrocarbons had led to the damage of the organs such as liver or lungs. What is more, cytochrome P4501A belongs to the super family of cytochrome P450 enzymes, which, among their many functions, can catalyse hydrocarbons' oxygenation, a first step in a whole degradation pathway of those compounds.

The first clean-up step used after the spill was an application of large amounts of dispersants in order to disperse the oil and therefore enhance its degradation. On the one hand this was found effective. On the other hand, however, the application of the potentially toxic dispersants used were said to have lethal consequences to some species (Anderson et al., 2014). Peterson et al. (2003) stated that "clean-up attempts can be more damaging than the oil itself", concluding that this kind of indirect source of pollution can lead to significant delay in recovery of the habitat.

The use of dispersants, however, was not the only method applied for the Exxon Valdez clean-up. Bioremediation techniques, first laboratory- and then field-tested have brought some satisfying results. A first approach was to implement hydrocarbon-degrading seed cultures (microorganisms able to degrade hydrocarbons), and although several of those turned out to have a positive effect on the oil degradation, some delayed or totally inhibited the biodegradation (Atlas, 1995). This negative effect can be explained due to the fact that hydrocarbon-degrading microorganisms are naturally present in marine, freshwater and soil reservoirs (Atlas, 1995) and the addition of non-indigenous microorganisms (often bacteria or fungi) can cause some unwanted antagonistic interactions between the microbes and limit the actual biodegradation process, in addition, the environmental conditions do not always suit the seed microorganisms, as they are not adapted to in situ conditions.

A second idea, which eventually appeared to be more successful, used the abovementioned fact of the microorganisms naturally inhabiting water and ground reservoirs, and therefore instead of seeding with non-indigenous microorganisms, fertilisers were applied. The role of these, was to naturally increase the amounts of phosphorus, nitrogen and mineral nutrients, which are crucial in biodegradation of hydrocarbons. This approach shortly turned out to be successful during the Exxon Valdez oil spill, showing significant effects in the habitats' recovery, compared to the untreated sites (Atlas, 1995).

Deepwater Horizon

When on 20th April 2010, a high pressure of oil and gas led to the Deepwater Horizon (DWH) drilling rig's explosion in the Gulf of Mexico, it quickly became clear, that this was one of the largest oil spills in history with its size exceeding even the Exxon Valdez accident (Baelum et al., 2012). Approximately 0.7 million tons of oil (4.4 million barrels) was released into the Gulf of Mexico within a period of 84 days until eventually, on June 15 of that year, the ruptured Macondo oil well (MC252) was finally sealed (Crone and Tolstoy, 2010).

This massive incident led to the death of 11 people (employees working on the Deepwater Horizon platform), large pollution of shorelines and death of many animals (including turtles, birds, mammals), which continued even six months after the spill (Paquette, 2013).

It is not the first time that the Gulf of Mexico has experienced oil contamination and a massive spill. The Gulf is a particularly rich source of oil, with a large annual natural seepage (Schmidt-Etkin, 2011), which can be considered as a sort of a "natural contamination". What is more, it had already faced a large oil spill, nearly 30 years before DWH. In early June of 1979, the oil well Ixtoc-1 exploded in the Bay of Campeche, Gulf of Mexico, releasing approximately 0.5 million tons of oil over a period of 10 months, until the well was finally sealed (Atlas, 1981; Kvenvolden and Cooper, 2003).

It has already been proven that such a continuous exposure of the relatively high concentrations of petroleum hydrocarbons leads to the enrichment of indigenous communities of hydrocarbon-degrading microorganisms (Geiselbrecht et al., 1998). The occurrence of large numbers of natural seeps in the Gulf of Mexico has likely led over time to the adaptation and partial pre-enrichment of hydrocarbon-degrading bacteria that are primed to respond quickly to a large influx of oil, such as had occurred with the Ixtoc I and

DWH oil spills in this region. The isolation and identification of these types of microorganisms in the Gulf of Mexico has been reported prior to the DWH spill. For example, Hollaway et al. (1980), studying the Buccaneer Oil Field in the north-west part of the Gulf of Mexico, revealed the presence of increased numbers of oil- and sulphur-degrading bacteria in this field when compared to a nearby uncontaminated control site. Some other interesting studies carried out by Geiselbrecht et al. (1998) were concentrated on searching for the PAH-degrading bacteria, such as *Cycloclasticus spp.* in the Gulf of Mexico.

As already mentioned, the DWH explosion resulted in the release of a large and unprecedented volume of oil into the Gulf of Mexico water column. The physical clean-up strategies (such as controlled burns, skimming, siphoning etc.) used to deal with the spill turned out to be successful in removing approximately 10% of the total oil released. The remainder of the oil had either volatilised into the atmosphere or had largely become dispersed into the water column and was treated with dispersants (Corexit 9500 and Corexit 9527) on the sea surface and by injection within the vicinity of the leaky wellhead at ca. 1,500 m depth. The latter is reported to have resulted in the formation of a deepwater oil plume – a "cloud" of dispersed oil droplets – that increased the bioavailability of the oil to indigenous microbial communities (Chakraborty et al., 2012).

2.1.4. Clean-up technologies

Large amounts of oil being annually released to the environment make the clean-up methods develop towards their efficiency, safety of use and minimised secondary pollution. Those technologies can be divided into three main categories: physical, chemical and biological. In this section the abovementioned techniques will be described together with some examples.

2.1.4.1. Physical Methods

- Booms. The very first action after an oil spill is directed to the oil collection, preventing it from spreading, preparing for the next operations or turning it to a specified direction. For these purposes, special floating barriers, called "booms" are used. These look like curtains immersed in water, with their upper layer (freeboard) floating above the water surface (Fingas, 2011c). Elastec/American Marine, which is the largest manufacturer of oil spill and environmental equipment in North

America, produce a few types of booms depending on their purpose of use, size of spill to be treated, area of spill etc. Among the different types available, one can find Foam Filled Oil Spill Booms (standard booms) that include fence and curtain booms. These are mostly used for inland purposes and also by ships during refuelling and the oil transfer. Curtain booms can be applied for inland and shallow calm waters. The next group that the company presents on their website is Inflatable Containment Boom, which is made of urethane and/or rubber materials and is said to be most suitable for vessel deployments (Elastec/American Marine). Figure 2 shows the boom being applied in the water.

Figure 2: Oil collected in a skimming boom.
Based on The Encyclopedia of Earth (2010).

- Sorbents. Another method successfully applied for clean-up purposes is the use of sorbents. The recovery of oil goes through either an absorption or adsorption mechanism and has a crucial role in the oil spill clean-up process. As varied as they are, there are several important purposes for the use of sorbents, which include:
    - as a first method for oil recovery in small spills (although in the past it was used for all types/sizes of the spills – see Torrey Canyon)
    - to discard the remaining oil from both water and land reservoirs
    - as a supporting technique for other clean-up methods
    - as a means of de-oiling shorelines.

  There are synthetic and natural sorbents, and both types are used in clean-up processes. The foremost are mostly made of polypropylene, polyethylene, polyesters, polyolefins and polyturethanes. Sorbents of natural origin are made of wood fibre,

bird feathers, peat, straw or vegetable fibre (Fingas, 2011c). Figure 3 presents the sorbent being released onto the oil-contaminated waters and its manual collection.

Figure 3: The use of a particular sorbent
Based on Fingas (2011c).

- *In-situ* burning. This technique can be used in the early stages of an oil spill, before its weathering and evaporation of its toxic volatile components, and can take place on the water surface or even in a marsh (Mullin and Champ, 2003). Before applying this *primary response method*, it is of high importance to establish a window-of-opportunity (a particular time/situation for an optimal use of a specific method or technique), which in the case of *in situ* burning, depends on oil characteristics (physic-chemical parameters of the oil, water contents, viscosity) and among others, the effectiveness of the oil's collection – mainly by using booms (Nordvik, 1995). According to Mullin & Champ (2003), this technique is particularly favourable when considering storage, transport, disposal or treatment of the spilled oil, as it largely decreases the costs of the listed activities.

- Manual clean-up. This method has been found to be particularly successful for small spills or limited areas, especially shorelines or shallow waters. It is however dangerous for those involved in the clean-up works (e.g. falls during the works, inhalation of the toxic fumes etc.).

Figure 4: Manual clean-up in Israel after the spill on 9th December 2014.
Based on Hopkins (2014)**.**

2.1.4.2. Chemical methods

- Dispersants. Soon after the Deepwater Horizon explosion, large amounts of dispersants were used in order to disperse the oil into smaller droplets and enhance its dissolution and bioavailability for microbial biodegradation processes (Chapman et al., 2007; Kujawinski et al., 2011). Chemically, dispersants are essentially surfactants (or detergents) that are often supplied dissolved in organic solvents (Lessard and Demarco, 2000) – this allows their effective application into contaminated waters or shorelines. Furthermore, the presence of surface-active agents is exceptionally important, as they contain both hydrophobic and hydrophilic moieties. When applied to oil-contaminated waters, the hydrophobic moiety (or lipid loving component) is orientated towards the oil, while the hydrophilic "head" is turned toward the water phase (Lessard and Demarco, 2000). Such arrangements of the surfactant's molecules lead to the significant reduction of the interfacial surface tension of oil droplets, thus increasing the availability of the oil to hydrocarbon-degrading microorganisms (Tsutsumi et al., 2000).

- Surface-washing agents (SWAs) or shoreline cleaners (SCs) are agents mostly used to improve the removal of oil from contaminated shorelines (Koran et al., 2009; Michel et al., 2001). Similarly to dispersants, SWAs also contain surfactants, although their purpose of use is quite different to dispersants (Rial et al., 2010). SWAs are applied to wash oil off solid surfaces rather than dispersing it. This divergence between dispersants and SWAs lies in the active compounds that are

used, which means that the surfactants in SWAs are characterised by a higher water solubility than the components of the dispersants (Fingas, 2011d).

- Emulsion breakers. Although the use of these agents might seem to be on the contrary to the application of dispersants, emulsion breakers play an important role in the primary clean-up processes, being strongly linked to the oil collection and *in situ* burning. Due to the high viscosity of the emulsified oil and its increased amounts, the storage, pumping and transport of such oil can not only be a few times more difficult, but also unnecessarily raise the costs of such operations (Nordvik et al., 1996). Therefore, the idea of using emulsion breakers is, as their name suggests, to break the emulsion forming between water and oil droplets. This process enables the reduction of water molecules collected and therefore the overall volume of the collected oil, making it ready either for the *in situ* burning or other operations.
- Solidifiers for the oil spill clean-up have been commercially available for over three decades, and were proven a good hydrocarbon removal from both aquatic and soil systems in laboratory-based experiments). They are composed of hydrophobic polymers, which, by reacting with the oil, form a stable solid mass that can easily be removed from a contaminated reservoir, leaving no traces of oil behind (Delaune et al., 1999; Rosales et al., 2010). According to Fingas (2008), there are numerous issues that limit the large scale application of the solidifiers. First of all, their toxicity has not been thoroughly studied yet, especially with regards to their long-term effects. Furthermore, no tests on biodegradability of neither solidifiers, nor the solidified oil were studied. What is more, the solidifiers appeared to be successful only in a small scale incidents and in the lab tests, thus leading to a conclusion that their use for larger spills could be out of control, expensive and difficult to provide the good mixing.

2.1.4.3. Bioremediation

The vast majority of the oil spill remediation technologies are based on the physical and chemical methods that can generally be classified as “primary methods”, meaning that they are applied in the first instance following an oil spill event. However, such methods cannot guarantee a 100 % efficiency in the removal of oil from the environment. The biological remediation is most often applied together with other methods, or as the last one, after using

the abovementioned techniques. Most often, however, the biological processes take place regardless of any other efforts undertaken.

According to the USEPA (EPA, 2015), bioremediation can be defined as the "use of living organisms to clean up oil spills or remove other pollutants from soil, water, or wastewater; use of organisms such as non-harmful insects to remove agricultural pests or counteract diseases of trees, plants, and garden soil". Following the "Citizen's Guide to Bioremediation" (EPA, 2012), bioremediation is considered as safer and cheaper method for the soil/marine reservoirs treatment. It does not require additional processes changing or greatly affecting the natural environment, such as drilling, digging, using chemicals etc.

The main processes of bioremediation can be divided in a few different ways. When considering the area, where the remediation takes place, these are:

- *In situ* bioremediation – this method is performed in the field at the site of contamination. It does not involve removal or transport of the contaminated material. The great advantage of this method is the reduction of the costs related to the collection and handling of the samples. (EPA, 2006).
- *Ex situ* bioremediation – this method involves the removal of the contaminated material, such as soil, to a different location where it is to be treated.

Another division can be made depending on the media added to the remediated sites (according to: EPA 2006; Singh et al. 2009):

- Intrinsic bioremediation – this kind of bioremediation occurs naturally, without any additives, and depends primarily on the ability of the autochthonous microbial communities to degrade the contaminants.
- Enhanced bioremediation – requires the application of some agent(s) that can positively stimulate the organisms to enhance the decontamination processes. This kind of bioremediation can be further classified depending on the abovementioned agents used for stimulation:
    - Biostimulation – when only chemical amendments are used (oxygen, macro- and micro-nutrients, vitamins etc.). This method involves such techniques as bioventing, biospraying, composting etc.

- Bioaugmentation, which involves the addition of some other microbial cultures, when the autochthonic microorganisms are unable to deal with the contaminants.

The methods listed above can be applied both *in situ* and *ex situ* (EPA, 2006; Singh et al., 2009).

Furthermore, when considering the oxygen demand, the bioremediation can follow either an aerobic (in the presence of oxygen) degradation scheme or an anaerobic pathway (in the absence of oxygen). This classification is of high importance when considering the biodegradation of the oil/hydrocarbon polluted areas, where the amounts of the oxygen can be greatly limited.

Last but not least, the type of organisms used for the bioremediation process can also influence the bioremediation process:

- Biodegradation involves the use of microorganisms such as bacteria and fungi, which by expressing the appropriate enzymes, are able to metabolise hydrocarbons, using them as sole carbon and energy sources. In other words, it is "a process by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment" (United States Environmental Protection Agency Office of Underground Storage Tanks. OSWER, 1997).
- Phytotechnologies that are based on the application of plants in order to reduce the contamination from the polluted areas. Among these, according to U.S. Environmental Protection Agency (EPA, 2010), one can list:
    - Phytodegradation – depending on the plants, which are able to take up and degrade the contaminants, using the internal metabolism.
    - Phytoextraction – based on the removal of plants that are able to take up and contain the contaminants.
    - Phytovolatilisation – involves the plants, which are able to take up, translocate and volatilise the pollutants, by using their respiration system (EPA, 2010).

2.2. Biodegradation

According to the International Union of Pure and Applied Chemistry (Duffus, 2003), biodegradation can be defined as the "*breakdown of a substance catalysed by enzymes in vitro or in vivo. This may be characterized for purposes of hazard assessment as:*

- *Primary. Alteration of the chemical structure of a substance resulting in loss of a specific property of that substance.*
- *Environmentally acceptable. Biodegradation to such an extent as to remove undesirable properties of the compound. This often corresponds to primary biodegradation but it depends on the circumstances under which the products are discharged into the environment.*
- *Ultimate. Complete breakdown of a compound to either fully oxidized or reduced simple molecules (such as carbon dioxide/methane, nitrate/ammonium and water). It should be noted that the products of biodegradation can be more harmful than the substance degraded*."

In other words, biodegradation is a complex process, during which the particular chemicals are turned into smaller molecules as a result of microbial metabolism of that compounds.

2.2.1. Characteristics of bacterial degradation of hydrocarbons

Petroleum hydrocarbons can be degraded by bacteria of different genera and origin (marine or terrestrial). The degradation process depends on many factors, among which the type and properties of the substrates are of high importance. What is more, not all hydrocarbon degraders are able to metabolise each group of petroleum compounds. Hence, when describing the biodegradation of hydrocarbons, it is helpful to consider as many aspects influencing this process as possible.

2.2.1.1. Factors affecting hydrocarbon biodegradation

Some of the main factors that can influence the biodegradation of petroleum hydrocarbons are described below.

- Temperature – although the biodegradation process may take place in very varied temperatures, there is a general division of the optimal temperatures for the efficient degradation, depending on the contaminated area (Figure 5).

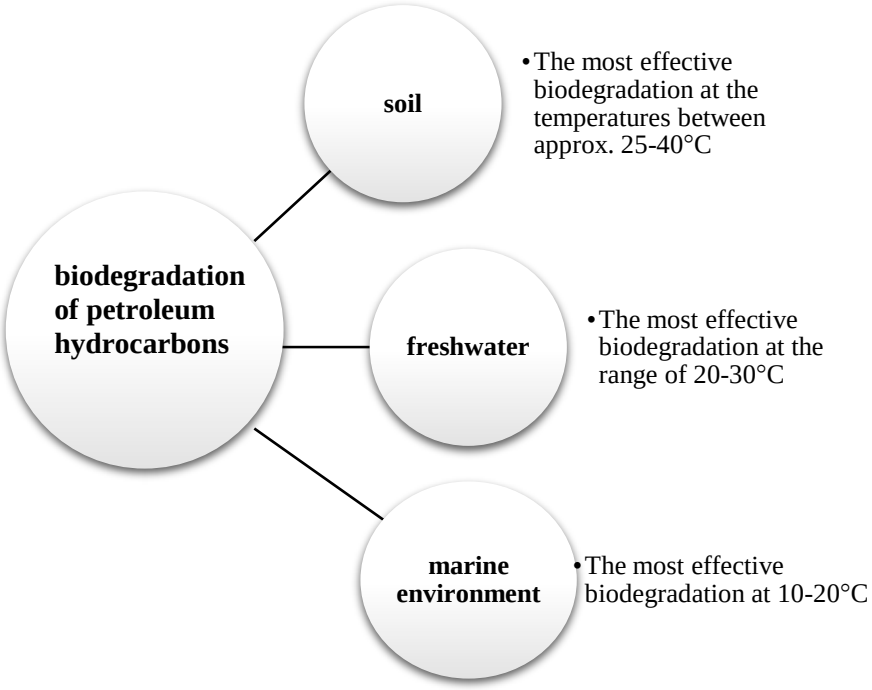


Figure 5: The rates of biodegradation of petroleum hydrocarbons in different reservoirs and temperatures.
According to Das and Chandran (2011).

What is more, there is a strong connection between the temperature and the crude oil composition, which was first reported by Atlas in 1975, eight years after the Torrey Canyon spill (Atlas, 1975). The author presented his findings, suggesting that at low temperatures the lighter crude oils were less likely to be degraded, as they contained some volatile, low-molecular-weight hydrocarbons, which turned to be toxic to the tested microorganisms. However, when the temperature was increased (from 10 to 20°C), the lightest hydrocarbons evaporated and the rest of the crude oil was degraded with satisfying results. The heavy crude oils, despite the lack of toxic volatile compounds, were degraded with much lower efficiency at 20°C, when compared to the lighter oils.

- Oil properties. As described in the paragraph above, the biodegradation of oil hydrocarbons depends not only on the environmental circumstances, but also on the type of the oil (light or heavy fractions). Furthermore, the amounts of the oil may also constitute a problem, when the concentration of the petroleum hydrocarbons is high enough to turn out to be toxic to the microbiota (Del'Arco and De França, 2001).

What is more, among many different features of hydrocarbons, one of the most important when considering their metabolism by microorganisms, is their hydrophobicity. The vast majority of hydrocarbons are characterised by a very limited or even lack of solubility in water, and generally the more hydrophobic the hydrocarbon species, the less bioavailable it will be to microorganisms for biodegradation.

Another important factor is the chemical structure of the hydrocarbons. Broadly speaking, they can be divided into aliphatic (saturated and unsaturated; straight chain or branched; short or long chain), aromatic (with or without substituents) and polycyclic aromatic hydrocarbons (PAHs; described above). Some strains are able to "specialise" in the degradation of aliphatic hydrocarbons, whilst others do so with hydrocarbons. The ability of a microorganism to metabolise these compounds depends on their genetic capacity to express the appropriate enzyme systems for their degradation. This, however, can significantly differ among bacterial classes or even between families of the same class. For example, bacteria of the family of *Rhizobiaceae* (class *Alphaproteobacteria*) were found able to degrade petroleum hydrocarbons, mainly PAHs, but also some polychlorinated biphenyls (PCBs) and aromatic heterocycles (Keum et al., 2006). On the other hand, members of the *Phyllobacteriaceae* within the same class have not been reported to degrade hydrocarbons.

- Enzymatic activity. As mentioned above, in order to be capable of metabolising hydrocarbons, as indeed any other chemical pollutant, microorganisms need to possess the appropriate enzyme systems. Such systems can be very different, depending on the compound to be degraded. Then, the individual enzymes catalyse step by step the respective reactions, as it is pictured in Figure 6 and are thoroughly described in the *chapter 2.3.* of this dissertation. With alkane degradation, for example, this starts with the initial oxidation of the substrate as catalysed by an alkane monooxygenase. The alcohol that is formed in this reaction is then transformed to an aldehyde (this step being catalysed by alcohol dehydrogenase) and the metabolic reactions continue leading to a beta oxidation (Rojo, 2009; Sabirova et al., 2006).

Early after the DWH oil spill (May 2010) the first analyses of the microorganisms involved in the hydrocarbon degradation were conducted. At that time the most abundant strains in the plume were classified as belonging to the *Oceanospirillales* order, usually linked to the aliphatic hydrocarbon degradation (Baelum et al., 2012; Yang et al., 2014). Those findings prompted the scientists to conduct further research on the exact role of *Oceanospirillales*, with a special attention to the functional genes and pathways (Mason et al., 2012). From a detailed examination of the metagenome sequence of the *Oceanospirillales* that was enriched during the DWH spill, Mason et al. (2012) reported the existence of a complete alkane and cycloalkane degradation pathways in the metagenome. These results confirmed this organism had contributed to the degradation of alkanes in the Macondo oil. PAH-degrading bacteria, such as *Cycloclasticus*, were also found enriched in the oil plume that formed in the Gulf of Mexico, but were much less abundant compared to the *Oceanospirillales* (Yang et al., 2014).

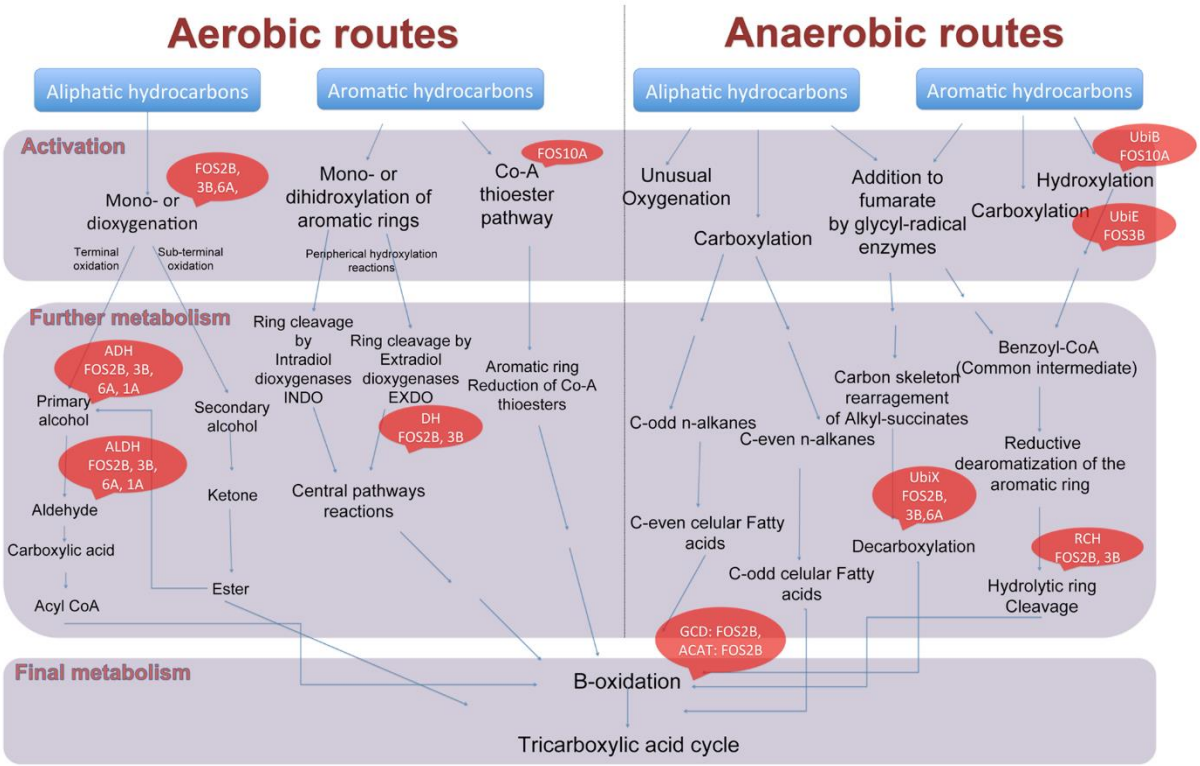


Figure 6: Comprehensive biochemical pathway for both aerobic and anaerobic bacterial degradation of hydrocarbon compounds.
According to Sierra-García et al. (2014).

- Cell surface properties play a very important role in hydrocarbon biodegradation processes, such as influencing the attachment of cells to the hydrocarbon substrates.

There is no detailed mechanism that could potentially be used to describe this process, mainly due to a number of different interpretations and features that are still unknown. However, there are some measureable features, that have gained a lot of interest in the recent years, being said to have an noteworthy impact on the biodegradation of hydrophobic compounds. These have been extensively studied by myself and the rest of the research team at Faculty of Chemical Technology at Poznan University of Technology in Poland. These are: hydrophobicity, zeta potential, surfactant enhancement and a production of biosurfactants.

- Nutrients. During their growth, microorganisms need vitamins and microelements to keep all the necessary functions, such as metabolism or cell divisions. Therefore, the presence of nutrients (especially phosphorus and nitrogen) in the area where the biodegradation is supposed to take place, is found valuable and fundamental for the process (Das and Chandran, 2011). The addition of nutrients (considered as a mean of bioaugmentation techniques) can effectively promote the biodegradation, as reported by Bento et al. (2005), where the scientists obtained 72% biodegradation of the petroleum hydrocarbons after just six weeks of the incubation.
- Presence of metals and chemical agents. Biological degradation of petroleum hydrocarbons taking place in areas co-contaminated with chemicals (such as surfactants, emulsifiers, chlorinated solvents, dispersants etc.) and metal ions is a complex process and requires a well-planned approach (Pérez et al., 2010). A special consideration of the problem covers the presence of heavy metals, as they cannot be degraded, and what is more – depending on their concentrations and properties – they may limit or totally inhibit the biodegradation because of their toxic influence on the microorganisms (Pérez et al., 2010). This may happen either due to the replacement of the required ions by toxic ones, or through their interactions with ligands (Bruins et al., 2000).

  The organic solvents can become more toxic to microorganisms even at low concentrations either by the osmotic stress or inhibition of the metabolic enzymes. This may constitute a significant problem, especially when the solvents are used as primary countermeasures after the oil spill. Therefore, it has been proposed that genetic engineering is used in order to obtain strains of high solvent tolerance and some unique catalytic features (Xu et al., 2006).

- Other factors. The efficiency of the biodegradation depends also on the area (water or soil) and its properties, such as pH, co-existing microflora and the presence of some other contaminants (Mohan et al., 2006). Additionally, it is worth considering how long a particular site was contaminated for. This can help to understand the differences between the microorganisms contaminated with petroleum hydrocarbons in contrast to the uncontaminated samples. Such observations were reported by our research team in Poland, when studying the influence of long term contact of a newly isolated *Achromobacter* sp. 4(2010) strain with diesel oil (Kaczorek et al., 2013b). The results we obtained indicated some significant changes compared to the untreated strain. These covered the higher biodegradation efficiency of the treated strain, along with a significantly increased hexadecane monooxygenase activity, genetic modifications and differences in the fatty acid profiles.

2.2.2. Microorganisms involved in biodegradation

A lot of progress has been made for the identification of oil-degrading bacteria, mainly due to a serious problem of petroleum contamination in the environment. The DWH oil spill was a great example of the use and development of gene-sequencing methods, helpful in an identification of the hydrocarbon-degraders from the sites contaminated by the spill. The research based on the obtained findings is helpful in the analyses of the biodegradation mechanisms, now mainly focused on enzymatic and surface properties of the microbiota involved in the process. What is more, a comprehensive and detailed knowledge on the type and properties of hydrocarbon-degraders is an invaluable contribution to the research focused on exploring and perfecting the bioremediation strategies of the polluted sites.

2.2.2.1. Marine strains

One of the major groups of bacteria capable of degrading petroleum hydrocarbons in the marine environment is *Cycloclasticus* (Kasai et al., 2002). Members of this genus play an important role, especially in the degradation of PAHs (including alkyl-substituted PAHs), such as alkylnaphthalene, anthracene, biphenyls, fluorene, naphthalene, phenanthrene and pyrene (Kasai et al., 2002). What is more, bacteria of the *Cycloclasticus* genus were, along with *Alteromonas* and *Colwellia*, isolated and identified from contaminated water samples collected during the DWH oil spill, and their PAH-degrading capabilities were characterised (Gutierrez et al., 2013).

An interesting observation was made first a month and then two months after the onset of the DWH spill, where the vast majority of hydrocarbon-degrading bacteria in the oil plume were found to belong to the Order *Oceanospirillales*, of which most are associated with aliphatic hydrocarbon degradation. However, in June, two months after the spill and a month after the first analysis of the plume, another examination was made, revealing that this time, the isolated microbiota in 95% of total was characterised by the sequences of the "specialist" PAH-degrader *Cycloclasticus*, and generalist-degrader *Colwellia* (Baelum et al., 2012; Hazen et al., 2010; Valentine et al., 2010). Other hydrocarbon-degrading bacteria, found during the abovementioned research conducted by Hazen et al. (2010) and Valentine et al. (2010), belonged to the genus of *Alteromonas*, *Halomonas* and *Pseudoalteromonas*.

What is interesting is that the tests repeated in September – already a few months after the leaky wellhead was officially capped – showed no detection of *Oceanospirillales* and *Colwellia*, but revealed an abundance of some methanotrophs, *Flavobacteria*, *Rhodobacterales* of the *Alphaproteobacteria* order (Redmond and Valentine, 2012). It is noteworthy that *Flavobacteria* are normally abundant in the marine environment, and are usually related to the degradation of high molecular weight organic matter. Stable-isotope probing (SIP) studies helped to identify methanol-degradation abilities of some *Flavobacterium* strains (Boden et al., 2008; Redmond and Valentine, 2012).

Such shifts in microbial communities over time can be viewed as a good indicator of the hydrocarbon exposure (Redmond and Valentine, 2012) and degradation progress. However, it is necessary to prove the oil-degradation properties of the isolated strains in order to have the maximum certainty of the conducted research and the final conclusions.

2.2.2.2. Terrestrial strains

The DWH oil spill had a negative influence, not only on the Gulf of Mexico waters, but also on some beach sands in the Gulf. Thorough research was conducted in order to analyse the gene sequences of hydrocarbon-degrading bacteria from oil contaminated beach sands, which uncovered microorganisms belonging to the genera *Alcanivorax*, *Marinobacter*, *Pseudoalteromonas*, *Pseudomonas* and *Vibrio* genera (Kostka et al., 2011).

A consideration of alkane biodegradation requires a division into three main groups: short chain alkanes (methane and corresponding alkanes), medium chain length alkanes (C_5-C_{10}) and long chain alkanes (>C_{11}). Methanotrophs are microorganisms capable of using methane

as a carbon and energy source, thanks to the methane monooxygenase system, which is responsible for the first step of degradation that involves oxidation of the substrate to a corresponding alcohol – in this case methanol. Due to some differences in their physiology and morphology, methanotrophs have been divided into two types. Type I includes *Methylobacter, Methylomicrobium, Methylomonas, Methylosphaera,* and *Methylococcus genera*, whereas Type II covers *Methylocystis, Methylosinus, Methylocella* and *Methylocapsa* (Hanson and Hanson, 1996; McDonald et al., 2005). Methanotrophs are very wide spread microorganisms and are present in different reservoirs such as soil, wetlands and marine environment (including marine sediments) and are said to play an important role in reducing the greenhouse effect by limiting the methane emission (Hanson and Hanson, 1996; Watanabe, 2001).

Some microorganisms are able to degrade other gaseous alkanes, but are not capable of degrading methane. *Pseudomonas butanovora* is an example of such a microorganism, using the butane monooxygenase system for the degradation of $C_2 – C_4$ alkanes. Some other soil strains, which are characterised by such features, belong to the genus of *Gordonia, Mycobacterium, Pseudonocardia, Mycobacterium* and *Nocardioides* (Rojo, 2009).

Hamamura et al. (2006) published research on soil samples taken from six different areas of the United States (Arizona, Indiana, Montana, Oklahoma, Oregon and Virginia) and contaminated (under control) with 2% (wt/wt) crude oil. The samples, after 50 days of cultivation, were then chemically and genetically analysed in order to examine their hydrocarbon-degrading potential and investigate the microbial population that was involved in the degradation process. A significant reduction of a contaminant was observed along with the development of some microbial population. What is more, the authors noticed that the soil type had an influence on the microorganisms involved in the hydrocarbon degradation process. It also turned out that the *Rhodococcus* bacterial strains were the most popular in all soil samples despite the soil's origin. Other strains identified in this study belonged to *Nocardioides*, *Collimonas* and *Acinetobacter* genus.

Other microorganisms involved in the petroleum contaminants (including long chain alkanes and PAHs) in soil are, for example, *Bacillus*, *Pseudomonas*, *Micrococcus* (Ghazali et al., 2004), *Sphingomonas* (Romantschuk et al., 2000), *Arthrobacter, Enterobacter, Flavobacterium* (Gannon et al., 1991), *Achromobacter* (Gannon et al., 1991; Sałek et al., 2013), and *Stenotrophomonas* (Kaczorek et al., 2013a). Andreoni et al. (2004) in their

research especially emphasised the crucial role of *Achromobacter*, *Methylobacterium*, *Rhizobium*, *Rhodococcus*, *Stenotrophomonas*, *Alcaligenes* and *Aquamicrobium* in the fastest phenanthrene degradation.

To summarise, it is difficult to generalise which bacterial strains might be involved in the degradation of hydrocarbons, as it strongly depends on the contaminated reservoirs (waters, soils, marchlands, wetlands, beach sands etc.) concomitant with several factors that can influence the degradation process.

2.3. Key role of oxygenases in bacterial degradation of hydrocarbons

Microorganisms able to metabolize hydrocarbons constitute an important group in both, marine and terrestrial reservoirs. In many cases, those organisms use alkanes and aromatics as sole carbon and energy sources. That leads to the question of how this can be possible, especially considering the low water solubility that hydrocarbons are characterized with. A hydrocarbon metabolism is not a straightforward process, as those compounds are inert and need activation before any degradation step (Rojo, 2010).

The vast majority of hydrophobic pollutants are degraded under aerobic conditions, hence, the information included in this chapter is directly related to such conditions. The first and the most crucial step in the whole complex metabolic process is the activation – an oxidation of the hydrophobic substrate, which is catalysed by hydroxylases – a group of enzymes able to oxidise the hydrocarbon thus leading to its increased bioavailability. Depending on the type of a hydrocarbon (aliphatic or aromatic) to be degraded, some different hydroxylases are required. That covers alkane and aromatic hydroxylases.

Alkane oxygenases can be further divided according to the hydrocarbon's chain length. Van Beilen and Funhoff (2007) suggest a simple division into three main categories: C_1–C_4 (methane to butane, oxidized by methane monooxygenase-like enzymes), C_5– C_{16} (pentane to hexadecane, oxidized by integral membrane non-heme iron or cytochrome P450 enzymes), and C_{17+} (longer alkanes, oxidized by essentially unknown enzyme systems).

Aromatic hydrocarbons may be oxidised by either alkane or aromatic oxygenases. While the monooxygenation of aromatics follows the mechanism of hydroxylation or epoxidation, leading to incorporation of one oxygen atom to the aromatic ring, the dioxygenation occurs either through a so called "double hydroxylation" or the intra-/extra-diol ring cleavage (Chaiyen, 2010).

2.3.1. Oxygenases

As already mentioned, the hydroxylation is the first and most important step and depending on the hydrocarbon, a corresponding enzyme is used. For instance, the hydroxylation of cyclopentanone is catalysed by 1,2-cyclopentanone monooxygenase, accordingly. Other examples include hexadecane monooxygenase, which catalyses the conversion of hexadecane to the corresponding alcohol, and methane is oxidised by a methane monooxygenase (MMO).

These abovementioned examples are only a small illustration of a wide variety of oxygenases, which can be characterised depending on the reactions' substrates, co-enzymes present, the type of the reaction to be catalysed or an active centre (a cluster that can contains metal ions involved in the electron transfer). Considering the hydroxylation of petroleum hydrocarbons, the characteristics of the active sites is the most important and will be presented in this paragraph.

Active sites

The hydroxylation of a substrate, already mentioned in this paragraph, is the most important reaction in hydrocarbon biodegradation. It is based on a C-H bond cleavage, which is possible due to binding and activation of the dioxygen at the enzyme's active site. Hydrogen is then reduced by the electrons provided by the metal atoms from the active site and by cofactors or a substrate itself (Decker and Solomon, 2005).

The metal ions in the active sites are mostly iron and copper. Depending on the metal atom and the structure of the enzyme's active centre, one can enumerate the following examples:

- Heme enzymes/systems
- Mononuclear non-heme iron systems
- Rieske centres
- Rieske non-heme iron oxygenases (RO)
- Mononuclear and binuclear (both coupled and non-coupled) copper systems (Decker and Solomon, 2005; Ferraro et al., 2005; Itoh, 2006).

Heme enzymes

A protoheme IX, or iron protoporphyrin, heme b, is the most common heme group found in the oxygenases (Figure 7). Like other active centres, its role differs depending on many factors such as substrate type (paraffin, acids, aromatic hydrocarbons, drugs etc.) and conditions, heme ligands and many others. The most characteristic mechanism for heme-containing oxygenases is based on an iron-dioxygen complex, which is a key intermediate for a successful catalysed reaction (Sono et al., 1996).

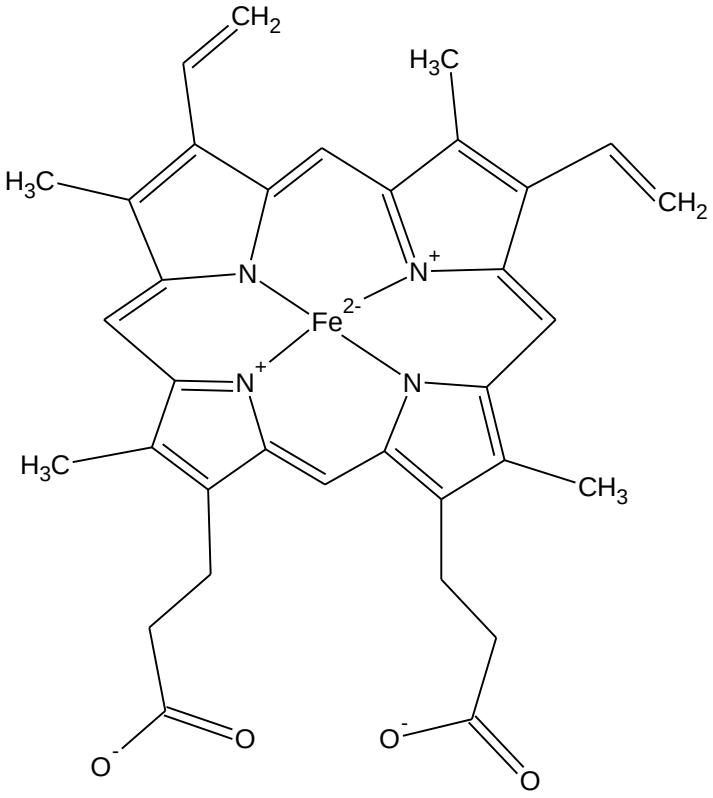


Figure 7: Structure of protoheme IX

The most popular representative of the heme oxygenases is cytochrome P450. This hemoprotein was discovered to be abundant in many organisms of all biological kingdoms (Hannemann et al., 2007). Eiben (2007) summarised some of the examples of such reactions, as follows: oxygenation of hydrophobic organic compounds (HOC), highly selective syntheses of hormones, signal transducers and some other secondary metabolites. The author suggested some interesting examples as ergosterol syntheses, insect- and phyto-hormones, development of fruit ripening and fragrance, as well as the flavour, and colour changes in plants. Additionally, P450s also play an important role in a large-scale production of some drugs.

Furthermore, as it is present in the human body, cytochrome P450 is involved in the metabolism of various xenobiotics and drugs. Table 4 presents some of the most common examples according to Anzenbacher (Anzenbacher and Anzenbacherová, 2001).

Table 4: Drugs and xenobiotics metabolised by cytochrome P450 in various compartments of the human body.

HUMAN ORGAN	SUBSTRATE
liver	aromatic amines, PAH, caffeine 1B1
	coumarin, steroids
	tolbutamide, diclofenac
lung, liver, brain, lymphocytes, heart	PAH (polycyclic aromatic hydrocarbons)
skin, brain, heart, lung, liver, kidney	PAH
brain	morphine
liver, heart	nicotine, omeprazole, diazepam
liver, kidney	retinoids
liver, brain, heart	antidepressants
liver, kidney, lung, brain, lymphocytes	different – Ca channel blockers, cyclosporin, acetaminophen, steroids

According to Hanneman (2007), P450s usually act like terminal monooxygenases, especially when considering the degradation of aliphatic hydrocarbons.

Mononuclear non-heme iron enzymes

There are two classes of mononuclear non-heme iron enzymes, reacting with dioxygen (Figure 8).

The mechanism of the reactions catalysed by pterin- and α-ketoglutarate (α-KG)-dependent enzymes goes through the reaction of iron(II) and a reduced cofactor with dioxygen, forming a Fe(IV)=O intermediate, which then reacts with a substrate by either hydrogen-abstraction or an electrophilic attack on a ring (Solomon et al., 2009).

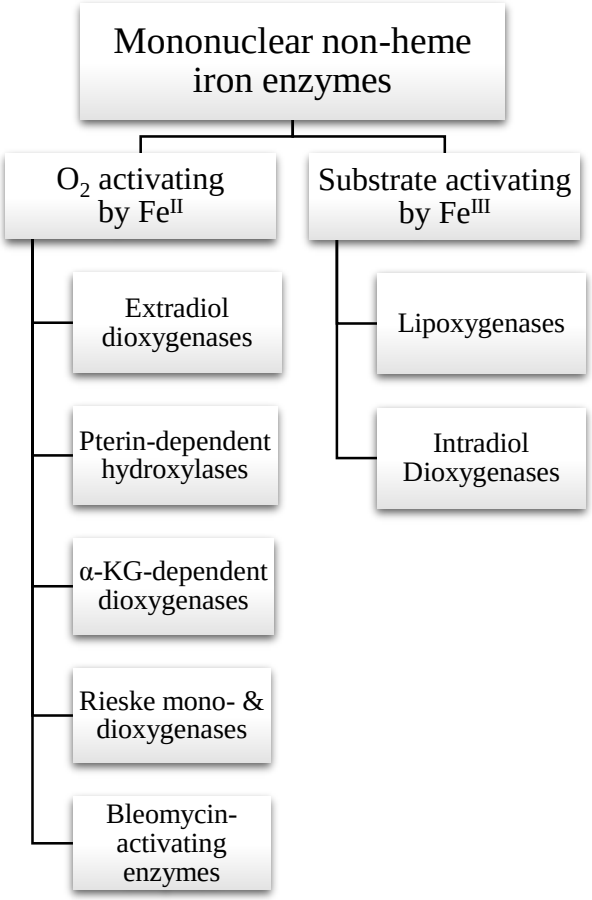


Figure 8: Classes of mononuclear non-heme iron enzymes.
According to Solomon et al. (2009).

A very interesting and now intensively studied example of a slightly different mechanism is the activation of bleomycin (BLM). This is an antibiotic able to cleave DNA by H-atom abstraction. It is used as a treatment for a variety of cancers, which gives it a special interest these days. The mechanism is based on the reaction of iron(II) with dioxygen together with an exogenous electron, resulting in a formation of the activated BLM (ABLM) (Solomon et al., 2009).

The other type of mononuclear non-heme iron enzymes is characterised by an oxidised iron active site, where iron(III) is used to activate the substrate for the reaction with dioxygen. Currently, the most well-studied enzymes of that class are lipoxygenases (LO) and intradiol dioxygenases (Pau et al., 2007; Solomon et al., 2009).

When it comes to lipoxygenases, the substrate-activation mechanism is not complicated. The active site of the enzyme consists of iron(II) coordinated by three histidine ligands, isoleucine, asparagine, and a water molecule. During the first step of the reaction, iron(II)

gets quickly oxidised to iron(III) and the water molecule is deprotonated giving a hydroxide. Further, the Fe(III)-OH form of the first step leads to the deprotonation of a substrate. That leads to a formation of a substrate radical and reduction of Fe(III)-OH to Fe(II)-H_2O form (Pau et al., 2007).

Rieske centres

There are many examples of different iron-sulphur clusters known in nature, and they are normally a part of those proteins that are engaged in electron transfer. There are a few types of such clusters, among which the [2Fe-2S] cluster is well known and popular when considering the oxygenase systems. It exists in enzymes isolated from both plants and microorganisms. The plant-origin iron-sulphur clusters are coordinated by four cysteine ligands, while bacterial-type Rieske clusters are coordinated by two histidine and two cysteine ligands (Ferraro et al., 2005).

Rieske non-heme iron oxygenases (RO)

According to Wackett (2002), there have been over 50 different Rieske oxygenases of bacterial origin identified so far. The vast majority of them are dioxygenases, however, there have been some monooxygenases found as well. Interestingly, in spite of some differences, these enzymes have shown some overlapping mechanisms and substrate specificities.

A special interest in Rieske oxygenases is observed in the environmental sciences, as these enzymes are involved in degradation of aromatic and aliphatic hydrocarbons. The best known enzyme belonging to this group of enzymes is naphthalene 1,2-dioxygenase, which catalyses the dihydroxylation of naphthalene, resulting in a formation of (1R,2S)-cis-1,2-dihydro-1,2-dihydroxynaphthalene (Chakrabarty et al., 2007).

The Rieske non-heme iron oxygenases provide an exceptionally important transport of the electrons and activation of the oxygen, which is then involved in the hydroxylation of the substrate.

There can be two- or three separate components (depending on the oxygenase) distinguished in a particular system, which participate in the electron transfer. These are: reductase, ferredoxin (absent in the two-component systems) and oxygenase. Usually, the reductase-component contains three main domains – the FAD-binding and the NAD(P)H-binding domains as well as a C-terminal domain. The ferredoxin is only present in the three-component Rieske oxygenases and takes part in transferring the electrons from reductase to

oxygenase unit. Last, but not least – an oxygenase component, which consists of the Rieske domain and the mononuclear iron domain. The role of the Rieske cluster is to accept the electrons from either the reductase or ferredoxin (depending on the system) components and pass them to the mononuclear iron, where the final product of the reaction is formed (Ferraro et al., 2005).

The mechanism of those reactions is presented in Figure 9. To summarise, in the first reaction (1), the NAD(P)H is oxidised to $NAD(P)^+$ at the reductase's NAD(P)H-biding site. During this reaction there are two electrons revealed, which are then kept in reductase's FAD domain (2). The electrons are stored in this domain as long as the reductase does not finish the reduction of one electron from ferredoxin's domain (3). This electron is further passed to the Rieske cluster in the oxygenase component (4). This reaction is repeated for each product molecule, which is formed on the mononuclear iron centre (5). For this scheme naphthalene was used as an example, and the reaction product is (1R,2S)-cis-1,2-dihydro-1,2-dihydroxynaphthalene.

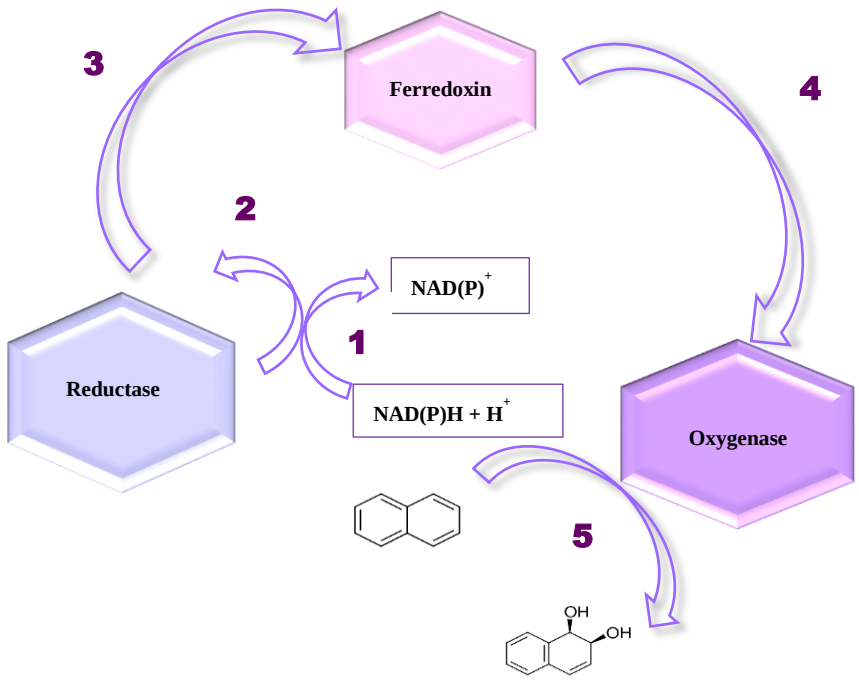


Figure 9: A three-component Rieske oxygenase system. According to Ferraro et al. (2005).

Copper-containing proteins

The copper-containing proteins are very common in nature, playing an essential role in oxygen activation and reduction (Chen et al., 2004). A special interest is put to the binuclear copper proteins, which are divided into two main groups – coupled and non-coupled copper active sites. Among the first group there are hemocyanin, tyrosinase and catechol oxidase, to name a few (Solomon et al., 1996).

The most common representatives of the second group (non-coupled binuclear copper proteins) are: peptidylglycine R-hydroxylating monooxygenase (PHM) and dopamine β-monooxygenase (DβM), both catalyzing a corresponding substrate hydroxylation. For the first one it is the glycine backbone C-H bond, and the second mentioned enzyme (DβM) is involved in reaction with dopamine benzylic C-H bond (Chen et al., 2004; Solomon et al., 1996).

Less is known, however, about the mononuclear copper systems. According to Itoh (2006), a relatively easy reaction between the mononuclear copper active site and another molecule of copper(I) complex, gives rather stable dicopper-dioxygen complexes. As an example of the reactions that the mononuclear copper active-oxygen systems are involved in, the author suggests the processes of 2,4,5-trihydroxylphenylalanine (TOPA) and some related organic cofactors in copper-containing amine oxidases and lysyl oxidase (Itoh, 2006). Also, Lieberman and Rosenzweig (2005) point to a co-existence of a mononuclear active site along with a binuclear copper site in particulate methane monooxygenase (pMMO).

Other alkanes, including long-chain alkanes and cycloalkanes, are metabolised by microorganisms using a wide variety of enzymes. As already mentioned, the hydroxylation is the first and most important step and depending on the hydrocarbon, a corresponding enzyme is used. For instance, the hydroxylation of cyclopentanone is catalysed by 1,2-cyclopentanone monooxygenase, and a hexadecane moonoxygenase catalyses the conversion of hexadecane to the alcohol.

2.3.2. Degradation pathways

The short-chain alkanes (methane, ethane, propane, butane, pentane and halogenated derivatives) are generally oxidized by methane monooxygenases (MMO), among which the pMMO (particulate methane monooxygenase) and sMMO (soluble methane monooxygenase) can be distinguished (van Beilen and Funhoff, 2007). The sMMO is at least a three-component enzyme with a di-iron cluster and often a flavin adenine

dinucleotide (FAD) (Shennan, 2006). Copper containing with three-subunits pMMO is a membrane-bound enzyme, usually compared to the sMMO in relation to its activity. However, while sMMO demonstrates a wide range of substrate specificity, a pMMO is limited to a small group of straight-chain alkanes and alkenes (Elliott et al., 1997; Lieberman and Rosenzweig, 2004). For a better understanding of that division in methane monooxygenases and their occurrence, the researchers mostly present a simple dependence on the copper concentration during the growth of bacterial strains. In other words, at low copper concentrations the monooxygenase activity is mostly expressed in the soluble fractions and the enzyme involved in the catalysis is a sMMO (Lieberman et al., 2003; Takeguchi et al., 1999). Similarly, when the copper-to-biomass concentrations are higher, the hydroxylation of the substrate takes place in the membrane fraction by pMMO (Takeguchi et al., 1999).

Following the short division of the hydrocarbon-degrading enzymes mentioned in paragraph 2.3., proposed by Van Beilen and Funhoff (2007), long chain alkanes like C_5– C_{16} and others, are most likely to be metabolised in the presence of membrane non-heme iron monooxygenases or cytochrome P450 enzymes. The general scheme of such degradation is presented in Figure 10.

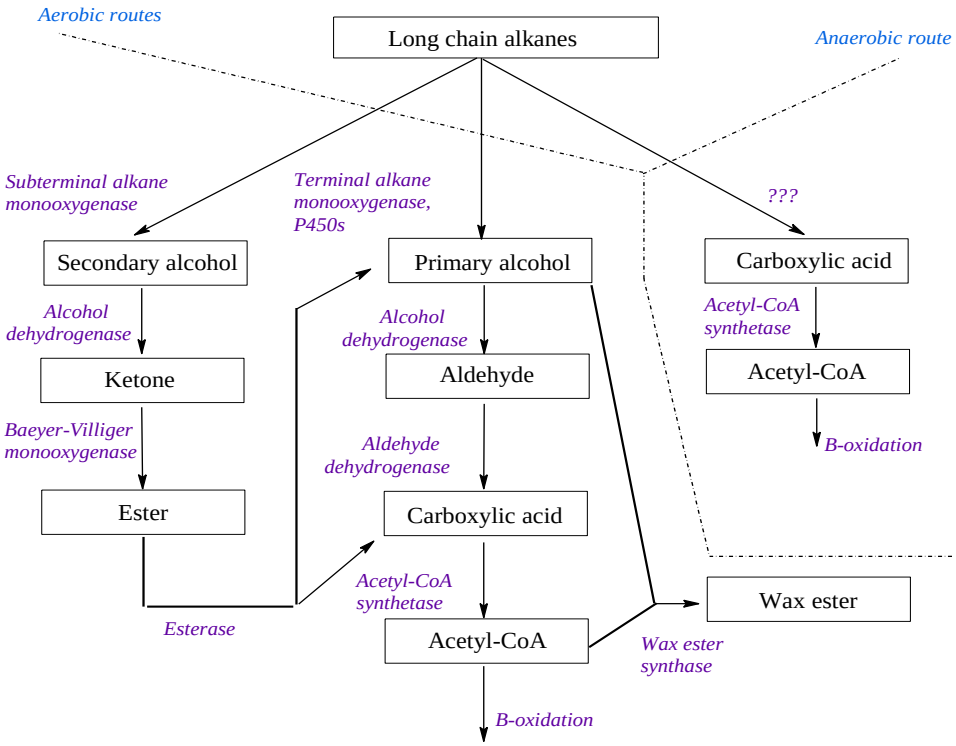


Figure 10: Aerobic and anaerobic degradation pathways of long chain alkanes. According to Wentzel et al. (2007).

As presented in Figure 10, the degradation of long-chain alkanes in aerobic conditions is possible within two different pathways. The first one proceeds via sub-terminal oxygenation and it is catalysed by alkane monooxygenases giving a secondary alcohol. The enzymes involved are said to be mono and/or di-nuclear non-heme iron monooxygenases (Wentzel et al., 2007), also classified by van Beilen and Funhoff (2007) as particulate alkane hydroxylases (pAH). The alcohol intermediate is further oxidised to a ketone (by alcohol dehydrogenase) and then to a corresponding ester (Baeyer Villiger monooxygenase). This product can then undergo a hydrolysis (catalysed by esterases), leading to the formation of a primary alcohol.

The same alcohol is obtained if a microorganism produces monooxygenases by directly oxidising an alkane to a primary alcohol. According to Wentzel et al. (2007), such monooxygenases mostly belong to the cytochrome P450 family. However, the authors also provide some examples of the overlapping substrate specificity in oxygenases. In other words, although those enzymes differ between each other regarding the catalytic centres (active sites), they are still able to catalyse the same reactions, yielding identical products. Therefore, it should be noted that the division of monooxygenases depending on an oxidation pathway may still be complicated and consequently should always take account of the microorganisms involved in the degradation process, as well as process' conditions (e.g. temperature).

Another noteworthy monooxygenase already mentioned in this section, is a so-called Baeyer-Villiger Monooxygenase (BVMO). BVMOs are flavoenzymes (flavin-containing enzymes, also named flavin-containing monooxygenases – FMOs) catalysing Baeyer-Villiger reactions (oxygenation of a cyclic or acyclic ketone to a corresponding ester or lactone) in a presence of dioxygen and NAD(P)H (Fraaije et al., 2002). Due to their properties and a high enantioselectivity, BVMOs have extensively been studied with regards to the large scale synthetic applications. Some of those enzymes have already been successfully introduced to the industry, allowing to omit the use of toxic and labile catalysts such as peroxides and preacids (Rodríguez et al., 2007).

Cyclohexanone monooxygenase (CHMO) is currently one of the best characterised representatives of BVMOs. It is a NADPH and oxygen-dependent flavoprotein of a high substrate tolerance, catalysing the oxygenation of a number of cyclic ketones (from four to eight carbon rings), as well as aromatic aldehydes and heteroatom-based reagents.

Nevertheless, the most studied reaction mechanism is still the oxygenation of the cyclohexanone to the caprolactone, which is a part of a cyclohexane degradation pathway (Figure 11) (Alphand et al., 2003; Sheng et al., 2001).

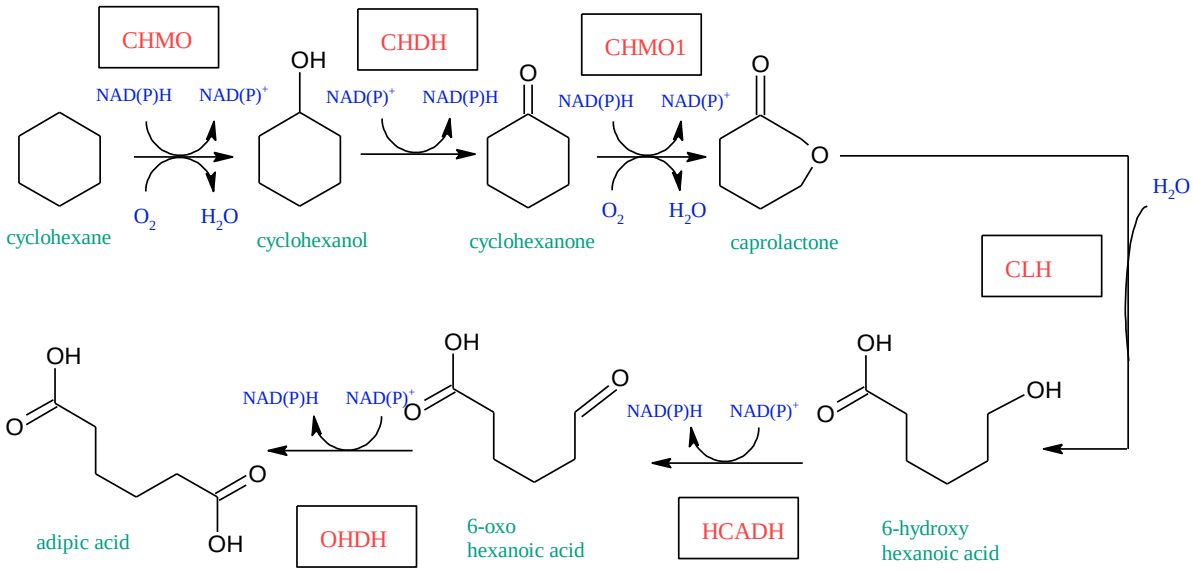


Figure 11: Cyclohexane degradation/oxidation pathway.
Based on Cheng et al. (2002).

Figure 11 presents a cyclic alkane degradation pathway, where cyclohexane was used as a model substrate. As for aliphatic alkanes, the first step of the substrate's degradation (in this case, cyclohexane) is its oxygenation, catalysed by a corresponding monooxygenase. According to Cheng et al. (2002), for most of the cycloalkanes their biological conversion leads to the formation of the (di)carboxylic acids and does not significantly differ even between the phylogenetically various bacterial strains, which are able to use those hydrocarbons as sole source of carbon and energy.

Unlike the similarities observed for degradation of short-, long-chain and cyclic alkanes, a bioconversion of aromatic hydrocarbons can involve more than one pathway, and which is largely dependent on the microorganism's metabolic abilities. Figure 12 presents a phenanthrene degradation pathway proposed by Stingley et al. (2004) for a bacterial strain *Mycobacterium vanbaalenii* PYR-1, that was shown to be able to degrade high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs). The authors conducted a thorough molecular study on the strain's genome, identifying genes responsible for phenanthrene degradation via phthalic acid route and thereby proving the *Mycobacterium* species are not only able to degrade phenanthrene via phthalate pathway but also via abovementioned phthalic acid path.

The enzymes engaged in phenanthrene's degradation according to the presented pathway are mostly dioxygenases and dehydrogenases, where the former are responsible for an initial attack on phenanthrene, leading to a formation of phenanthrene 3,4-dihydrodiol. The compound is subsequently reduced by dihydrodiol dehydrogenase giving a 3,4-dihydroxyphenanthrene which is then further oxidised by an extradiol dioxygenase.

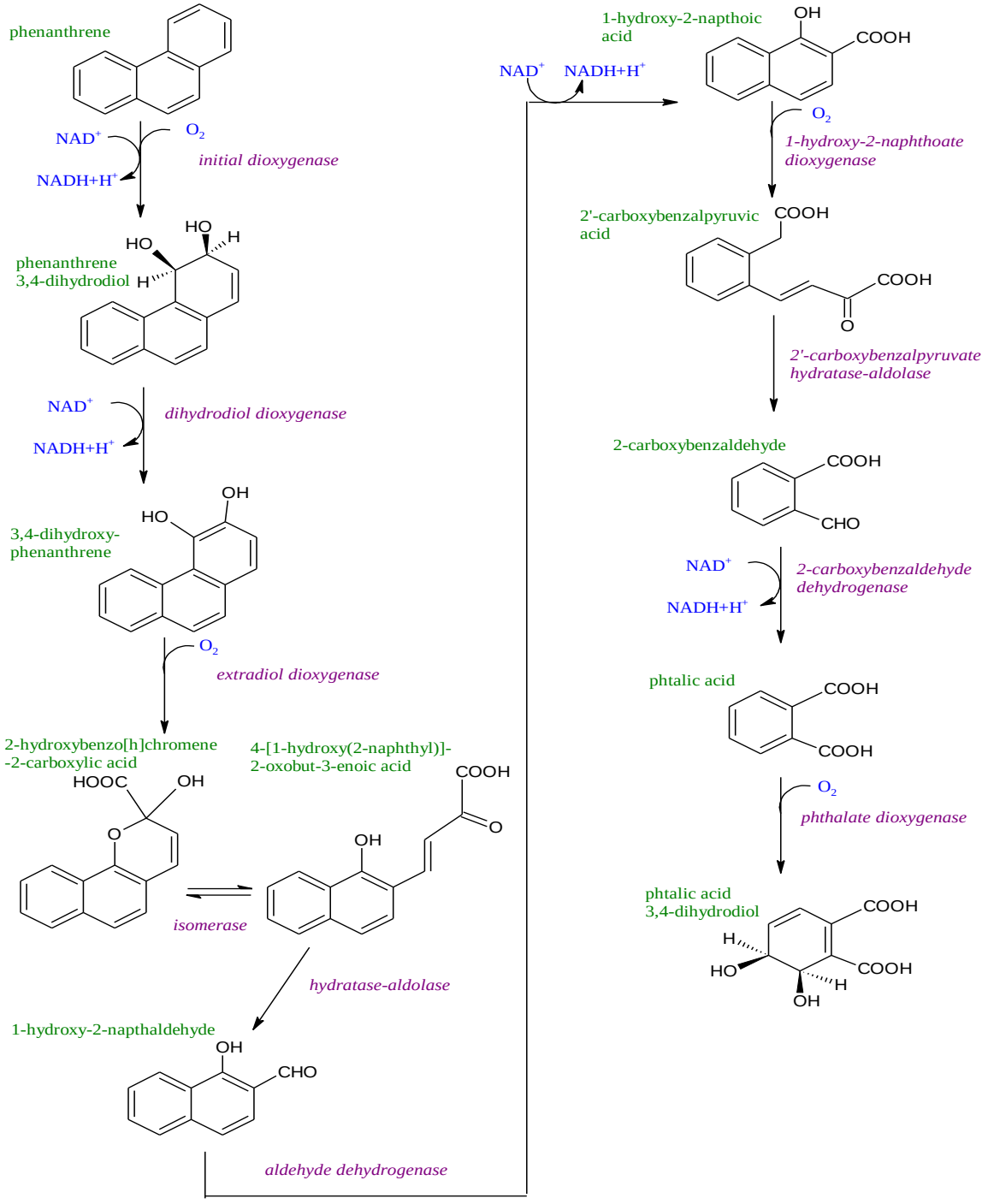


Figure 12: An enzymatic degradation pathway of phenanthrene. According to Stingley et al. (2004).

2.4. Cell Surface Properties of Bacteria During Biodegradation

A very limited or total lack of solubility of hydrocarbons in water considerably reduces their bioavailability and biodegradation (Megharaj et al., 2011). However, despite this limitation, the microorganisms possess some adhesive properties, which enable them to attach to different surfaces – including hydrophobic organic compounds. According to DeFlaun et al. (1999), those properties depend on a few main factors such as cell surface charge (that is connected with the electrostatic interactions between a bacterial cell and the substrate surface) and the bacterial cell hydrophobicity.

Bacterial attachment to different surfaces, although extensively studied for many years, in some aspects still remains unclear (Abbasnezhad et al., 2011; Liu et al., 2004; Obuekwe et al., 2009). Among the important aspects of this process, the hydrophobic-hydrophilic interactions between the microbial cell surfaces and substratum molecules have been identified to play an essential role in the adhesion mechanism.

Another important aspect in this regard of the adhesion process is the surface charge of microbial cells. It is impossible to directly measure the charge accumulated at the bacterial cell surface. However, the zeta potential that is calculated from measureable electrophoretic mobility of the particles (e.g. microorganisms) during the application of an electric field, has gained a lot of interest and is used to measure the charge of microbial cell surfaces.

2.4.1. Hydrophobicity

The bacterial cell surface hydrophobicity (CSH) is considered to be one of the most important factors regulating the adhesion of microorganisms to surfaces, such as oil droplets, Teflon, glass, activated sludge, dental implants, teeth and many other solid and gel or liquid surfaces (Chakraborty et al., 2010; Cowan et al., 1992; Jucker et al., 1996; Zita and Hermansson, 1997). The hydrophobic character of the bacterial cell surfaces is said to promote the bioavailability of the hydrocarbons due to the increase in the affinity between the microbial cells and hydrocarbons.

2.4.1.1. Methods for measuring cell surface hydrophobicity

Whilst many scientific methods have been developed for assessing the various features and properties of microorganisms, there is still doubt over which procedure could be the most useful for describing cell-surface hydrophobicity of microbial cells. There are three most common methods that have been applied, and although all seem to have pros and cons, they

cannot be compared with each other. These methods are: BATH (**B**acterial **A**dhesion **T**o **H**ydrocarbons), CAM (**C**ontact **A**ngle **M**easurement) and HIC (**H**ydrophobic **I**nteraction **C**hromatography) (Palmer et al., 2007).

- BATH – also known as MATH (**M**icrobial **A**dhesion **T**o **H**ydrocarbons), this method was first described by Mel Rosenberg and colleagues in 1980 as a quick and simple method for measuring the adhesion of bacteria to liquid hydrocarbons (Rosenberg et al. 1980). Soon after the paper was published, the method became popular (Rosenberg, 1981) and is still successfully applied by other researchers around the world (Geertsema-Doornbusch et al., 1993; Górna et al., 2011; Palmer et al., 2007; Rosenberg, 2006; Sałek et al., 2015; Zoueki et al., 2010). The BATH method is also the most popular means to determine cell surface hydrophobic/hydrophilic feature, mainly because of its simplicity, low-cost and quick results (Rosenberg, 2006). Over the past two decades, the method has been extensively studied, mainly due to the new insights of bacterial properties (such as surface charge etc.). A standard assay by this method usually involves spectrophotometric measurements of the absorbance of the bacterial cell suspension before and after the addition of a liquid hydrocarbon. However, Zoueki et al. (2010) proposed a modification to the method, because of the formation of hydrocarbon droplets (or emulsion), which, as the authors suggested, could have negatively influence measurements. Therefore, they recommended taking measurements of the bottom aqueous suspension under the microscope and direct cell counts in order to obtain the number of cells that were not involved in the adhesion to the oil droplets. This modification, however, has not gained as much popularity and application, mainly due to the fact that cell counts could be inaccurate and it was difficult to compare with any other results obtained via the standard method. Nonetheless, the above-described problem was not the only one that put researchers into doubts. Another factor reported by Busscher et al. (1995) and van der Mei et al. (1993) that was reported to influence BATH (or MATH) measurements is electrostatic interactions. In their work, van der Mei et al. (1993) concluded that BATH measurements are in fact the result of "hydrophobicity and electrostatic interactions" and suggested that the right measurement of hydrophobicity is when electrostatic interactions are absent; for e.g., close to the

cells' isoelectric point. Other researchers, with regard to this aspect also suggested conducting BATH measurements at high ionic strength (Palmer et al., 2007).

Using the BATH method, Bellon-Fontaine et al. (1996) explained that the basis of the cells' attachment to different liquid organic compounds lies in the abovementioned acid-base interplay – note that in their work they referred to the method as MATS (Microbial Adhesion To Solvents). As examples, the authors had used hexadecane (which is not involved in giving and accepting electrons), chloroform (weak electron acceptor) and diethyl ether (a weak electron donor). The strains with the electron-donating character were more likely to attach to chloroform rather than to hexadecane, and not attach to diethyl ether in this case working as a repellent according to the electrostatic mechanisms.

To summarise, the BATH (or MATH) method is useful and sensitive when considering both hydrophobic and electrostatic interactions, which as a consequence, is a factor showing the complicated compilation between van der Waals and Lewis acid-base forces (Busscher et al., 1995; Geertsema-Doornbusch et al., 1993; Hamadi and Latrache, 2008).

- CAM is more commonly known as a technique in the determination of acid-base interactions rather than just a simple assay for the hydrophobicity of microbial cells. The CAM method is based on measuring the contact angles of microbial lawns that need to be dried prior to use. Normally, the drying process takes up to 60 minutes until there is only bound water present in the lawn (van der Mei et al., 1998). Bearing in mind the findings that followed BATH or MATH method, Hamadi and Latrache (2008) presented a comparison of MATH/MATS and CAM in determining surface properties of microbial cells. The authors did not find any correlation between hydrophobicity estimated using contact angle measurement and MATH/MATS.

  It has been suggested that CAM measurements should be a better indicator of the cell surface hydrophobicity as it is determined on a dry lawn of microorganisms, while BATH, MATH or MATS is estimated in the aqueous phase. This, however, should not always be considered as the advantage of the method, especially with regard to environmental sciences, where it is important to establish different features referring as close as possible to the natural environment.

- HIC was first used in 1972 when two independent researchers, Yon and Shaltiel, performed the chromatographic purification of proteins on a synthesised matrix that was, in both cases, a hydrocarbon-coated agarose (Jennissen, 2000). Since then, the term of HIC has been widely used according to the observed interactions between the matrix and the molecules of the solutions that needed to be purified.

  Based on the same idea of interplay, in the mid-70's HIC was first used for determining the microbial hydrophobicity, where cells of high hydrophobicity attached to the column packing, while those of hydrophilic properties were eluted (Palmer et al., 2007).

  Smyth et al. (1978) presented the use of HIC (with phenyl and octyl Sepharose as the matrix) to evaluate the hydrophobicity of various *E. coli* strains. The authors noted the interplay of the cell attachment to the matrix and the ionic strength of the solution, in which the cell pellets were suspended. In other words – the increasing ionic strength of the solution (in that case NaCl) promoted the attachment of cells to the phenyl Sepharose matrix. Wiencek et al. (1990) reported similar observations when testing the hydrophobicity of the spores of *Bacillus* and *Clostridium*. The authors suggested that the high ionic strength was inevitable in order to reduce the electrostatic repulsion between the matrix and the spores. The researchers also observed a good correlation between MATH and HIC results of the spores' hydrophobicity. Nowadays HIC is mainly used for protein purification (Jennissen, 2002).

2.4.2. Zeta Potential

According to Saito et al. (1997), zeta potential is defined as the electrical potential of the bacteria-solution interface. It is not possible to directly measure zeta potential, but it can be calculated from the measurements of the electrophoretic mobility of the cells (or particles, in general) in an electric field (Wilson et al., 2001).

More precisely, zeta potential is located somewhere close to the so-called shear plane (or a plane of shear, plane of slip), which is a distance from the cell surface to the point where the solvent (or other) molecules are not attached to the cell and are not moving along with it (Wilson et al., 2001) – see Figure 13.

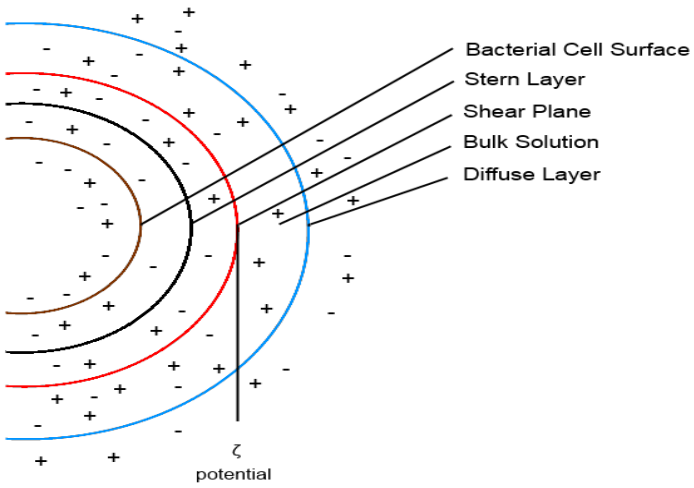


Figure 13: Diverse layers surrounding a bacterial cell.
According to Wilson et al. (2001).

Although most of the bacterial strains are characterised by a negative zeta potential, its charge may change under various pH conditions and even reach positive values. Cowan et al. (1992) explained that such an alteration of the detected charge is a result of the protonation of the amino, carboxyl and phosphate groups (which are located on the cell surface) in the acidic conditions (pH around 2-4), where around pH of 4 there might be a balance between the dissociated amino and acidic groups, and the charge values can be closer to zero. A further increase of pH leads to the nearly complete dissociation of the latter groups, resulting in the negative zeta potential.

The abovementioned pH dependency of zeta potential has been observed for many various bacterial strains (Cowan et al., 1992; Jucker et al., 1996; Mohanty and Mukherji, 2012; Palmer et al., 2007; Wilson et al., 2001) and despite the changes in the zeta potential values, which depend on some other bacterial cell properties (Gram-positive or negative, production of LPS and EPS etc.), it can be concluded that at the physiological pH, the zeta potential always reaches negative values.

Chakraborty et al. (2010), in their study on the surface properties of *Burkholderia* strains in the presence of NAPLs (non-aqueous phase liquids), showed that an increase of the zeta potential values consequently led to increased adherence of cells to hexadecane, where the highest adherence was observed where the zeta potential was close to zero. The authors

explained that as the hexadecane droplets are known to possess a negative charge at a neutral pH, the increase of zeta potential facilitated the adherence due to the electrostatic forces. Medrzycka (1991) explained that the abovementioned negative charge of the hydrocarbon droplets is associated with the hydroxyl (OH^-) and bicarbonate (HCO^-_3) ions from the water, where at the neutral or close to neutral pH, the concentration of bicarbonate ions significantly exceeds the amount of hydroxyl ions.

In 2008, Soni and co-workers published their findings on zeta potential of *E. coli*, *Salmonella* sp. and *Pseudomonas* sp. in drinking water, depending on their physiological stage and nutrient exposure. The results demonstrated that zeta potential of *Salmonella* sp. was the highest in all nutrient levels and physiological stages (rich, minimal, starved and dead), while the zeta potential of *Pseudomonas* sp. was the lowest in all systems. Based on those findings it can be concluded that the zeta potential is not only a function of pH and ionic strength but, when considering the living organisms – also the nutrient availability and growth stages and, what is important – bacteria as well.

2.5. The role of surfactants in biodegradation of petroleum hydrocarbons

Surfactants belong to the group of the surface-active agents that have the ability to concentrate at the interfaces between two non-miscible substances (such as water and oil) thanks to their amphiphilic (bifold) structure consisting of both, hydrophobic and hydrophilic moieties.

The action of the surfactants is connected with their self-assembly at the interfaces and the formation of monolayers and/or aggregates, which lead to an overall decrease of the surface and interfacial tensions.

2.5.1. General classification of surfactants

Most often surfactants are classified according to their polar head group. In this respect, there are four main groups of surfactants:

- Anionic – the polar group of these surfactants can be carboxylate, phosphate, sulphate or sulphonate. These are the most common surface-active agents, largely used in various branches in industry. It has been estimated that they cover 70% of the total production of surfactants in the world, and the most well-known

representative of these is soap (an alkylbenzene sulphonate, such as sodium stearate). The most popular counterions are ammonium, calcium, potassium and sodium, as well as some protonated alkyl amines (Kronberg et al., 2014).

- Cationic – of which the majority is composed of nitrogen, which carries the surfactant's charge. The most common are the amine and quaternary ammonium agents, and their use is often limited by pH. This concerns especially amine-based products, which are used as surfactants in the protonated state (low pH).
- Non-ionic – these are the second largest group of surfactants. The polar (hydrophilic) moiety is usually composed of polyhydroxyl or polyether units. The latter term is used for the units made of oxyethylene, obtained by the polymerisation of the ethylene oxide. The ethoxylation may take place in alkaline pH on any substrate with the active hydrogen (for example: fatty alcohols or alkylphenoles). The polyhydroxyl groups may undergo the ethoxylation as well, giving as a result ethoxylated fatty acid esters such as commonly known Tween (Kronberg et al., 2014).
- Zwitterionic – these types of surfactants contain both positive and negative charges. The positive charge is mostly carried by ammonium, while the negative one may have various origins, among which carboxylate is most common. Sometimes they are referred to as amphoteric, although this term should not be used as a synonym because the charge of amphoteric surfactants may undergo some changes depending on the pH, while most of the zwitterionics retain their charge despite pH variations. The most generally known representatives of this group are simple N-alkyl derivatives of amino acids, for example betaine or glycine (Kronberg et al., 2014). Due to their formula, zwitterionic surfactants are broadly used in the dermatology and pharmacy, as they are unlikely to cause any eye and skin irritations, therefore they can be found in the shampoos and other cosmetic products (Kronberg et al., 2014).

2.5.2. Adsorption and aggregation of surfactants

The ability of surfactants to adsorb and or aggregate has already been described as a characteristic feature of these molecules. While adsorption is commonly associated with the formation of the layers of the surfactant's molecules at the particular interface, the aggregation is represented by the composition of aggregates, called micelles in the solutions. The micellization is a process, during which the hydrophobic "tail" of the surfactant is being

kept away from any contact with water. The formed micelles are often referred to as the reservoirs of the unimers (free, unassociated molecules of the surfactant), which have a direct impact of some of the surfactant's properties such as wetting or foaming. What is more, the micelles are not super-stable and hence the exchange of the molecules between them and the solution can be very fast, taking up to only a millisecond or even less (Kronberg et al., 2014).

The aggregation and therefore the formation of the micelles leads to the changes in the physical properties of the surfactant. The concentration, at which this process occurs is called the **C**ritical **M**icelle **C**oncentration (**CMC**) (Misra and Somasundaran, 2008).

2.5.3. Natural surfactants

Time after time, the term "natural surfactant" has faced many modifications regarding its meaning. In a strict sense, the term covers only those surfactants obtained directly from the natural sources (plants, animals, microorganisms) and where no organic synthesis methods were involved. Lecithin that is obtained from the eggs or soybean can be considered as a natural surfactant in this respect (Holmberg, 2001). Another example is saponin (or saponins), which is a glycoside derived from many plants such as *Quillaja saponaria* bark and is commercially available (Sigma-Aldrich). Essentially, naturally-derived surfactants are referred to as biosurfactants. More commonly they are obtained from microorganisms (mainl from bacteria and yeast), and they can be characterised by either having a low or high molecular weight (LMH or HMW) and in how they interact with oil substrates. The LMW ones are commonly referred to as biosurfactants as their activity is often to lower the surface or interfacial tension between two immiscible liquids. On the other hand, those of HMW are commonly referred to as bio-emulsifiers as they are capable of emulsifying oils into small droplets and creating stable emulsions. In recent years, a closer look has been taken at rhamnolipid biosurfactants, often called just "rhamnolipids". These are non-toxic and highly biodegradable **rhamno**se-containing glyco**lipids**, produced mainly by bacteria (such as *Pseudomonas aeruginosa*) and have several important applications in the cosmetic and chemical industry, they are also widely used in the environmental remediation (Maier and Soberón-Chávez, 2000).

A less strict meaning of "natural surfactant" allowed for adding another group of surfactants to this list. These are obtained/synthesised from natural raw materials. A good example of such is alkyl polyglucosides (APGs), which can be obtained from natural and renewable

sources such as wheat, coconut, potatoes, corn etc. (Sałek et al., 2013). The general formula of APGs is often presented as $\mathbf{C_mG_n}$, where **m** stands for the number of carbon atoms and **n** represents the amount of glucose units (which form a hydrophilic "head" of the surfactant) (Ryan and Kaler, 2001).

Currently, surfactants, which contain only one unit of a natural origin can be called natural. In other words, if a surfactant contains either hydrophilic head unit or a hydrophobic tail unit of a natural origin, it may be referred to as natural. A good representative of the former is N-dodecanoylarginine metyl ester salt, which contains a polar head group based on the amino acid arginine. An example of the latter are fatty amide ethoxylates or sterol/phytosterol ethoxylates (Holmberg, 2001).

2.5.4. Biodegradation of hydrocarbons in the presence of surfactants

A major interest of surfactants in the bioremediation processes is their ability to increase of the solubility, and hence bioavailability, of hydrocarbons. According to Makkar and Rockne (2003), there are three main mechanisms that explain the abovementioned changes in hydrocarbons' properties, which are presented in Figure 14.

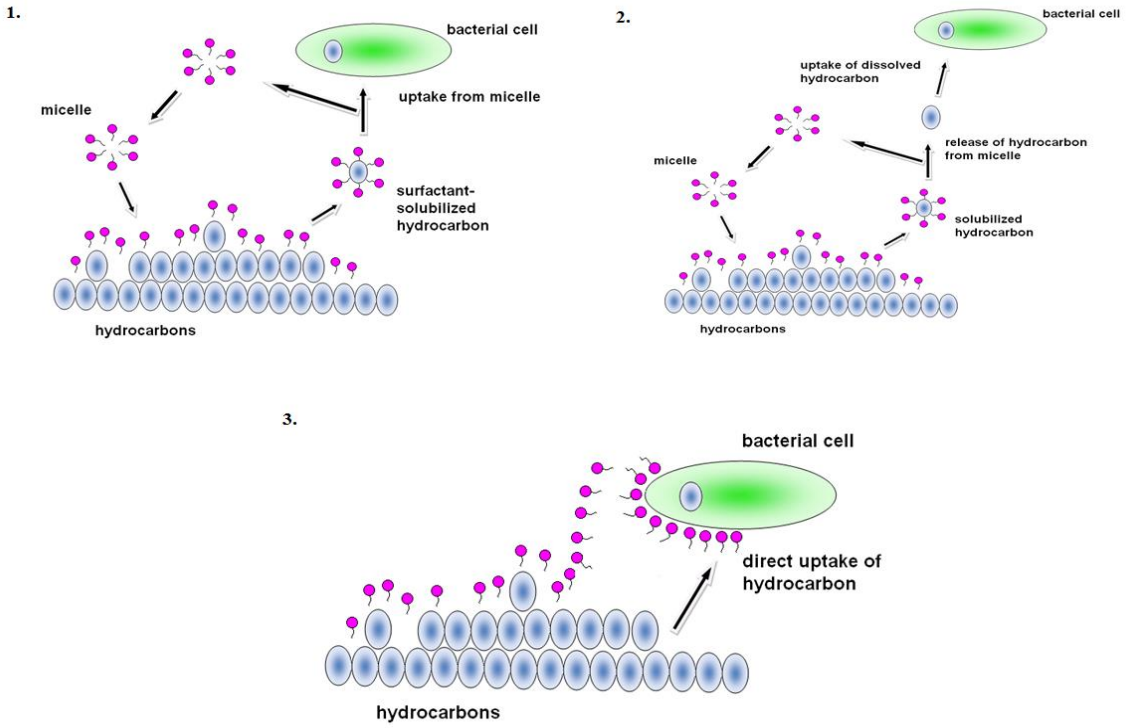


Figure 14: Three main mechanisms on the role of surfactants in increasing the bioavailability of hydrocarbons
According to Makkar and Rockne (2003).

The first mechanism, as seen in the Figure 14 presents a direct uptake of the hydrocarbon molecule from a micelle. The second one shows the uptake of the hydrocarbon from the aqueous phase after releasing from the micelle. The last, third mechanism presents a facilitated uptake of the hydrocarbon thanks to a cell-surfactant-hydrocarbon system, where surfactant's molecules change the bacterial cell surface hydrophobicity (Makkar and Rockne, 2003).

Volkering et al. (1995), when testing the influence of four non-ionic surfactants on the biodegradation of PAHs, such as naphthalene and phenanthrene, they found that the presence of Triton X-100, Tergitol NPX, Brij 35, and Igepal CA-720 led to an increased solubility of the hydrocarbons. This had a positive effect on the biodegradation of the abovementioned compounds, which additionally turned out not to be toxic to the bacterial strains involved in that study.

Kim et al. (2001) investigated the effect of three non-ionic surfactants – i.e. Triton X-100, Tween 80 and Brij 30 – on the biodegradation and solubility of selected PAHs (naphthalene and phenanthrene). The results revealed that the solubility of the hydrocarbons was proportional to the surfactants' concentrations above their CMCs. All three surfactants increased the biodegradation of both naphthalene and phenanthrene, however there were some minor differences in the time of mineralisation of those hydrocarbons.

When it comes to natural surfactants, an interesting study was presented by Whang et al. (2008). Their research was aimed at the use of surfactin and rhamnolipids in the biodegradation of oil-contaminated sites. Both surfactants increased the solubility of diesel oil and remarkably enhanced its biodegradation. However, when surfactin's concentration exceeded 40 mg L^{-1}, a decrease in biomass production and diesel oil degradation was observed. This effect, however, was not observed for rhamnolipids.

Despite the vast majority of reports showing a generally positive effect of surfactants on biodegradation of petroleum hydrocarbons, there was a number of publications revealing entirely opposite observations (Chen et al., 2000; Grimberg et al., 1995; Laha and Luthy, 1991; Stelmack et al., 1999). Therefore, detailed studies on the physico-chemical properties, including toxicity and influence to biodegradation by surfactants is of high importance prior to their field or commercial application.

3. MATERIALS & METHODS

3.1. Bacterial strains

For the purpose of the comparison of marine and terrestrial strains, eight bacterial strains were isolated from the petroleum contaminated reservoirs (four strains each): marine and terrestrial.

3.1.1. Marine Strains

The marine bacterial strains: *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105; *Cycloclasticus* sp. strain TK-8 were isolated and identified by Gutierrez et al. (2013) from the surface- and plume water samples that were collected during the Deepwater Horizon Oil Spill in the Gulf of Mexico. *Polycyclovorans algicola* TG408 was isolated from the laboratory culture of the marine diatom *Skeletonema costatum* (CCAP 1077/1C) using the enrichment with polycyclic aromatic hydrocarbons as the sole carbon and energy source (Tony Gutierrez et al., 2012).

3.1.2. Terrestrial Strains

The terrestrial strains: *Achromobacter denitrificans* sp. 4(2010), *Pseudomonas stutzeri* strain 9, *Rahnella* sp. EK12, and *Stenotrophomonas maltophilia* strain 6 were isolated from the soil contaminated with the petroleum hydrocarbons in Poland.

The isolated strains were phenotypically characterised (by using the standard techniques such as Gram staining, the determination of the shape, size and colour of the colonies on the agar plates etc.) as described in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994; Sałek et al., 2015).

The 16S rRNA gene sequence of the tested strains was deposited in the GeneBank database of NCBI under the following accession numbers:

- HM246520.1 for *Achromobacter* sp. 4(2010) strain
- HM246521.1 for *Stenotrophomonas maltophila* strain 6
- JN006140.1 for *Pseudomonas stutzeri* strain 9
- JQ409469.1 for *Rahnella* sp. EK12 (Kaczorek et al., 2013b; Sałek et al., 2015).

3.1.3. Bacterial culture conditions

3.1.3.1. Marine strains

The marine bacterial strains *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 are PAH-degrading bacteria that were isolated from sea surface oil slick samples collected during the Deepwater Horizon oil spill (Gutierrez et al. 2013b). *Polycyclovorans algicola* TG408 is an obligate PAH-degrading bacterium that was isolated from a laboratory culture of the cosmopolitan marine diatom *Skeletonema costatum* (Gutierrez et al., 2013). Strains TK46(2) and TK105 were kept on ZM/10 agar plates, whereas strains TK8 and TG408 were kept on the ONR7a agar plates amended with sodium pyruvate. ZM/10 is a 10-fold dilution of Zobell's marine medium 2216. ONR7a medium is a synthetic seawater medium (Dyksterhouse et al., 1995). The strains were streaked every three or four weeks onto fresh agar plates.

Unless otherwise specified, all liquid culture experiments that are described in the following chapters were initiated by inoculation from colonies on a plate. For this, a loop full of cells, from an isolated colony on an agar plate, was transferred into a sterile Erlenmeyer flask containing 10 mL (or more, depending on the task) of sterile ONR7a amended with sodium pyruvate. This medium was often used for experiments that used strains TK-8 and TG408. In experiments with strains TK-46(2) and TK-105, Zobell's marine medium 2216 was often used. After inoculation, the cultures were left for approx.. 48 h – or longer, depending on the strain – at 25 °C with shaking (120 rpm). The cells were then centrifuged (4,000 × *g*; 10 min), washed at least three times with 0.01 M (pH = 7.2) phosphate buffered saline (PBS) prior to re-suspending in the same buffer to be used as inoculum experimentation.

3.1.3.2. Terrestrial strains

The terrestrial strains used in this study *Achromobacter* sp. 4(2010), *Pseudomonas stutzeri* strain 9, *Rahnella* sp. EK12, *Stenotrophomonas maltophilia* strain 6 are hydrocarbon degrading strains isolated from soil contaminated with petroleum hydrocarbons and were kept on standard nutrient agar plates and were streaked every three weeks onto the fresh plate (Kaczorek et al., 2013b; Sałek et al., 2013).

The inoculum was prepared similarly to the one described above. A bacterial colony taken carefully from the plate was transferred into the sterile Erlenmeyer flask with 10 mL (or more – depending on the task) of sterile mineral medium described by Kaczorek et al. (2010). After inoculation, the cultures were left for approx. 48 h at 25 °C with shaking (120

rpm). The cells were then centrifuged (4,000 × *g*; 10 min), washed at least three times with 0.01 M (pH = 7.2) phosphate buffered saline (PBS) prior to re-suspending in the same buffer to be used as inoculum experimentation.

3.2. List of Chemicals

The list of the most important chemicals used in the described assays is presented in the Table 5.

Table 5: The list of the most important chemicals used in the described assays

TYPE	NAME	BRAND	PURITY
SURFACTANTS	Triton X-100	Sigma-Aldrich	~95%
	Glucopon 215 UP	BASF	~65%
	Lutensol GD 70	BASF	~70%
CO-FACTORS	NADH	Sigma-Aldrich	≥94%
	FAD	Sigma-Aldrich	≥95%
LC-MS	Ammonium acetate	Sigma-Aldrich	≥99%
	Methanol	Sigma-Aldrich	≥99%
HYDROCARBONS	Catechol	Sigma-Aldrich	≥99%
	Hexadecane	Sigma-Aldrich	≥99%
	Sodium pyruvate	Sigma-Aldrich	≥99%
	Diesel oil	BP Poland and BP Scotland	
PROTEIN STANDARD	Lysozyme	Sigma-Aldrich	≥90%
PROTEIN DYE	Coomassie Brilliant Blue G	Sigma-Aldrich	98%
SOLVENT	Dichloromethane (DCM)	Merck	≥99%

3.3. Enzymatic Assays

3.3.1. Preparation of cell free extracts

Terrestrial and marine bacterial strains were grown at, respectively, 30 °C and 21 °C for 7 days in 500 ml Schott Duran bottles containing: culture medium, mineral salts, yeast extract (terrestrial strains) and hexadecane or diesel oil as a carbon source. The cultures were then centrifuged (5,000 × *g*; 15 min) at 4 °C and the cell pellets washed twice with 0.01 M PBS (pH 7.2). Cell pellets were then re-suspended in a fresh volume of PBS and disrupted using a sonicator at a frequency of 20 kHz. Sonication was performed for 6 x 15 sec intervals on each cell suspension, with a 30 sec break in between each sonication interval. Unbroken cells and cell debris were removed by centrifugation (13,000 × *g*; 50 min) at 4 °C, and the resultant cell lysate was used in the enzyme assays described below.

3.3.2. Determination of enzymatic activity

3.3.2.1. Long chain alkane monooxygenase

Activity for long chain alkane monooxygenase was assayed using the method of Iwaki et al. (2006). For this, activity was measured spectrophotometrically by following the decrease in absorbance of NADH at 340 nm. The standard mixture, of total volume 1.5 ml, contained the following at final concentrations: 330 µL of PBS (0.01 M, pH 7.2), 120 µL of 4 mM NADH (4 mM), 450 µL of FAD (44 µM), 300 µL of deionized water and 297 µL of the cell free extract. The reaction was started by the addition of 3 µl of hexadecane to the reaction mixture. Specific activities were expressed as U per milligram of protein. The protein concentrations were determined by the method of Bradford (1976) using lysozyme as a standard.

3.3.2.2. Catechol 1,2- and 2,3-dioxygenases

The activity of catechol 1,2-dioxygenase was measured spectrophotometrically by following the procedure described by Wojcieszyńska et al. (2011). Briefly, activities were calculated from the measurements of the formation of *cis,cis*-muconic acid from catechol at 260 nm over time. The reaction mixture, of total volume 1 mL, comprised the following: 67 µL of Na_2EDTA (20 mM), 893 µL of phosphate buffer pH 7.2 and 20 µL of cell-free extracts. The reaction was started when 20 µL of catechol (50 mM) was added to the reaction mixture.

The activity of **catechol 2,3-dioxygenase** was determined by measuring the absorbance increase for the 2-hydroxymuconic semialdehyde (the meta-cleavage product) at 375 nm over time, as per the method of Guzik et al. (2012). The reaction mixture contained 960 µL of phosphate buffer at pH 7.2 and 20 µL of cell-free extracts. The reaction was started when 20 µL of catechol (50 mM) was added to the reaction mixture.

3.3.3. The influence of surfactants, pH and temperature on the activity of the long chain alkane monooxygenase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase.

The influence of pH on the activity of long chain alkane monooxygenases, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase was determined using 0.01 M PBS prepared at different pH ranging from 4.8 to 8.1. This was added to the standard reaction mixture in place of the deionized water, following the same method as described for the standard measurements above.

In order to determine the influence of temperature on the enzymatic activity, the abovementioned enzymes and substrate solutions were pre-incubated in the temperatures ranging from 20 to 50 °C, mixed and followed the reaction in the same temperature (Wojcieszyńska et al., 2011b).

The effect of surfactants on the enzymatic activity was established using the cell free extracts isolated after 7 days of incubations of the bacterial strains with surfactants used as sole carbon and energy sources.

3.4. Cell Surface Properties

3.4.1. Microbial Adhesion To Hydrocarbons

Microbial CSH was assessed according to the MATH method described in detail by Kaczorek et al. (2013a). The cultures were grown on different carbon sources: hexadecane, diesel oil, glucose, and three surfactants (Lutensol GD 70, Glucopon 215 and Triton X-100). The substrates were added at various concentrations (6, 60, 120, 240 and 360 mg L^{-1}) either independently or used in combination with diesel oil. After 7 days of cultivation, the cells were centrifuged (8,000 × *g*; 5 min) and washed three times with a PUM buffer. Triplicate incubations for each strain and substrate tested were performed, and the MATH was calculated using the following formula:

Hydrophobicity (%) = (1 – OD_{600} of aqueous phase after mixing with hexadecane/OD_{600} of initial aqueous phase) × 100

3.4.2. Zeta potential

The electrophoretic mobility was automatically measured, and the zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation (Miyake et al., 1990). The measurements were performed at 21 °C with a ZetaPlus instrument (Brookhaven Instruments Co., USA). Bacterial strains were grown on Lutensol GD 70, Glucopon 215 and Triton X-100 separately, using five concentrations for each surfactant: 6, 60, 120, 240 and 360 mg L^{-1}. Additionally, the strains were also grown with each of these surfactants in combination with diesel oil. All the cultures were incubated for 7 days at 30 °C (for terrestrial strains) or 21 °C (for marine strains). Bacterial cells were centrifuged (8,000 × *g*; 10 min) and washed three times with the PUM buffer to remove any residual hydrocarbon or surfactant substrates. The cells were then suspended in the same buffer to a total volume of ?? ml.

3.5. Biodegradation Assays

3.5.1. Biodegradation of diesel oil

Bacterial strains were grown in Schott Duran bottles (500 mL) containing 100 mL culture medium amended with 1% (w/v) diesel oil. For some experiments, 6, 60, 120, 240 and 360 mg L^{-1} of surfactant (Glucopon 215, Lutensol GD 70, Triton X-100) was also added to the medium in order to evaluate its effect on biodegradation of the diesel oil. Samples were incubated with shaking (120 rpm) at 30 °C (terrestrial strains) or 21 °C (marine strains) for 7 days. The cultures were then centrifuged to separate the biomass (8,000 × *g*; 10 min). The resultant supernatant fraction was isolated and to 30 ml of brine and 36% HCl were added until the pH reached a value of 1.0. The acidified solution was then triple extracted with DCM. The organic phase was dried with anhydrous magnesium sulphate. The solvent was then evaporated using a rotary evaporator. The oil residues were weighed and recorded as the total oil remaining from the respective incubations for each bacterial strain tested. The final results were calculated with respect to blank samples.

3.5.2. Biodegradation of naphthalene

The biodegradation of the model PAH – naphthalene was determined using a quantitative spectrophotometric method (Gutierrez et al., 2013).

In these assays an acid-washed (0.1 M HCl) and steam-sterilised glass test tubes (16x100 mm) with screw caps containing Teflon silicone septa were used. The stock solution of naphthalene was prepared by dissolving 0.18 g of naphthalene in 20 mL of acetone to obtain the concentration of approximately 9000 mg L^{-1}.

For every strain, two sets of three test tubes were prepared, each containing 2.9 mL of ONR7a medium. One set was prepared for the 7 day-biodegradation assay and the other for the 14-day biodegradation assay. All test tubes were then inoculated with the tested strain. The uninoculated controls, acid-killed controls and tubes that were inoculated, but without any added PAH, were prepared as well. The test tubes were kept in the dark with shaking (100 rpm) at 21 °C.

In order to estimate the biodegradation levels of naphthalene, after 7 or 14 days of incubations the extraction with ethyl acetate was performed. Briefly, 2 mL of ethyl acetate was added to each tube and then vortexed for 30 seconds. Aliquots of the non-aqueous top layer were diluted with ethyl acetate in quartz cuvettes for spectrophotometric analysis at 275 nm for naphthalene. The concentrations of remaining naphthalene were calculated using the Lambert-Beer equation, knowing the molar absorptivity coefficient equal to $5.7x10^3$ L mol^{-1} cm^{-1} (Thomas and Burgess, 2007).

3.6. The liquid chromatography-tandem mass spectrometry of polyglucosides

A chromatographic system UltiMate 3000 RSLC from Dionexwas used in order to determination whether the alkyl polyglucoside-based surfactants (Glucopon 215 and Lutensol GD 70) would be degraded by the marine and terrestrial strains used in the study. For this, 5 µL samples were injected into a Hypersil GOLD column (100 mm × 2.1 mm I.D.; 1.9 µm) from Thermo Scientific (Waltham, MA, USA) that was equipped with a 2.1 mm I.D. filter cartridge (0.2 µm) from the same supplier. The mobile phase consisted of 5×10^{-3} mol L^{-1} ammonium acetate in water (A) and methanol (B) that was run at a flow rate of 0.2 mL min^{-1} at 35 °C. Glucopon 215 was analysed using gradient elution starting from 70% of phase B, then changed to 95 % of phase B after 10 min. Lutensol GP 70 was analysed using a gradient of phase B that started at 50 % and reached 85 % after 5 min, and then changed to 95 % at 6 min and maintained at this concentration for another 2 min. A pre-run time of

4 min was used between injections. The chromatographic system was connected to an API 4000 QTRAP triple quadrupole mass spectrometer from AB Sciex (Foster City, CA, USA). The LC column effluent was directed to the electrospray ionization source (Turbo Ion Spray). The Turbo Ion Spray source operated in positive ion mode. The time for each mass transition detected in the MS/MS multiple reaction monitoring mode was set to 30 milliseconds. All alkyl glucosides were detected using the following settings for the ion source and mass spectrometer: curtain gas at 20 psi; nebulizer gas at 40 psi; auxiliary gas at 40 psi; temperature at 400 °C; collision gas medium; ion spray voltage at 4500 V; and declustering potential at 50 V. The detected mass transitions and specific parameters for each analyte are summarized in Table 6.

3.7. The liquid chromatography-tandem mass spectrometry of octylphenol ethoxylates

A chromatographic system UltiMate 3000 RSLC from Dionexwas used in order to determination whether the octylphenol ethoxylate-based surfactant (Triton X-100) would be degraded by the marine and terrestrial strains used in the study.. Five microliters of samples were injected into a phenyl-hexyl column (50×3 mm I.D.; 1.8 μm) from Agilent Technologies (Santa Clara, CA, USA). The mobile phase employed in the analysis consisted of 5×10^{-3} mol L^{-1} ammonium acetate in water and methanol at a flow rate of 0.3 mL min^{-1} at 35 °C. Triton X-100 was analysed using a gradient elution starting from 70% of methanol changed to 95 % of methanol in 3 min and maintained at 95 % for 5 min. A pre-run time of 4 min was applied before the next injection. The chromatographic system was connected to the API 4000 QTRAP triple quadrupole mass spectrometer from AB Sciex (Foster City, CA, USA). The LC column effluent was directed to the electrospray ionization source (Turbo Ion Spray). The Turbo Ion Spray source operated in a positive ion mode. The octylphenol ethoxylates were analysed in multiple reaction monitoring mode. The dwell time for each mass transition was set to 50 ms. The following settings for the ion source were used: curtain gas 20 psi, nebulizer gas 40 psi, auxiliary gas 40 psi, temperature 350 °C, ion spray voltage 4500 V and declustering potential 50 V. The collision gas was set to medium. The detected mass transitions and specific parameters for each analyte are summarized in Table 8.

Note: tasks 3.7 and 3.8 were performed in The Institute of Chemistry and Electrochemistry and Poznan University of Technology together with dr. Agnieszka Zgoła-Grześkowiak.

3.8. Determination of critical micelle concentration (CMC)

In order to establish the CMC of the three surfactants used in the studies (Triton X-100, Lutensol GD 70 and Glucopon 215), the surface tensions were measured using the du Noüy ring technique with a platinum ring mounted on a Krüss K12 tensiometer. All the experiments were performed at 22 ± 1 °C. The instrument was calibrated using deionised Milli-Q water (Milipore Corp., Austria) at resistance 18.2 MΩcm, of which the surface tension was equal to ~74.0 ± 0.5 (mN m^{-1}).

The results obtained for surface tension for each surfactant analysed were plotted against their concentrations. The CMC was calculated was a result of the extrapolation of the plots to the breakpoint (Jiang et al., 2011).

4. RESULTS AND DISCUSSION

4.1. Statistical analysis

A multiple one-way ANOVA analysis was used to determine any significant differences ($P \leq 0.05$) in the obtained result. The calculations were performed using Statistica® 10 programme.

4.2. Physical and chemical properties of surfactants and biodegradation

Three surface active agents were used throughout these studies:

- Glucopon 215
- Lutensol GD 70
- Triton X-100.

Their critical micelle concentration, biodegradability and influence on various factors, such as biodegradation of diesel oil and PAH, hydrophobicity, zeta potential and enzymatic activity were determined.

The two terrestrial strains – *Achromobacter* sp. 4(2010) and *Stenotrophomonas maltophilia* strain 6 were chosen as the model microorganisms for detecting the biodegradation of alkyl polyglucosides and octylphenol ethoxylates.

4.2.1. Critical micelle concentration (CMC)

The critical micelle concentration (CMC) of the three tested surfactants was determined based on the measurements of the surface tension (following the du Noüy ring technique), using a Krüss K100 tensiometer with a platinum ring.

Figure 15 presents the obtained results:

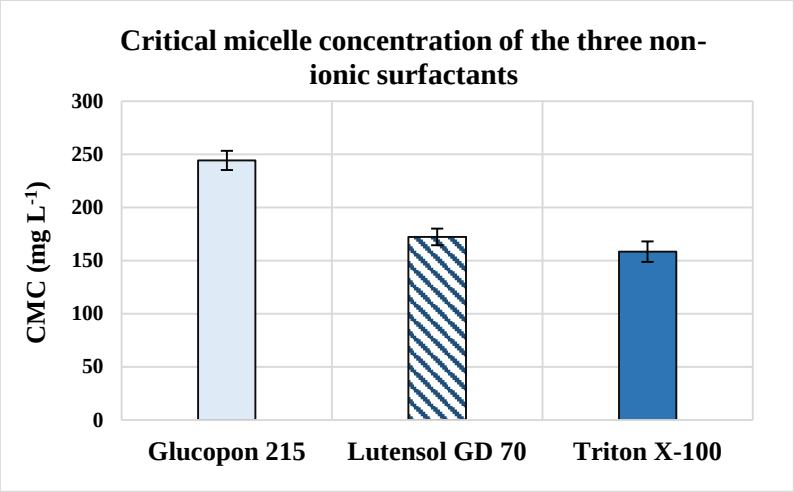


Figure 15: The CMC of Glucopon 215, Lutensol GD 70 and Triton X-100.

Among the analysed surfactants, **Triton X-100** was characterised by the lowest CMC (158.4 ± 9.6 mg L^{-1}). According to literature reports, the critical micelle concentration may vary strongly, depending on the laboratory techniques used for its estimation, as well as other factors such as temperature. The CMC of Triton X-100 is reported to achieve values between 130 and 200 mg L^{-1} (Liu et al., 2001; Wyrwas et al., 2011; Zhu et al., 2003).

Lutensol GD 70 belongs to a large family of Lutensol surfactants, produced by BASF and although this group is widely known, the CMC of the Lutensol GD 70 has not yet been studied. The results obtained within this study revealed that the CMC of the abovementioned surfactant was equal to 172.3 ± 7.8 mg L^{-1}. Due to the lack of reported CMC values for Lutensol GD 70 in literature, a comparison with other reported CMC values for other surfactants of the Lutensol group is made. Ghouas et al. (2012) showed that the CMC of Lutensol ON 30 was equal to 200 mg L^{-1}, while the CMC of Lutensol FSA10 was only 10 mg L^{-1} (Hartwig et al., 2008). Even smaller values for the CMC, of as little as 4.9 mg L^{-1} were obtained by Hecht et al. (2013) for Lutensol AT 50. According to the product information from BASF (2005), the CMC of Lutensol XL may vary from 400 mg L^{-1} to

1000 mg L^{-1}, and Lutensol XP from 800 to 5000 mg L^{-1}, depending on the degree of ethoxylation.

Glucopon 215 showed a critical micelle concentration of 244.3 ± 9.2 mg L^{-1}. Similar to Lutensol, Glucopon represents a range of various surfactants, which differ between one another with regard to their physic-chemical properties (BASF, 2015). Bravo Rodríguez et al. (2006) published their findings on the CMC of the three Glucopon surfactants. The values of CMC were equal to 241, 28 and 73 mg L^{-1} for Glucopon 215, Glucopon 600 and Glucopon 650 respectively.

4.2.2. LC-MS/MS of polyglucosides

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) of the APGs was performed in order to determine the biodegradability of Glucopon 215 and Lutensol GD 70. The parameters for detection of the analytes are presented in Table 6.

Table 6: Parameters of mass spectrometric detection characteristic to particular analytes (MRM 1 – analytical multiple reaction monitoring transition, MRM2 – confirmatory multiple reaction monitoring transition)

Glucoside	Precursor ion $[M+NH_4]^+$ (m/z)	MRM transitions (precursor ion m/z → product ion m/z)			
		MRM 1	Collision energy (V)	MRM 2	Collision energy (V)
Glucopon 215					
C_8Glu	310.5	310.5 → 163.4	14	310.5 → 145.4	18
$C_{10}Glu$	338.5	338.5 → 163.4	15	338.5 → 145.4	19
$C_{12}Glu$	366.5	366.5 → 163.4	15	366.5 → 145.4	19
$C_{14}Glu$	394.5	394.5 → 163.4	16	394.5 → 145.4	19
C_8Glu_2	472.5	472.5 → 163.4	21	472.5 → 145.4	30
$C_{10}Glu_2$	500.5	500.5 → 163.4	21	500.5 → 145.4	30
$C_{12}Glu_2$	528.5	528.5 → 163.4	21	528.5 → 145.4	30
$C_{14}Glu_2$	556.5	556.5 → 163.4	21	282.5 → 145.4	30
C_8Glu_3	634.6	634.6 → 163.4	28	634.6 → 145.4	40
$C_{10}Glu_3$	662.6	662.6 → 163.4	28	662.6 → 145.4	40
Lutensol GP 70					
C_4Glu	254.5	254.5 → 163.4	13	254.5 → 145.4	17
C_4Glu_2	416.5	416.5 → 163.4	21	416.5 → 145.4	28
$C_{10}Glu$	338.5	338.5 → 163.4	15	338.5 → 145.4	19
$C_{10}Glu_2$	500.5	500.5 → 163.4	21	366.5 → 145.4	30
$C_{10}Glu_3$	662.5	662.5 → 163.4	30	662.5 → 145.4	38
$C_{10}Glu_4$	824.6	824.6 → 163.4	41	824.6 → 145.4	48
$C_{10}Glu_5$	986.7	986.7 → 163.4	51	986.7 → 145.4	58
$C_{10}Glu_6$	1148.8	1148.8 → 163.4	61	1148.8 → 145.4	68

Having established the parameters, the biodegradation of Glucopon 215 and Lutensol GD 70 by *Achromobacter* sp. 4(2010) and *Stenotrophomonas maltophilia* strain 6 after 7 days of incubations was determined. The summary of the results is presented in Table 7.

Table 7: Results from the 7 day biodegradation of Glucopon 215 and Lutensol GP 70 with and without diesel oil using Achromobacter sp. 4(2010) and Stenotrophomonas maltophila strain 6 bacteria.

Glucoside	Biodegradation (%) ± SD			
	Achromobacter sp. 4(2010)		*Stenotrophomonas maltophilia* strain 6	
	with diesel oil	without diesel oil	with diesel oil	without diesel oil
Glucopon 215				
C_8Glu	49.3 ± 2.6	99.9 ± < 0.1	96.8 ± 0.6	97.3 ± 0.2
$C_{10}Glu$	62.1 ± 1.1	99.8 ± < 0.1	97.5 ± 0.3	97.8 ± 0.3
$C_{12}Glu$	82.8 ± 0.9	99.6 ± 0.2	98.5 ± 0.2	98.6 ± 0.2
$C_{14}Glu$	84.3 ± 2.1	98.2 ± 0.5	97.9 ± 0.2	98.1 ± < 0.1
C_8Glu_2	30.1 ± 4.1	75.5 ± 0.1	72.4 ± 0.8	77.0 ± 1.1
$C_{10}Glu_2$	54.9 ± 0.6	87.4 ± < 0.1	87.0 ± < 0.1	89.9 ± 0.4
$C_{12}Glu_2$	66.6 ± 3.9	91.8 ± 0.9	89.2 ± 1.5	92.9 ± 1.1
$C_{14}Glu_2$	78.5 ± 11.5	96.9 ± 0.8	89.5 ± 2.2	95.6 ± 3.8
C_8Glu_3	6.6 ± 6.8	43.8 ± 8.8	26.7 ± 7.3	52.3 ± 3.5
$C_{10}Glu_3$	15.7 ± 0.5	55.7 ± 0.2	47.3 ± 1.0	56.8 ± 4.8
Lutensol GD 70				
C_4Glu	7.0 ± 5.9	94.5 ± < 0.1	81.6 ± 6.5	90.6 ± 4.6
C_4Glu_2	40.7 ± 6.8	71.3 ± 1.7	66.4 ± 14.3	83.0 ± 10.7
$C_{10}Glu$	79.2 ± 2.8	98.2 ± 0.5	97.6 ± 0.2	97.5 ± 0.4
$C_{10}Glu_2$	66.1 ± 3.6	95.4 ± 5.8	91.1 ± 0.4	89.6 ± 1.6
$C_{10}Glu_3$	23.8 ± 1.7	61.1 ± 0.3	61.2 ± 1.6	53.5 ± 2.8
$C_{10}Glu_4$	19.3 ± 1.2	64.0 ± 1.6	65.0 ± 6.5	67.8 ± 0.1
$C_{10}Glu_5$	18.2 ± 0.1	62.3 ± 2.5	58.1 ± 1.5	57.3 ± 0.2
$C_{10}Glu_6$	19.5 ± 13.8	62.5 ± 0.1	47.5 ± 4.6	52.2 ± 1.8

According to Garcia et al. (1997) and Qin et al. (2006), akyl polyglucosides can undergo a microbial degradation, however its results may strongly depend on the surfactants' chemical structures, more precisely, if the alkyl chain is linear or branched. Garcia et al. (1997) evaluated the biodegradation of the selected APGs with the linear alkyl chain, with the number of carbon atoms ranging from C_8 to C_{16}. The results revealed that the microbiological removal of the APGs may reach nearly 100% (97.5 ± 2.0%).

As stated in the European Patents assigned to BASF (BASF SE 67056 Ludwigshafen (DE)) (Garnier et al., 2009; Li et al., 2013), both Glucopon 215 and Lutensol GD 70 have linear alkyl chains (Fig. 16 and 17).

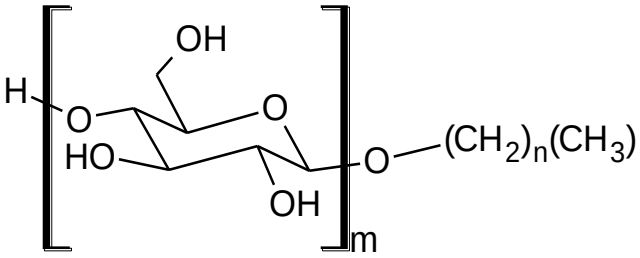


Figure 16: Glucopon 215 formula, where m = 1-3 and n = 7-9. Adapted from Li et al. (2013).

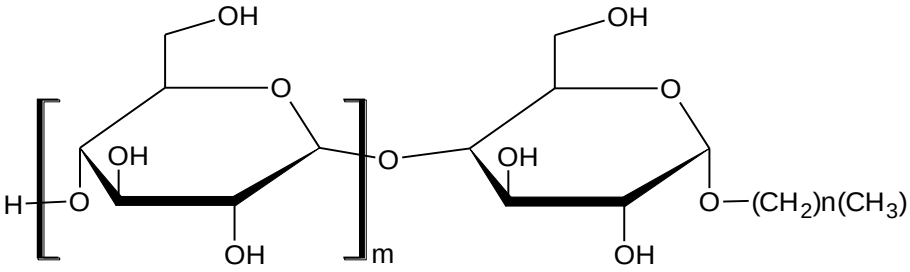


Figure 17: The formula of Lutensol GD 70, where m = 0-3 and n = 4-20. According to Garnier et al. (2009).

Based on the results presented in Table 7, in the presence of diesel oil the two glucosides of **Glucopon 215** – C_{12}Glu and C_{14}Glu were degraded with very high efficiency (reaching 82.8 and 84.3 % respectively) by *Achromobacter* sp. 4(2010). When the surfactant was used as a sole carbon source, the degradation of the abovementioned glucosides rose, along with the two other glucosides – C_8Glu and C_{10}Glu, reaching nearly 100 %.

The correlations between the chain length in combination with the number of saccharide (methyl galactopyranoside) moieties (from 1 to 3) and the degradation of glucosides from Glucopon 215 with and without diesel oil by *Achromobacter* sp. 4(2010) are presented Fig. 18a and 18b respectively.

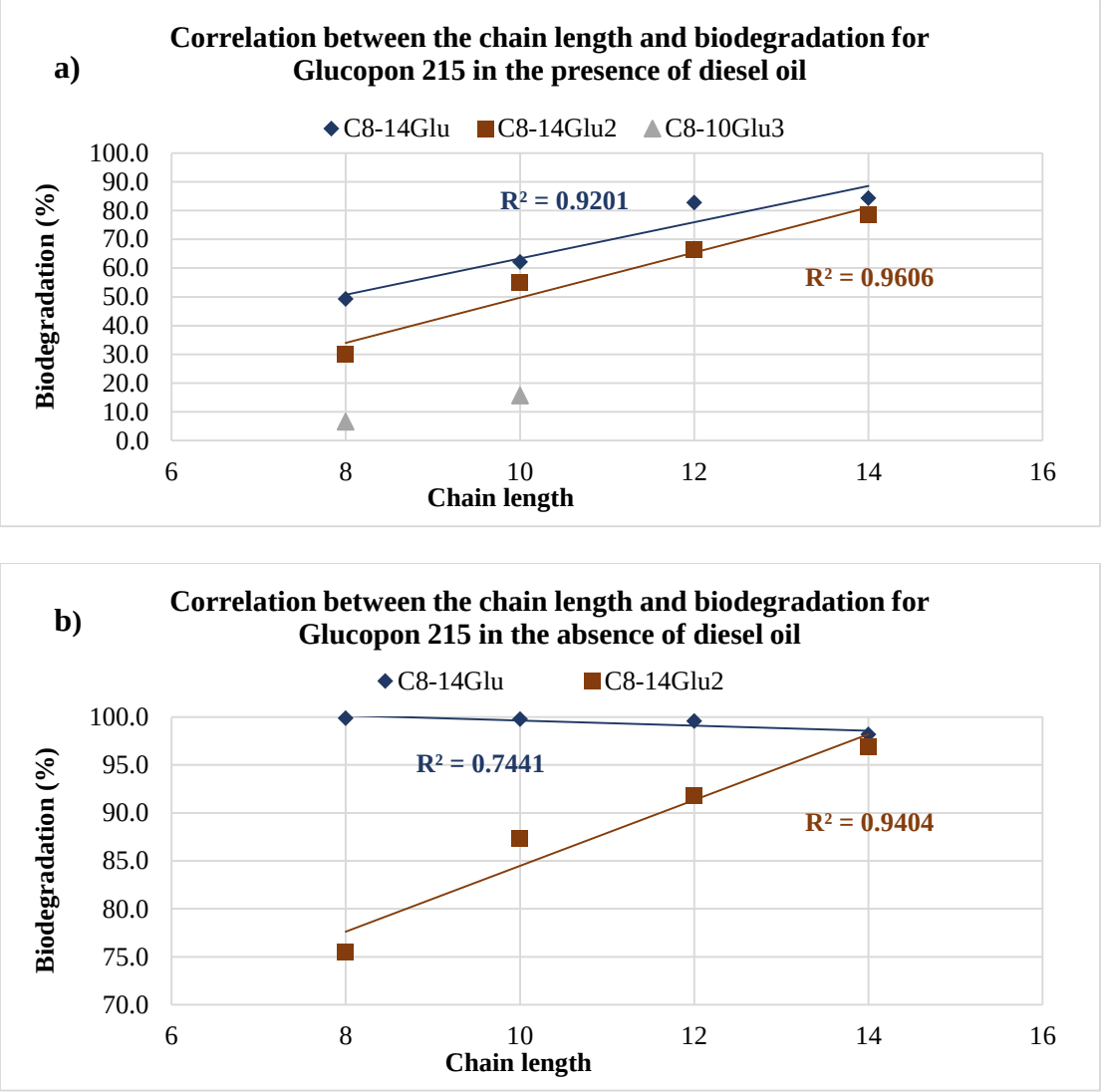


Figure 18: Correlation between chain length and biodegradation by *Achromobacter* sp. 4(2010) for Glucopon 215 in the presence (a) and absence of diesel oil (b).

To summarise the correlations, when diesel oil is present in the system, the *Achromobacter* sp. 4(2010) microorganisms were more likely to degrade components of longer alkyl chains of glucosides (**C_{14}**Glu – 84 %). The relation between the alkyl chain length and biodegradation was linear, with the coefficient of determination (R^2) equal to 0.9201 (Fig. 18a, trend line marked in blue). The glucosides were degraded in a similar way, when composed of two saccharide moieties. The linear trend with the R^2 equal to 0.9404 (marked in red) could be determined.

The results obtained for $C_{8\text{-}14}Glu_2$ when the surfactant was used as sole carbon source (Fig. 18b, marked in red) were a bit lower, but showed the similar trends. However, the highest biodegradation was seen for $C_{8\text{-}14}$Glu, that is, with only one saccharide moiety. Unlike in the

first case (Fig. 18a, with diesel oil added, blue line), in this case the longer the alkyl chain was, the lower biodegradation was observed.

The second tested strain – *Stenotrophomonas maltophilia* strain 6 was able to degrade all of the glucosides with very high yields. The lowest results were observed for C_8Glu_3 and $C_{10}Glu_3$, where in the presence of diesel oil, the degradation reached 26.7 and 47.3 % respectively. When the surfactant was used without diesel, the degradation of the abovementioned glucosides was slightly higher, but still lower than for the ones with one or two sugar molecules in the formula.

The correlation between the alkyl chain length, number of saccharide components and the glucoside degradation is presented in Fig. 19 a and b.

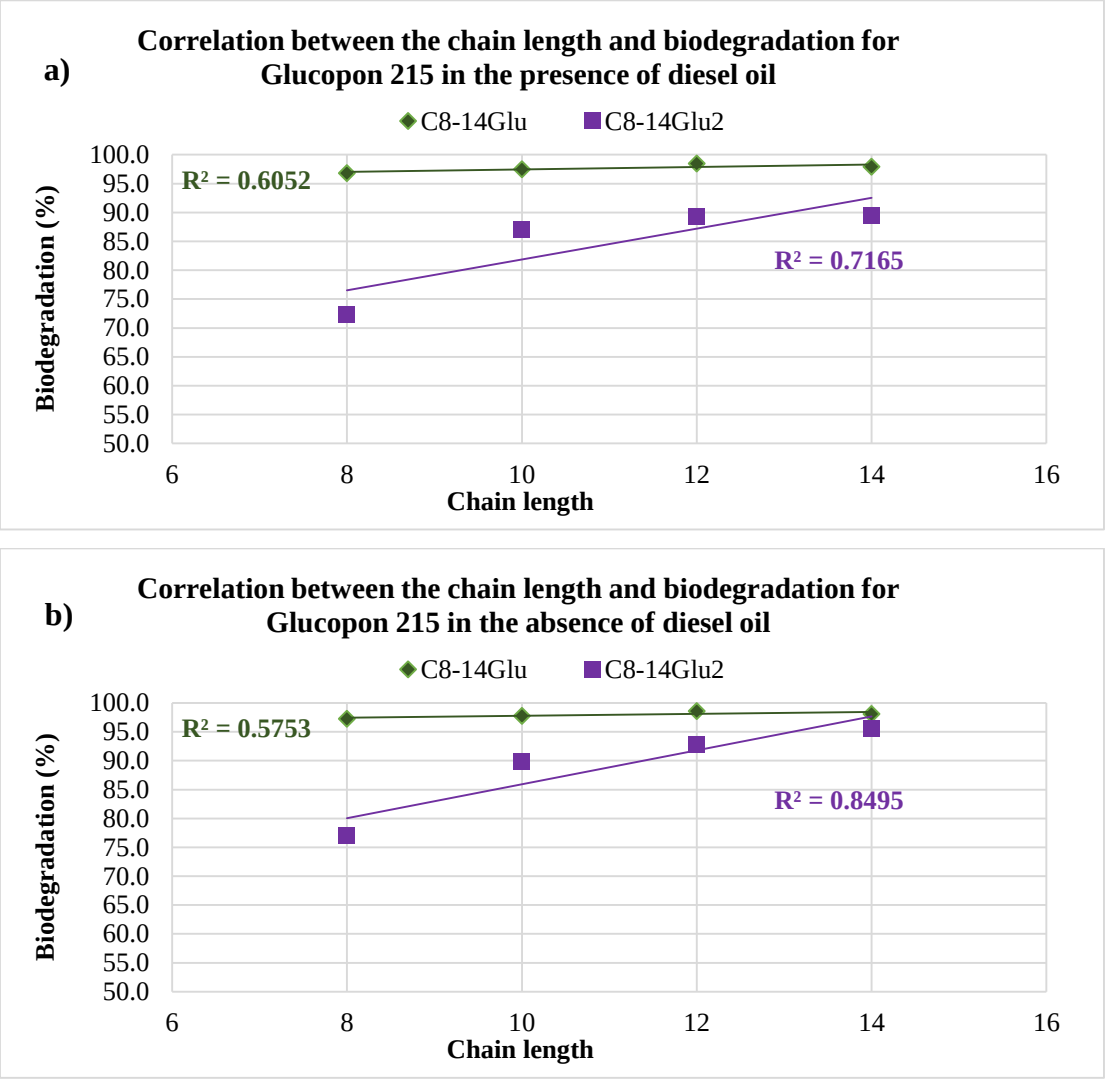


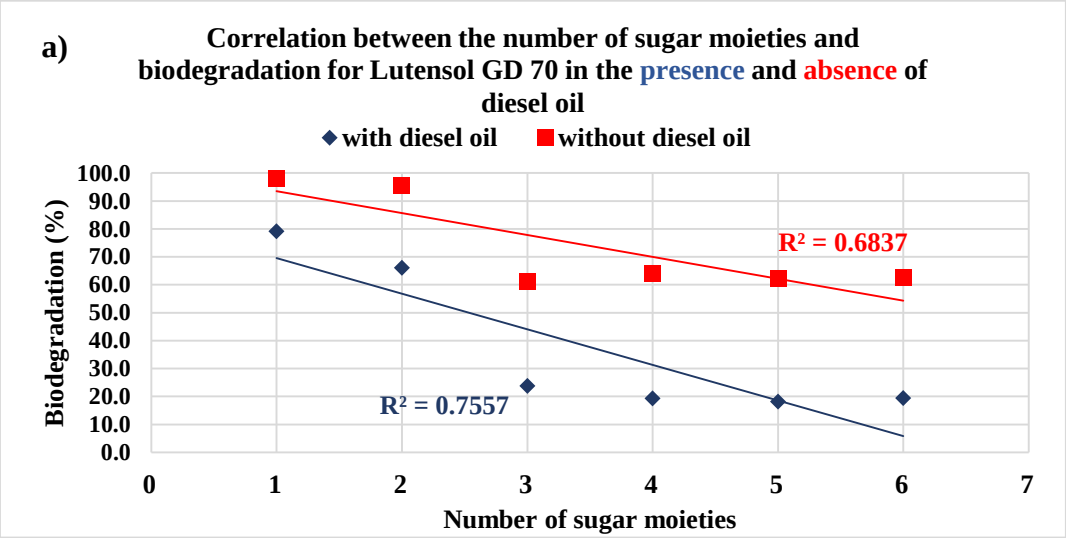
Figure 19: Correlation between chain length and biodegradation by *Stenotrophomonas maltophilia* strain 6 for Glucopon 215 in the presence (a) and absence of diesel oil (b).

As seen in Fig. 19a, the coefficient of determination for $C_{8\text{-}14}$Glu is equal to 0.6052, which means that the differences in biodegradation of the four glucosides were not significant. In other words, the glucosides were nearly equally degraded regardless the alkyl chain length. However, when the glucoside contained two methyl galactopyranoside moieties, a relation between the biodegradation and structure of the glucoside could be noticed, with R^2=0.7165. In addition, the same trends could be observed in the systems without diesel oil (Fig. 19b).

For the second surfactant, Lutensol GD 70, in mixture with diesel oil, the least favourable for degradation by *Achromobacter* sp. 4(2010) glucoside was C_4Glu. However, when there was no diesel in the system, the same glucoside was found to be degraded in nearly 95 %.

In addition, *Stenotrophomonas maltophilia* strain 6, likewise Glucopon, was capable of degrading all of the glucosides with the yields reaching and exceeding 50%. The highest results were obtained for C_{10}Glu with and without diesel oil (97.6 % and 97.5 % respectively).

Since the glucoside components of Lutensol GD 70 are different than of Glucopon 215, other correlation models were suggested. Fig. 20a. presents the correlation between the biodegradation and the number of saccharide moieties for *Achromobacter* sp. 4(2010) and Fig. 20b for *Stenotrophomonas maltophilia* strain 6.



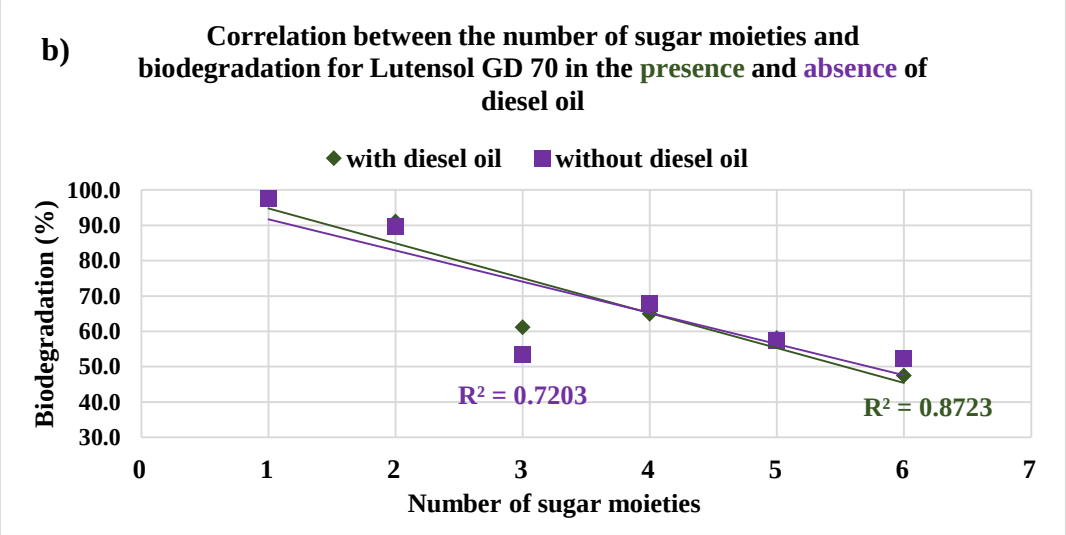


Figure 20: The correlation between biodegradation and the number of saccharide moieties for *Achromobacter* sp. 4(2010) (a) and for *Stenotrophomonas maltophilia* strain 6 (b).

Based on the correlations presented in Fig. 20, in both cases (with and without diesel oil), the degradation trend for *Achromobacter* sp. 4(2010) was nearly the same. The increasing number of sugar moieties led to the decrease in biodegradation of glucosides – from 98.2 % for $G_{10}Glu$ to 62.5 % for $C_{10}Glu_6$ – with no diesel in the system, and from 79.2 for $G_{10}Glu$ to 19.5 % for $C_{10}Glu_6$ in the presence of diesel. The fact that the vast majority of glucosides with 6 molecules of saccharides was degraded with high extent (up to 19.5 %) can be a result of the sugar moieties being good carbon and energy sources for the microbial cells. Therefore, the surfactant could be used by the microorganisms either as the competitive (to petroleum hydrocarbons) or supplemental carbon source.

Surprisingly, the results obtained for *Stenotrophomonas maltophilia* strain 6 (Fig. 20b) revealed that the biodegradation of glucosides seems to be independent of the presence of diesel oil. This may suggest that the microorganisms of this strain could use the surfactant as the first carbon source, degrading the most favourable molecules and then turning to diesel oil.

4.2.3. LC-MS/MS of octylphenol ethoxylates

Triton X-100 is an octylphenol ethoxylate, a non-ionic surfactant, of the formula presented in the Fig. 21. It is widely used in the various branches of science and industry, e.g. molecular biology and biochemistry, paper and textile industry, household cleaners, etc.

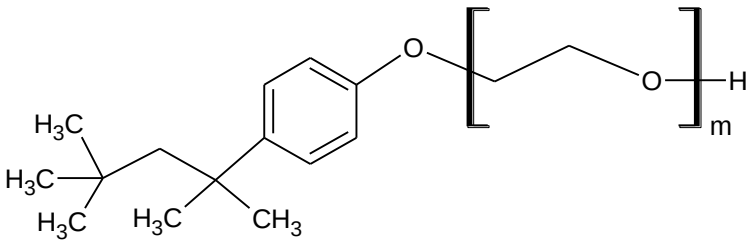


Figure 21: Triton X-100 formula, where m=9-10.

The liquid chromatography-tandem mass spectrometry of the octylphenol ethoxylates (OPEOs), the components of Triton X-100, was performed in order to determine the biodegradability of this surfactant. The parameters for detection of the analytes are presented in Table 8.

Table 8: Parameters of mass spectrometric detection characteristic to particular analytes (MRM – multiple reaction monitoring). OPEO1-19 - octylphenol ethoxylates containing from 1 to 19 ethoxy groups.

Triton X-100	MRM Transitions (Precursor Ion M/Z → Product Ion M/Z)	Collision Energy (V)
OPEO1	268.2 → 113.0	13
OPEO2	312.2 → 183.0	9
OPEO3	356.3 → 227.0	17
OPEO4	400.3 → 272.0	21
OPEO5	444.3 → 316.0	25
OPEO6	488.4 → 360.0	26
OPEO7	532.4 → 133.0	33
OPEO8	576.4 → 133.0	35
OPEO9	620.4 → 133.0	37
OPEO10	664.5 → 133.0	38
OPEO11	708.5 → 133.0	40
OPEO12	752.5 → 133.0	41
OPEO13	796.5 → 133.0	42
OPEO14	840.6 → 133.0	46
OPEO15	884.6 → 133.0	46
OPEO16	928.6 → 133.0	49
OPEO17	972.6 → 133.0	52
OPEO18	1016.7 → 133.0	53
OPEO19	1060.7 → 133.0	55

The results obtained throughout this study are presented in the Table 9.

Table 9: The biodegradation of the octylphenol ethoxylates (OPEOs) by Achromobacter sp. 4(2010) and Stenotrophomonas maltophilia strain 6 in the presence and absence of diesel oil.

octylphenol ethoxylates	Biodegradation (%) ± SD			
	Achromobacter sp. 4(2010)		*Stenotrophomonas maltophilia* strain 6	
	with diesel oil	without diesel oil	with diesel oil	without diesel oil
OPEO5	86.9 ± 2.6	14.5 ± 3.2	82.6 ± 9.1	16.7 ± 2.9
OPEO6	92.3 ± 12.1	9.7 ± 0.5	77.4 ± 9.9	3.4 ± 0.8
OPEO7	84.6 ± 8.9	9.9 ± 1.9	70.9 ± 6.8	2.6 ± 0.4
OPEO8	71.4 ± 6.2	3.6 ± 1.0	55.5 ± 6.3	2.1 ± 0.6
OPEO9	63.0 ± 8.3	5.5 ± 0.5	49.3 ± 2.1	6.0 ± 2.3
OPEO10	55.6 ± 1.7	2.1 ± 0.3	44.4 ± 1.6	4.8 ± 2.0
OPEO11	48.9 ± 5.4	0.6 ± 0.1	40.8 ± 5.3	4.2 ± 1.1
OPEO12	45.2 ± 8.7	3.0 ± 0.5	39.8 ± 4.7	0.9 ± 0.0
OPEO13	41.8 ± 6.5	2.7 ± 0.8	39.4 ± 6.2	3.9 ± 0.8
OPEO14	37.2 ± 7.1	3.5 ± 1.1	34.4 ± 0.5	0.6 ± 0.0
OPEO15	30.7 ± 10.2	8.9 ± 1.1	29.7 ± 2.6	1.9 ± 0.5
OPEO16	27.4 ± 5.7	8.0 ± 0.7	27.7 ± 3.4	6.5 ± 1.3
OPEO17	21.5 ± 6.0	2.0 ± 0.2	23.0 ± 2.1	3.8 ± 0.9
OPEO18	22.6 ± 4.3	4.2 ± 0.7	21.9 ± 2.0	4.2 ± 1.0
OPEO19	11.4 ± 2.5	3.3 ± 1.4	15.9 ± 1.7	9.9 ± 1.2

To summarise, in the presence of diesel oil both of the strains were able to degrade octylphenol ethoxylates that contained from 1 to 19 ethoxy groups. The least degradable (only ~11-13 %) ethoxylate contained 19 ethoxy groups, while OPEO5 (containing only 5 ethoxy groups) turned out to be the most biodegradable ethoxylate (86.9 and 82.6 % for *Achromobacter* sp. 4(2010) and *S. maltophilia* strain 6 respectively).

Very different results however, were observed in the absence of diesel oil in the tested systems. The biodegradation of OPEO5 reached only 14.5 and 16.7 % for *Achromobacter*

sp. 4(2010) and *S. maltophilia* strain 6 respectively. What is more, in both cases, the biodegradation of the rest of ethoxylates did not exceed 10 %.

Broadly speaking, octylphenol ethoxylates ($OPEO_n$) along with nonylphenol polyethoxylates ($NPEO_n$) are the most common ethoxylates, belonging to the so called alkylphenol ethoxylates ($APEO_n$ or APE), a group of surfactants broadly used in various commercial and industrial products, such as detergents, dispersants, emulsifiers, etc. (Chen et al., 2005). Due to their broad usage, an emphasis has been put on their presence in the environment. Their degradation products have gained a lot of interest, being found in effluents of many municipal sewage treatment plants and untreated wastewater (e.g. paper mill sewage) (Klečka et al., 2007; Ying et al., 2002).

Pseudomonas sp. TX1 was proposed by Lin et al. (2010) as the first bacterial strain able to grow on $OPEO_n$, back to date. Based on the metabolite analysis, the researchers concluded that the mechanism involved in ethoxylate degradation was a sequential cleavage of the ethoxylates. Furthermore, the authors considered models of a chain shortening (oxidative and non-oxidative biodegradation) depending on the product formation, and suggested that the oxidative biodegradation had most likely occurred in this case. The fastest decrease (with regard to the concentration) of ethoxylates was observed for OPEO12 and OPEO13, 8 hours after the innoculation), while the other ethoxylates, OPEO5-11, were found to be degraded after 25 hours.

Nishio et al. (2002) identified 10 isolates of *Pseudomonas putida* and 1 isolate of *Burkholderia cepacia* from paddy soil that was said to be contaminated with OPPEOs (octylphenol polyethoxylates), which are used as a wetting and dispersing agent in pesticides. The 10-day incubation of each isolate with OPPEOs revealed that all of the strains were able to degrade the polyethoxylates with almost the same results, with OPEO3 and OPEO2 as the final products of biodegradation. The authors emphasised that in all cases, the degradation of OPPEO started from the end of the polyethoxy chain.

4.3. Biodegradation of diesel oil

The following chapter presents the results obtained for the four terrestrial strains: *Achromobacter* sp. 4(2010), *Pseudomonas stutzeri* strain 9, *Rahnella* sp. EK12, *Stenotrophomonas maltophilia* strain 6, as well as for the four marine bacterial strains:

Alteromonas sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105, *Cycloclasticus* sp. strain TK-8 and *Polycyclovorans algicola* TG408.

If not specified in the graphs, the concentration of all tested surfactants was 120 mg L^{-1}.

Fig. 22 presents the degradation of diesel oil in the presence of surfactants for *Achromobacter* sp. 4(2010). The highest degradation (67.37 ± 0.74%) was observed when Glucopon 215 was added at the concentration equal to 240 mg L^{-1}. A further increase of the surfactant's concentration did not lead to any significant change in the degradation process.

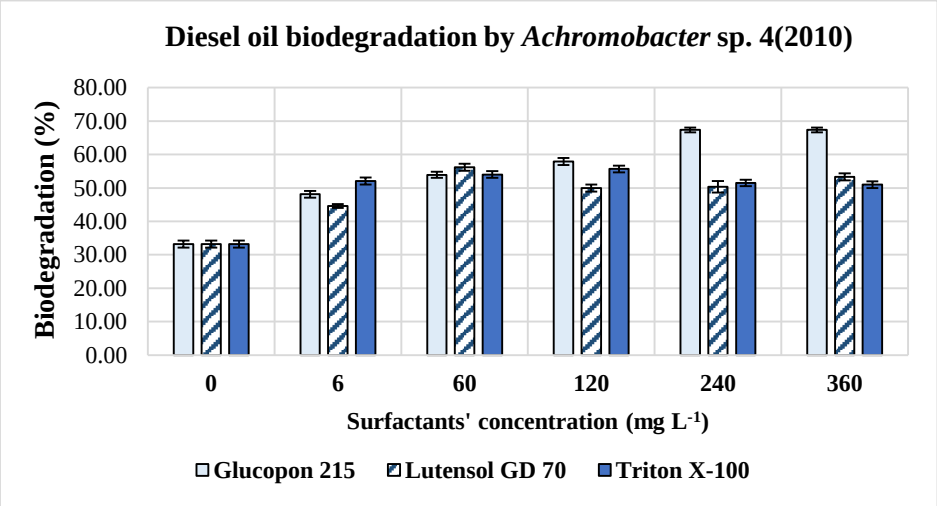


Figure 22: Degradation of diesel oil by *Achromobacter* sp. 4(2010) in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 (c) at varied concentrations (6-360 mg L^{-1}).

For another strain – *Stenotrophomonas maltophilia* strain 6, the results indicated that all surfactants had a positive effect on the biodegradation, and although the highest result was observed for Lutensol GD 70 (conc. 360 mg L^{-1}), the overall increasing trend could be observed for each surfactant.

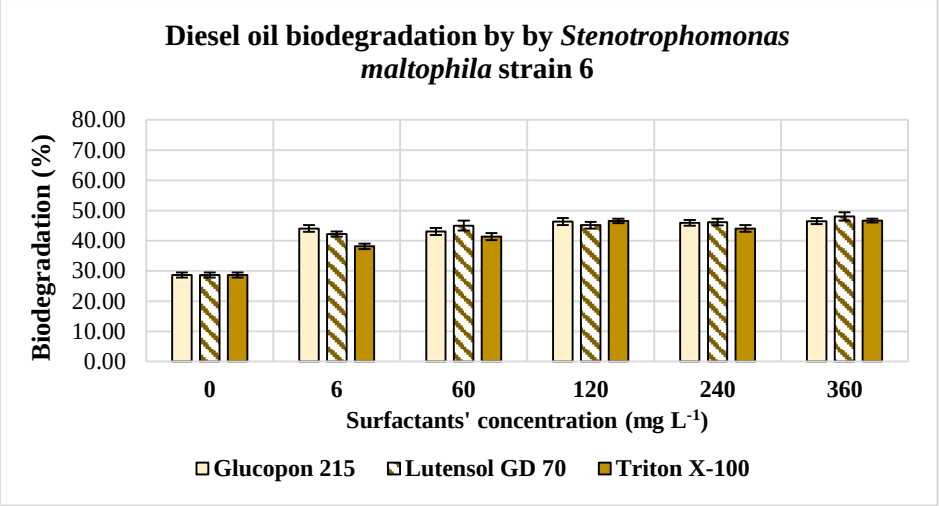


Figure 23: Degradation of diesel oil by *Stenotrophomonas maltophilia* strain 6 in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

Fig. 24 presents the results of diesel oil degradation by the next terrestrial strain - *Pseudomonas stutzeri* strain 9. In this case, the surfactant that influenced the biodegradation significantly was Glucopon 215 at 360 mg L^{-1}.

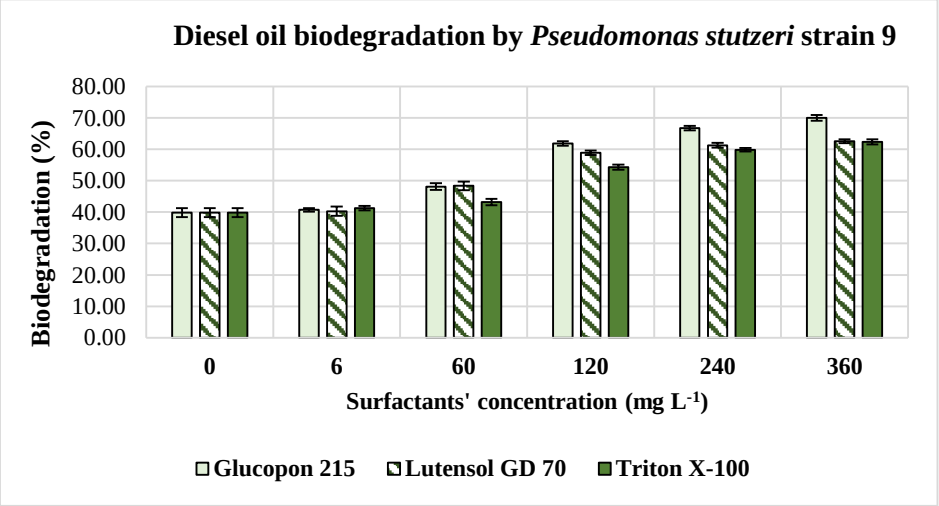


Figure 24: Degradation of diesel oil by *Pseudomonas stutzeri* strain 9 in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

The initial diesel oil degradation by *Rahnella* was equal to 34.1%, and the addition of Lutensol GD 70 at 360 mg L^{-1} led to 30% increase in biodegradation, reaching 62.6 % (Fig. 25).

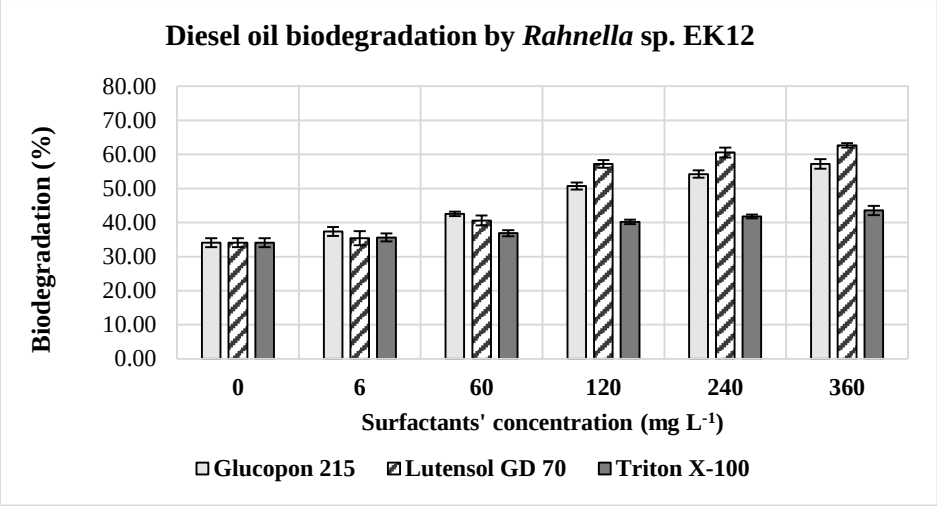
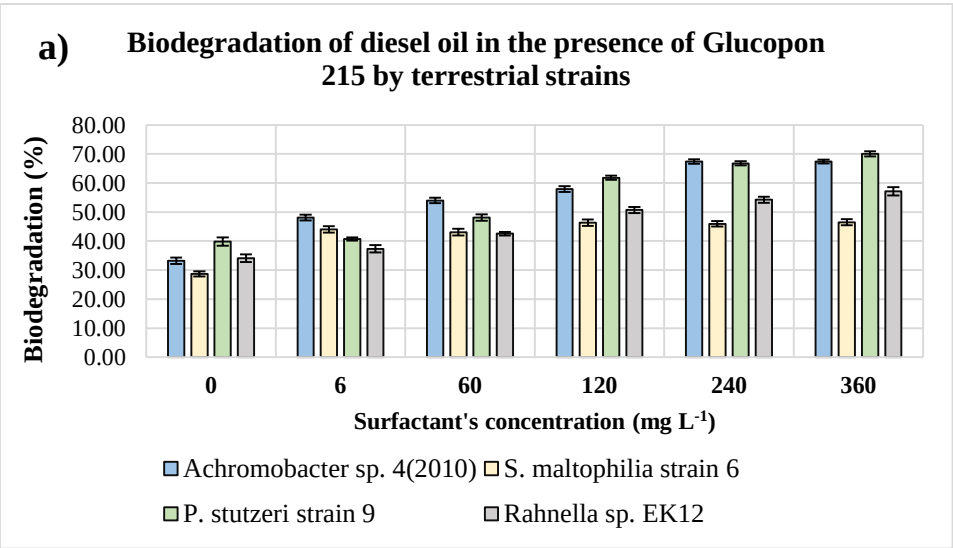
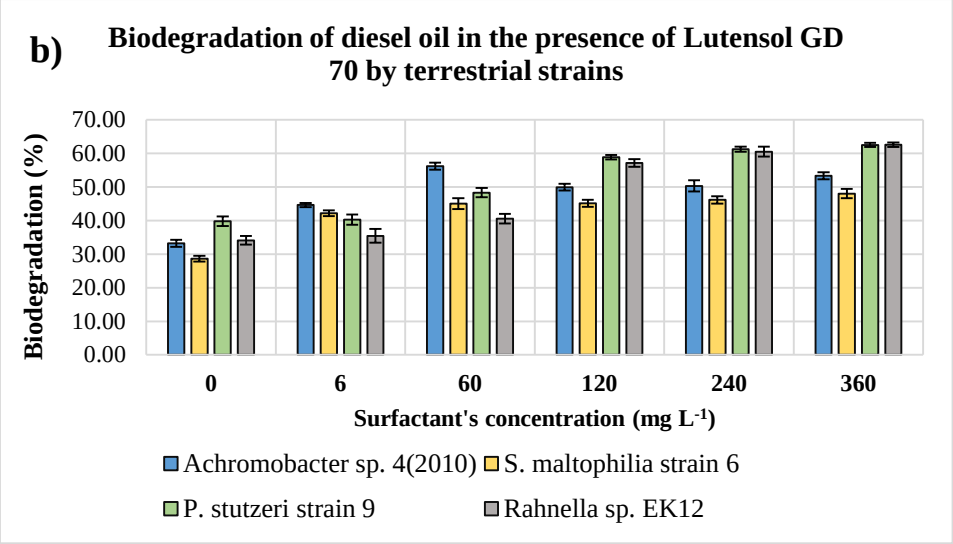


Figure 25: Degradation of diesel oil by *Rahnella* sp. EK12 in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

In order to compare and contrast the influence of each surfactant on biodegradation of diesel oil by the four terrestrial strains, the additional three graphs were prepared (Fig. 26 a-c).





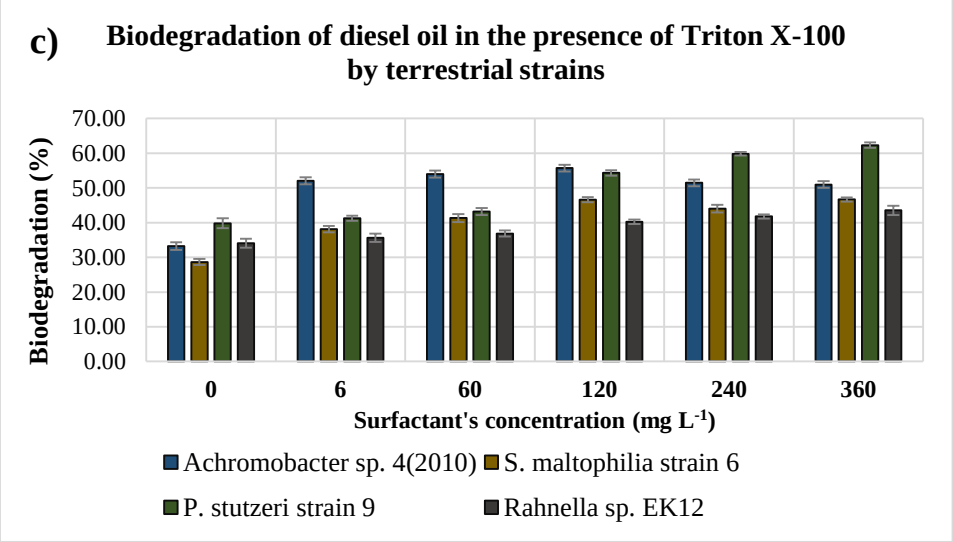


Figure 26: The biodegradation of diesel oil in the presence of Glucopon 215 (a), Lutensol GD 70 (b) and Triton X-100 (c) by the isolated terrestrial strains.

To summarise, each from the tested surfactants had a positive effect on the biodegradation of diesel oil by terrestrial strains. In the case of Glucopon 215, the topmost results were obtained for *Achromobacter* sp. 4(2010) and *Pseudomonas stutzeri* strain 9. The comparison of the obtained results with literature reports is very difficult as Glucopon 215 has not been tested with regard to the biodegradation of petroleum hydrocarbons. However, this surfactant was well studied when it comes to its physical and chemical properties, such as its stability under different pH and temperatures (Vaz et al., 2012).

In the case of Lutensol GD 70 (Fig. 26b), the results which displayed the most similarity (~69%) were achieved by *Pseudomonas stutzeri* strain 9 and *Rahnella* sp. EK12 within the surfactant's concentration ranging between 120 and 360 mg L^{-1}, thus close to the estimated

CMC (see section 4.2.1.). Chrzanowski et al. (2006) applied Lutensol GD 70 (conc. 150, 300 and 600 mg L^{-1}) to their studies on biodegradation within a model mixture of hydrocarbons (dodecane and hexadecane 1:1) by *Candida maltosa* EH 15 over time. In this case, the highest biodegradation (71.4 %) was observed after 7 days for the surfactant's concentration equal to 150 mg L^{-1}. Therefore, they show that when the concentration of Lutensol GD 70 is close to or slightly exceeds the CMC, the increase in biodegradation of hydrocarbons is much higher.

Triton X-100 is one of the most studied surfactant with regard to the biodegradation purposes. Both its positive and negative influence on the degradation of petroleum hydrocarbons have been reported (Sałek et al., 2015). The critical micelle concentration estimated in my studies for Triton X-100 was equal to 158.4 mg L^{-1}. A strong relation between the CMC and surfactant's influence on diesel oil biodegradation could be observed for *Achromobacter* sp. 4(2010). In this case, the highest degradation (55.7 %) was observed at Triton's concentration 120 mg L^{-1}. However, a further increase in the surfactant's concentration caused the decrease in diesel's biodegradation. A very similar relation for this strain was also observed in the presence of Glucopon 215, with its CMC of 244.3 ± 9.2 mg L^{-1}. In that case the highest biodegradation was achieved for the surfactant's concentration between 240 and 360 mg L^{-1}.

The first marine strain that was studied – *Alteromonas* sp. TK-46(2) showed a very high diesel oil degradation ability even when not supplemented with any of the tested surfactants (66.9 %). However, their addition at various concentrations led to a further increase in biodegradation efficiency of the strain.

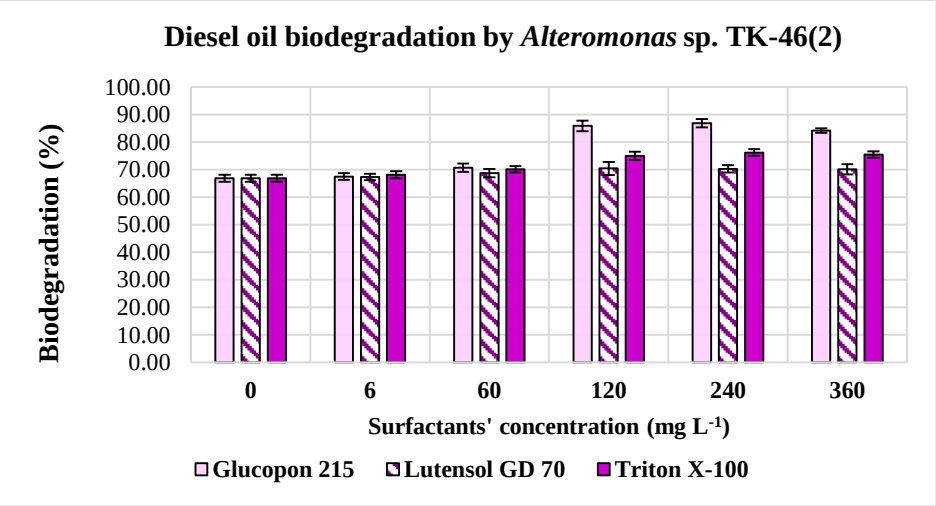


Figure 27: Degradation of diesel oil by *Alteromonas* sp. TK-46(2) in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

Pseudoalteromonas sp. TK-105 was characterised with the initial degradation ability equal to 47.2 %. The addition of surfactants resulted in the increase in biodegradation results, reaching 78.0 % for Glucopon 215 at 240 mg L^{-1} (Fig. 28).

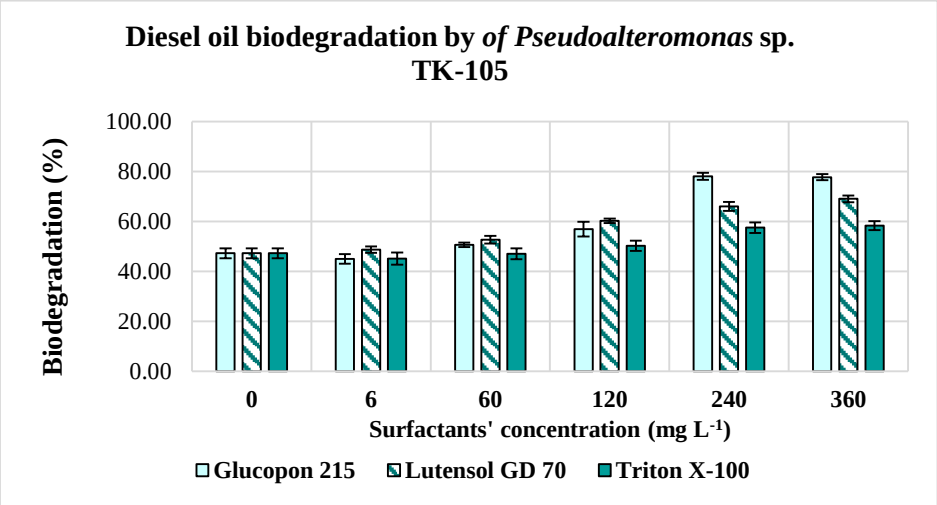


Figure 28: Degradation of diesel oil by *Pseudoalteromonas* sp. TK-105 in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

The lowest biodegradation among the marine strains was recorded for *Cycloclasticus* sp. TK8 strain, where the in the results of the unsupplemented system was equal to 20.4 %. At higher concentrations of surfactants only a small increase was observed (to 32.5 % for Glucopon 215).

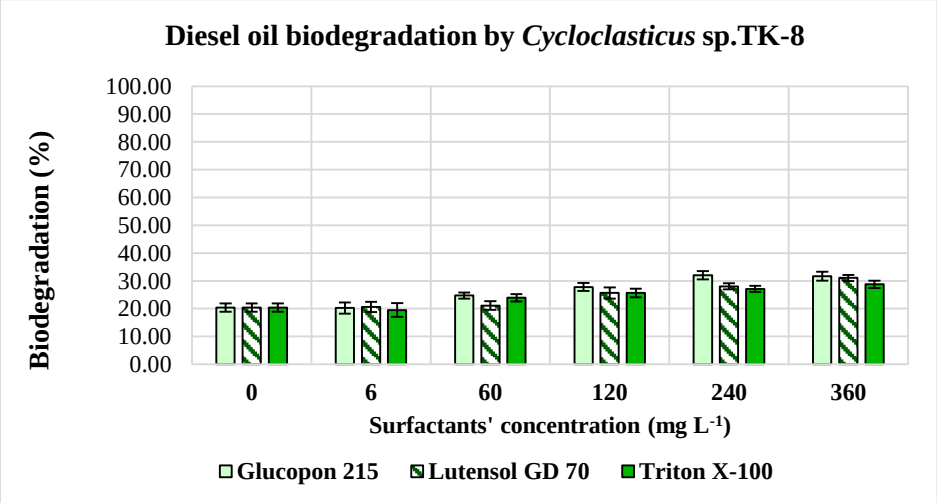


Figure 29: Degradation of diesel oil by *Cycloclasticus* sp. TK-8 in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

Polycyclovorans algicola TG408 was able to degrade diesel oil in over 50 % without any of the surface active agents (Fig. 30). However, when added, Glucopon 215 turned out to be

most effective, increasing the biodegradation to over 85 %. The least efficient surfactant in this case was Triton X-100, leading to only 8 % increase in degradation.

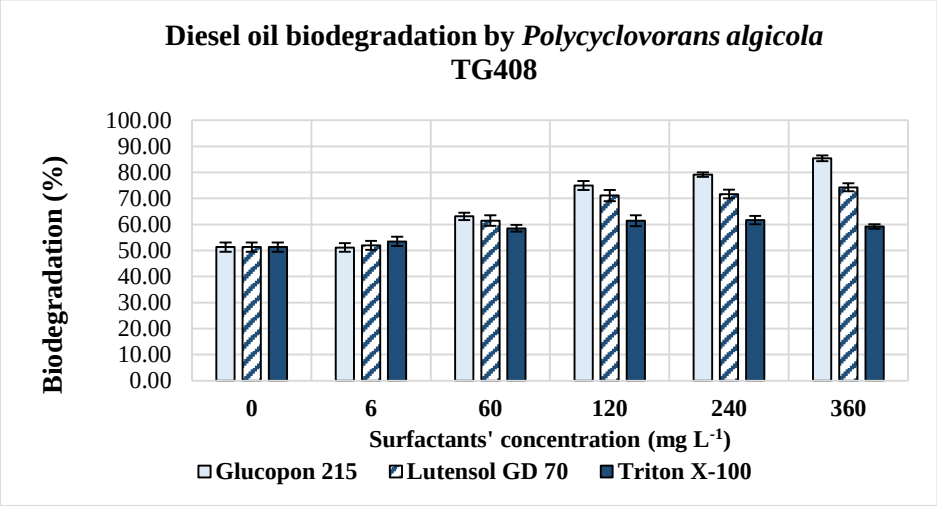
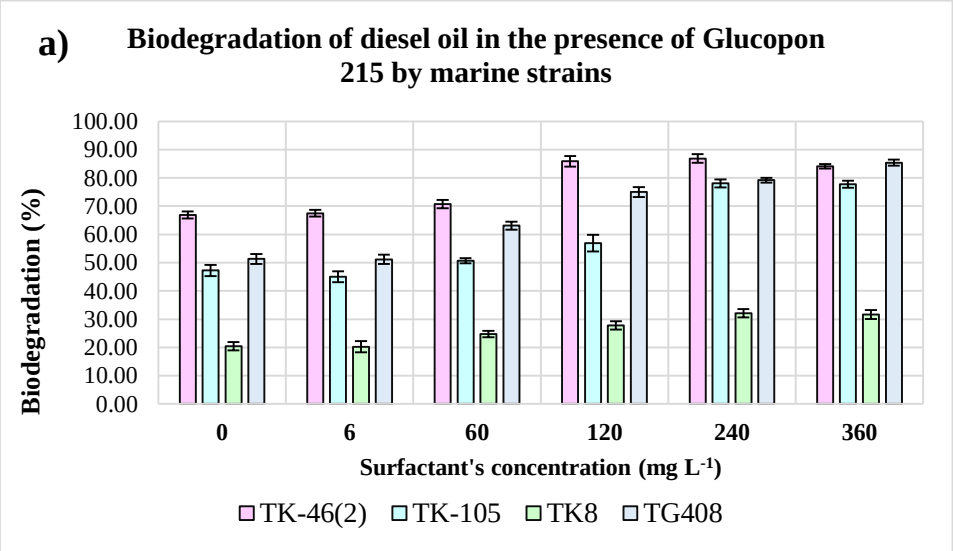


Figure 30: Degradation of diesel oil by *Polycyclovorans algicola* TG408 in the presence of Glucopon 215, Lutensol GD 70 and Triton X-100 at varied concentrations (6-360 mg L^{-1}).

Fig, 31 was prepared to present the influence of each surfactant separately on diesel oil biodegradation by the four marine strains.



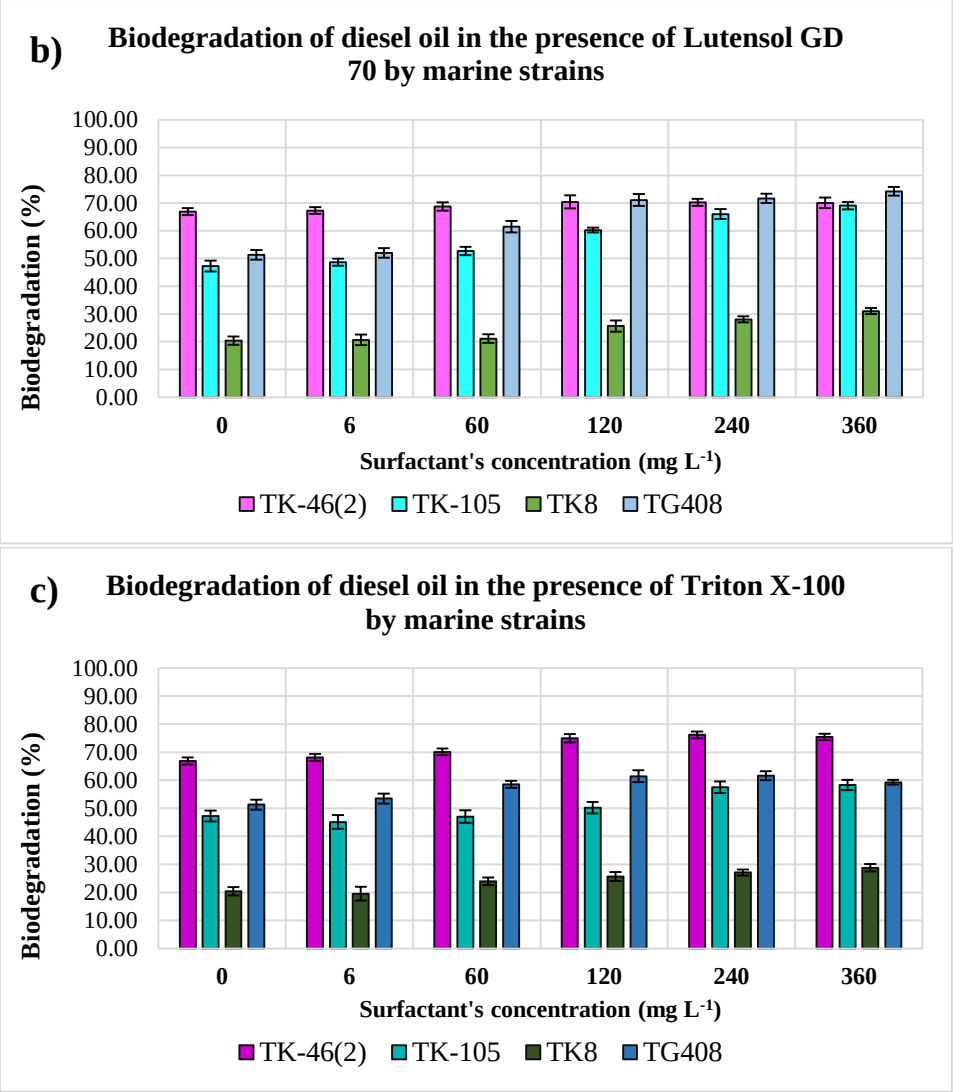


Figure 31: Biodegradation of diesel oil in the presence of Glucopon 215 (a), Lutensol GD 70 (b) and Triton X-100 (c) by the isolated marine strains.

According to the presented figures, among all the strains and surfactants, the highest biodegradation was observed for the strain *Alteromonas* sp. TK-46(2) in the presence of Glucopon 215 (86.83 ± 1.55 % at 240 mg L^{-1}). In addition, this surfactant at the same concentration of as high as 240 mg L^{-1} turned out to be the most effective, regarding the increase in biodegradation, for *Pseudoalteromonas* sp. TK-105 and *Cycloclasticus* sp.TK-8, while the highest degradation by *Polycyclovorans algicola* TG408 was noticed at Glucopon's concentration equal to 360 mg L^{-1} (85.37 ± 1.09 %).

The other natural surfactant, Lutensol GD 70 enhanced biodegradation of diesel oil most effectively at the concentration equal to 350 mg L^{-1} for *Pseudoalteromonas* sp. TK-105, *Cycloclasticus* sp. TK-8 and *Polycyclovorans algicola* TG408. In the case of *Alteromonas*

sp. TK-46(2), the increase in biodegradation was hardly noticed and seemed to be independent of the surfactant's concentration.

Likewise with Glucopon 215, in the presence of Triton X-100, among all the strains, the highest biodegradation results were observed for *Alteromonas* TK-46(2), exceeding 70 % for the surfactant's concentrations ranging from 120 to 360 mg L^{-1}.

Although the four tested marine strains have mostly been known for their PAH degrading properties (Geiselbrecht et al., 1998; Gutierrez et al., 2015; Hedlund and Staley, 2006; Jin et al., 2012), the herein research revealed also their ability to degrade diesel oil as sole carbon and energy source. The strain *Cycloclasticus* TK-8 turned out to be the least effective diesel degrader and the addition of either surfactant only slightly enhanced the biodegradation. As an aromatic hydrocarbon degrading microorganism, it is of high probability, that *Cycloclasticus* TK-8 was only able to degrade the aromatic components of diesel oil.

To summarise, the highest results for diesel oil biodegradation among the terrestrial strains were observed for *Pseudomonas stutzeri* strain 9, while in the case of the marine strains *Polycyclovorans algicola* TG408 was characterised with the highest results reaching nearly 85 %. For both of the strains Glucopon 215 appeared to influence the biodegradation of diesel oil most efficiently. At the surfactant's concentration as high as 360 mg L^{-1} the biodegradation of diesel oil reached 69.88 % and 85.37 % for P. stutzeri strain 9 and *Polycyclovorans algicola* TG408 respectively. Fig. 32 presents the comparison between the two abovementioned strains with regards to the biodegradation of diesel oil.

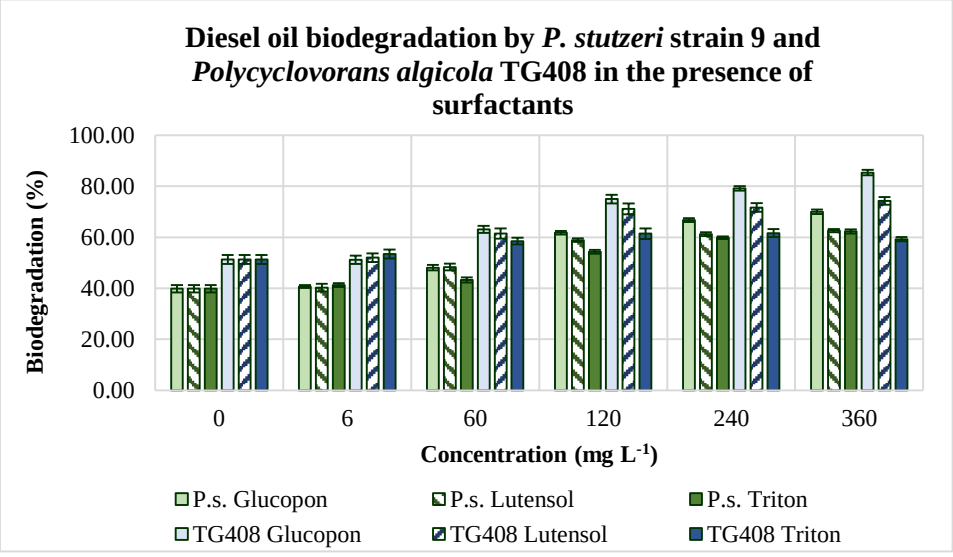
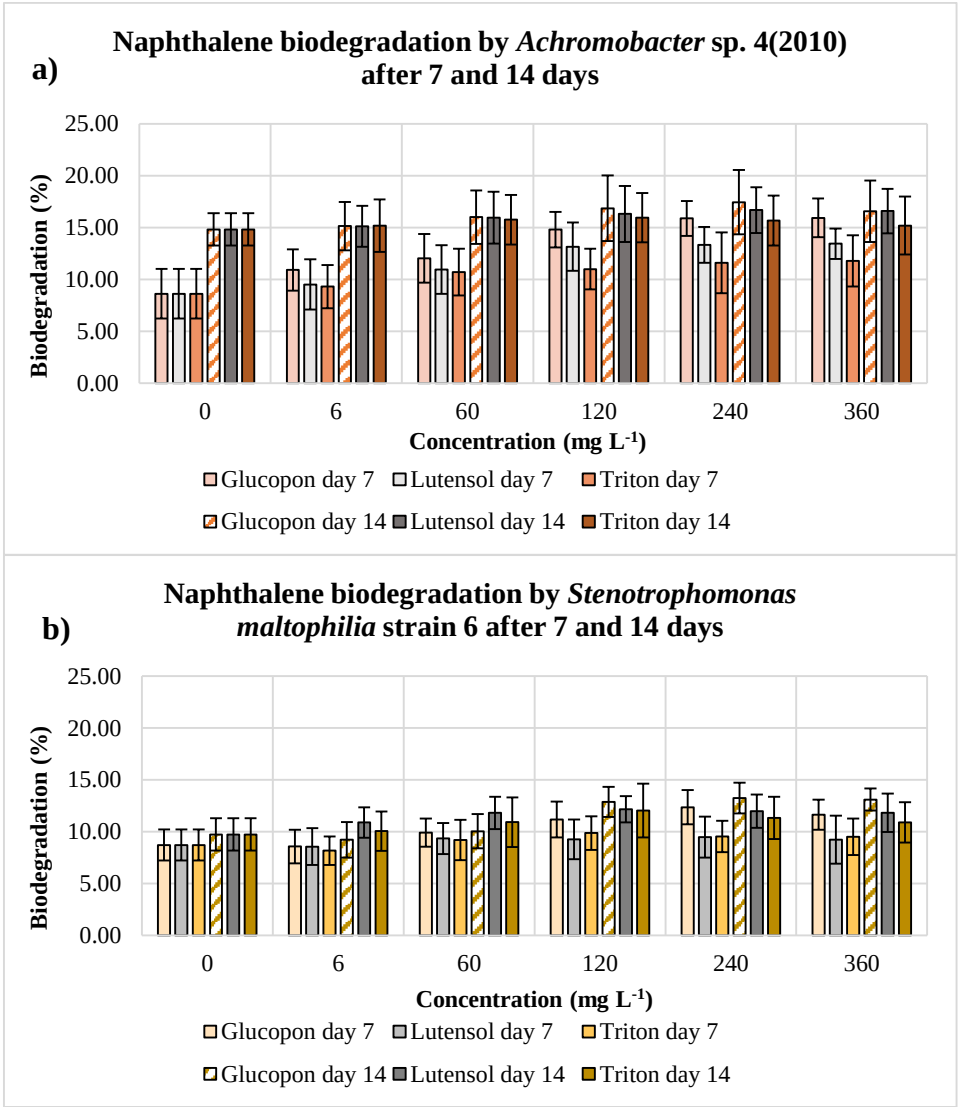


Figure 32: A comparison between terrestrial and marine strains that were characterised with the highest diesel oil biodegradation in their class of origin.

4.4. Naphthalene biodegradation

The biodegradation of one of the polycyclic aromatic hydrocarbons, i.e. naphthalene, was determined for marine and terrestrial strains after 7 and 14 days of incubation.

Fig 33 presents the data collected for the terrestrial strains, with each graph demonstrating the results obtained for each of the strains individually.



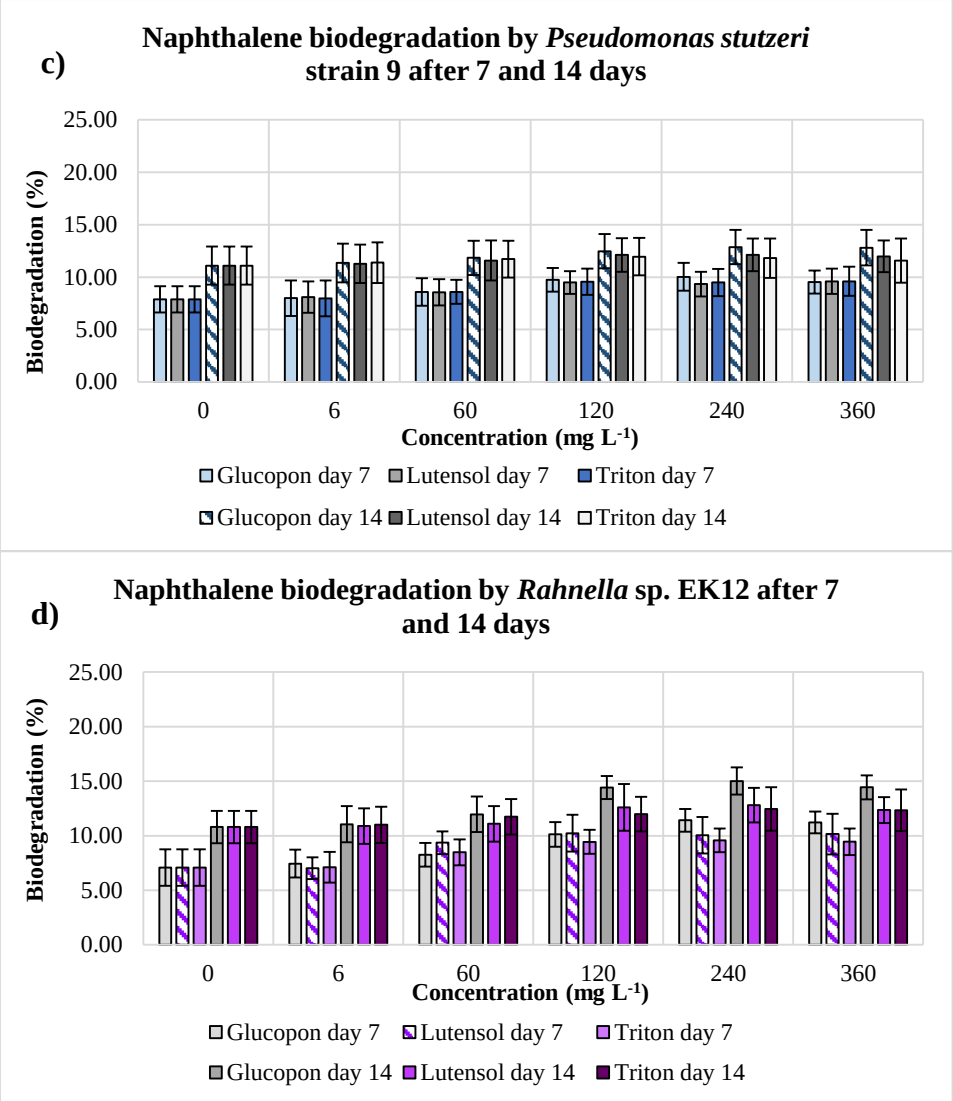


Figure 33: Naphthalene biodegradation after 7 and 14 days of incubation by *Achromobacter* sp. 4(2010) (a), *Stenotrophomonas maltophilia* strain 6 (b), *Pseudomonas stutzeri* strain 9 (c), *Rahnella* sp. EK12 (d).

Among terrestrial strains, *Achromobacter* sp. 4(2010) exhibited best biodegradation activity towards naphthalene. The initial degradation (with no surfactants) was equal to 9.61 %, and after 7 days of incubation it reached nearly 16 % in the presence of Glucopon 215 at 360 mg L^{-1}. After 14 days naphthalene was reduced in nearly 15 % in the absence of surfactants, while the incubation with Glucopon 215 at 240 mg L^{-1} led to the increase in biodegradation to 17.44 %. Only slightly lower results were obtained in the presence of Lutensol GD 70 and Triton X-100 (16.67 and 15.66 % respectively).

The ability of *Achromobacter* strains to degrade PAHs has already been reported several times. The microorganisms were found in different reservoirs (water, soil) contaminated with PAHs (Andreoni et al., 2004; Cutright and Lee, 1994; Dellagnezze et al., 2014). Dave

et al. (2014) investigated the potential of *Achromobacter xylosoxidans* towards degradation of low molecular weight (LMW) and high molecular weight (HMW) PAHs. Based on their results, naphthalene was degraded in over 35 %, while the addition of Triton X-100 led to nearly 40 % of the hydrocarbon's mineralisation. The authors also demonstrated that all of the tested PAHs were degraded to some extent, and the presence of Triton X-100 additionally increased the biodegradation in all cases.

Juhasz et al. (2000) demonstrated a high LMW and HMW PAH degradation ability of *Stenotrophomonas maltophilia* strain VUN 10,003. The authors estimated a significant decrease in the concentrations of the selected hydrocarbons: pyrene (98 %), fluoranthene (45 %), benz[*a*]anthracene (26 %), benzo[*a*]pyrene (22 %), dibenz[*a,h*]anthracene (22 %) and coronene (55 %) along with the corresponding reduction in the mutagenicity of culture and soil extracts. Therefore, they suggested *Stenotrophomonas maltophilia* strain VUN 10,003 would be effective in the detoxification of PAH contaminated reservoirs.

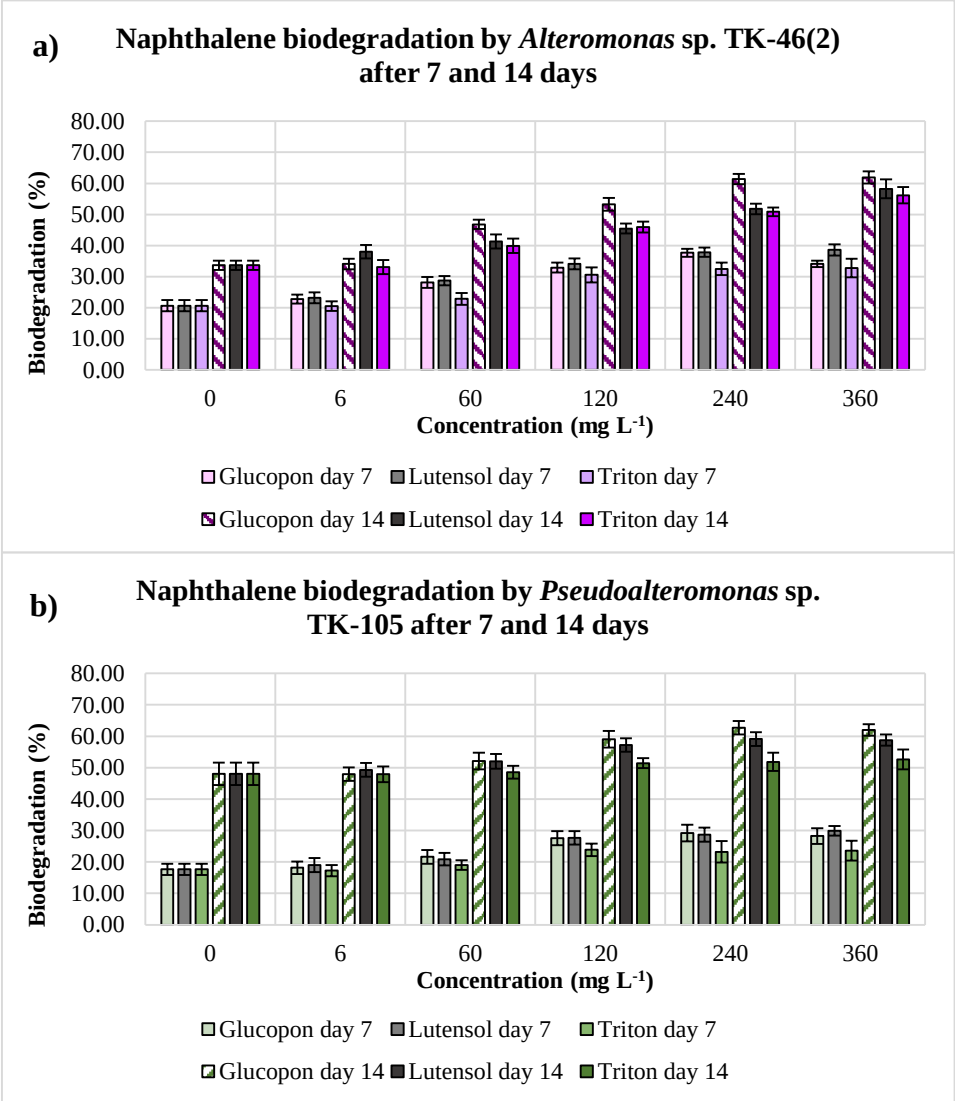
The tests conducted within this thesis indicated that *Stenotrophomonas maltophilia* strain 6 was able to degrade naphthalene both in the presence and absence of surfactants. When no surface active agent was added, the biodegradation reached 8.7 % after 7 days and 9.72 % after 14 days. The highest increase in biodegradation after one week was observed in the presence of Glucopon 215, achieving the maximum 12.34 % at surfactant's concentration as high as 240 mg L^{-1}. Similarly, after a fortnight the highest level of biodegradation, 13.24 % was obtained for Glucopon 215 at 240 mg L^{-1}. In this case, however, there was no significant change in biodegradation observed over the time of 7 and 14 days ($P > 0.05$).

Unlike *S. maltophilia*, the influence of incubation time on naphthalene biodegradation by *Pseudomonas stutzeri* strain 9 and *Rahnella* sp. EK12 could be observed (Fig. 33c.). In both cases the addition of Glucopon 215 (240 mg L^{-1}) led to the highest increase in biodegradability of naphthalene.

Regarding the naphthalene biodegradation, it can be concluded that Glucopon 215 turned out to be the most effective agent, which enhanced the degradation for all terrestrial strains tested within this study.

Among the marine strains, *Cycloclasticus* sp. TK-8 proved to be a good example of the influence of incubation time on biodegradation of naphthalene (Fig. 34c). After 7 days (in the absence of surfactants), the mineralisation of naphthalene reached 17.54 %, while after

14 days under the same conditions the hydrocarbon degradation was estimated to be as high as 57.89 %, which is 40.35 % higher in comparison to the first week of incubation. The maximal biodegradation (~63 %) by strain TK-8 was recorded after 14 days, in the presence of Glucopon 215 in the system. In this case there was no significant difference between the degradation level and the concentration of the surfactant (very similar results were obtained for the other surfactant concentrations used, ranging from 120 to 360 mg L^{-1}).



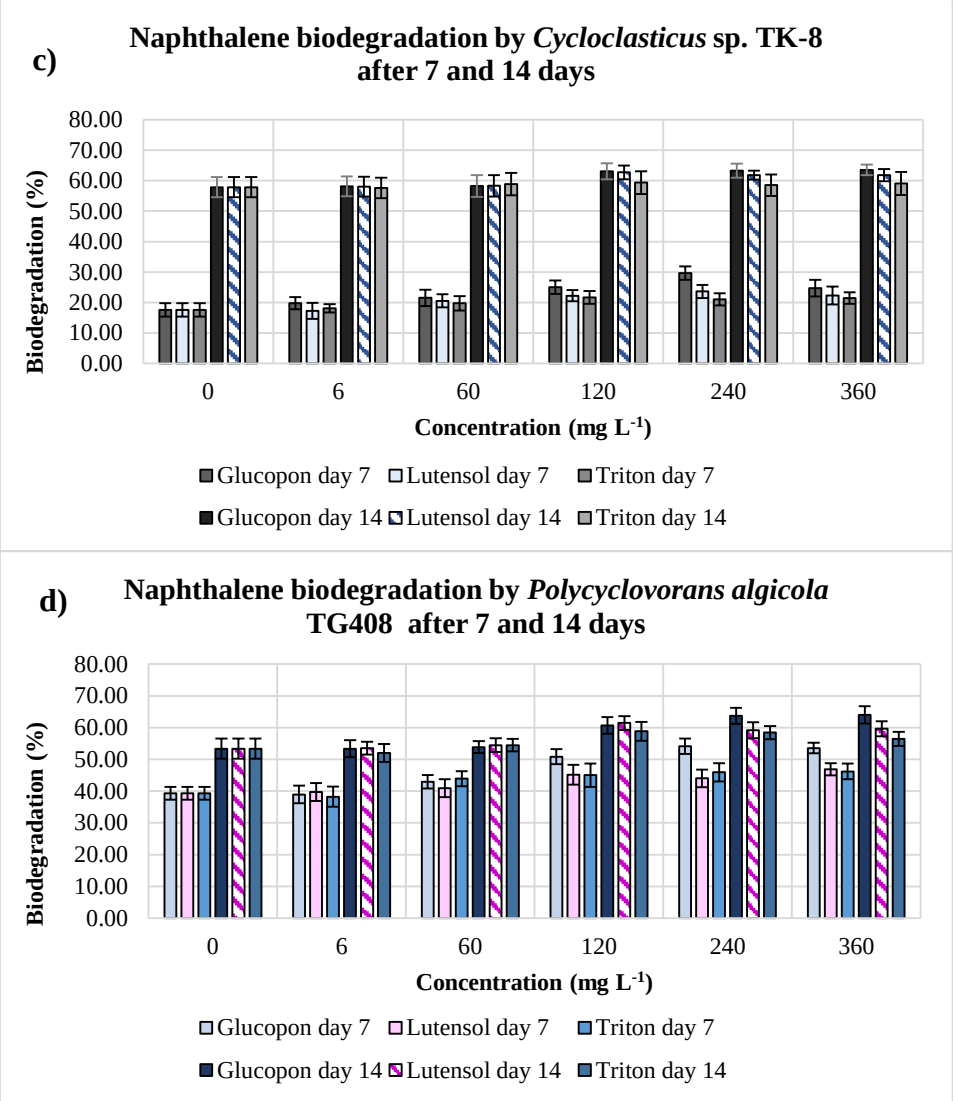


Figure 34: Naphthalene biodegradation after 7 and 14 days of incubation by *Alteromonas* sp. TK-46(2) (a), *Pseudoalteromonas* sp. TK-105 (b), *Cycloclasticus* sp. TK-8 (c), *Polycyclovorans algicola* TG408 (d).

The 14-day incubation was also found to lead to an increase of naphthalene biodegradation by *Alteromonas* sp. TK-46(2), *Pseudoalteromonas* sp. TK-105, *Polycyclovorans algicola* TG408 (statistically significant when compared to the 7-day incubation time – $P < 0.05$). For all of the strains Glucopon 215 was seen to promote the biodegradation most efficiently. The least effective surfactant in this case was Triton X-100, although the differences between the degradation in the presence of Glucopon 215 and Triton X-100 were only up to 10 % ± 2.5 % ($P < 0.05$).

Bacteria of the genus *Cycloclasticus* were reported to play a very important role on biodegradation of PAH on several occasions, especially after the Nakhodka oil spill (Maruyama et al., 2003; Yamamoto et al., 2003) and Deepwater Horizon oil spill (Yang et

al., 2014). Geiselbrecht et al. (1998) showed their results on *Cycloclasticus* PAH degradation range and revealed that the strains of this genus degraded the following substrates: naphthalene, 1-methyl- naphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, phenanthrene, fluorene, and anthracene completely after 7 days of incubation. In comparison, Kasai et al. (2002) revealed that the *Cycloclasticus* strains were found to degrade the PAH of the number of rings from 1-3 most effectively after 14 and 28 days of incubation. Those experiments, however, were performed for relatively low concentrations of naphthalene (Geiselbrecht et al.: 5 mg L^{-1}, Kasai et al.: 1 mg L^{-1}) where the complete degradation of a substrate was most likely to occur. Nonetheless, Miyasaka et al. (2006) conducted research on the naphthalene biodegradation with concentrations as high as 50 mg L^{-1} over a duration of 150 days. They reported that after 90 days of incubation the naphthalene concentration decreased to 10 mg L^{-1} and remained constant for the next 60 days until the end of the degradation experiment.

Alteromonas strains, likewise *Cycloclasticus*, were reported to belong to the topmost (dominant) PAH degrading microorganisms. The *Alteromonas* genus, which was first identified by Baumann in the early 1980s, has been isolated from many ecosystems so far and since its important role in PAH degradation was discovered, strains of this genus have gained a lot of interest by researchers. *Alteromonas* sp. SN2 strain was isolated from the crude oil-contaminated coastal sediment of South Korea and its biodegradation of four selected PAH was evaluated over time (Jin et al., 2012). Based on this research, 30 mg L^{-1} of naphthalene was fully degraded after 20 days of incubation, while this same amount of anthracene was reduced to 3 mg L^{-1} after 45 days. Zaidi et al. (2003) demonstrated the ability of the previously isolated *Alteromonas* sp. (from Guayanilla coastal water - Zaidi and Imam (1999)) to degrade biphenyl, naphthalene, phenanthrene and pentachlorophenol (PCP).

Bacteria of the *Pseudoalteromonas* genus, despite being metabolically diverse, were, in vast majority reported to be able to degrade PAHs (Hedlund and Staley, 2006) and were found in many water reservoirs such as the river Thames estuary (McKew et al., 2007), deep sea sediments of the Middle Atlantic Ridge (Cui et al., 2008), deep sea sediments of Southwest Indian Ridge (Yuan et al., 2015) and South Mid-Atlantic Ridge (Gao et al., 2015), as well as in the Arctic Ocean (Dong et al., 2014). The *Pseudoalteromonas* isolates from all of the abovementioned reservoirs were proved to be able to grow on (metabolised) PAH.

To conclude, among all eight strains that were tested throughout the study, there were two strains of different origin chosen: *Achromobacter* sp. 4(2010) (terrestrial) and *Cycloclasticus* sp. TK-8, as they were characterised with the highest naphthalene biodegradation.

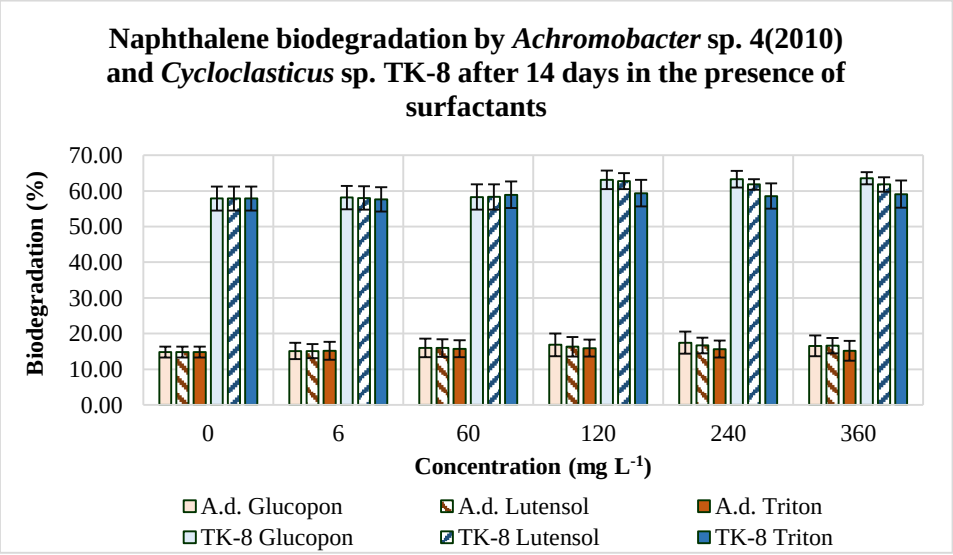


Figure 35: A comparison between terrestrial and marine strains that were characterised with the highest naphthalene biodegradation in their class of origin.

Fig. 35 shows that although *Achromobacter* sp. 4(2010) was characterised with the highest biodegradation of naphthalene among other terrestrial strains, the obtained results are even 40 % smaller than those recorded for the marine strain *Cycloclasticus* sp. TK-8. To summarise, based on the Fig. 33-35, the marine strains can be considered as better PAH degraders than terrestrial strains.

4.5. Cell surface properties

This chapter presents the results of MATH and zeta potential of marine and terrestrial strains. In order to make the discussion clearer, there is one figure for each strain, presenting the influence of each surfactant separately on both, MATH and zeta potential.

4.5.1. MATH

The cell surface hydrophobicity (CSH) of the tested bacterial strains was conducted in the presence of two different sources of carbon and energy: sodium succinate (terrestrial and marine strains TK-46(2) and TK-105) or sodium pyruvate (marine strains TK-8 and TG408) and diesel oil (both marine and terrestrial strains).

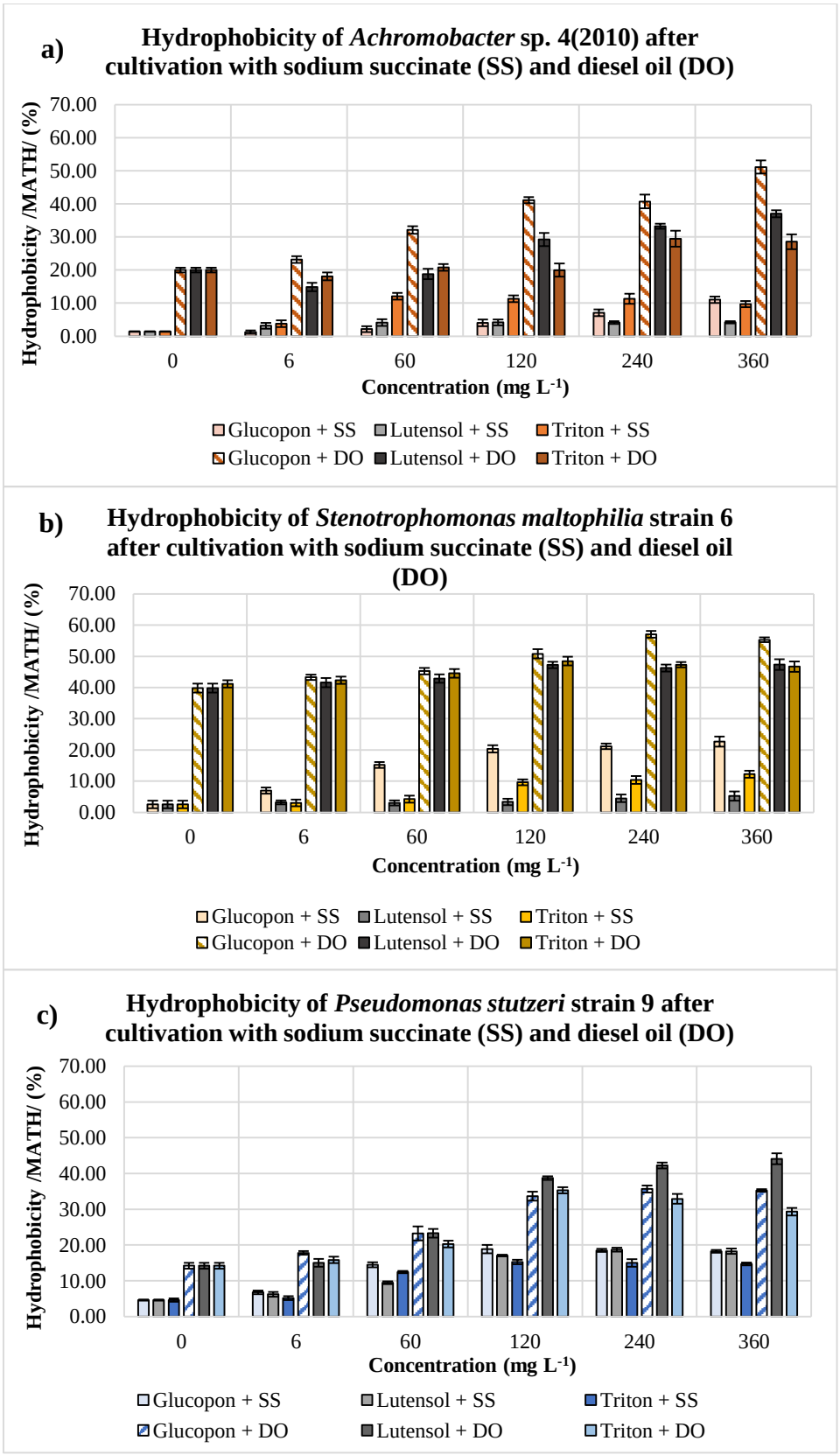
As seen in Fig. 36a, the highest hydrophobicity of *Achromobacter* sp. 4(2010) – equal to 51.11 % – was detected in the presence of diesel oil together with Glucopon 215 at the

concentration 360 mg L^{-1}. The initial hydrophobicity (no surfactants) was only 1.42 % after the incubation with sodium succinate and 20.01 % in the presence of diesel oil. The addition of surfactants to the systems with diesel oil had a significant influence on the increase of the hydrophobicity, especially for the surfactants' concentrations between 120 and 360 mg L^{-1}.

Stenotrophomonas maltophilia strain 6 (Fig. 36b) was characterised with very high hydrophobicity that was equal to 39.83 %, in the presence of diesel oil (and without surfactants) in comparison to the system with sodium succinate (hydrophobicity reaching only 2.35 %). When supplemented with surfactants, the hydrophobicity of the bacterial cells from the diesel oil system increased, reaching a maximum as high as 57.03 % in the presence of Glucopon 215 at 240 mg L^{-1}. The differences between the results observed for Lutensol GD 70 and Triton X-100 were hardly noticeable and statistically insignificant ($P > 0.05$).

After a 7-day incubation with diesel oil, the hydrophobicity of *Pseudomonas stutzeri* strain 9 (Fig. 36c) was nearly 10 % higher than the result obtained for the incubation with sodium succinate (14.20 % and 4.58 % respectively). In the presence of surfactants, the highest hydrophobicity (44.09 %) was observed for the system "bacteria-diesel oil-Lutensol GD 70" at Lutensol's concentration equal to 360 mg L^{-1}. What is interesting, the addition of surfactants also led to the increase of the cell surface hydrophobicity in the systems with sodium succinate, however, the results did not exceed 20 %.

Unlike the three abovementioned terrestrial strains, *Rahnella* EK12 was characterised by a significant increase of hydrophobicity in the presence of Triton X-100, with the highest result (44.92 %) observed for the surfactant's concentration equal to 360 mg L^{-1}. In comparison, neither Glucopon 215 nor Lutensol GD 70 led to CSH greater than 28 %.


a)
Hydrophobicity of *Achromobacter* sp. 4(2010) after cultivation with sodium succinate (SS) and diesel oil (DO)
Hydrophobicity /MATH/ (%)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
6
60
120
240
360
Concentration (mg L-1)
Glucopon + SS
Lutensol + SS
Triton + SS
Glucopon + DO
Lutensol + DO
Triton + DO
b)
Hydrophobicity of *Stenotrophomonas maltophilia* strain 6 after cultivation with sodium succinate (SS) and diesel oil (DO)
Hydrophobicity /MATH/ (%)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
6
60
120
240
360
Concentration (mg L-1)
Glucopon + SS
Lutensol + SS
Triton + SS
Glucopon + DO
Lutensol + DO
Triton + DO
c)
Hydrophobicity of *Pseudomonas stutzeri* strain 9 after cultivation with sodium succinate (SS) and diesel oil (DO)
Hydrophobicity /MATH/ (%)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
6
60
120
240
360
Concentration (mg L-1)
Glucopon + SS
Lutensol + SS
Triton + SS
Glucopon + DO
Lutensol + DO
Triton + DO

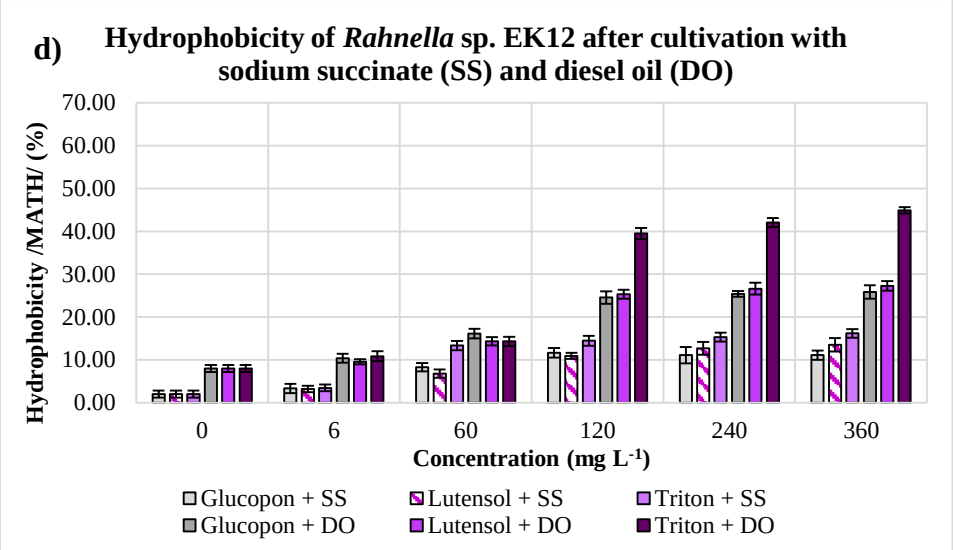


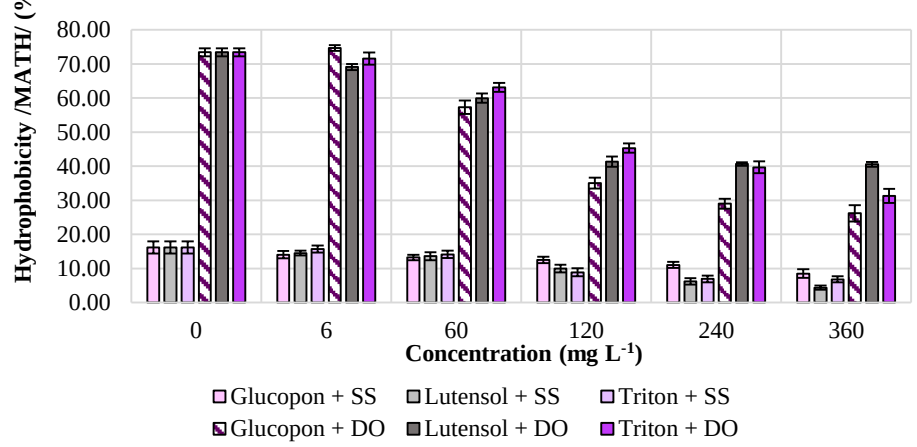
Figure 36: Hydrophobicity of *Achromobacter* sp. 4(2010) (a), *Stenotrophomonas maltophilia* strain 6 (b), *Pseudomonas stutzeri* strain 9 (c), *Rahnella* sp. EK12 (d) in grown separately with sodium succinate (SS) and diesel oil (DO).

Among the marine strains (Fig. 37), the highest CSH was observed for *Alteromonas* sp. TK-46(2) in the system "bacteria-diesel oil" – 73.44 %. The addition of surfactants resulted in the overall decrease of the hydrophobicity, especially in the case of Glucopon 215, where at surfactant's concentration of 360 mg L^{-1} the CSH was equal to 26.18 %. The initial hydrophobicity of the strain (system with sodium succinate only) was high compared to the terrestrial strains.

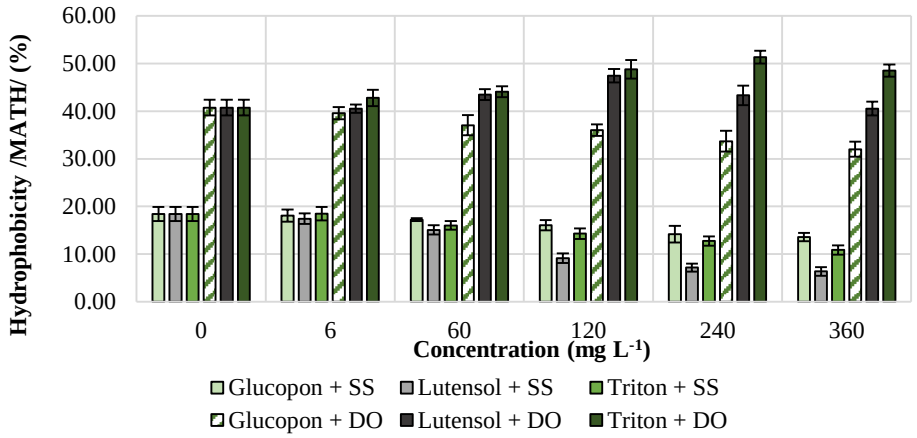
Another strain that was characterised with a very high hydrophobicity in the "bacteria-diesel oil" system was *Polycyclovorans algicola* TG408, reaching 65.87 % (Fig. 37d). The addition of surfactants to this system resulted in a gradual decrease in the hydrophobicity. This effect was, however, opposite to the influence that the two alkyl polyglucoside-based surfactants had on the CSH in the system "bacteria-sodium pyruvate", where the cellular hydrophobicity rose from 14.21 % to the final 34.31 % and 23.79 % for Glucopon 215 and Lutensol GD 70 at 360 mg L^{-1} respectively. In the case of Triton X-100 the hydrophobicity decreased from initial 14.21 % to 10.73 % at 360 mg L^{-1}.

The highest CSH results of *Pseudoalteromonas* sp. TK-105 were recorded for the "bacteria-diesel oil" system in the presence of Triton X-100 at 240 mg L^{-1} and was equal to 51.31 %, while Glucopon 215 resulted in the decrease in hydrophobicity, finally reaching 32.01 % at 360 mg L^{-1}. However, when the surfactants were added to the system "bacteria-sodium succinate", a decrease in CSH was observed, with the lowest value (6.37 %) observed for Lutensol GD 70 at 360 mg L^{-1}.

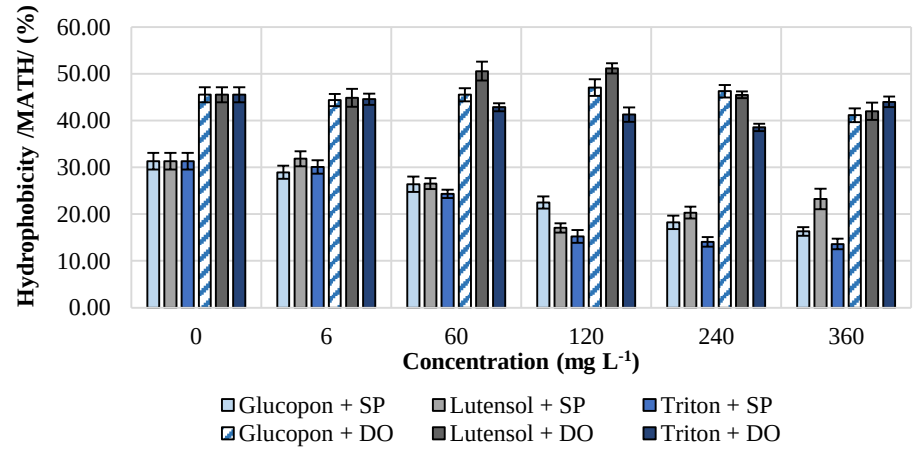
a)


Hydrophobicity of *Alteromonas* sp. TK-46(2) after cultivation with sodium succinate (SS) and diesel oil (DO)
Hydrophobicity /MATH/ (%)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
6
60
120
240
360
Concentration (mg L-1)
Glucopon + SS
Lutensol + SS
Triton + SS
Glucopon + DO
Lutensol + DO
Triton + DO

b)


Hydrophobicity of *Pseudoalteromonas* sp. TK-105 after cultivation with sodium succinate (SS) and diesel oil (DO)
Hydrophobicity /MATH/ (%)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
6
60
120
240
360
Concentration (mg L-1)
Glucopon + SS
Lutensol + SS
Triton + SS
Glucopon + DO
Lutensol + DO
Triton + DO

c)


Hydrophobicity of *Cycloclasticus* sp. TK-8 after cultivation with sodium pyruvate (SP) and diesel oil (DO)
Hydrophobicity /MATH/ (%)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
6
60
120
240
360
Concentration (mg L-1)
Glucopon + SP
Lutensol + SP
Triton + SP
Glucopon + DO
Lutensol + DO
Triton + DO

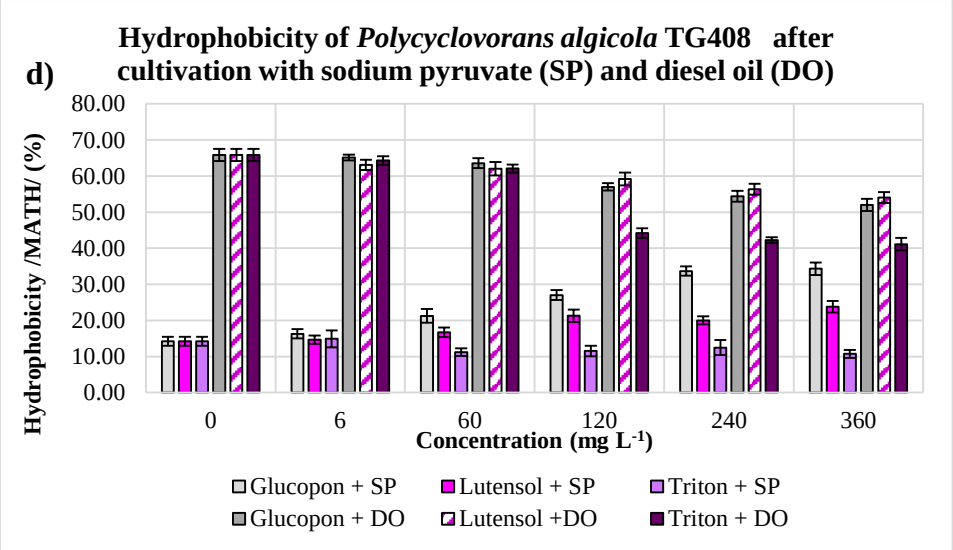


Figure 37: Hydrophobicity of *Alteromonas* sp. TK-46(2) (a), *Pseudoalteromonas* sp. TK-105 (b), *Cycloclasticus* sp. TK-8 (c), *Polycyclovorans algicola* TG408 (d) grown separately with sodium succinate (SS) or sodium pyruvate (SP) and diesel oil (DO).

Cell surface hydrophobicity of bacterial cells is considered to be the most important parameter in the mechanism of bacterial adhesion to the surfaces (Chakraborty et al., 2010). It is also thought to play an important role in the biodegradation of hydrocarbons, where the direct contact between the cell and hydrocarbon (oil) droplets is a driving force in the uptake and biodegradation, although a direct, linear correlation between the hydrophobicity and biodegradation is rarely observed. In other words, high CSH does not always mean that the hydrophobic substrate will be degraded (Chakraborty et al., 2010).

Bacterial CSH is also considered as a dynamic parameter, as there are factors, which may have a significant influence on it, such as surfactants, other hydrophobic compounds etc. (Kaczorek et al., 2013a). For example, according to Vadillo-Rodríguez et al. (2004) the adhesion of *Lactobacillus* strains does not always progress as expected based on the hydrophobicity measurements. The authors also suggested that the changes in the bacterial cell surface hydrophobicity and the adhesion of microorganisms to the various surfaces can be considered as the response of the cells to the changes in the parameters such as pH or ionic strength.

Surfactants, as already mentioned, are considered as the parameters influencing the CSH. The direct interaction between the bacterial cells and surface active agents may depend on various aspects, such as a type of a surfactant and microorganism as well as the hydrophobic substrate (Mohanty and Mukherji, 2007).

The role of surfactant in enhancing the biodegradation of hydrocarbons is based on increasing the contact area between the cell surface and a hydrocarbon molecule. In some cases however, the use of surfactants may limit or totally inhibit the biodegradation of the hydrocarbons (see chapter 4.5.2), especially above their CMC (Li and Chen, 2009; Wang, 2011). This however cannot be considered as a rule as it depends on many factors, which need to be considered throughout the research.

Due to the fact that the hydrophobicity and biodegradation of the HOCs turned out to be related with each other based on the research conducted within this Ph.D. thesis, a following chapter (4.5.2.) presents the correlations between the abovementioned parameters in the presence of surfactants at concentrations varying from 0 to 360 mg L^{-1}.

4.5.2. Correlation between CSH and hydrocarbon biodegradation

Biodegradation of hydrophobic organic compounds (HOC) in the environment is very often limited due to the minimal or complete lack of water solubility of these compounds. Therefore, their bioavailability is one of the most important factors in the biodegradation process, as the contact of a microorganism with a hydrophobic substrate is a crucial step for its degradation.

The microorganisms that are characterised with a hydrophobic properties of their cell surface are favourable for the hydrocarbon biodegradation because the direct contact between the substrate and microorganism is achievable and therefore the degradation of the compound is expected to be possible. However, bacterial strains with their natural low hydrophobicity can also successfully metabolise the HOCs. This is possible by either addition of surfactants or an indirect modification of the CSH (Kaczorek et al., 2013b).

The following Figures 38-40 show the correlations between the biodegradation of diesel oil and naphthalene and the CSH of the terrestrial and marine strains. The strains, for which the coefficient of determination (R^2) was smaller than 0.5 ($R^2 \leq 0.5$) are not included.

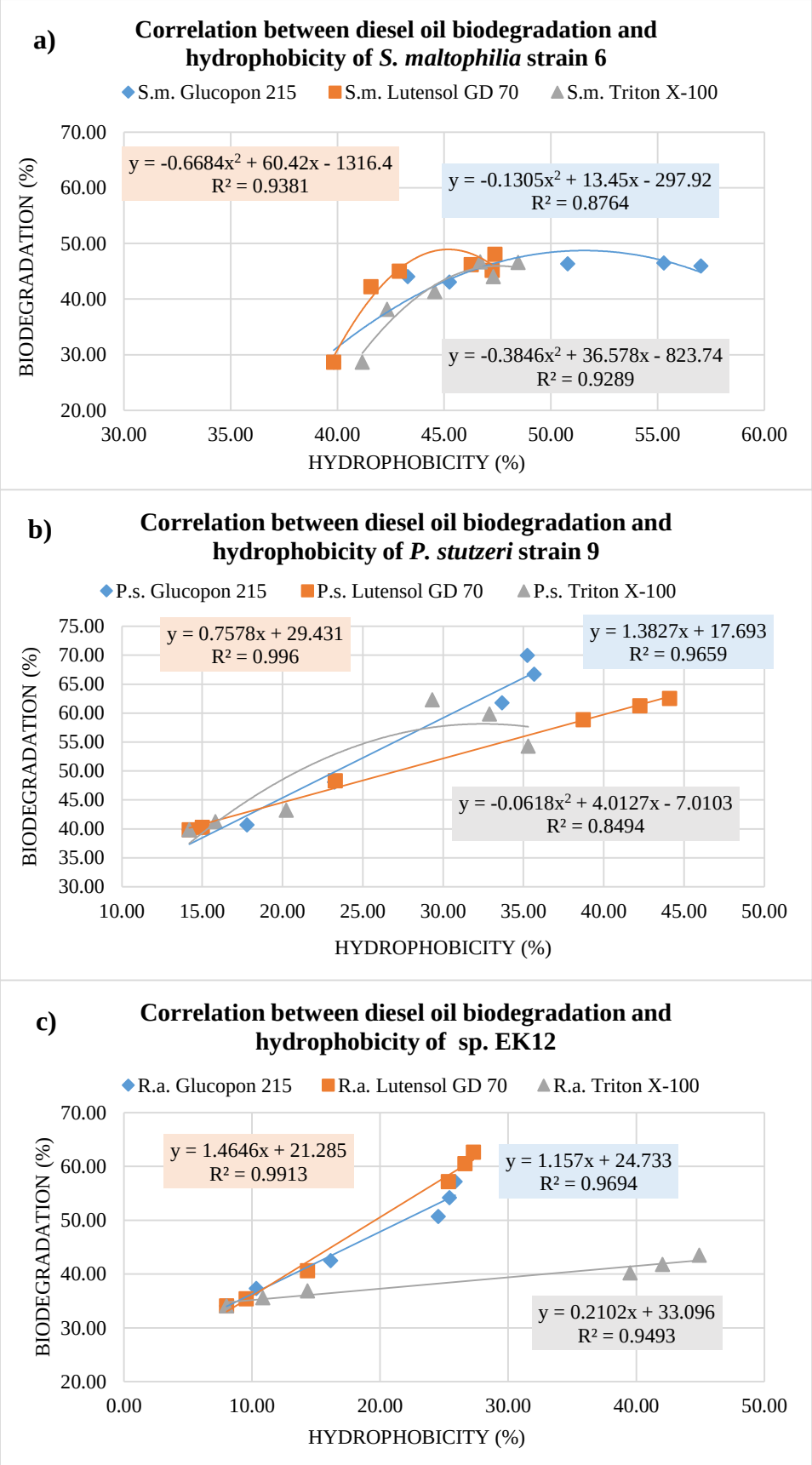
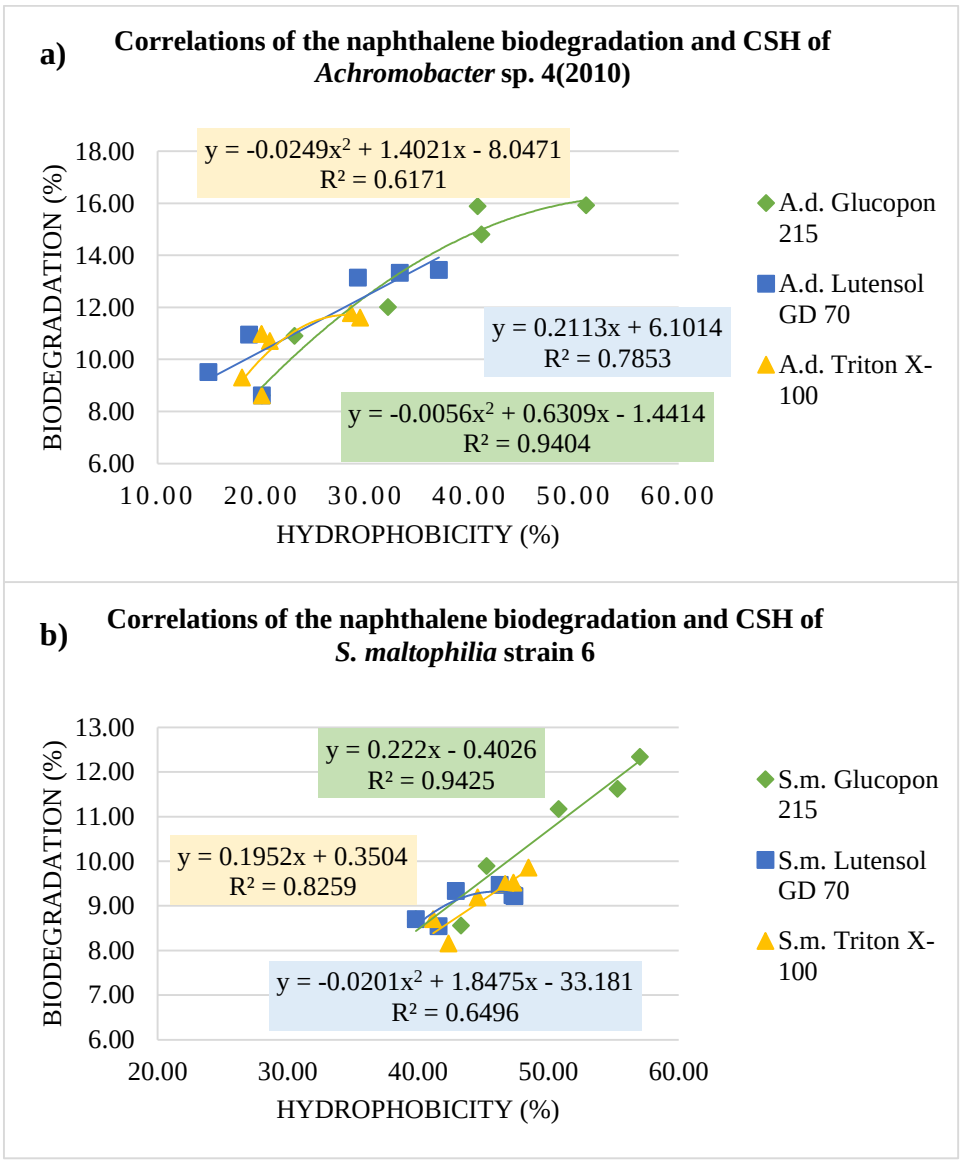


Figure 38:The correlations of diesel oil biodegradation and cell surface hydrophobicity of *S. maltophilia* strain 6 (a), *P. stutzeri* strain 9 (b) and *Rahnella* sp. EK12 (c).

As it is shown in the Fig. 38, the determination of correlations between the cell surface hydrophobicity and biodegradation of diesel oil was possible for three out of four terrestrial strains. In the case of *Stenotrophomonas maltophilia* strain 6, the relationship between the two abovementioned parameters was a polynomial function for all tested surfactants. On the contrary, the functions characterising correlations for *Rahnella* sp. EK12 were in all three cases linear. For *Pseudomonas stutzeri* strain 9 the functions connecting diesel oil biodegradation and CSH in the presence of Glucopon 215 and Lutensol GD 70 were linear, however, in the presence of Triton X-100 the function was polynomial.



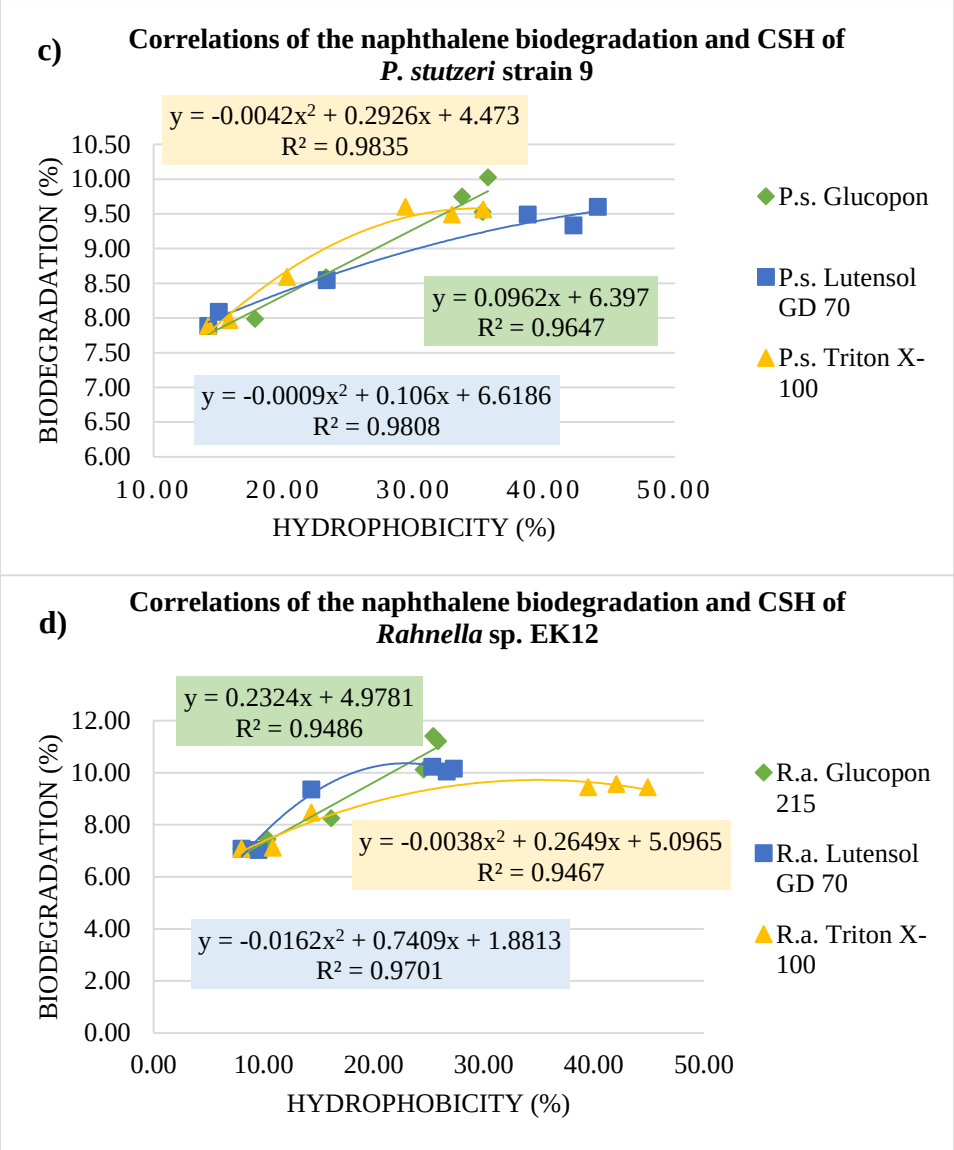
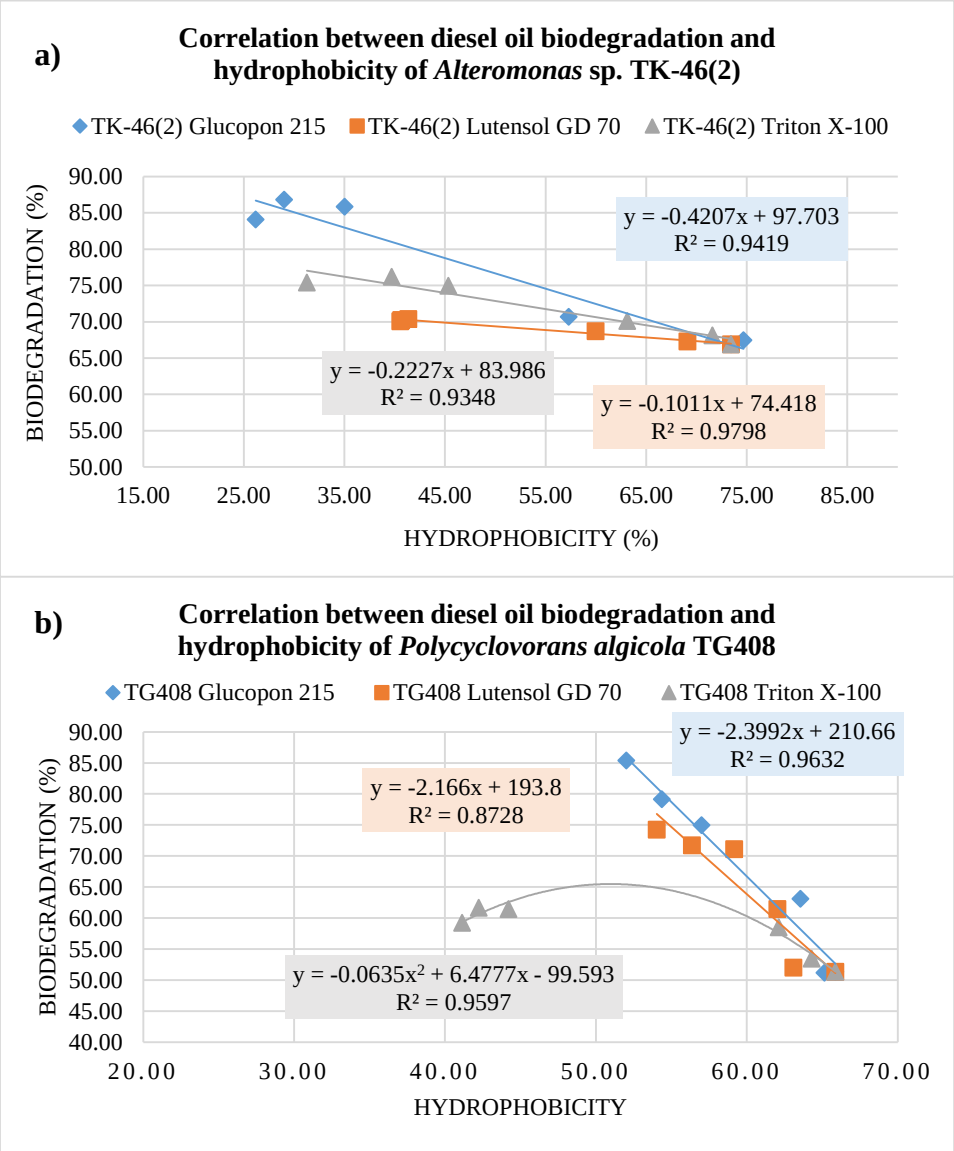


Figure 39: The correlations of the 7-day-biodegradation of naphthalene and cell surface hydrophobicity of *Achromobacter* sp. 4(2010) (a), *S. maltophilia* strain 6 (b), *P. stutzeri* strain 9 (c) and *Rahnella* sp. EK12 (d).

The correlations between the 7-day-biodegradation of naphthalene and cell surface hydrophobicity of the terrestrial strains were possible to establish. The functions, likewise for diesel oil, had mixed forms, which means that within one strain both linear and polynomial forms of functions were possible. The highest coefficients of determination were found for *P. stutzeri* strain 9 and *Rahnella* sp. EK12 strains ($R^2 \geq 0.94$), which means that in the case of these two strains the relation between naphthalene degradation and CSH is very strong.

Fig. 40 presents the similar correlations obtained for the marine strains. In this case the graphs for diesel oil and naphthalene biodegradation vs CSH are presented in one figure, as

the correlations for *Pseudoalteromonas* sp. TK-105 and *Cycloclasticus* sp. TK-8 were not found.



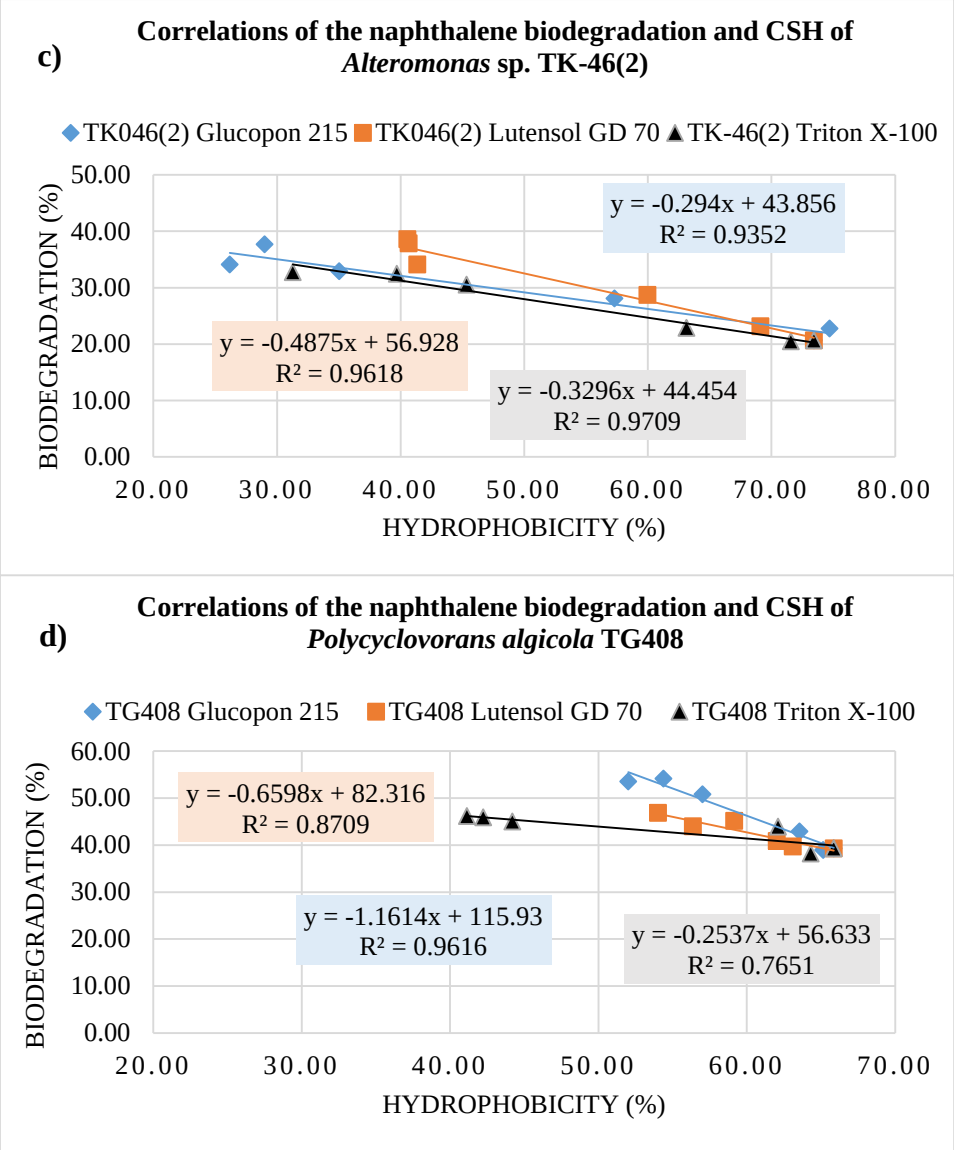


Figure 40: The correlations between the diesel oil biodegradation and cell surface hydrophobicity of *Alteromonas* sp. TK-46(2) (a) and *Polycyclovorans algicola* TG408 (b) as well as the correlations for the 7-day-biodegradation of naphthalene and cell surface hydrophobicity od *Alteromonas* sp. TK-46(2) (c) and *Polycyclovorans algicola* TG408 (d).

Like already mentioned, in the case of marine strains, the correlations of hydrocarbon biodegradation and cell surface hydrophobicity were only found for two, out of four strains.

The coefficients of determination for *Alteromonas* sp. TK-46(2) were found to be high ($R^2 \geq 0.93$) for both diesel oil and naphthalene biodegradation systems in the presence of all three surfactants and the functions were linear.

In the case of diesel oil biodegradation by *Polycyclovorans algicola* TG408 coefficient of determination was the lowest in the presence of Lutensol GD 70 ($R^2 = 0.8728$) for the linear

function. The polynomial function was found for the system with Triton X-100, where R^2 was equal to 0.9597.

The correlations observed for the biodegradation of naphthalene had, in all cases, linear functions and the highest coefficients of determination ($R^2 \geq 0.93$) were recorded for *Alteromonas* sp. TK-46(2).

Although for most of the tested strains a direct relationship between the hydrophobicity and HOC degradation could be observed in the presence of surfactants, it should not be taken as a rule. Hydrophobicity, as already mentioned is a dynamic parameter and strongly depends on many factors. Churchill et al. (1995) tested the influence of three non-ionic surfactants: Triton X-45, Triton X-100 and Triton X-165 on the solubilisation and biodegradation of phenanthrene by three bacterial strains: two *Pseudomonas saccharophilia* and *Pseudomonas putida.* The tested surfactants had a positive influence on the biodegradation of phenanthrene by the two *P. saccharophilia* strains by increasing the rates of degradation. However, the same surfactants totally inhibited the biodegradation of phenanthrene by *P. putida*.

Guha and Jaffé (1996) observed an inhibitory effect of the non-ionic surfactant Tergitol NP-10 on the phenanthrene biodegradation by a mixed bacterial culture (with a proven ability to degrade PAH). The same surfactant was reported by Grimberg et al. (1996) to increase the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16.

Boonchan et al. (1998) reported the influence of Tergitol NP-10 on crystalline and DMF-solubilised pyrene, revealing that the surfactant slightly enhanced biodegradation of the crystalline hydrocarbon and significantly increased the degradation of a solubilised pyrene. This research proved that the same surfactant may have different effect on the biodegradation of different forms of a hydrocarbon.

One of the possible explanations of such phenomena is the almost complete micellization of the hydrocarbon molecules by surfactant, making the hydrocarbon difficult to access by microbial cells. A similar conclusion was drawn by Li and Chen (2009), who studied the biodegradation of phenanthrene by a bacterial strain *Neptunomonas naphthovorans*, in the presence of three non-ionic surfactants (Tergitol 15-S-7, Tergitol 15-S-9 and Tergitol 15-S-12) at and above their CMC. The obtained results showed that the addition of surfactants at concentrations above their CMC resulted in a reduced biodegradation of phenanthrene due to lower availability of the hydrocarbon in a micellar phase.

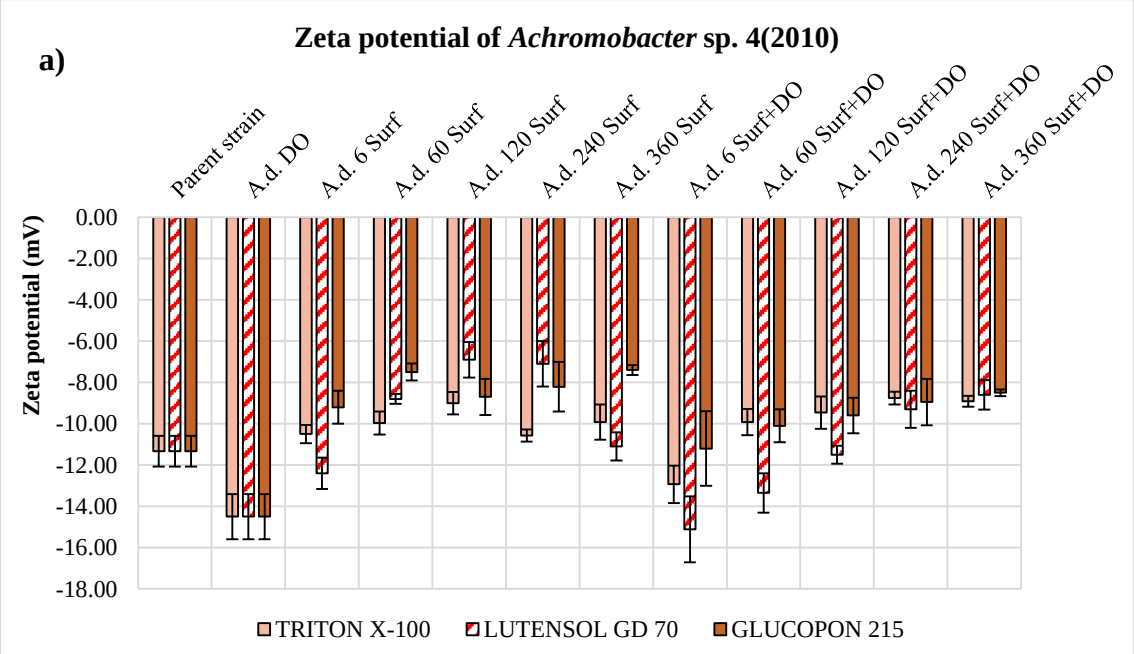
However, the results obtained within this thesis show that the increased concentrations (also above of the surfactants' CMCs) of Glucopon 215, Lutensol GD 70 and Triton X-100 did not have any inhibitory effect neither on biodegradation of diesel oil, nor on naphthalene biodegradation.

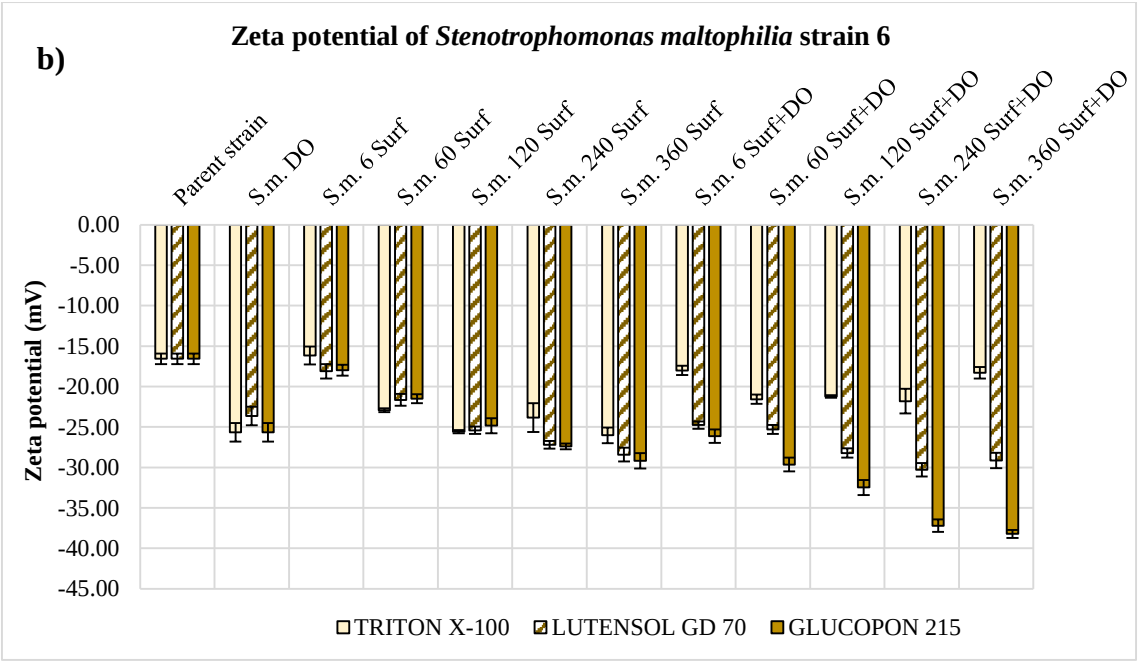
Wang (2011) suggested that the biodegradation of hydrocarbons "hidden" in a micelle is a dynamic and complex process and there is no direct method that could allow for the determination those dynamic conditions and their overall influence on the bioavailability of a hydrocarbon in a micelle.

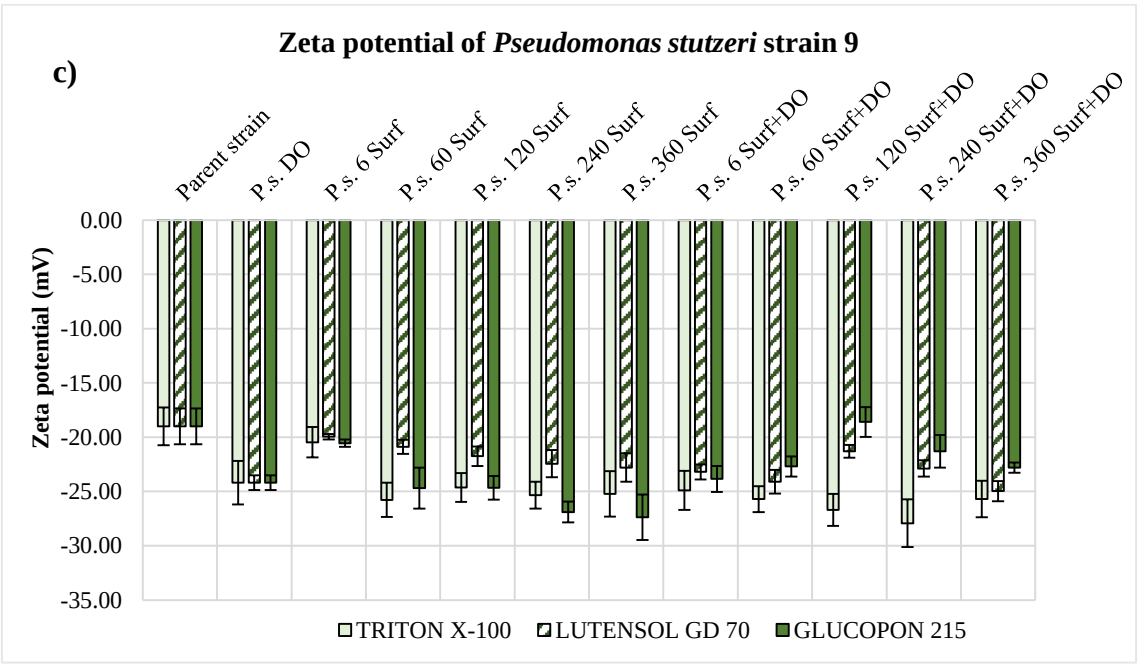
Broadly speaking, the influence of surfactants on the hydrophobicity and therefore biodegradation of hydrophobic organic compounds is a very individual aspect and depends on many abovementioned factors.

4.5.3. Zeta potential

The results of the zeta potential for terrestrial (Fig. 41) and marine strains (Fig. 42) are presented below.




b)
Zeta potential of *Stenotrophomonas maltophilia* strain 6
Parent strain
S.m. DO
S.m. 6 Surf
S.m. 60 Surf
S.m. 120 Surf
S.m. 240 Surf
S.m. 360 Surf
S.m. 6 Surf+DO
S.m. 60 Surf+DO
S.m. 120 Surf+DO
S.m. 240 Surf+DO
S.m. 360 Surf+DO
Zeta potential (mV)
0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
-40.00
-45.00
TRITON X-100
LUTENSOL GD 70
GLUCOPON 215


c)
Zeta potential of *Pseudomonas stutzeri* strain 9
Parent strain
P.s. DO
P.s. 6 Surf
P.s. 60 Surf
P.s. 120 Surf
P.s. 240 Surf
P.s. 360 Surf
P.s. 6 Surf+DO
P.s. 60 Surf+DO
P.s. 120 Surf+DO
P.s. 240 Surf+DO
P.s. 360 Surf+DO
Zeta potential (mV)
0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
TRITON X-100
LUTENSOL GD 70
GLUCOPON 215

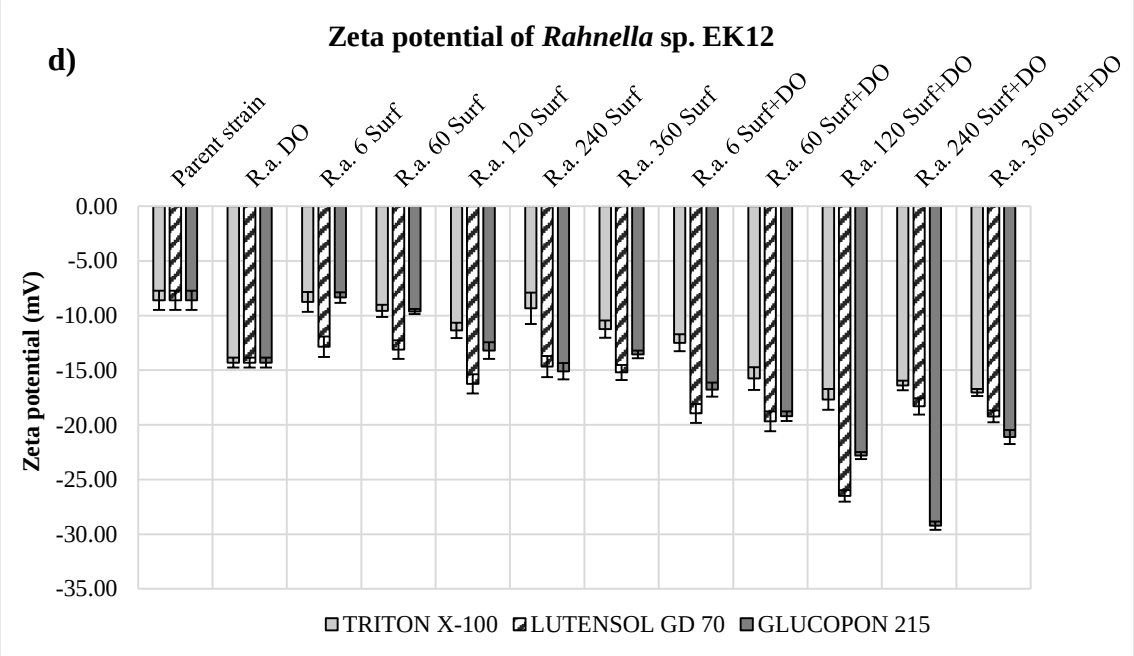


Figure 41: Zeta potential of *Achromobacter* sp. 4(2010) (a), *Stenotrophomonas maltophilia* strain 6 (b), *Pseudomonas stutzeri* strain 9 (c), *Rahnella* sp. EK12 (d).

As seen in Fig. 41, among the terrestrial strains the lowest zeta potential values were observed for *Stenotrophomonas maltophilia* strain 6, with the minimal result (-38.21 mV) obtained for the system "bacterial strain-Glucopon 215-diesel oil" at the surfactant's concentration as high as 360 mg L^{-1}. What is interesting, Jucker et al. (1996) reported the clinical isolate – *Stenotrophomonas maltophilia* 70401, which was observed to have a positive zeta potential at physiological pH. This strain, however, was isolated in from a patient with a suspected urinary infection. That means that, although belonging to the same genus, the *S. maltophilia* 70401 reported by Jucker et al. and isolated from a human body and *S. maltophilia* strain 6, isolated from the petroleum contaminated soil possessed two, very different features.

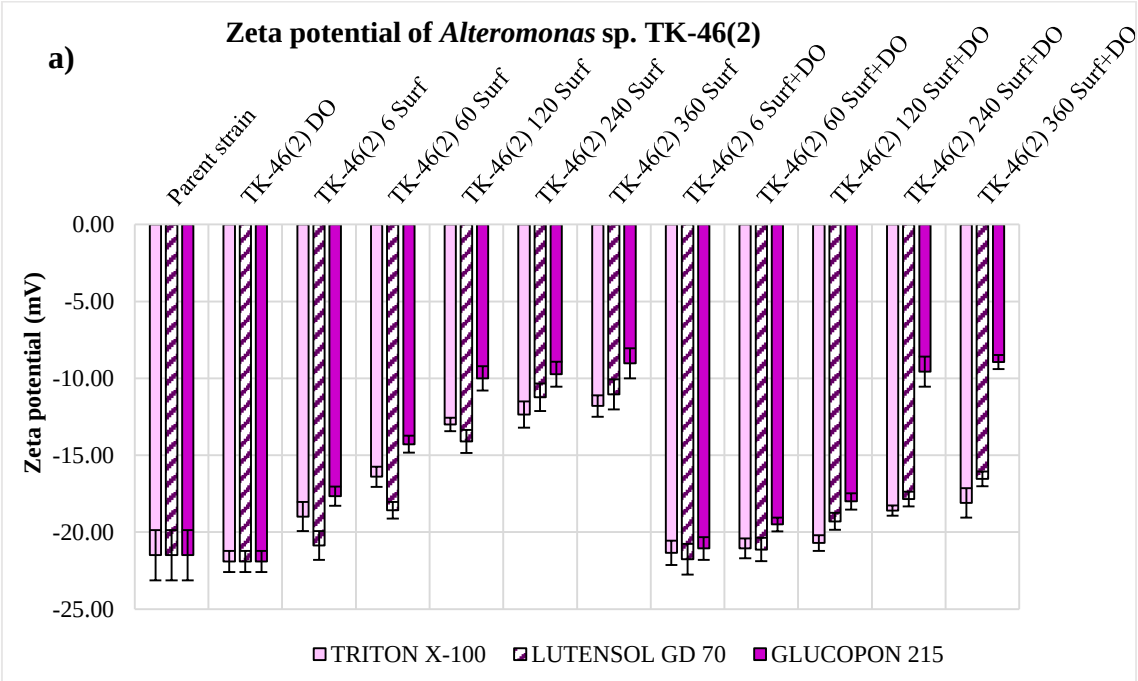
In contrast, the highest zeta potential was seen for *Rahnella* sp. EK12 (Fig. 41d) and was equal to -8.60 (mV) for the parent strain (strain grown in standard conditions, with sodium succinate, without diesel oil and surfactants).

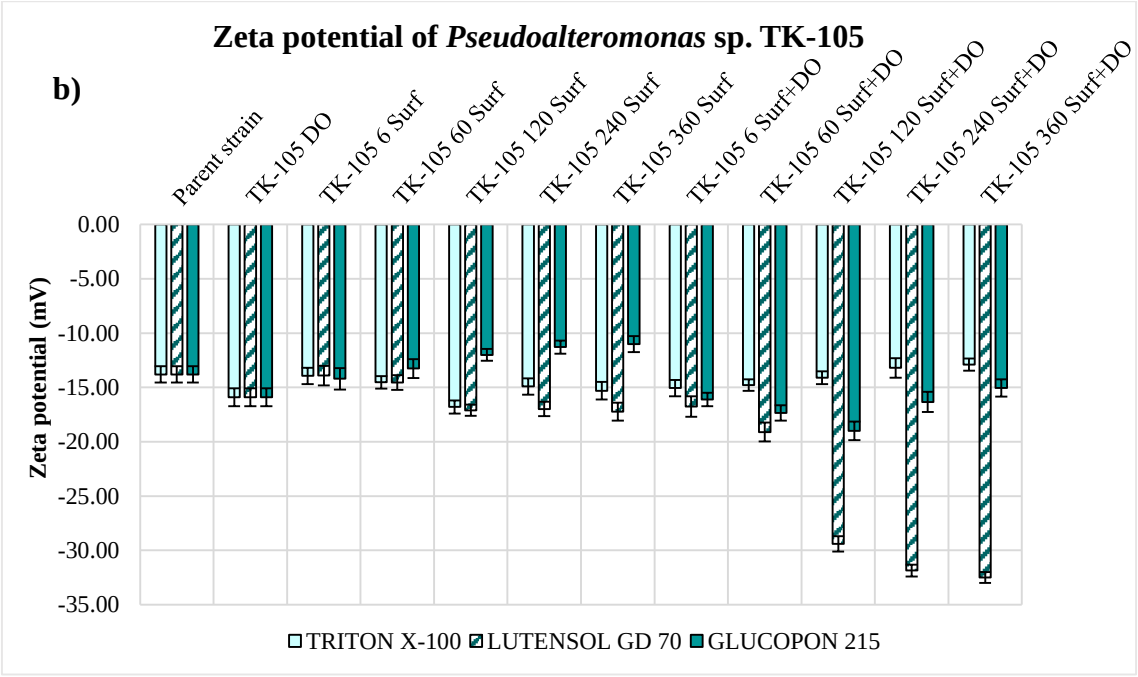
The addition of surfactants in their increasing concentrations resulted in the overall decrease in zeta potential.

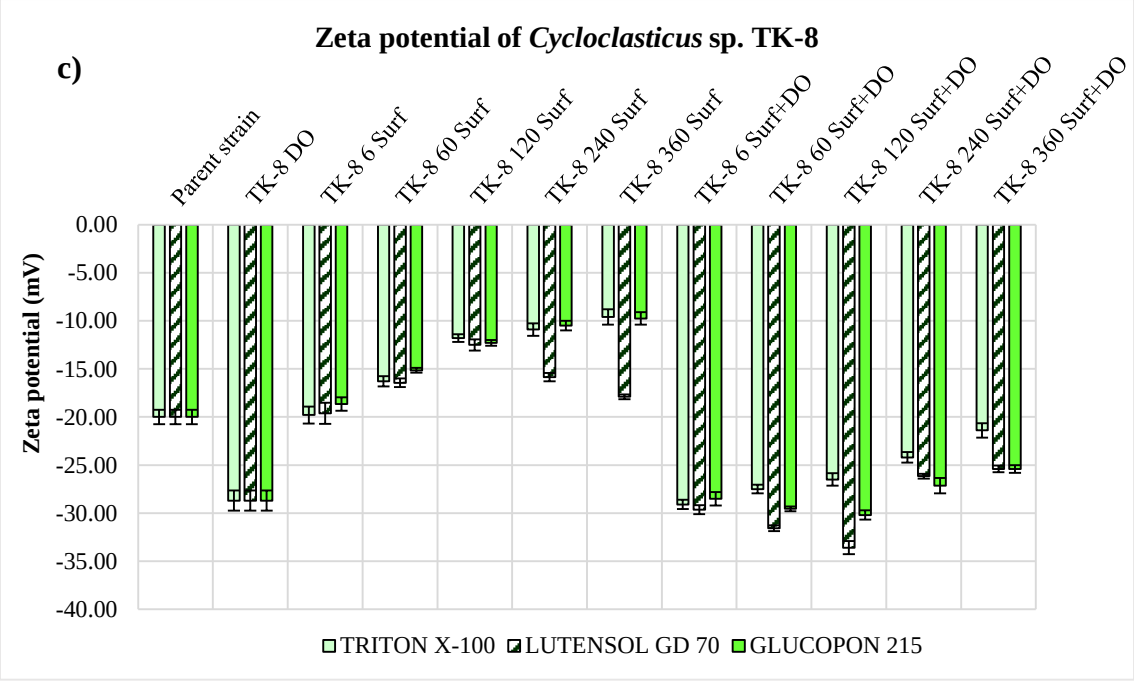
The zeta potential estimated for the marine strains is shown in Fig. 42.

The lowest results were observed for *Polycyclovorans algicola* TG408 (Fig. 42d) in the system with diesel oil and the addition of surfactants increased the values only slightly.

The highest values of zeta potential were found for *Pseudoalteromonas* sp. TK-105 (-9.02 mV) in the system: “bacteria-Glucopon 215”, the results however, decreased in the presence of diesel oil.







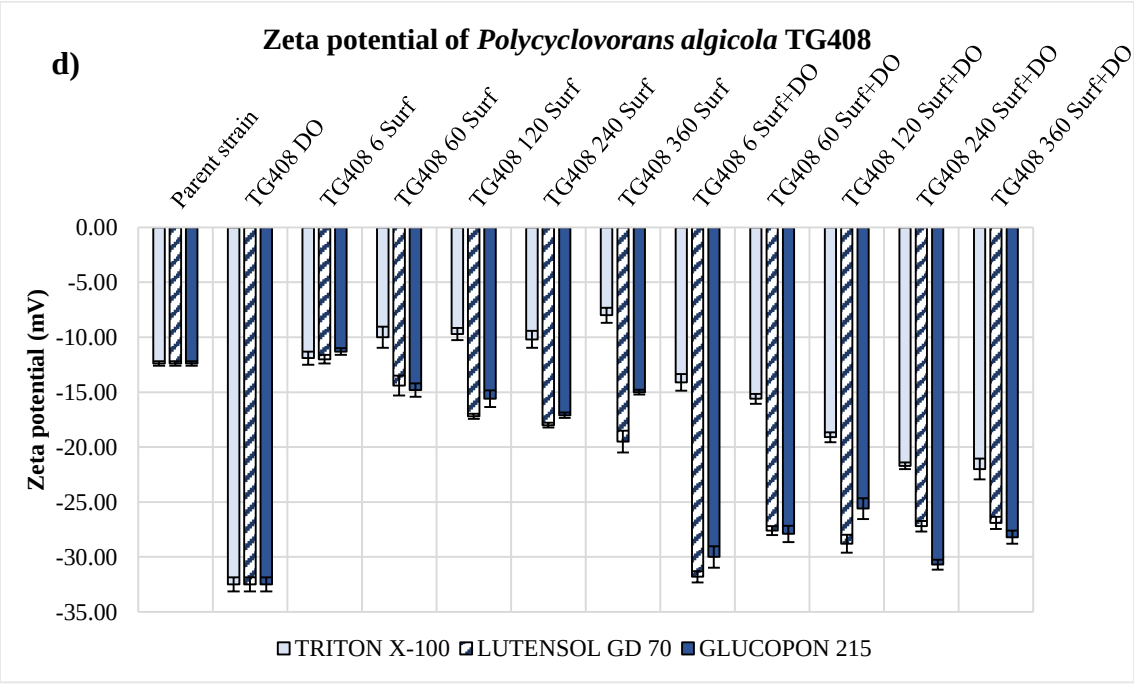


Figure 42: Zeta potential of *Alteromonas* sp. TK-46(2) (a), *Pseudoalteromonas* sp. TK-105 (b), *Cycloclasticus* sp. TK-8 (c), *Polycyclovorans algicola* TG408 (d).

Likewise for terrestrial strains, the values of the zeta potential were varied and strongly depended on the bacterial strain used in the analyses, which draws a conclusion that the surface charge might be a very individual feature of the microorganism and its origin.

According to Palmer et al. (2007) and Jucker et al. (1996), a possible explanation for such phenomenon is that the bacterial cell surface charge depends on the dissociation of acidic

groups such as carboxyl, phosphate and amino groups along with alkaline groups found on the cell surface.

Although many researchers link the zeta potential with hydrophobicity (Abbasnezhad et al., 2011, 2008; Busscher et al., 1995; Jucker et al., 1996; Palmer et al., 2007) and therefore biodegradation of hydrocarbons, the results obtained within the research for this Thesis revealed that the good correlations between the abovementioned parameters were only found for a few strains and systems. It is also worth noticing, that the correlations had mostly of 2^{nd} and 3^{rd} degree-polynomial functions.

The following tables present the estimated correlations between zeta potential and cell surface hydrophobicity as well as zeta potential vs. biodegradation of diesel oil by marine and terrestrial strains.

Table 10: The correlations between zeta potential and CSH of the tested strains when cultivated without diesel oil.

Strain	System	Equation	R^2
***Achromobacter* sp. 4(2010)**	A.d.+Glucopon 215	$y = -0.0233x^2 + 0.4995x - 10.207$	$R^2 = 0.3769$
	A.d.+Lutensol GD 70	$y = 1.5286x^2 - 7.4576x - 3.862$	$R^2 = 0.5315$
	A.d.+Triton X-100	$y = -0.0212x^2 + 0.4158x - 11.84$	$R^2 = 0.5881$
***S. maltophilia* strain 6**	**S.m.+Glucopon 215**	$\mathbf{y = -0.0296x^2 + 0.1598x - 17.096}$	$\mathbf{R^2 = 0.9818}$
	S.m.+Lutensol GD 70	$y = 1.691x^2 - 17.455x + 16.745$	$R^2 = 0.8206$
	S.m.+Triton X-100	$y = 0.16x^2 - 3.2001x - 9.662$	$R^2 = 0.8538$
***P. stutzeri* strain 9**	**P.s.+Glucopon 215**	$\mathbf{y = 0.0218x^2 - 1.0259x - 14.685}$	$\mathbf{R^2 = 0.9178}$
	P.s.+Lutensol GD 70	$y = 0.011x^2 - 0.4868x - 17.179$	$R^2 = 0.9533$
	P.s.+Triton X-100	$\mathbf{y = 0.1129x^2 - 2.7652x - 8.9495}$	$\mathbf{R^2 = 0.9929}$
***Rahnella* sp. EK12**	R.a.+Glucopon 215	$y = -0.112x^2 + 0.9304x - 10.074$	$R^2 = 0.8996$
	R.a.+Lutensol GD 70	$y = 0.0723x^2 - 1.5871x - 6.7593$	$R^2 = 0.8245$
	R.a.+Triton X-100	$y = -0.0147x^2 + 0.1153x - 8.857$	$R^2 = 0.598$
***Alteromonas* sp. TK-46(2)**	TK-46(2)+Glucopon 215	$y = -0.248x^2 + 4.3594x - 27.847$	$R^2 = 0.921$
	TK-46(2)+Lutensol GD 70	$\mathbf{y = -0.049x^2 + 0.031x - 9.9036}$	$\mathbf{R^2 = 0.9814}$
	TK-46(2)+Triton X-100	$\mathbf{y = -0.1216x^2 + 1.9149x - 19.695}$	$\mathbf{R^2 = 0.9691}$
***Pseudoalteromonas* sp. TK-105**	**TK-105+Glucopon 215**	$\mathbf{y = -0.0607x^2 + 1.2915x - 17.339}$	$\mathbf{R^2 = 0.9595}$
	TK-105+Lutensol GD 70	$\mathbf{y = 0.0076x^2 + 0.1198x - 18.396}$	$\mathbf{R^2 = 0.9732}$
	TK-105+Triton X-100	$y = 0.0768x^2 - 2.0736x - 1.701$	$R^2 = 0.6063$
***Cycloclasticus* sp. TK-8**	**TK-8+Glucopon 215**	$\mathbf{y = -0.0298x^2 + 0.7075x - 13.373}$	$\mathbf{R^2 = 0.9892}$
	TK-8+Lutensol GD 70	$y = 0.0187x^2 - 1.3597x + 4.6854$	$R^2 = 0.8645$
	TK-8+Triton X-100	$\mathbf{y = 0.0045x^2 - 0.7609x - 0.7594}$	$\mathbf{R^2 = 0.9908}$
***Polycyclovorans algicola* TG408**	TG408+Glucopon 215	$y = 0.0135x^2 - 0.8738x - 1.8787$	$R^2 = 0.8002$
	TG408+Lutensol GD 70	$\mathbf{y = 0.034x^2 - 2.0607x + 10.426}$	$\mathbf{R^2 = 0.9627}$
	TG408+Triton X-100	$y = 0.1997x^2 - 5.9972x + 32.928$	$R^2 = 0.8915$

Table 11: The correlations between zeta potential and CSH in the presence of diesel oil.

Strain	System	Equation	R^2
***Achromobacter* sp. 4(2010)**	A.d.+Glucopon 215	$y = -0.0064x^2 + 0.6061x - 23.079$	$R^2 = 0.8701$
	A.d.+Lutensol GD 70	$y = 0.2988x - 19.686$	$R^2 = 0.9547$
	A.d.+Triton X-100	$y = -0.0546x^2 + 2.9704x - 48.988$	$R^2 = 0.4965$
***S. maltophilia* strain 6**	**S.m.+Glucopon 215**	$y = -0.0079x^2 + 0.0002x - 12.712$	$R^2 = 0.9571$
	S.m.+Lutensol GD 70	$y = -0.0769x^2 + 6.1343x - 147.5$	$R^2 = 0.7621$
	S.m.+Triton X-100	$y = -0.2602x^2 + 23.577x - 553.31$	$R^2 = 0.3336$
***P. stutzeri* strain 9**	P.s.+Glucopon 215	$y = -0.0082x^2 + 0.5739x - 31.094$	$R^2 = 0.5366$
	P.s.+Lutensol GD 70	$y = -0.0062x^2 + 0.3753x - 28.173$	$R^2 = 0.1747$
	P.s.+Triton X-100	$y = 0.0025x^2 - 0.2477x - 21.413$	$R^2 = 0.7565$
***Rahnella* sp. EK12**	R.a.+Glucopon 215	$y = 0.006x^2 - 0.7592x - 8.9362$	$R^2 = 0.7228$
	R.a.+Lutensol GD 70	$y = 0.062x^2 - 2.4669x + 0.8147$	$R^2 = 0.5148$
	R.a.+Triton X-100	$y = 0.0052x^2 - 0.3681x - 10.788$	$R^2 = 0.7313$
***Alteromonas* sp. TK-46(2)**	TK-46(2)+Glucopon 215	$y = 0.0084x^2 - 1.0799x + 13.044$	$R^2 = 0.8839$
	TK-46(2)+Lutensol GD 70	$y = 0.0041x^2 - 0.5845x - 0.7505$	$R^2 = 0.8665$
	TK-46(2)+Triton X-100	$y = 0.0019x^2 - 0.2853x - 10.941$	$R^2 = 0.8981$
***Pseudoalteromonas* sp. TK-105**	TK-105+Glucopon 215	$y = 0.1422x^2 - 10.406x + 172.37$	$R^2 = 0.769$
	TK-105+Lutensol GD 70	$y = -0.0032x^2 - 0.7992x + 15.771$	$R^2 = 0.1406$
	TK-105+Triton X-100	$y = -0.0166x^2 + 1.7865x - 61.116$	$R^2 = 0.8794$
***Cycloclasticus* sp. TK-8**	TK-8+Glucopon 215	$y = 0.0749x^2 - 7.2467x + 145.87$	$R^2 = 0.6527$
	TK-8+Lutensol GD 70	$y = -0.0128x^2 + 0.4088x - 20.278$	$R^2 = 0.8118$
	TK-8+Triton X-100	$y = -0.0552x^2 + 4.1946x - 104.28$	$R^2 = 0.1557$
***Polycyclovorans algicola* TG408**	TG408+Glucopon 215	$y = -0.0817x^2 + 9.5147x - 303.61$	$R^2 = 0.5042$
	TG408+Lutensol GD 70	$y = -0.0384x^2 + 4.1359x - 138.2$	$R^2 = 0.7738$
	TG408+Triton X-100	$y = -0.1401x^2 + 14.924x - 401.76$	$R^2 = 0.4967$

Table 12: The correlations between zeta potential and biodegradation of diesel oil.

Strain	System	Equation	R^2
***Achromobacter* sp. 4(2010)**	**A.d.+Glucopon 215**	***$y = -0.003x^2 + 0.4724x - 26.89$***	***$R^2 = 0.9953$***
	A.d.+Lutensol GD 70	*$y = -0.0085x^3 + 1.1459x^2 - 50.604x + 711.9$*	*$R^2 = 0.897$*
	A.d.+Triton X-100	*$y = -0.0094x^2 + 1.0377x - 38.599$*	*$R^2 = 0.5864$*
***S. maltophilia* strain 6**	S.m.+Glucopon 215	*$y = -0.1389x^2 + 9.8447x - 193.76$*	*$R^2 = 0.7306$*
	S.m.+Lutensol GD 70	*$y = 0.0074x^3 - 0.9282x^2 + 37.718x - 518.12$*	*$R^2 = 0.6726$*
	S.m.+Triton X-100	*$y = 0.0161x^3 - 1.8861x^2 + 72.397x - 931.46$*	*$R^2 = 0.8919$*
***P. stutzeri* strain 9**	**P.s.+Glucopon 215**	***$y = -0.0012x^3 + 0.187x^2 - 9.1136x + 119.89$***	***$R^2 = 0.9569$***
	P.s.+Lutensol GD 70	*$y = -0.0045x^3 + 0.6777x^2 - 33.511x + 520.16$*	*$R^2 = 0.8728$*
	P.s.+Triton X-100	*$y = 0.0005x^3 - 0.0563x^2 + 2.0433x - 45.348$*	*$R^2 = 0.7797$*
***Rahnella* sp. EK12**	R.a.+Glucopon 215	*$y = 0.0252x^2 - 2.7508x + 50.786$*	*$R^2 = 0.7273$*
	R.a.+Lutensol GD 70	*$y = 0.0417x^2 - 4.1689x + 78.6$*	*$R^2 = 0.6537$*
	R.a.+Triton X-100	*$y = -0.4018x - 0.06$*	*$R^2 = 0.6084$*
***Alteromonas* sp. TK-46(2)**	TK-46(2)+Glucopon 215	*$y = 0.4921x - 54.37$*	*$R^2 = 0.6671$*
	TK-46(2)+Lutensol GD 70	*$y = 0.2273x^2 - 29.997x + 967.51$*	*$R^2 = 0.7501$*
	TK-46(2)+Triton X-100	*$y = 0.0311x^2 - 4.1283x + 115.4$*	*$R^2 = 0.755$*
***Pseudoalteromonas* sp. TK-105**	TK-105+Glucopon 215	*$y = 0.0123x^2 - 1.511x + 27.525$*	*$R^2 = 0.8266$*
	TK-105+Lutensol GD 70	***$y = 0.0186x^2 - 2.9936x + 84.857$***	***$R^2 = 0.9749$***
	TK-105+Triton X-100	*$y = 0.0021x^2 - 0.0322x - 18.107$*	*$R^2 = 0.8296$*
***Cycloclasticus* sp. TK-8**	TK-8+Glucopon 215	*$y = 0.076x^2 - 3.7816x + 16.955$*	*$R^2 = 0.7853$*
	TK-8+Lutensol GD 70	*$y = 0.1489x^2 - 7.1598x + 54.36$*	*$R^2 = 0.6168$*
	TK-8+Triton X-100	***$y = 0.1119x^2 - 4.6068x + 18.454$***	***$R^2 = 0.9918$***
***Polycyclovorans algicola* TG408**	TG408+Glucopon 215	*$y = -0.0093x^2 + 1.3328x - 74.999$*	*$R^2 = 0.4728$*
	TG408+Lutensol GD 70	*$y = -0.0157x^2 + 2.1749x - 102.42$*	*$R^2 = 0.8918$*
	TG408+Triton X-100	***$y = 0.1258x^3 - 21.863x^2 + 1263.3x - 24292$***	***$R^2 = 0.9304$***

Only the equations with the high coefficients of determination ($R^2 \geq 0.91$), were written in bold as they indicate the best correlated results. In the case of terrestrial strains the best correlations between zeta potential and CSH were obtained for *Achromobacter* sp. 4(2010)

and *Stenotrophomonas maltophilia* strain 6 both in the presence and absence of diesel oil (Table 10 and 11).

The marine strains were observed to show good correlations between zeta potential and CSH only in the absence of diesel oil (Table 10). When the strains were incubated with diesel oil, hardly any correlations between the surface charge and hydrophobicity were observed.

The strong relationship between the zeta potential and biodegradation of diesel oil in the presence of surfactants was observed for *Pseudoalteromonas* sp. TK-105, *Cycloclasticus* sp. TK-8 and *Polycyclovorans* algicola TG408.

4.6. Enzymatic activity

The activity of the two types of oxygenases was estimated throughout this study – long chain alkane monooxygenase and catechol dioxygenase. Although hexadecane was used as a model long chain alkane substrate in all tests on alkane monooxygenases, it would be tricky to call those enzymes as hexadecane monooxygenases as no chromatographic or spectroscopic analyses were applied to prove or contradict it. Furthermore, long chain alkane monooxygenases are said to have a broad range of substrate selectivity and can catalyse the transformations of more than one long chain alkanes (Duetz et al., 2001; Li et al., 2002).
In the case of catechol dioxygenases – for all eight tested strains the catechol 2,3-dioxygenases were detected. However, the differences in the activities of the enzymes isolated from marine and terrestrials strains were high and turned out to be significant from the statistical point of view ($P < 0.05$).
The abbreviations used in the presented figures are as follows:

- A.d. – Achromobacter sp. 4(2010)
- S.m. – Stenotrophomonas maltophilia strain 6
- P.s. – Pseudomonas stutzeri strain 9
- R.a. – Rahnella sp. EK12
- TK-46(2) – Alteromonas sp. TK-46(2)
- TK-105 – Pseudoalteromonas sp. TK-105
- TK-8 – Cycloclasticus sp. TK-8
- TG408 – Polycyclovorans algicola TG408
- DO – diesel oil
- G215 – Glucopon 215

- L – Lutensol GD 70
- T – Triton X-100

4.6.1. Long chain alkane monooxygenase

The activity of long chain alkane monooxygenases of *Achromobacter* sp. 4(2010) was not detected when the strain was grown on sodium succinate, however, when incubated with diesel oil, the activity reached 0.989 U mg^{-1}. The presence of surfactants in the system without diesel oil, monooxygenase activity was detected, especially for Glucopon 215 and Lutensol GD 70. Both surfactants contain the alkyl chains in their structures, and as indicated in the chapter 4.2.2. the strain was capable of degrading the alkyl polyglucosides. In the case of the system with surfactants and diesel oil the increase in monooxygenase activity was observed, with the highest activity recorded in the presence of Lutensol GD 70 (Fig. 43).

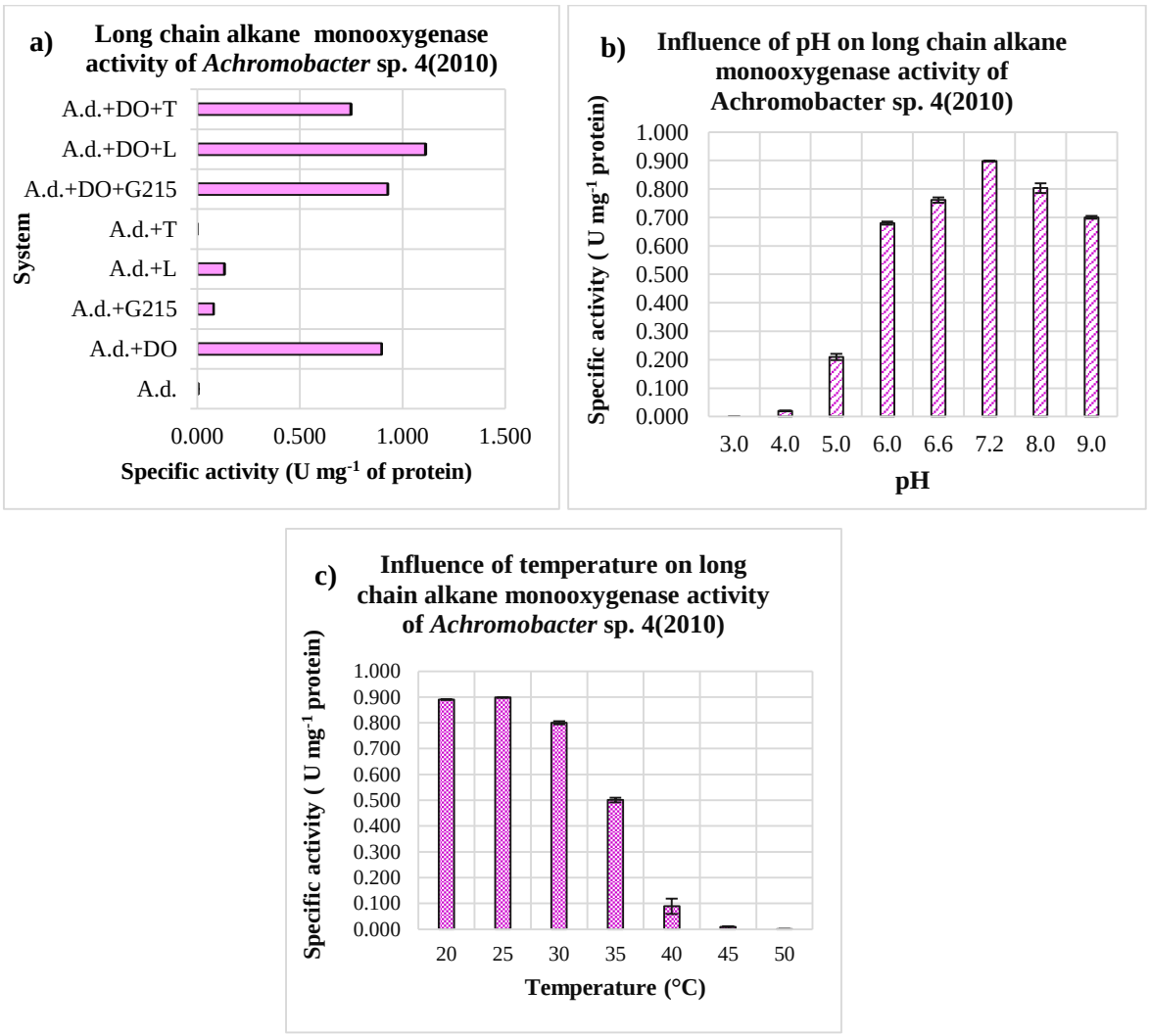


Figure 43: Long chain alkane monooxygenase activity of *Achromobacter* sp. 4(2010) under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The optimal pH and temperature of long chain alkane monooxygenase activity for *Achromobacter* sp. 4(2010) are 7.2 and 25 °C respectively (Fig. 43b and c).

The highest monooxygenase activity of *Stenotrophomonas maltophilia* strain 6 was detected in the system bacteria-diesel oil-Lutensol GD 70 (0.987 U mg^{-1} of protein). In the absence of diesel oil, the activity of monooxygenase was also the highest for Lutensol GD 70 in comparison to the other surfactants.

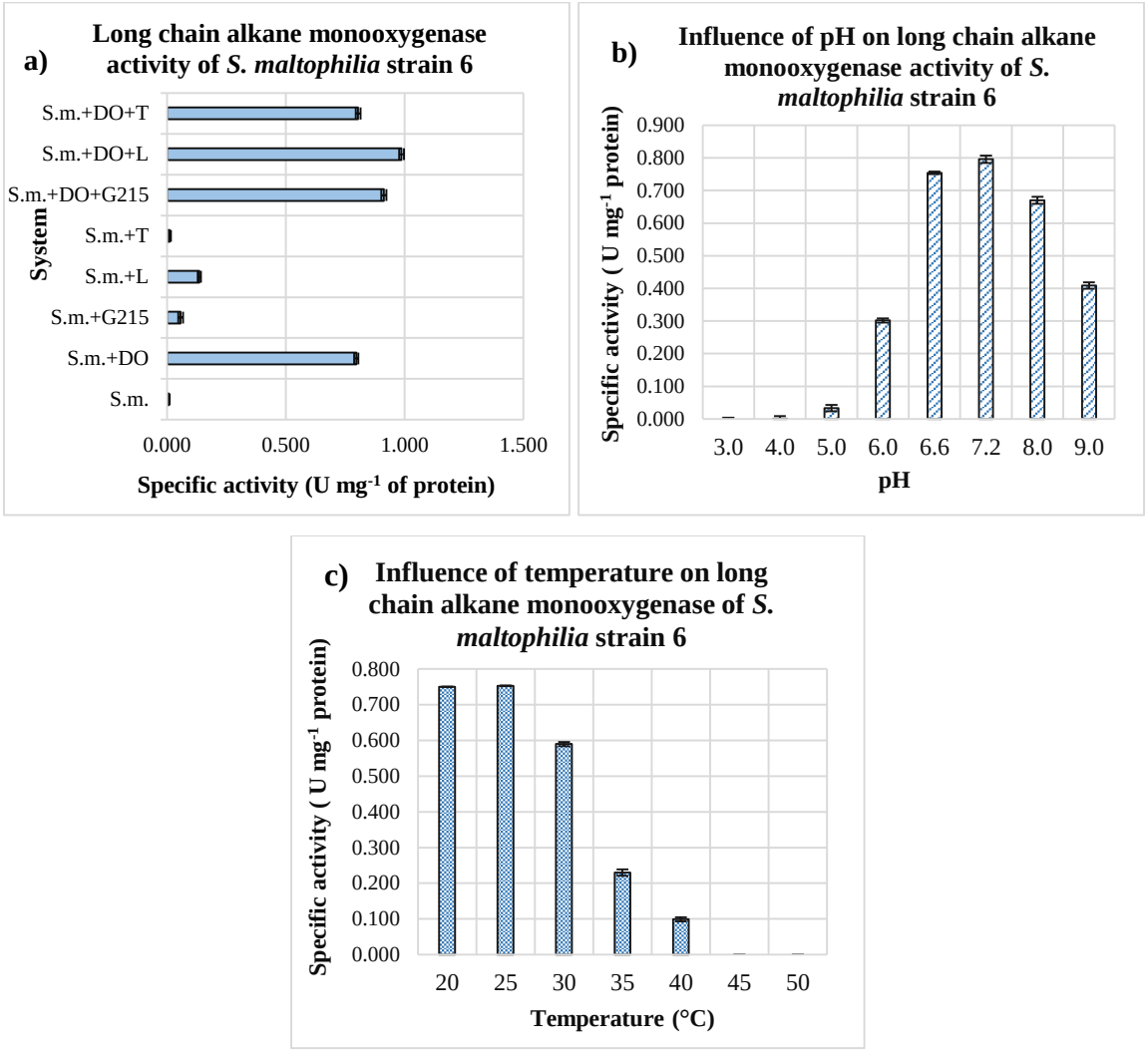


Figure 44: The activity of the long chain alkane monooxygenase of *Stenotrophomonas maltophilia* strain 6 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

No significant differences ($P < 0.05$) were observed for the monooxygenase activity in 20 and 25 °C. Likewise for *Achromobacter* strain, pH equal to 7.2 was most favourable for the enzyme's highest activity.

In the case of both *Pseudomonas stutzeri* strain 9 and *Rahnella* sp. EK12 (Fig. 45 and Fig. 46 respectively) alkane monooxygenases, the addition of surfactants did not inhibit the enzymatic activity, but slightly increased it. The highest activity of alkane monooxygenase

of *P. stutzeri* strain 9 was observed ad pH = 6.6, while the optimal range of temperature was between 20 and 30 °C.

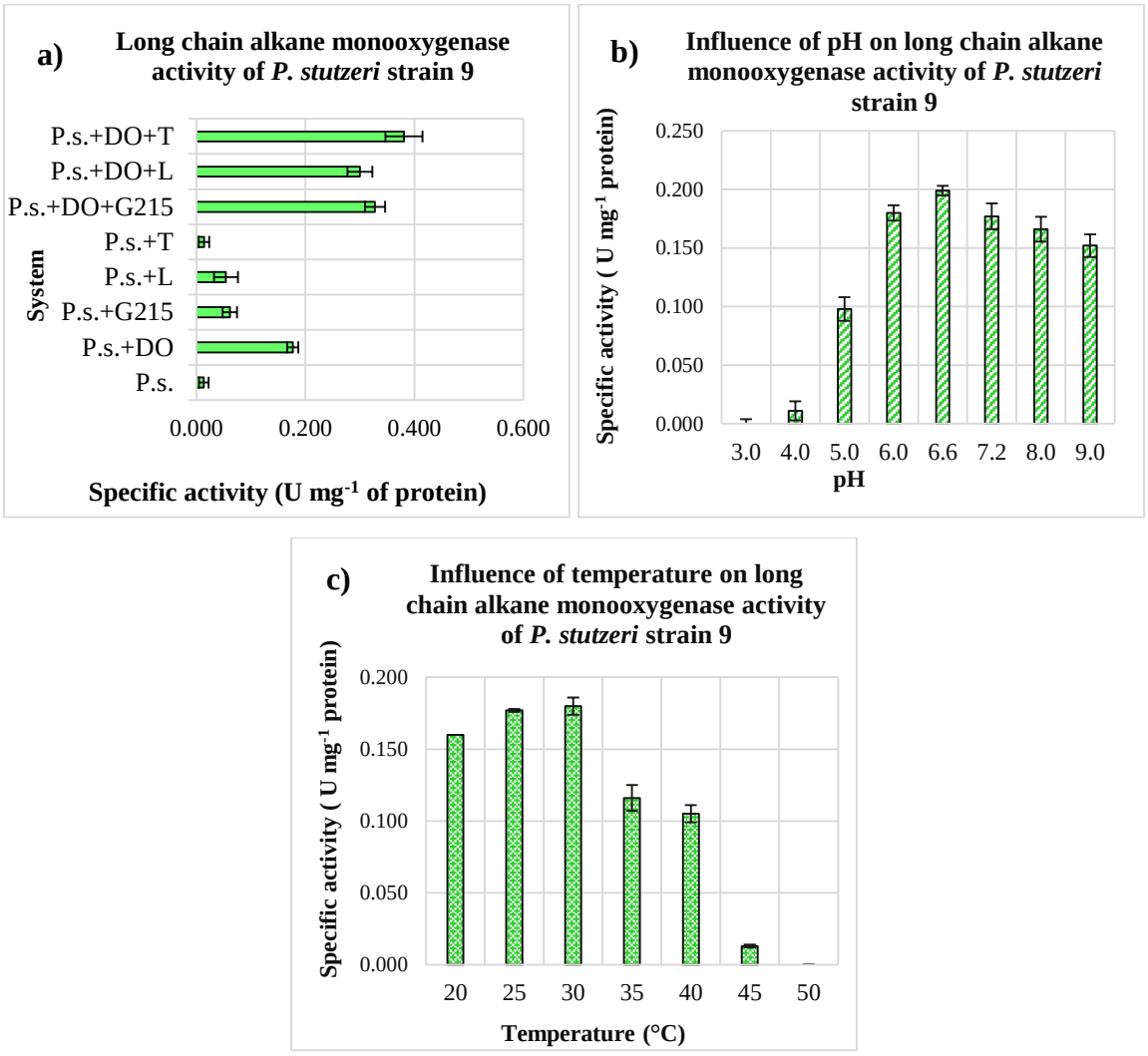


Figure 45: The activity of the long chain alkane monooxygenase of *Pseudomonas stutzeri* strain 9 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The optimal pH range for *Rahnella* sp. EK12 (Fig. 46c) was observed between 6.6 and 7.2, with the highest monooxygenase activity observed at pH = 7.2. The most favourable temperature turned to be 30 °C, although there was no significant difference between 20 and 30 °C ($P > 0.05$).

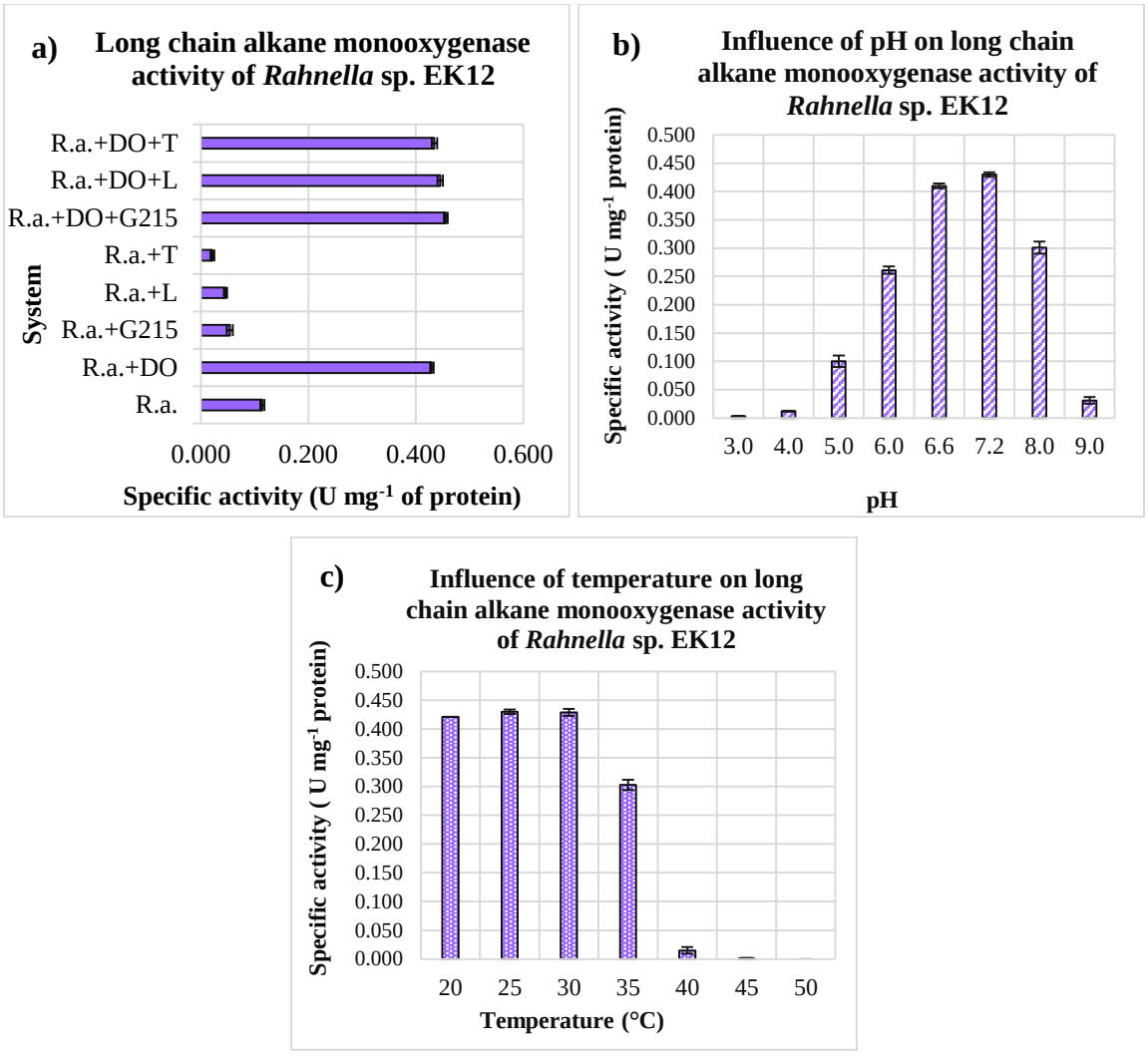


Figure 46: The activity of the long chain alkane monooxygenase of *Rahnella* sp. EK12 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The following four figures present the activities of long chain alkane monooxygenases isolated from the marine strains.

The activity of long chain alkane monooxygenase for both, *Alteromonas* sp. TK-46(2) and *Pseudoalteromonas* sp. TK-105 was the highest in the systems: "bacteria-diesel oil-surfactants" (Fig. 47a and 48a). In neither case was the inhibitory effect of surfactants observed, however, none of the strains showed the ability to degrade the tested surface active agents.

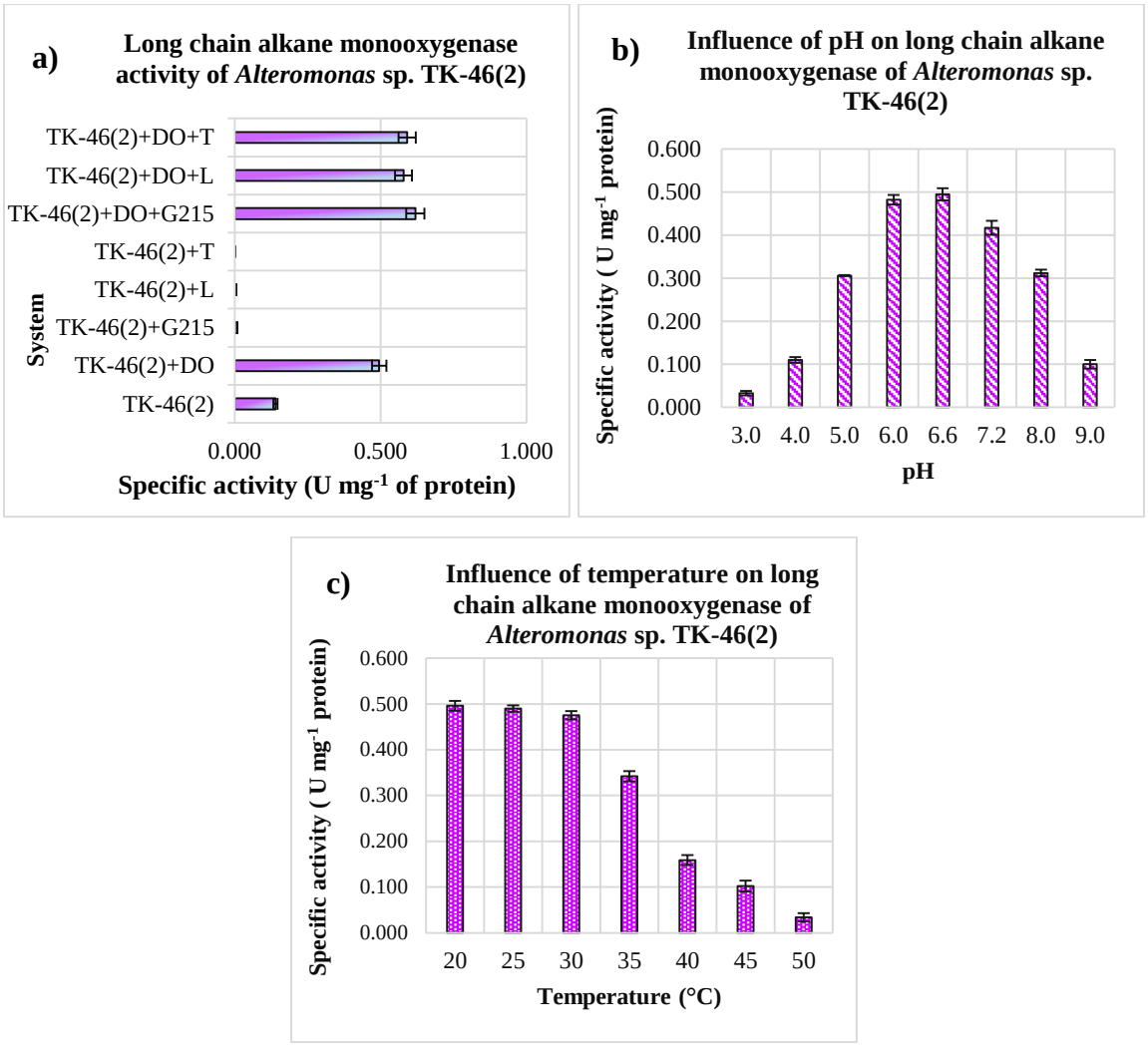


Figure 47: The activity of the long chain alkane monooxygenase of *Alteromonas* sp. TK-46(2) under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The optimal pH for *Alteromonas* sp. TK-46(2) monooxygenase was in the range of 6.0-6.6, with no significant difference between those two values (Fig. 47b). A similar pH range was observed for *Pseudoalteromonas* sp. TK-105 monooxygenase, however, in this case the highest enzymatic activity was observed at pH=6.6 (Fig. 48b). The most favourable temperatures to obtain the highest monooxygenase activity of *Alteromonas* sp. TK-46(2) ranged between 20 and 30 °C, with only slight differences between those values (Fig. 47c). The optimal temperatures for the highest monooxygenase activity of *Pseudoalteromonas* sp. TK-105 were 20 and 25 °C (Fig. 47c).

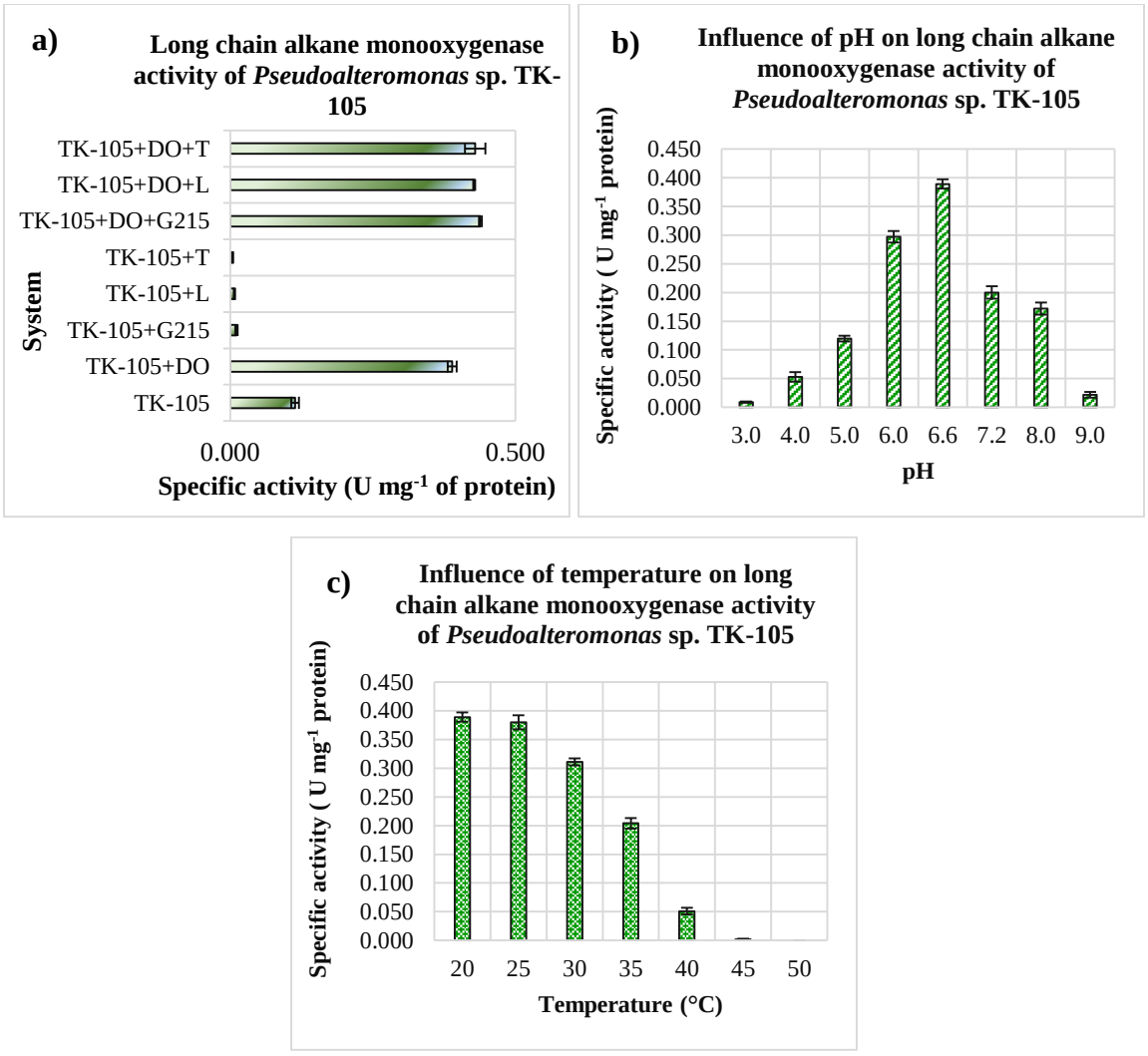


Figure 48: The activity of the long chain alkane monooxygenase of *Pseudoalteromonas* sp. TK-105 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

As seen in Fig. 49a, the highest long chain alkane monooxygenase activity of *Cycloclasticus* sp. TK-8 in the presence of diesel oil. The addition of surfactants to the "bacteria-diesel oil" resulted in the decrease of the enzymatic activity. This could have been a result of the monooxygenase activity shown for single surfactants, when used as carbon sources. Such observations may indicate that the strain is able to degrade the limited amounts of surfactants and the decreased enzymatic activity was a result of a competitive inhibition of the activity by the hexadecane and surfactants.

The pH, at which the highest monooxygenase activity was observed was equal to 6.6. At pH ranging from 3.0 to 4.0 and 8.0-9.0 no activity was detected. That means that this marine strain might prefer slightly acidic conditions. The optimal temperature was 20 °C, while over 90 % loss of the activity was observed at 45 °C and no enzyme activity was detected at 50 °C.

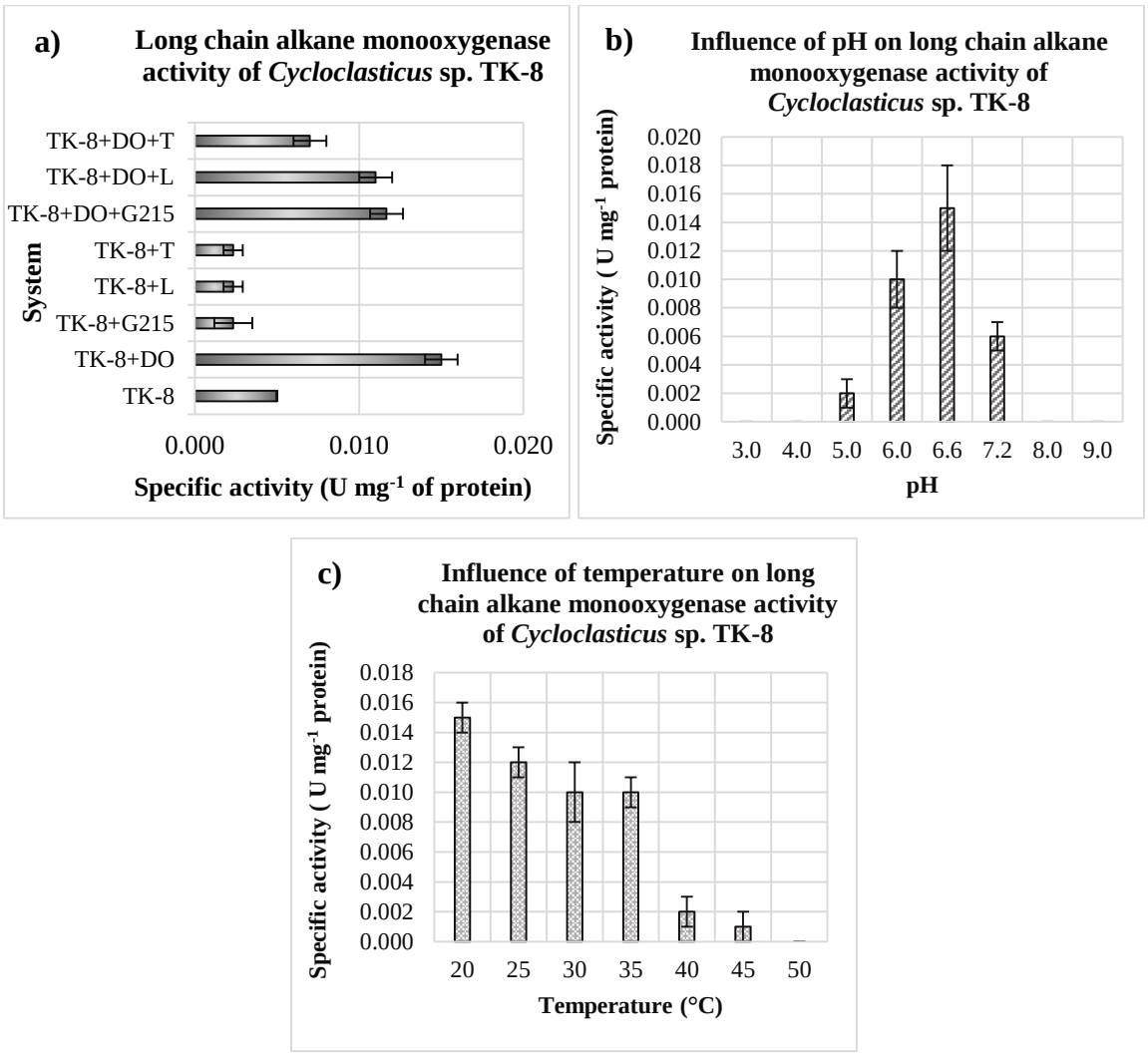


Figure 49: The activity of the long chain alkane monooxygenase of *Cycloclasticus* sp. TK-8 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

It is important to notice that the overall values of the long chain alkane monooxygenase activity of *Cycloclasticus* sp. TK-8 were much lower in comparison to all of the terrestrial strains and the remaining three marine strains - *Alteromonas* sp. TK-46(2), *Pseudoalteromonas* sp. TK-105, *Polycyclovorans algicola* TG408. Those low results of the enzymatic activity seem to be in an agreement with the minimal biodegradation of diesel oil, which was observed for this strain.

Long chain alkane monooxygenase isolated from *Pseudomonas algicola* TG408 reached its maximal activity at pH equal to 6.0 at 20-25 °C (Fig. 50b and c). The addition of surfactants to the mixture with diesel oil did not inhibit the enzymatic activity but slightly increased it (Fig. 50a).

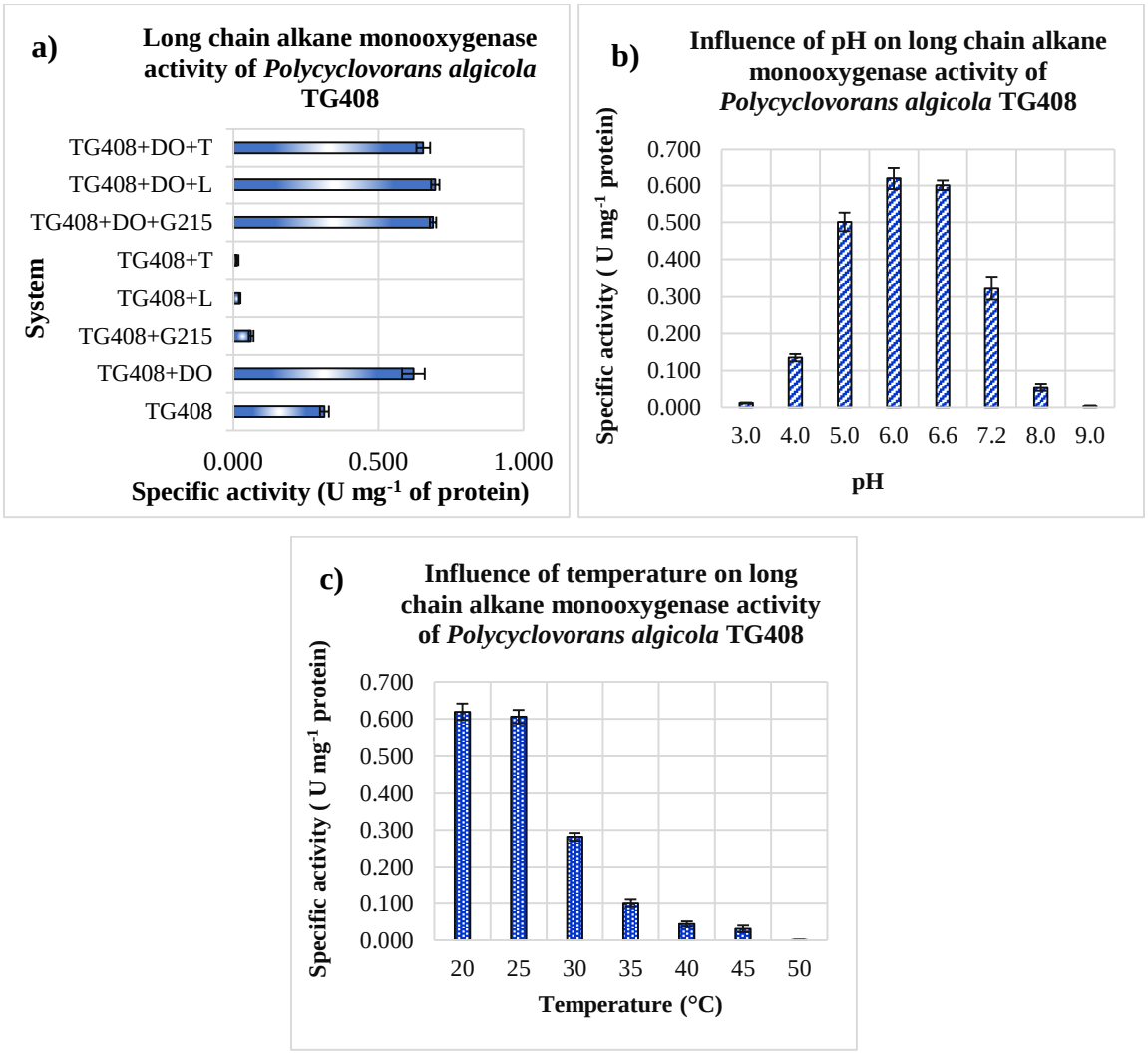


Figure 50: The activity of the long chain alkane monooxygenase of *Polycyclovorans algicola* TG408 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

Among the terrestrial strains *Achromobacter* sp. 4(2010) was characterised with the highest long chain alkane monooxygenase activity, where the topmost result was equal to 1.112 (U mg^{-1} of protein) detected in the system "bacteria-diesel oil-Lutensol GD 70" (A.d.+DO+L). The optimal pH range for the monooxygenases isolated from the terrestrial strains, was between 6.6 and 8.0, with the highest activity mostly observed at pH = 7. The temperature optimum for those enzymes was found between 20 and 30 °C.

Long chain alkane monooxygenase isolated from *Polycyclovorans algicola* TG408 showed the highest activities when compared to the other marine strains. The highest activity was determined in the system bacteria-diesel oil-Lutensol GD 70 (TG408+DO+L) and was equal to 0.696 (U mg^{-1} of protein).

The optimum pH for the monooxygenases of the marine strains was slightly acidic and varied between 5.0 and 6.6, with the highest results obtained for pH ≤ (6.0 – 6.6). The highest enzymatic activities were detected at the temperatures ranging from 20 to 30 °C with the maximal results obtained at 20 °C.

Both pH and temperature are factors that play a crucial role in the activity of the enzymes. The activity of all of the long chain alkane monooxygenases isolated from terrestrial strains strongly depended on both – temperature and pH.

Enzymes, which chemically are classified as proteins, at some specific temperature and pH values not only lose their tertiary and secondary structures but also their activity due to the denaturation processes. The functional pH range of the enzymes is determined by the acid ionisation constant (pKa) of their functional groups, especially in the active sites. The shape of the curve picturing the relation between the enzymatic activity (or stability) and pH can give useful information on the functional groups in the enzyme's active site – and most importantly on the character of the hydrogen bonds (Smith et al., 2007). Nielsen and Mccammon (2003) revealed that the determination of the ionisation constants is helpful in identifying the proton donor and catalytic nucleophile, which by no means is crucial to understand the mechanisms of the reactions taking place in the catalytic centre of the enzyme.

Temperature, like already mentioned, is another the factor, which can lead to the denaturation of the enzymes, having a direct influence on the vibrational and rotational energies of the bonds, which enable the formation of stable three-dimensional conformations of proteins. Thus, the extreme temperatures lead to the destruction of the abovementioned three-dimensional conformations and the complete loss of the enzymatic activity (Smith et al., 2007).

Hartmans et al. (1990) isolated a styrene monooxygenase (from bacterial cells) with high temperature optimum ranging from 30 to nearly 40 °C and pH ≈ 7.2.

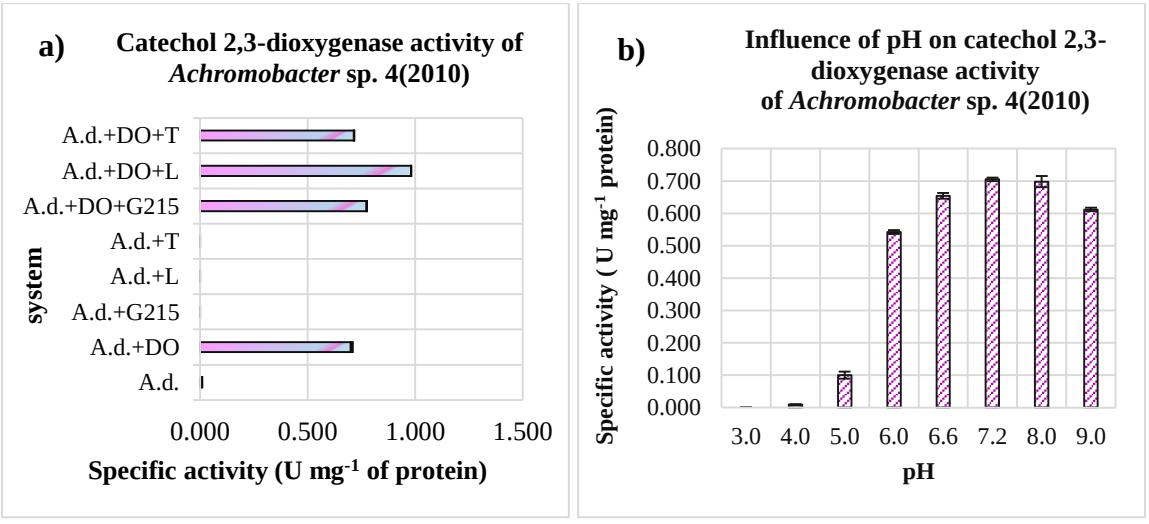
The chlorophenol 4-monooxygenase isolated from a bacterial strain *Burkholderia cepacia* AC1100 by Gisi and Xun (2003) was characterised with the optimal pH equal to 6. Nearly 40 % loss of the activity was observed at pH = 7.4. The highest activity of the enzyme was seen at 30 °C and 70 % of the activity was still retained by the enzyme at 45 °C.

In 1998 Payne et al. isolated, purified and characterised an EDTA monooxygenase from EDTA-degradting bacterium denoted as BNC1. Their research showed the optimal temperature for the monooxygenase as equal to 35 °C, however at 40 °C nearly 92 % of the maximal activity was still observed. The most favouralbe pH was 7.7 and at pH = 8.1 nearly 70 % of the activity was retained by the enzyme.

There are however, reports of the monooxygenases with the optimal pH and temperature values being very low or high. Those enzymes are usually isolated from microorganisms able to live in the extreme environmental conditions such as methane monooxygenase from thermoacidophilic bacterium of *Verrucomicrobia* phylum, with the optimal pH 2.0-2.5 and temperature 45-55 °C (Dunfield et al., 2007; Islam et al., 2008).

4.6.2. Catechol dioxygenases

The highest catechol 2,3-dioxygenase of *Achromobacter* sp. 4(2010) was observed in the presence of Lutensol GD 70 and diesel oil ("A.d.+DO+L" system) – 0.981 (U mg^{-1} of protein). Very similar results were obtained in the systems with other two surfactants – Glucopon 215 and Triton X-100. The pH range for the enzyme seemed to be very broad from 6.0 to 9.0, however no activity was detected in the strong acidic conditions. Likewise with pH, the optimal temperature varied between 20 and 35 °C, however a dramatic loss (over 80 %) was observed at 40 °C and the enzyme lost totally its activity at 50 °C (Fig. 51c).



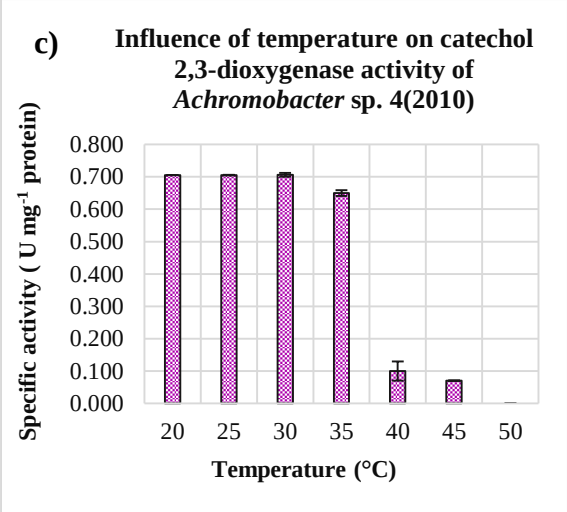
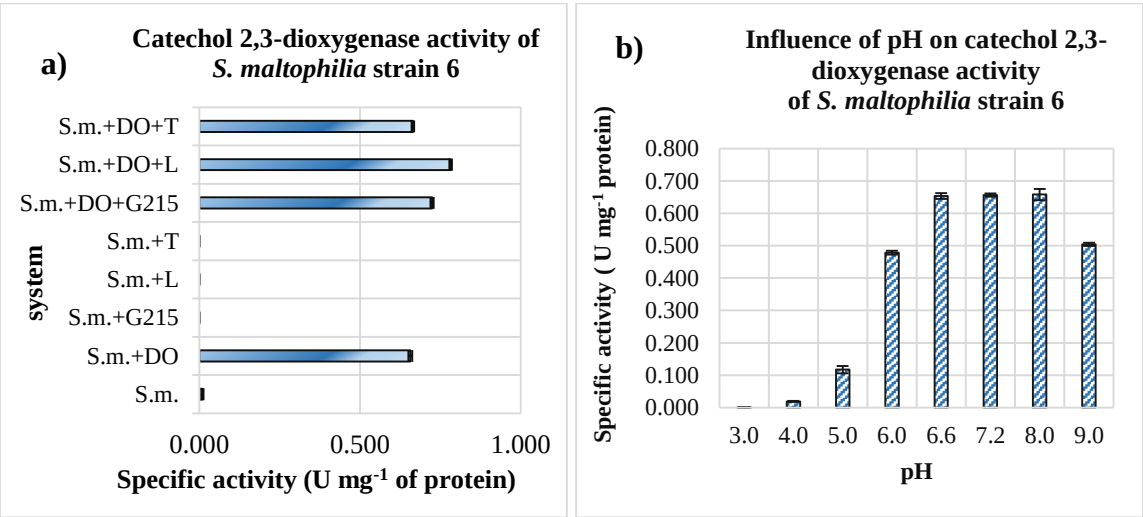


Figure 51: Catechol 2,3-dioxygenase activity of *Achromobacter* sp. 4(2010) under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

Lower, but keeping the same trends were the enzymatic activities measured for catechol 2,3-dioxygenase of *Stenotrophomonas maltophilia* strain 6 (Fig. 52). Again, the highest activity was observed for the system "S.m.+DO+L" and was equal to 0.782 (U mg^{-1} of protein). A very broad pH range was also observed with a particular preference of slightly alkaline conditions (6.6 to 9.0). The topmost results under varied temperatures were recorded in the range from 20 to 40 °C, but no activity was detected at 50 °C and the enzyme was thought to be already denatured.



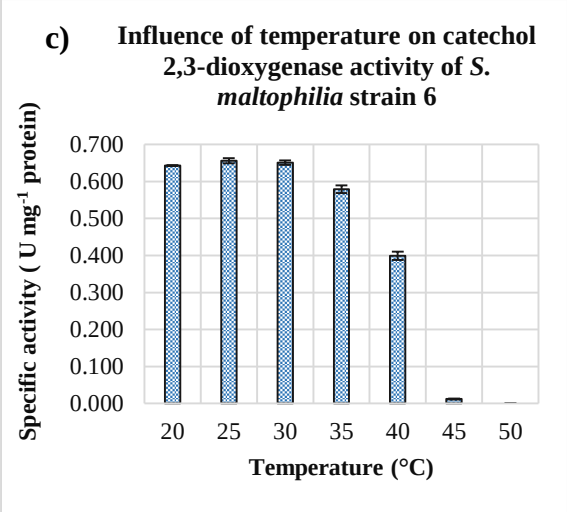
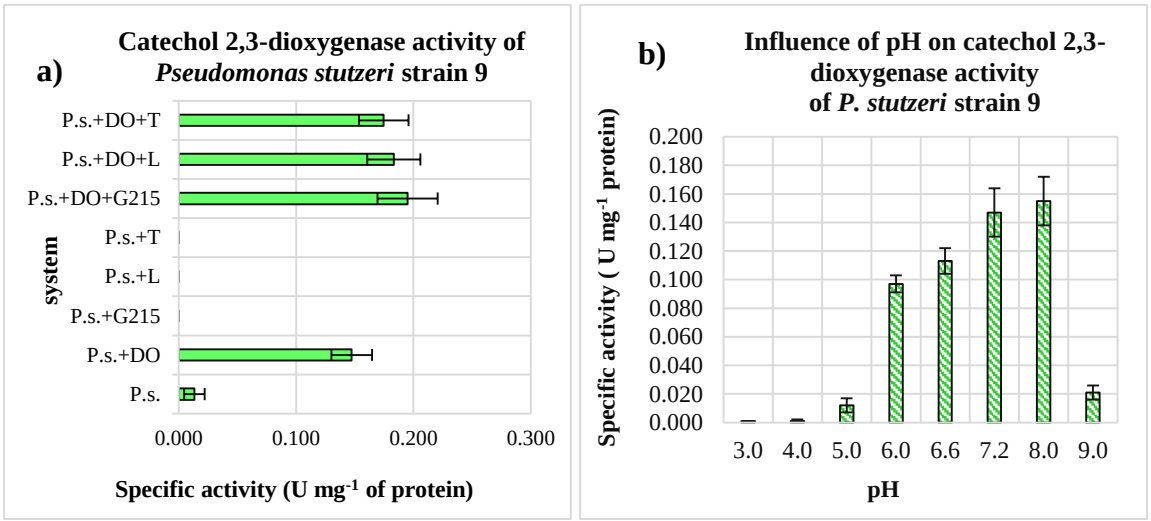


Figure 52: The activity of the catechol 2,3-dioxygenase of *Stenotrophomonas maltophilia* strain 6 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The lowest catechol 2,3-dioxygenase activities were observed in the case *Pseudomonas stutzeri* strain 9 (Fig. 53). The best results were noticed in the systems containing diesel oil and surfactants, especially Glucopon 215 (0.195 U mg^{-1}). Slightly alkaline pH and temperature of 20 to 25 °C turned to be most favourable in order to achieve the highest enzymatic activity.



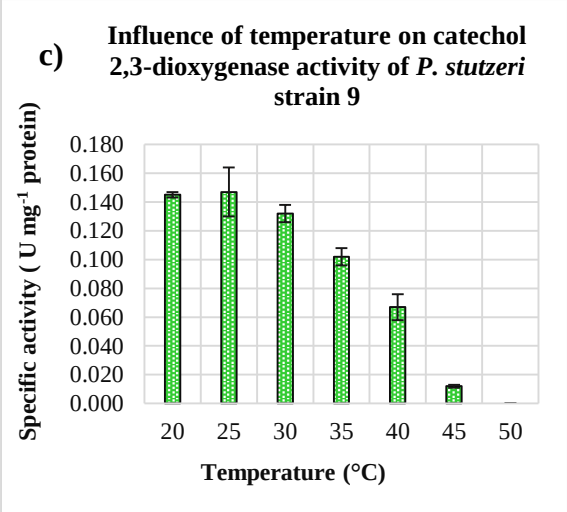
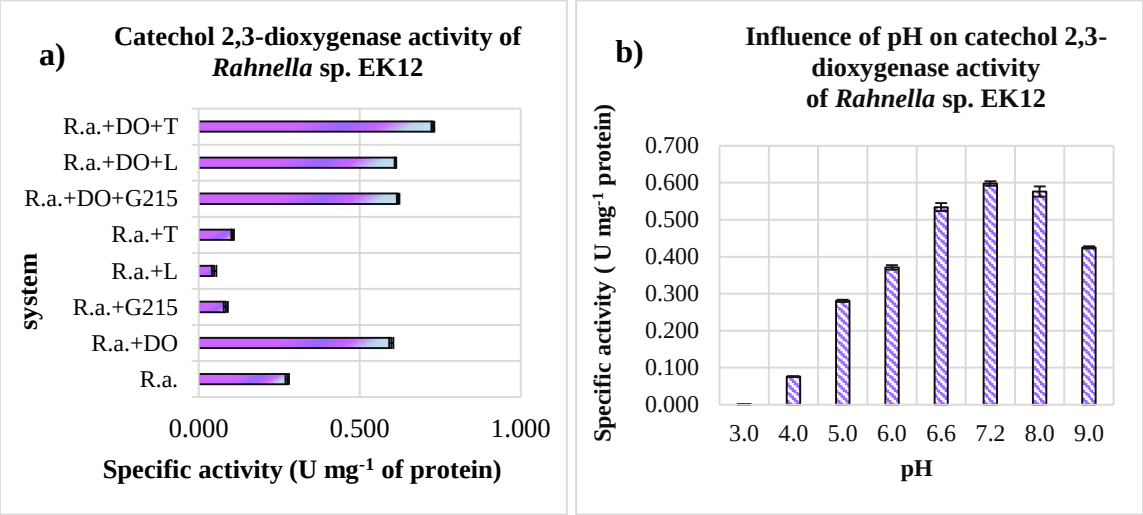


Figure 53: The activity of catechol 2,3-dioxygenase of *Pseudomonas stutzeri* strain 9 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

Catechol 2,3-dioxygenase of *Rahnella* sp. EK12 was characterised by the highest activity obtained for the system: "bacteria-diesel oil- Triton X-100". In the case of pH and temperature, the optimal conditions were pH = 7.2 and T = 25 °C.



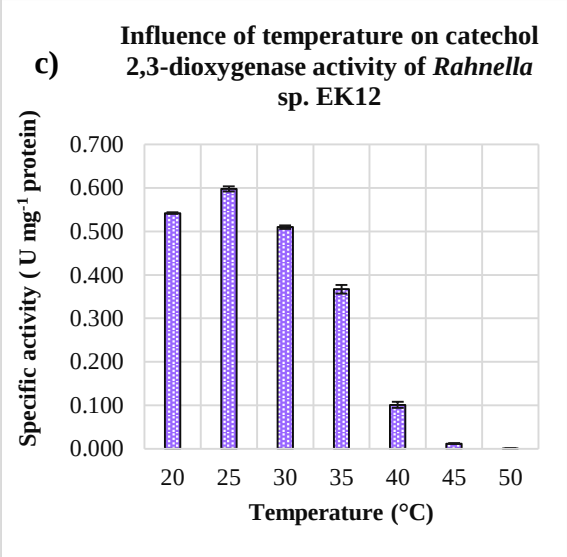
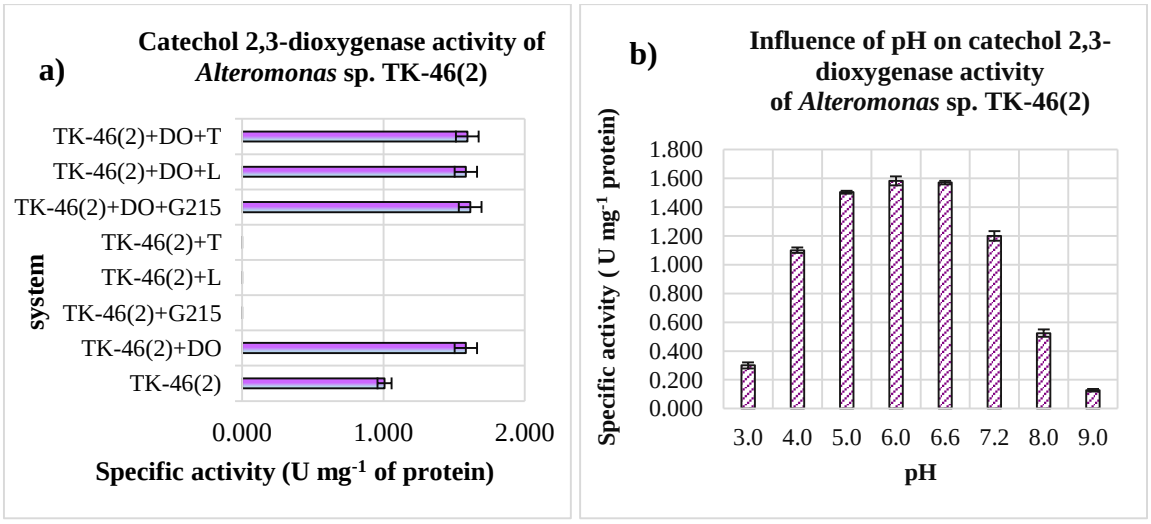


Figure 54: The activity of catechol 2,3-dioxygenase of *Rahnella* sp. EK12 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The following Figures 55 – 58 present the results obtained for the measurements of the activity of catechol 2,3-dioxygenase isolated from marine strains.

Fig. 55 shows the enzymatic activity of the catechol 2,3-dioxygenase from *Alteromonas* sp. TK-46(2). The enzyme was most efficient in the acidic conditions (pH ranging from 5.0 to 6.6) and at temperatures oscillating between 20 and 30 °C. The highest activity was observed in the presence of diesel oil and the addition of non-ionic surfactants neither significantly increased ($P > 0.05$) nor decreased the enzymatic activity.



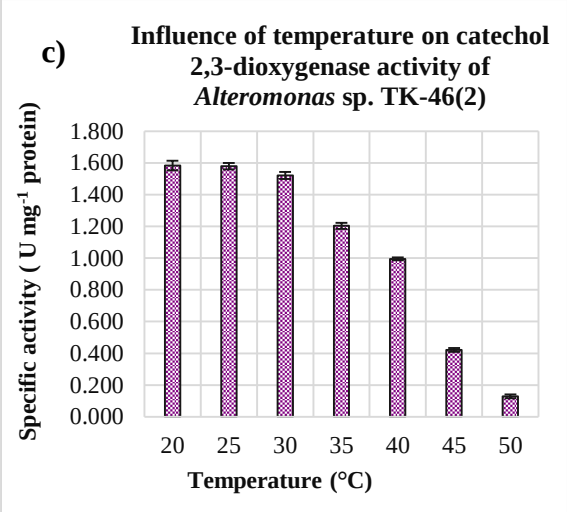
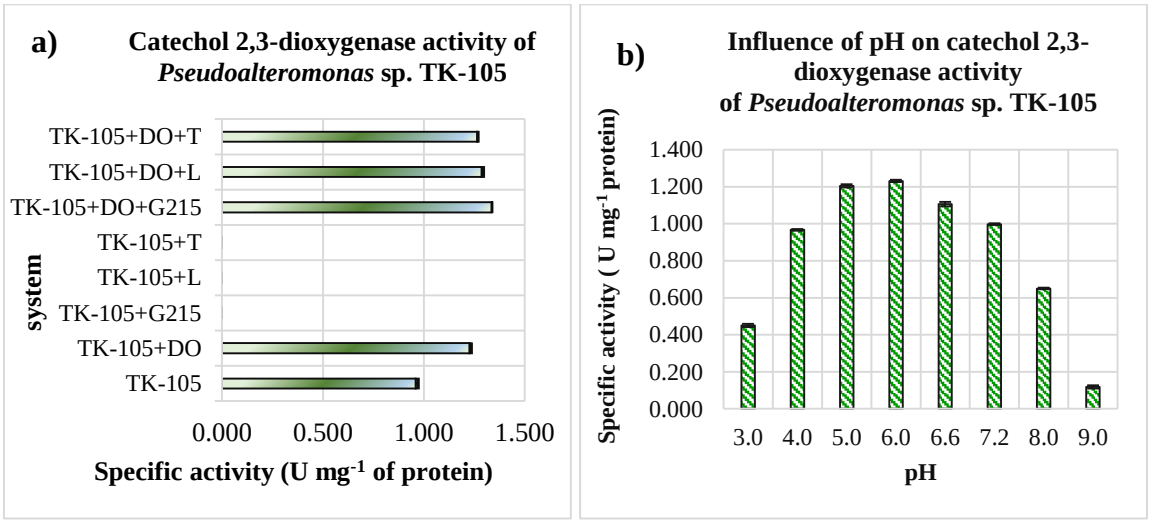


Figure 55: The activity of catechol 2,3-dioxygenase of *Alteromonas* sp. TK-46(2) under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

As seen in Fig. 56, no activity of a dioxygenase isolated from *Pseudoalteromonas* sp. TK-105 was detected when the non-ionic surfactants were used as sole carbon and energy sources, however, when added to the mixtures with diesel oil, the increase in the activities was observed for all three surfactants, with the highest result observed in the system with diesel oil and Glucopon 215. Likewise in the case of Alteromonas sp. TK-46(2), the pH range was wide, covering in the majority the acidic conditions (pH < 4.0 – 7.2>). The optimal temperature was 20 – 25 °C, however 10 % of the highest activity was still retained at 50 °C.



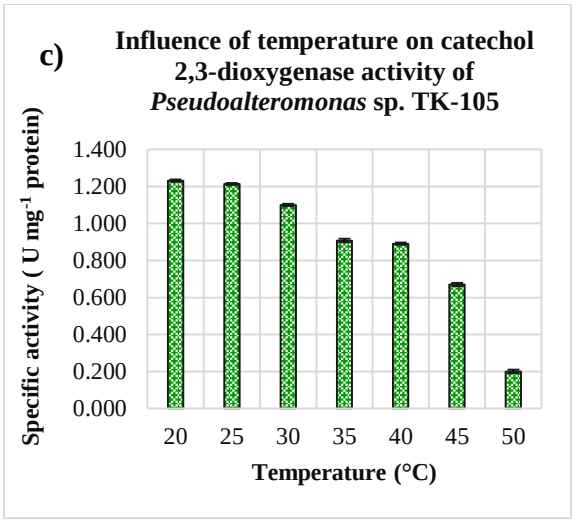
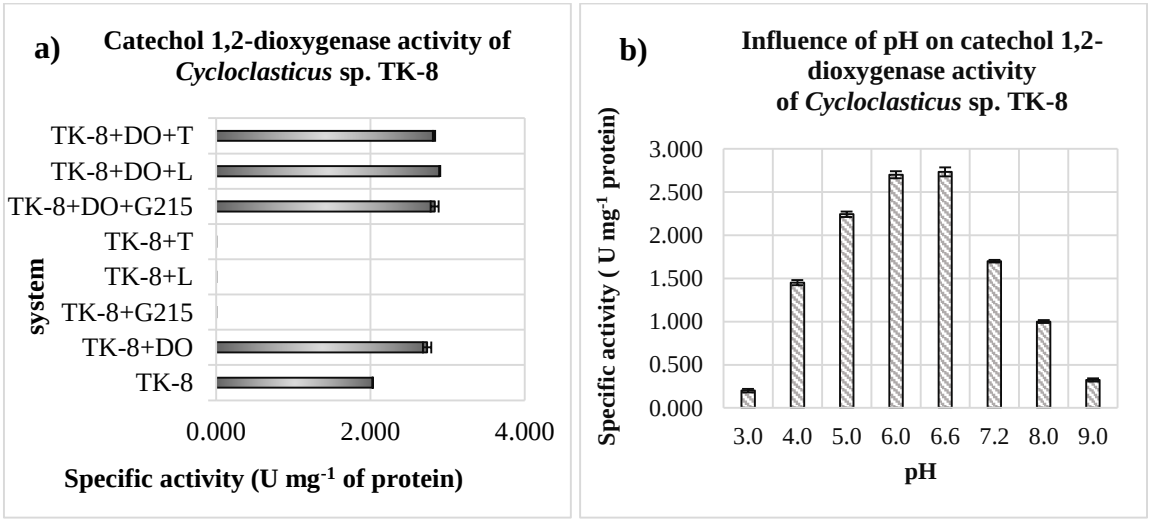


Figure 56: The activity of catechol 2,3-dioxygenase of *Pseudoalteromonas* sp. TK-105 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

In the case of catechol 1,2-dioxygenase isolated from *Cycloclasticus* sp. TK-8 and catechol 2,3-dioxygenase from *Polycyclovorans algicola* TG408 (Fig. 57 and Fig. 58 respectively), the observed trends in the changes of enzymatic activities under the influence of surfactants, pH and temperatures were virtually the same.



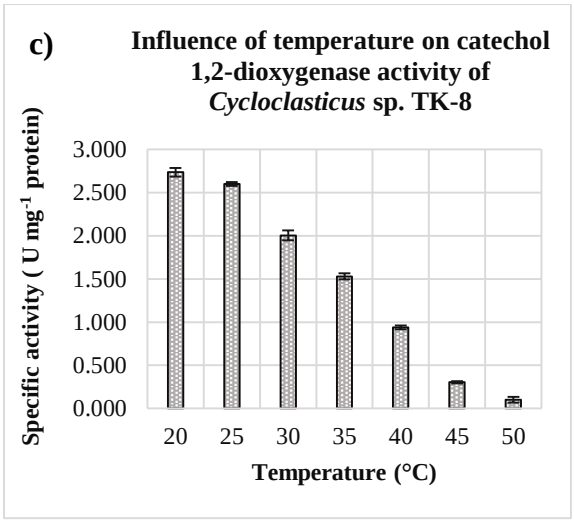
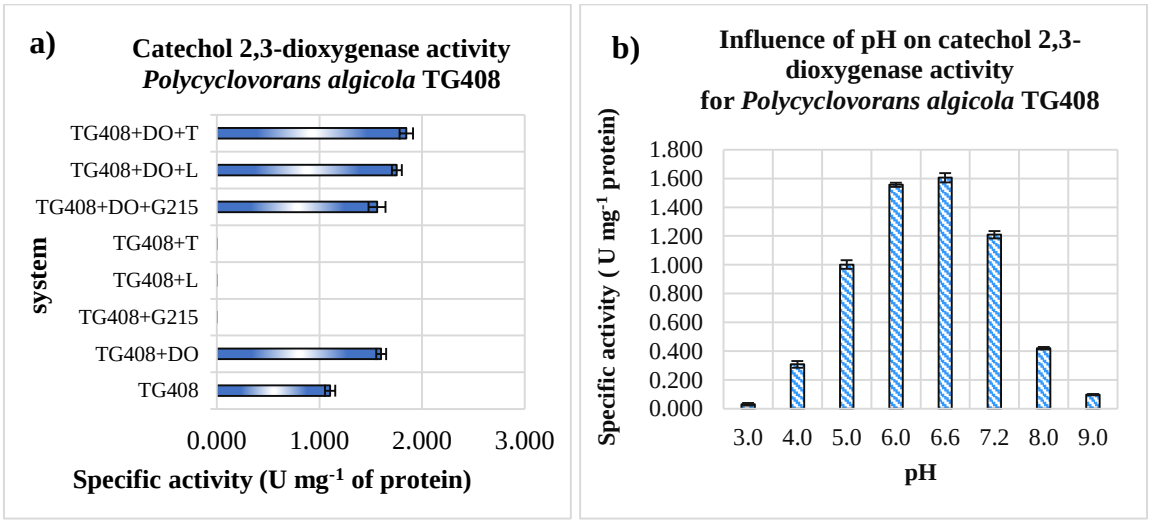


Figure 57: The activity of catechol 1,2-dioxygenase of *Cycloclasticus* sp. TK-8 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

The optimal pH for the catechol 1,2-dioxygenase from *Cycloclasticus* sp. TK-8 and *Polycyclovorans algicola* TG408 varied between 5.0 and 6.6, with the highest activities observed at pH = 6.6. In the case of temperature, the most favourable was 20 °C.



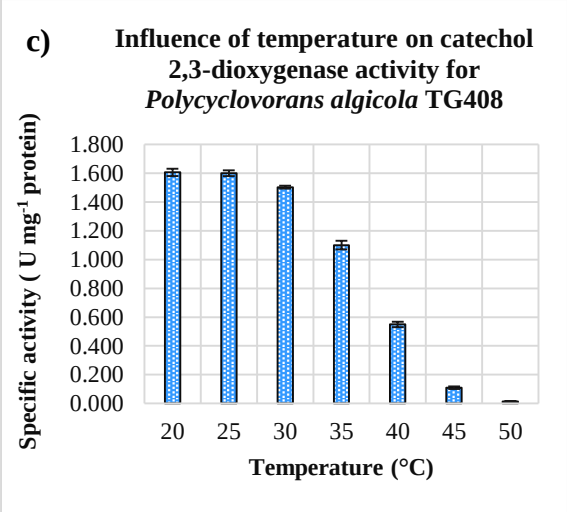


Figure 58: The activity of catechol 2,3-dioxygenase of *Polycyclovorans algicola* TG408 under the influence of surfactants and diesel oil (a), pH (b) and temperature (c).

To summarise, although the catechol 2,3-dioxygenase of *Achromobacter* sp. 4(2010) was found to have the highest activity when compared with the enzymes isolated from other three terrestrial strains, the catechol dioxygenases (both 1,2- and 2,3-dioxygenases) obtained from marine strains were characterised by higher values of the activity and much broader pH and temperature ranges in comparison with the terrestrial strains. The dioxygenase of the highest activity under all conditions was isolated from the marine strain *Cycloclasticus* sp. TK-8, for which the highest result was obtained in the system: "bacteria-diesel oil-Lutensol GD 70". What is more, in the case of the optimal pH – the dioxygenases of marine origin remained more stable under the acidic conditions and, although the most favourable temperature was around 20 °C, the enzymes seemed not to denature even at temperatures exceeding 40 °C.

As already mentioned in the previous chapter, pH is one of the most important factors in all biochemical reactions. Therefore, the maintenance of the optimal pH is crucial to all cells. The metabolism and other cellular cells mostly depend on the enzymatic activity, which pH has a great influence on. If the pH is unfavourable for the enzymes to keep their activities, the cellular metabolism will be limited or completely inhibited, which is equal to the cell death (Garrett and Grisham, 2013).

Gutierrez et al. (2012) were first to estimate the activity of the catechol 2,3-dioxygenase from the newly isolated marine strain *Polycyclovorans algicola* TG408, which was equal to 3.330 (U mg^{-1} of protein). Although the authors did not determine the optimal pH and temperatures for the abovementioned enzyme, they provided information of the newly

isolated strain, revealing its temperature of growth ranging from 10 to 30 °C, with the optimal 30 °C. The range of pH was between 6.5 to 8.5 with the optimal pH = 8.3. The results obtained by the researchers seem to be in the contradiction to the outcome of the analyses performed within this thesis. The possible explanation of such differences may depend on the laboratory conditions. The tests on the enzymatic activities were performed at Poznan University of Technology, which does not have any direct source of fresh sea water and the artificial sea water was used. Second of all, the reported in the paper studies are dated back to the year 2012. The analyses for this thesis were conducted in 2014. The microbial responses to the various conditions and time can be very different and unpredictable, and in this case some changes in the biochemistry of the microbial cells could have taken place.

5. CONCLUSIONS

1. The aim of this study was to increase a general knowledge and understanding on the differences between the marine and terrestrial hydrocarbon-degrading bacterial strains with regard to their capacities to interact (via surface and enzymatic properties) and degrade petrochemical pollutants.
2. The research conducted within this Ph.D. project led to the final conclusions:
3. The highest results for diesel oil biodegradation among the terrestrial strains were observed for *Pseudomonas stutzeri* strain 9, and *Polycyclovorans algicola* TG408 among the marine strains. For both of the strains Glucopon 215 appeared to influence the biodegradation of diesel oil most efficiently. At the surfactant's concentration as high as 360 mg L^{-1} the biodegradation of diesel oil reached 69.88 % and 85.37 % for *P. stutzeri* strain 9 and *Polycyclovorans algicola* TG408 respectively.
4. Although *Achromobacter* sp. 4(2010) was characterised with the highest naphthalene biodegradation among other terrestrial strains, the obtained results for this strains were more than 40 % smaller than those recorded for the marine strain *Cycloclasticus* sp. TK-8.
5. The cell surface hydrophobicity of the terrestrial strains was the highest for *Achromobacter* sp. 4(2010) in the presence of diesel oil together with Glucopon 215 at the concentration 360 mg L^{-1}. The initial hydrophobicity (in the absence of diesel oil and surfactants) was only 1.42 % after the incubation with sodium succinate and 20.01 % after the incubation with diesel oil. The addition of surfactants to the systems

with diesel oil had a significant influence on the increase of the hydrophobicity, especially for the surfactants' concentrations between 120 and 360 mg L^{-1}.

6. Among the marine strains, the highest CSH was observed for *Alteromonas* sp. TK-46(2) in the system "bacteria-diesel oil" – 73.44 %. The addition of surfactants resulted in the overall decrease of the hydrophobicity, especially in the case of Glucopon 215, where at surfactant's concentration of 360 mg L^{-1} the CSH was equal to 26.18 %.
7. The determination of correlations between the cell surface hydrophobicity and biodegradation of diesel oil was possible for three out of four terrestrial strains. In the case of *Stenotrophomonas maltophilia* strain 6, the relationship between the two abovementioned parameters was a polynomial function for all tested surfactants, for *Rahnella* sp. EK12 – linear and for *Pseudomonas stutzeri* strain 9 the functions in the presence of Glucopon 215 and Lutensol GD 70 were linear, however, in the presence of Triton X-100 the function was polynomial.
8. The correlations between the 7-day-biodegradation of naphthalene and cell surface hydrophobicity of the terrestrial strains were possible to establish for all four strains. The functions, likewise for diesel oil, had mixed forms, which means that within one strain both linear and polynomial functions were possible. The highest coefficients of determination were found for *P. stutzeri* strain 9 and *Rahnella* sp. EK12 strains ($R^2 \geq 0.94$), which means that in the case of these two strains the relation between naphthalene degradation and CSH is very strong.
9. In the case of marine strains, the correlations of hydrocarbon biodegradation and cell surface hydrophobicity were only found for two, out of four strains. The coefficients of determination for *Alteromonas* sp. TK-46(2) were found to be high ($R^2 \geq 0.93$) for both diesel oil and naphthalene biodegradation systems in the presence of all three surfactants and the functions were linear. In the case of diesel oil biodegradation by *Polycyclovorans algicola* TG408, the coefficient of determination was the lowest in the presence of Lutensol GD 70 ($R^2 = 0.8728$) for the linear function. The polynomial function was found for the system with Triton X-100, where R^2 was equal to 0.9597.
10. The correlations observed for the biodegradation of naphthalene had, in all cases, linear functions and the highest coefficients of determination ($R^2 \geq 0.93$) were recorded for *Alteromonas* sp. TK-46(2).
11. The values of zeta potential for both, marine and terrestrial strains, were varied and strongly depended on the bacterial strain used in the analyses, which draws a

conclusion that the surface charge might be a very individual feature of the microorganism and its origin.

12. The best correlations between zeta potential and CSH were obtained for *Achromobacter* sp. 4(2010) and *Stenotrophomonas maltophilia* strain 6 both in the presence and absence of diesel oil. The marine strains were observed to show good correlations between zeta potential and CSH only in the absence of diesel oil. When the strains were incubated with diesel oil, hardly any correlations between the surface charge and hydrophobicity were observed. The strongest relationship between the zeta potential and biodegradation of diesel oil in the presence of surfactants was, however, observed for the marine strains: *Pseudoalteromonas* sp. TK-105, *Cycloclasticus* sp. TK-8 and *Polycyclovorans* algicola TG408.
13. Among the terrestrial strains *Achromobacter* sp. 4(2010) was characterised with the highest long chain alkane monooxygenase activity, where the topmost result was equal to 1.112(U mg^{-1} of protein) detected in the system "bacteria-diesel oil-Lutensol GD 70" (A.d.+DO+L).
14. The optimal pH range for the monooxygenases isolated from the terrestrial strains, was between 6.6 and 8.0, with the highest activity mostly observed at pH = 7. The temperature optimum for those enzymes was found between 20 and 30 °C.
15. Long chain alkane monooxygenase isolated from *Polycyclovorans algicola* TG408 showed the highest activities when compared to the other marine strains. The highest activity was determined in the system bacteria-diesel oil-Lutensol GD 70 (TG408+DO+L) and was equal to 0.696 (U mg^{-1} of protein).
16. The optimum pH for the monooxygenases of the marine strains was slightly acidic and varied between 5.0 and 6.6, with the highest results obtained for pH ≤ (6.0 – 6.6). The highest enzymatic activities were detected at the temperatures ranging from 20 to 30 °C with the maximal results obtained at 20 °C.
17. Although the catechol 2,3-dioxygenase of *Achromobacter* sp. 4(2010) was found to have the highest activity when compared to the enzymes isolated from other three terrestrial strains, the catechol dioxygenases (both 1,2- and 2,3-dioxygenases) obtained from marine strains were characterised by higher values of the activity and much broader pH and temperature ranges in comparison with the terrestrial strains. The dioxygenase of the highest activity under all conditions was isolated from the marine strain *Cycloclasticus* sp. TK-8 (catechol 1,2-dioxygenase).

18. In the case of the optimal pH – the dioxygenases of marine origin remained more stable under the acidic conditions and, although the most favourable temperature was around 20 °C, the enzymes seemed not to denature even at temperatures exceeding 40 °C

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7. STRESZCZENIE

Ciągły popyt na produkty ropopochodne prowadzi do corocznego uwalniania ogromnych ilości wspomnianych substancji do środowiska naturalnego. Powoduje to zagrożenie dla wszystkich organizmów żywych. Dlatego też, wszelkie metody oczyszczania są stale rozwijane w kierunku ich efektywności, bezpieczeństwa i zminimalizowania powstawania zanieczyszczeń wtórnych. Biodegradacja uważana za naturalny proces oczyszczania środowiska, od ostatnich dwóch dekadach cieszy się wciąż rosnącym zainteresowaniem. Proces ten polega na zastosowaniu mikroorganizmów, takich jak bakterie i grzyby, które poprzez produkcję odpowiednich enzymów, zdolne są do metabolizowania zanieczyszczeń ropopochodnych, wykorzystując je jako źródło węgla i energii.

Wyciek ropy naftowej z platformy wiertniczej Deepwater Horizon, był doskonałym przykładem wykorzystania i rozwoju metod pomocnych w identyfikacji mikroorganizmów (o wysokiej zdolności do degradacji związków ropopochodnych) z terenów skażonych przez wyciek. Uzyskane wyniki, okazały się niezwykle pomocne w analizach mechanizmów biodegradacji. Obecne badania, koncentrują się głównie na właściwościach enzymatycznych i powierzchniowych mikroorganizmów zaangażowanych we wspomniany proces. Zatem, uzyskanie wyczerpującej i szczegółowej wiedzy na temat rodzaju i właściwości mikroorganizmów zdolnych do rozkładu związków ropopochodnych jest nieocenionym wkładem dla dalszych badań związanych z unowocześnianiem i doskonaleniem bioremediacji zanieczyszczonych terenów.

Bardzo ograniczony lub całkowity brak rozpuszczalności węglowodorów w wodzie, znacznie zmniejsza ich biodostępność i biodegradację. Niemniej jednak, pomimo tego ograniczenia, mikroorganizmy posiadają właściwości powierzchniowe, które umożliwią im adhezję do różnych powierzchni - w tym hydrofobowych związków organicznych. Wspomniane właściwości zależą od kilku głównych czynników, takich jak ładunek na powierzchni komórki (który związany jest z elektrostatycznym oddziaływaniem między komórką bakterii a powierzchnią) i hydrofobowość powierzchni komórkowej.

Innym ważnym aspektem biodegradacji jest aktywność enzymów uczestniczących w przemianie węglowodorów. W pierwszym i najważniejszym etapie tego procesu, katalizowanego przez enzymy z klasy oksydoreduktaz (mono- i dioksygenazy), dochodzi do wprowadzenia atomów tlenu do węglowodoru, który następnie jest substratem dla dalszych etapów rozkładu. Niniejsza praca przedstawia analizę aktywności enzymatycznej

monooksygenaz alkanów długołańcuchowych oraz dioksygenaz katecholowych (1,2- oraz 2,3-), które izolowano w postaci surowych frakcji enzymatycznych z wybranych do badań szczepów bakteryjnych.

Do badań w ramach niniejszej Rozprawy Doktorskiej wybrano osiem szczepów bakteryjnych, które wyizolowane zostały z terenów skażonych substancjami ropopochodnymi. Do morskich szczepów bakteryjnych należą *Alteromonas* sp. strain TK-46 (2), *Pseudoalteromonas* sp. strain TK-105 oraz *Cycloclasticus* sp. strain TK-8, które zostały wyizolowane z próbek oleju zebranych podczas wycieku ropy naftowej z platformy wiertniczej Deepwater Horizon. Czwarty szczep, *Polycyclovorans algicola* TG408 wyizolowano z hodowli laboratoryjnej z okrzemków morskich *Skeletonema costatum*.

Szczepy: *Achromobacter* sp. 4 (2010), *Pseudomonas stutzeri* strain 9, *Rahnella* sp. EK12 oraz *Stenotrophomonas maltophilia* strain 6 zostały wyizolowane z gleby zanieczyszczonej związkami ropopochodnymi na terenie Polski.

W badaniach zastosowano trzy dostępne na rynku niejonowe środki powierzchniowo czynne: Glucopon® 215 i Lutensol® GD 70 (naturalne) i Triton X-100 (syntetyczny). Wymienione surfaktanty nie zahamowały procesów biodegradacyjnych ani w przypadku oleju napędowego, ani naftalenu.

Uzyskane wyniki wskazują, że szczepy morskie charakteryzują się wysoką aktywnością dioksygenaz katecholowych oraz dużą zdolnością do degradacji naftalenu, który został zastosowany jako modelowy wielopierścieniowy węglowodór aromatyczny. Dla wyżej wymienionych enzymów określono również optymalne warunki pH i temperatury.

Analiza właściwości powierzchniowych wykazała istnienie silnej zależności pomiędzy hydrofobowością powierzchni bakteryjnej oraz biodegradacją węglowodorów w przypadku szczepów izolowanych z gleb. Charakteryzowały się one również wyższą, w porównaniu do szczepów morskich, aktywnością monooksygenazy alkanów długołańcuchowych.

8. AUTHOR'S SCIENTIFIC ACHIEVEMENTS

Publications

1. **Karina Sałek**, Ewa Kaczorek, Urszula Guzik, Agnieszka Zgoła-Grześkowiak. Bacterial properties changing under Triton X-100 presence in the diesel oil biodegradation systems: from surface and cellular changes to mono- and dioxygenases activities. Environ Sci Pollut Res. 2015, 22: 4305-4315.

2. Jakub Zdarta, **Karina Sałek**, Agnieszka Kołodziejczak-Radzimska, Katarzyna Siwińska-Stefańska, Karolina Szwarc-Rzepka, Małgorzata Norman, Łukasz Klapiszewski, Przemysław Bartczak, Ewa Kaczorek and Teofil Jesionowski. Immobilization of amano lipase A onto Stöber silica surface: process characterization and kinetic studies. Open Chemistry 2015. 13: 138–148.

3. **Karina Sałek**, Agnieszka Zgoła-Grześkowiak, Ewa Kaczorek, Modification of surface and enzymatic properties of Achromobacter denitrificans and Stenotrophomonas maltophilia in association with diesel oil biodegradation enhanced with alkyl polyglucosides, Colloids and Surfaces B: Biointerfaces 111 (2013) 36-42

4. Ewa Kaczorek, **Karina Sałek**, Urszula Guzik, Teofil Jesionowski, Zefiryn Cybulski, Biodegradation of alkyl derivatives of aromatic hydrocarbons and cell surface properties of a strain of Pseudomonas stutzeri, Chemosphere 90 (2013) 471–4782.

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6. Ewa Kaczorek, **Karina Sałek**, Urszula Guzik, Beata Dudzińska-Bajorek, Andrzej Olszanowski, The impact of long-term contact of Achromobacter sp. 4(2010) with diesel oil – Changes in biodegradation, surface properties and hexadecane monooxygenase activity, International Biodeterioration & Biodegradation 78 (2013) 7–16.

7. Urszula Guzik, Katarzyna Hupert-Kocurek, **Karina Sałek**, Danuta Wojcieszyńska, Influence of metal ions on bioremediation activity of protocatechuate 3,4-dioxygenase from Stenotrophomonas maltophilia KB2. World Journal of Microbiology and Biotechnology. World Journal of Microbiology and Biotechnology 29 (2013) 267–273

Grants

- August 2013 – August 2015 a grant awarded by Polish National Science Centre (NCN) for a research project entitled: Isolation and purification of oxygenases - steps taken towards characteristics of enzymes involved in biodegradation of petroleum hydrocarbons and their further application.
- September 2014 – February 2015 a grant awarded by Poznań University of Technology for the best young researchers – The Engineer of The Future 2014/2015.
- January – September 2013 Erasmus Work Placement, Heriot Watt University, Edinburgh, United Kingdom.

International experience

- September – December 2015 Full time PhD Student and a main grant executor at Heriot Watt University, Edinburgh, Scotland. Language – English only.
- January – September 2013 Full time PhD Student and a main grant executor at Heriot Watt University, Edinburgh, Scotland. Language – English only.

Doctoral scholarships

- May 2014 - April 2015, Scholarship project entitled "The supporting scholarships for PhD students in the fields that are considered strategic from the point of view of the development of Greater Poland" 8.2.2 Sub-Regional Innovation Strategies, Action 8.2, Priority VIII Human Capital Operational Programme
- October 2013 - June 2014, A scholarship for best PhD students awarded by the Rector of Poznan Univesity of Technology
- October 2012 - June 2013, A scholarship for best PhD students awarded by the Rector of Poznan Univesity of Technology
- November 2011 - August 2012, Scholarship project entitled "The supporting scholarships for PhD students in the fields that are considered strategic from the point of view of the development of Greater Poland" 8.2.2 Sub-Regional Innovation Strategies, Action 8.2, Priority VIII Human Capital Operational Programme
- January 2012 - September 2012, Pro-quality scholarship awarded by the Rector of the University of Technology

- October 2011 - June 2012, Scholarship for outstanding academic performance and academic achievements awarded by the Dean of the Faculty of Chemical Technology

Awards

- April 2015 Best Oral Presentation Award – First Place at the 1st International Science Conference “Chemistry, Environment and Nanotechnology”, Gdańsk, Poland
- February 2012 Prize for the best speech at the XVIII National Symposium for PhD Students "Environmental Biotechnology”, Wisła, Poland
- September 2011 Distinction received for the best oral presentation at the First National Congress of Young Biotechnologists "ATRINBIOTECH", Katowice, Poland

Activities

- January 2014 – January 2015 Member of the Student and Doctoral Research Team – BioInicjatywa at Poznań University of Technology
- March 2012 Participation in organization of „Joint Conference of German Mass Spectrometry Society and Polish Mass Spectrometry Society” held by The Institute of Bioorganic Chemistry of Polish Academy of Sciences. Poznan, Poland.
- 2007 – 2014 Organization of Researchers Night at Poznan University of Technology, Poznan, Poland
- 2007 – 2010 Member of the Chemistry Student Research Team