Hungarian University of Agriculture and Life Sciences

Doctoral School of Environmental Sciences

AEROBIC AND MICROAEROBIC ENRICHMENT OF XYLENE DEGRADING BACTERIA

DOI: 10.54598/003580

Ph.D. Thesis

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GÖDÖLLŐ, HUNGARY
2023

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LIST OF ABBREVIATIONS

BTEX	benzene, toluene, ethylbenzene and xylenes
PCR	polymerase chain reaction
dNTP:	deoxynucleotide triphosphates
T-RFLP	Terminal Restriction fragment length polymorphism
OTU	Operational taxonomic unit
dDDH	Digital DNA–DNA hybridization
GC-MS	Gas Chromatography Mass Spectrometry
OrthoANI	Orthologous average nucleotide identity
DSMZ	German Collection of Microorganisms and Cell Cultures
C23O	catechol 2,3-dioxygenase
sp .	species
spp .	species (plural)

1 INTRODUCTION

Though there are several alternative energy sources available for mankind but unfortunately still, today, fossil fuels are the main sources of energy to satisfy the increasing energy need of the human population. The use of fossil fuels leads to several consequences mainly related to global warming, like melting glaciers, rising seas, increasing global average temperature etc. Moreover, air pollution caused by the burning of fossil fuels is responsible for nearly one in every five deaths worldwide. Hence, the world is trying to turn down the use of fossil fuels. Even in the 26th UN Climate Conference (COP26) in Glasgow (2021), world leaders explicitly plan to reduce the use of fossil power (coal, oil, gas). Among fossil power, petroleum oil is the major supplier of energy source on which the world economy is dependent. Petroleum derivatives are also used as raw materials for several industries. Due to this widely used nature, accidental and negligent contamination is also very common, occurring during their disposal, extraction, recovery, refining, storage, transport and use, thereby contaminating particularly soil and aquifers. There are several incidents of such contaminations that took place during the last century; some of the most talked about incidents that raise concerns are: the accident of the cargo ship Amoco Cadiz in 1978, where around 223,000 tonnes of oil spillled into the sea off the coast of Brittany, in 1989, a tanker named Exxon Valdez ran aground off the coast of Alaska, spilling 7,000 tons of crude oil into the sea, on April 20, 2010, there was another oil disaster took place in the Gulf of Mexico that proved to be more serious than ever. The explosion contaminated the zone with 700,000 tons of crude oil. As one of the rapidly growing economies of the European Union, Hungary is also experiencing petroleum derivatives as the most common environmental pollutants like the rest of the world. However, there are several laws in place to limit such contaminations, but unfortunately, they cannot completely restrict such incidents. That is why we need to be prepared with advanced processes in place to treat such conditions to save the ecosystem and mankind from their toxic and carcinogenic effects. The mono-aromatic hydrocarbons, such as the carcinogenic benzene, and others like toluene, ethylbenzene and *o*-, *m*-, and *p*- xylene (BTEX compounds) are among the most frequent groundwater contaminants in Hungary, as well. These compounds are always detectable in petroleum hydrocarbon-contaminated environments. Due to their relatively high-water solubility, soil contamination can considerably threaten subsurface

aquifers, the main drinking water resource. Therefore, the cleanup of the polluted ecosystems is always obligatory by law in Hungary. Amongst the remediation techniques, the bioremediation technique is the most relevant, environmentally sound and cost-effective method to treat large-scale BTEX contaminated ecosystems. Since microbial degradation of these mono-aromatic hydrocarbons readily occurs under aerobic conditions, oxygen supply into the contaminated ecosystem is a frequently applied method to enhance aerobic degradation of the contaminants. This is necessary because hydrocarbons stimulate the metabolic activity of indigenous aerobic microbial populations resulting in a rapid depletion of dissolved oxygen in subsurface environments. Thus, the development of extensive microaerobic to anaerobic zones in these environments can be observed. Although a broad diversity of microorganisms can degrade mono-aromatic hydrocarbons even in the absence of oxygen, benzene, along with *p*-and *o*-xylene, are among the least degradable BTEX compounds under anaerobic conditions (Rabus and Widdel, 1995; Foght, 2008). Though there are several studies have been conducted regarding both aerobic and anaerobic degradation, somehow, the microaerobic degradation aspect got neglected. As the contaminated subsurface environment is generally oxygen limited in nature, so it is always worth studying the microaerobic degraders that harbour specific enzymes encoded by unique functional genes needed to transform contaminants under low dissolved oxygen concentration. Nevertheless, unfortunately, very little is known about how different microbial communities evolve under different levels of dissolved oxygen availability. It is known that trace amounts of oxygen can support aerobic degradation of benzene (Yerushalmi *et al.*, 2002; Aburto *et al.*, 2009) and that certain functional genes (catechol dioxygenases) playing a key role in the degradation of aromatic compounds are transcribed even under microaerobic conditions (Táncsics *et al.*, 2012).Moreover, microbial communities of BTEX-contaminated microaerobic ecosystems are usually dominated by Betaproteobacteria. Besides, in these environments, catechol 2,3- dioxygenase (C23O) genes that encode subfamily I.2.C-type extradiol dioxygenase enzymes are represented in large diversity. Therefore, it is conceivable that a much smaller level of aeration of the contaminated subsurface environment would be sufficient to enhance aerobic degradation of the contaminants. In light of environmental sustainability, it would be beneficial to reveal those structural changes in the microbial communities induced by the contamination and concurrent processes, such as decreased oxygen availability.

1.1 Key questions and aims of the research

1. One of the main research questions of this research is whether the low availability of dissolved oxygen is the main factor which causes the dominancy of Betaproteobacteria in BTEX contaminated subsurface environments. Could other bacterial groups and other types of C23Os become dominant without oxygen limitation?

2. The second main aim of the project is to isolate bacterial strains from the aromatic hydrocarbon-degrading enrichment cultures and to look for new C23O and other relevant functional genes (e.g., toluene monooxygenases) possibly taking part in the degradation of the contaminants either microaerobic or fully aerobic conditions.

3. Thirdly, to find out how distinct microbial communities evolve in case of a xylene contamination under hypoxic or aerobic condition.

2 LITERATURE REVIEW

2.1 BTEX as an environmental contaminant

During the last decades, due to heavy industrialization, the use of fossil fuel products has severely increased the environmental contamination by different hydrocarbons (Odell, 2000). Mismanagement of contaminants in use have caused widespread contamination of air, soil and water. Human practices such as, transportation, oil refining, urban development, etc., have changed a large number of ecosystems (Halpern *et al.*, 2007). According to the European environment agency, the primary soil contaminants in Europe are hydrocarbons (European Environment Agency, 2005), that can contaminate the soil and subsurface environment and, as a consequence, contaminate the groundwater reserve and aquatic ecosystems. The US EPA has also classified these compounds as priority contaminants of natural resources (Husain, 2008). The primary source of hydrocarbon contamination in the environment is petroleum. Petroleum is a blend of complex compounds, mainly hydrocarbons and other organic compounds. Hydrocarbons are formulated either by the biosynthetic activity of microorganisms and plants (enzymatic reduction of fatty acid molecules) (Schirmer *et al.*, 2010) or by prolonged geochemical processes that transform biological compounds under high temperature and pressure. BTEX are one of the prominent classes of pollutants that naturally occur in crude oil. They are among the most abundant chemicals produced worldwide by the petrochemical industry, with benzene alone accounting for ~50 million metric tons. BTEX are generally colourless, highly volatile substances (Coates *et al.*, 2002), mostly liquid at room temperature, moderately soluble in water and soluble in organic solvents. These compounds are highly mobile in the environment, and always there is a threat that these compounds could end up in drinking water.

All four members of BTEX compounds exhibit both high health risks to humans and the environment. Among them, xylene is one of the mono-aromatic hydrocarbons that occurs naturally in coal tar and petroleum and is also a component of smoke from most combustion sources. There are three xylene isomers exist: *ortho*-xylene, *meta*-xylene, and *para*-xylene; abbreviated *o*-, *m*-, and *p*-xylene, respectively. Molecular formula of xylene is C_8H_{10}, also known as $C_6H_4(CH_3)_2$. The molecular

structures of the three xylene isomers are represented in **Fig. 1**. Xylene is a colourless liquid with a sweet odour and is volatile, flammable, and explosive in the air. The source of xylene in the atmosphere is mainly due to emissions from industries (e.g., petroleum refineries, chemical plants), automotive exhausts, and due to volatilization from their use as a solvent. Xylenes are somewhat mobile in soil and may be absorbed there. Xylenes may seep into groundwater, where they may linger for several years. Xylenes are stable to hydrolysis and oxidation in the aquatic environment (Chemical, Reporting and Right-to-know, 1997; US EPA-United States Environmental Protection Agency, 2012)

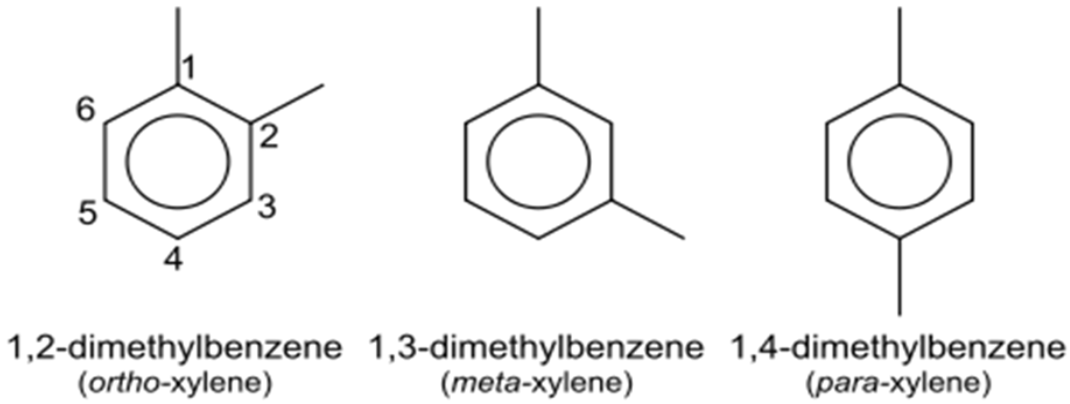


Figure 1: The molecular structures of the three xylene isomers

The majority of commercially available xylene is derived synthetically from petroleum and, to a lesser extent, from coal. Mixed xylenes produced from petroleum contain approximately 20 percent *o*-xylene, 44 percent *m*-xylene, 20 percent *p*-xylene, 15 percent ethylbenzene, and 1 percent other hydrocarbons (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). High-purity mixed xylenes are used as a solvent in chemical manufacture, agricultural sprays, adhesives, paints, and coatings. Xylene is also a key ingredient of aviation fuel and gasoline and is used as a feedstock material in the chemical, plastic, and synthetic fiber industries. In addition, xylene isomers are also used in the manufacturing of various polymers (Peters, 1991; Howard, 2017). *Ortho*-xylene is exclusively used in making phthalic anhydride, which is an aromatic acid anhydride commercially available as white, free-flowing flakes used mainly in the manufacture of plasticizers, unsaturated polyester resins, and alkyd resins. Furthermore, *o*-xylene is used in the manufacture of bactericides, herbicides, and lube oil additives, *m*-xylene is used in the manufacture of isophthalic acid that is used to make specialized resins. Finally, *p*-xylene is the crucial ingredient for making dimethyl

terephthalate and terephthalic acid (DMT/TPA), subsequently used as a raw material of polyethylene terephthalate (PET) that is used in polyester fibers, molded plastics, films, and blown beverage bottles. (USEPA, 1994). A significantly less percentage (approximately less than 1%) of mixed xylene derived from coal during the carbonization of coal in coke ovens—the demand for pure isomers, especially `*p*-xylene, is higher compared to the demand for mixed xylene. However, separation of individual isomers is difficult for xylene isomers through conventional distillation because of the closeness of their boiling points; Hence, by using the differences in freezing points, xylene isomers are separated (Hancock, 1982). Exposure to VOCs, like xylene, has remarkable harmful health effects on living beings. Generally, direct interaction with xylene compounds happens through breathing, eye, oral, and dermal route. In most countries like the United States, the health authorities suggest a threshold level of xylene as 100 ppm in the industrial area of xylene production and use and 14 ppm in the normal atmosphere (Langman, 1994). Majorly xylene released from petroleum refineries and automobile exhausts in the environment eventually may percolate into the groundwater reservoir resulting in entering into the food chain. Xylene is primarily metabolized in the liver by oxidation of methyl groups resulting in hippuric acid (Ogata *et al.*, 1970; Šedivec and Flek, 1976), which is released through urine. However, excess amounts or higher doses of xylene could harm the liver, and even metabolites damage the hepatocyte. Fewer fractions of xylene are also excreted through exhalation. The hazardous effect and its intensity also depend on different factors such as exposure route, duration and also depends on the individual. Occupational exposure to xylene is mainly observed in histopathology laboratories, petrochemical and steel manufacturing, and leather and rubber industries, as shown in **Fig. 2.**

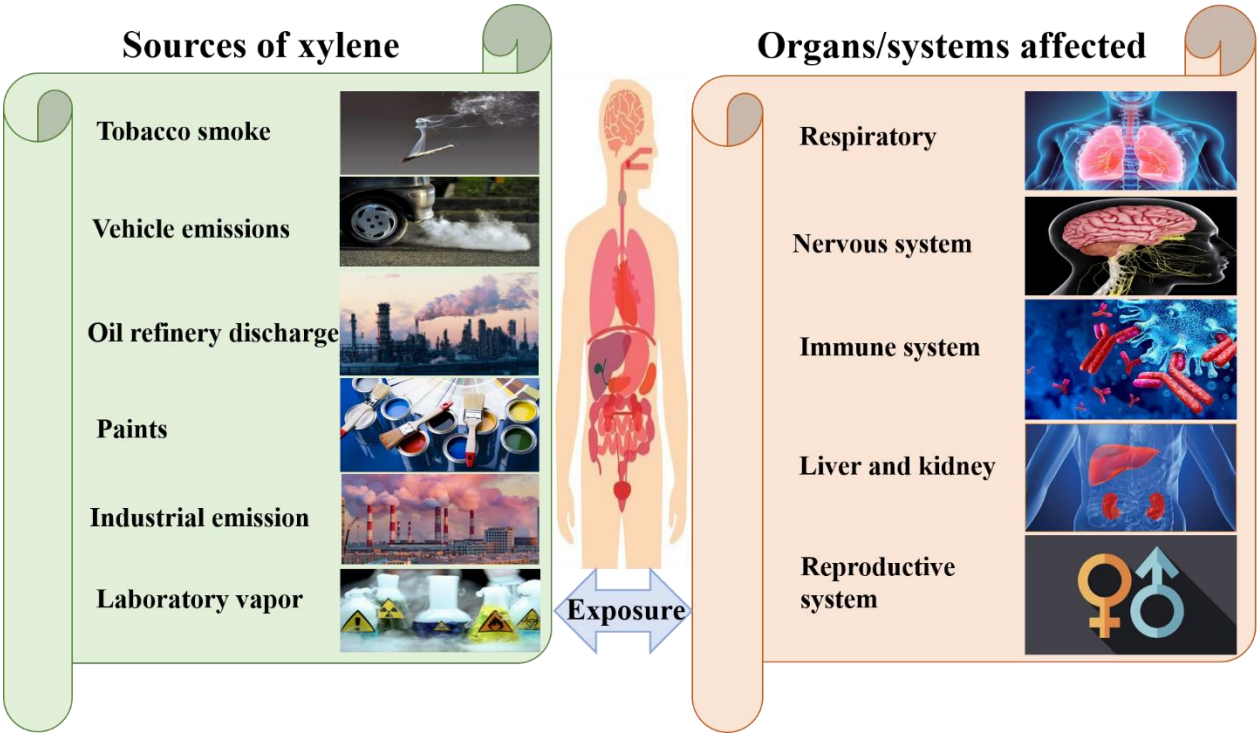


Figure 2: Sources and human heath hazards of xylene isomers

Jewel processing workers, who are exposed to xylene, benzene and toluene are found to be more prone to DNA damage in the peripheral blood cells (Huang *et al.*, 2010). They also suffer from fatigue, conjunctivitis, deterioration in memory and hand degreasing. Limited exposure to mixed xylene leads to incoordination, memory loss, coryza, catamenai disruption, dermatitis and pharyngitis. Different studies have reported symptoms like nausea, vomiting, poor appetite and gastric discomfort of the gastrointestinal tract (GIT) in workers exposed to xylene vapours (Klaucke *et al.*, 1982; Nersesian *et al.*, 1985; Uchida *et al.*, 1993). Although, above mentioned symptoms subsided after the cessation of xylene contact. Goldie (1960) reported that xylene exposure caused gastric discomfort and nausea. Excessive or over exposure to xylene can lead to edema of the lungs, which is a threat to life due to the accumulation of fluid in the lung. A significant increase occurred in irritation of the nose and esophagus in workers subjected to long-term mixed xylene (Uchida *et al.*, 1993).Comparison of individual isomers of xylene effects on respiratory system of both rat and mice showed that *o*- and *m*-xylene have more prolonged effects than that of *p*-xylene (Korsak *et al.*, 1990). Various studies have found an increased acidity of kidney tubules (Martinez *et al.*, 1989), declined creatinine in the urine, and hematuria due to xylene exposure (Morley *et al.*, 1970). Studies reported that direct eye

contact with heated xylene could lead to hemorrhagic eye, conjunctiva, intolerance to light, irritation and incomplete loss of epithelium (Ansari, 1997).

2.2 Hydrocarbon bioremediation techniques

To transform and reduce the occurrence of aforementioned contaminants from the environment (from soil and groundwater resources), several remediation methods are used, which can be divided into two major groups: *ex-situ* and *in situ*. In *ex-situ*, the contaminated subject is cleaned up out of place. *In situ*, the contaminated environment is treated in its original location using several physical-chemical and degradation methods. Due to the cost factor, *ex-situ* procedures are not commonly preferred. However, in some cases of severe contamination, where urgent treatment is needed, this technique is an obvious choice over time-consuming methods. The *in-situ* remediation is relatively slow but usually involves the least environmental intervention and the least financial outlay. One of the most essential processes for the removal of pollutants is biodegradation. The bioremediation technology was invented by George M. Robinson (1960) when the natural microbial population was used to remove petroleum and other hydrocarbon pollutants from the environment in a more cost-effective way than other remediation technologies (Ulrici, 2000; Sonawdekar, 2012; Okoro and Akpabio, 2015). For both *in situ* and *ex situ* cleanup, biological remediation techniques have demonstrated astounding effectiveness. They are capable of remediating or degrading aromatic hydrocarbons and various organic contaminants to non-toxic compounds without any long-term adverse effects on the impacted environments (Lim *et al.*, 2016). The separation and destruction of the contaminated environments could be performed using several *in-situ* and *ex-situ* remediation treatment technologies like biological, chemical, physicochemical, thermal, electric, electromagnetic and ultrasonic treatment methods (Reddy, 2013; Rodrigo *et al.*, 2014; Chang *et al.*, 2016).These remediation techniques can contain, sequester, separate, extract, remove, destroy, transform and mineralize contaminants in the polluted environment into less harmful, non-hazardous, less reactive forms. Even decrease the concentration of the contaminants. (Maletić *et al.*, 2013). There are various restoration and remediation treatment methods for soil, sediments, surface water, and groundwater contaminated with petroleum hydrocarbon compounds.

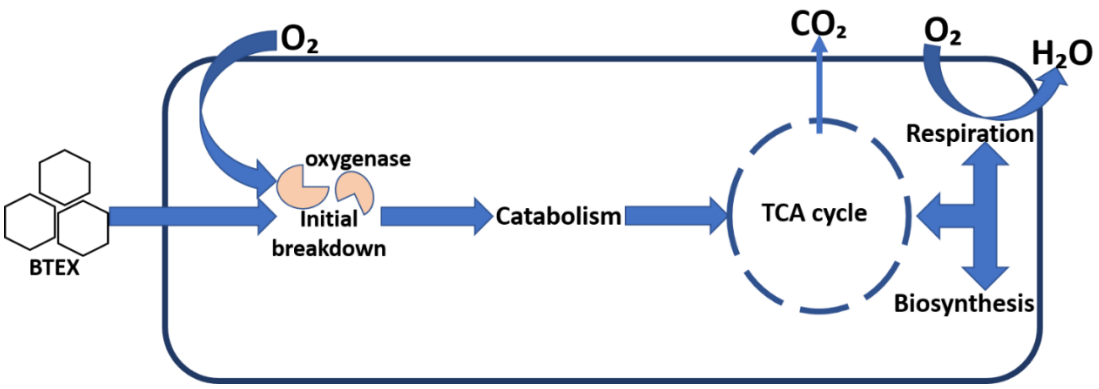


Figure 3: Simplified outline of microbial BTEX breakdown

Bioremediation (*in-situ* and *ex-situ*) is environmental-friendly, sustainable, and cost-efficient method for restoration and cleaning of contaminants in the soil and water. By using hydrocarbon-degrading microorganisms like bacteria, fungus, petroleum hydrocarbon pollutants are naturally broken down in this process. It removes and neutralizes contaminants in the environment into non-toxic or simpler compounds such as carbon di-oxide and water (**Fig 3**) through oxidation under aerobic conditions by the microorganisms with nutrient supply and optimization of the limiting factors for their biological activities (Chaillan *et al.*, 2004; Kostka *et al.*, 2011; Dzionek *et al.*, 2016; Wu *et al.*, 2017; Yanti, 2018). The interest of researchers in bioremediation employing specific microbes for petroleum hydrocarbon breakdown is increasing. Some of the most commonly isolated petroleum hydrocarbon degrading bacteria belong to the genus *Pseudomonas* which efficiently degrade petroleum hydrocarbon into simpler compounds (Wongsa *et al.*, 2004). Bioremediation of petroleum hydrocarbon has gained its popularity after the incident of Exxon Valdez spill in 1980 (Hoff, 1993). Bioattenuation (*in-situ* and *ex-situ*) is the naturally occurring process, including a variety of physical and biochemical processes, to remove, transform, neutralize and reduce the mass, volume, concentration and toxicity of the contaminant (Agarry and Latinwo, 2015; Abatenh *et al.*, 2017). When alternative remediation techniques cannot be used, bioattenuation might be used in polluted areas with low concentrations of pollutants. (Vásquez-Murrieta *et al.*, 2016). In biostimulation (*in-situ* and *ex-situ*) technique, the addition of any stimulatory materials, bulking agents, nutrients amendments, bio-surfactants, biopolymers and slow release fertilizers was done to enhance and support microbial growth and enzymatic activities of the autochthonous microorganisms in the contaminated soil for remediation activities (Agarry and Latinwo, 2015; Carr *et al.*, 2015; Lim *et al.*,

2016). Bioaugmentation (*in situ* and *ex-situ*) involves the addition of exogenous microbial cultures, autochthonous microbial communities, or genetically engineered microbes with a specific catabolic ability to degrade contaminants to increase the rate of degradation (Abdulsalam *et al.*, 2010; Nzila *et al.*, 2016; Nwankwegu and Onwosi, 2017) Bioventing (*in situ*) is the injection of air (oxygen) into the contaminated soil in order to increase the in situ degradation and to minimize volatile contaminants to the atmosphere (Camenzuli and Freidman, 2015; Trulli *et al.*, 2016). Biosparging (*in situ*) involves the injection of air (oxygen) and nutrient to stimulate the growth of indigenous microorganisms and thereby enhance the transformation of the contaminants (Azubuike *et al.*, 2016). Biosparging is used to reduce the contaminant concentration adsorbed to the soil, as well as contaminants dissolved in the groundwater. Bioslurry (*ex situ*) involves the treatment of contaminated soil in controlled bioreactors such as sequencing batch, feed-batch, continuous and multistage bioreactors (Azubuike *et al.*,2016; Megharaj and Naidu ,2017; Zappi *et al.*,2017). In this treatment process, nutrients are added to enhance microbial activities to degrade the contaminants. Biotransformation (*in situ* and *ex-situ*) is a biotechnological process that involves structural modifications in the chemical components, aided by microorganisms or enzyme systems, to form molecules with high polarity. This process transforms organic compounds from one form to another in order to reduce the toxicity and persistence of the contaminants (Størdal *et al.*, 2015). Landfarming (*in situ* and *ex-situ*) is an engineered bioremediation system, which employs tilling, ploughing and spreading of the impacted soil in the land surface to stimulate aerobic microbial activities with addition of nutrients. It is suitable for treatment of soil contaminated with low molecular weight petroleum hydrocarbons, volatile organic compounds (VOCs) and variety of other organic compounds (Guarino *et al.*, 2016; Brown *et al.*, 2017). Composting (*in situ* and *ex situ*) is a controlled microbial aerobic biochemical degradation of organic waste and its conversion into a stabilized organic material that can be used as soil conditioners for remediation of soil contaminated with organic compounds (Cai *et al.*,2016; Ren *et al.*, 2017).

Other than the way mentioned above, there are other biological remediation techniques also used,like, vermicomposting or vermi remediation by utilization of earthworms, Mycoremediation by the use of fungi, Phytoremediation using green plantsto to remove or extract contaminants in the soil, sediments, surface water and groundwater (Ekperusi and Aigbodion., 2015; Chachina *et al*, 2016; Anderson and Juday, 2016).

2.3 Factors affecting the degradation of hydrocarbons

Several factors influence the success of a bioremediation process. Limitations related to bioremediation processes may be due to the recalcitrance of contaminants, the incomplete pollutant transformations and the slow biodegradation rate. It is commonly observed that during bioremediation by bioaugmentation method, microbial viability decreases because of microbial stress caused by decreased nutrient availability, changes in environmental factors such as temperature, pH, salinity, the toxicity of pollutants and their intermediates, and even competition between the inoculated and indigenous bacterial community. The efficiency of microbial bioremediation can depend on the following major factors. When these factors are favourable to the microorganisms, biodegradation reaches a maximum level.

Temperature is one of the most important factors that influence biodegradation by affecting the physical and chemical compositions of pollutants (Atlas, 1981). It was observed that low temperatures decrease the rate of degradation in general; one of the reasons for that might be due to reduced enzymatic activity (Bisht *et al.*,2015). Another reason could be due to an increase in the viscosity of the substance, availability of the substances to microbes decrease or may be due to reduced evaporation of volatile components, pollutants can have a toxic effect on microbes (Leahy and Colwell, 1990). However, there are known psychrophilic bacterial species that can grow and degrade BTEX effectively under low temperatures, which are practically crucial for bioremediation purposes because in a contaminated subsurface environment temperature factors are usually not within the optimal range. Although , biodegradation of BTEX can take place on a wide range of temperatures, the rate of degradation increases with the rise in temperature, probably due to higher metabolic activity in bacterial strains. Several studies have been conducted to understand the effect of temperature on microbial degradation, one of such experment by Dibble and Bartha (1979) showed that the biodegradation capability of microbes increases with an rise in temperature. Hydrocarbon metabolism rate was found to be maximum in high temperatures between 30 °C to 40 °C (Bossert, et al., 1984).

Oxygen concentration has been found to be a limiting factor for the biodegradation of pollutants. The availability of oxygen in the ecosystem depends on several factors, like oxygen consumption rates of the indigenous microbes, soil type, presence of organic materials, presence of pollutants that can lead to oxygen depletion. (Haritash and Kaushik, 2009). Some organisms require oxygen as the main electron acceptor for aerobic bioremediation; on the other hand, some do not require oxygen for

degradation, but in general aerobic degradation is much faster than anaerobic degradation (Macaulay, 2015). This due to the fact that the first step in the microbiological degradation of hydrocarbons is usually the oxidation of contaminants with oxygenase enzymes in the presence of oxygen (Leahy and Colwell, 1990). In an anaerobic environment the help of alternative electron acceptors like nitrate, sulfate, Fe (III), Mn (IV), chlorate, perchlorate, arsenate and CO_2 is needed. Some studies showed that anaerobic microbial degradation of contaminants occurs at negligible rates (Haritash and Kaushik , 2009). Whereas McNally et al. (1998) showed that the aerobic biodegradation rate of pollutants was higher compared with the anaerobic biodegradation.

The availability of nutrients for the microbes is also an important factor for successful contaminants biodegradation (Atlas, 1981). The optimal C / N and C / P ratios are essential for the proper metabolism of microorganisms. Some of these nutrients can be limiting factors, thus impacting the biodegradation rate. Hence in oligotrophic environments, the degradability is either slow or negligible. Normally in a contaminated environment, carbon comes from an organic source (the contaminant); hydrogen and oxygen are from the water (Kalantary *et al.*,2014) but in such environment, drastic increase of carbon source and decrease of nitrogen and phosphorus concentration observed that affect the biodegradation rate. In the marine environments availability of nitrogen and phosphorus are low and in the wetlands majority of the nutrients are competitively taken up by the plants. Thats why, supplementation of nutrients is necessary to promote the biodegradation of contaminants (Hesnawi and Adbeib, 2013). Thus, nitrogen and phosphorus are also often added to boost the degradation. On the other hand, the concentration of excess nutrients can also inhibit biodegradation activity (Atlas, 1981; Leahy and Colwell, 1990).

The pH is also one of the most crucial variables that can affect the rate of biodegradation significantly. The pH affects functions like cell membrane transport, enzyme activities.(Bonomo *et al.*,2001). In general, the pH of the ecosystems may vary widely. The majority of heterotrophic bacteria favour a pH range of neutral to alkaline for optimum growth. However, hydrocarbon-degrading bacteria are known, that is adapted to extreme alkaline environments (pH, 7.5-10.6) (Sarnaik and Kanekar, 1995, 1999). Dibble and Bartha (1979) reported that pH 7.8 is the optimum pH for degradation in oil sludge samples. Pawar (2015) found that pH 7.5 was most appropriate for the hydrocarbon degradation in soil.

There are several other factors that can affect the biodegradation rate like salinity, the activity of water, toxicity of metabolites, composition of microbial community.Ward and Brock (1978) reported that degradation rates substantially reduce with the increase of salinity due to decline in microbial

metabolism rates. Qin et al. (2012) pointed out that salinity has a severe effect on the bioremediation and biodegradation process, and it also can influence changes in microbial growth and diversity. The biodegradation in terrestrial ecosystems may be restricted depending on the water available for metabolism and microbial growth. Dibble and Bartha (1979), in their experiment, showed that biodegradation was optimal with 30–90% water saturation in oil sludge. The availability of water directly impacts the movement and growth of microorganisms. One of the major factors impacting hydrocarbon degradation is the availability of microorganisms (bacteria, fungi and some algae) that have the ability to catabolise pollutants.

2.4 Microbiology of BTEX degradation

Monoaromatic hydrocarbons (BTEX) are recalcitrant in nature due to their stability; the reason behind this is the presence of an aromatic planar ring allowing a low reactivity and degradation rate. Microorganisms are known to play a critical role in the degradation of these toxic compounds. Microorganisms use these toxic monoaromatic pollutants as source of carbon and energy and subsequently transform them into simpler, less harmful compounds that then enter into central catabolic pathways. Hence, this detoxification process contributes to the global carbon cycle. During the process of complex biochemical conversion, the microorganism gains energy and building blocks in return. Hydrocarbon-degrading microorganisms are ubiquitous in the environment (Atlas and Hazen, 2011) due to the presence of the necessary enzyme complex that is needed for the degradation, but their percentage in the community is known to be highly variable (Jones et al, 1970; Pinholt *et al.*, 1979; Hollaway *et al.*, 1980) Oxygen limited aromatic hydrocarbon contaminated environments are commonly dominated by genera belonging to the order Burkholderiales (formerly the class of Betaproteobacteria), including the families *Comamonadaceae* and *Rhodocyclaceae* (Fahy *et al.*, 2006; Alfreider and Vogt, 2007; Nestler *et al.*, 2007; Martínez-Lavanchy *et al.*, 2015). There are several bacterial genera exhibited to be efficient hydrocarbon degraders. Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria isolated from the marine environment, while Bossert et al.(1984) listed 22 bacterial genera isolated from soil. Based on the published reports, the most important hydrocarbon-degrading bacteria are *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Comamonas, Micrococcus, Nocardia, Rhodococcus, Hydrogenophaga, Rhodoferax, Sphingomonas* and *Pseudomonas* (Kim and Oriel, 1995; Essam *et al.*, 2010; Fuchs et al. 2011; Wasi et al., 2013). The list of known organisms capable of degrading hydrocarbons is continuously expanding, with almost 200 bacterial, fungal and algal genera recorded (Van Beilen *et*

al., 2003; Yakimov et al. 2007). Among these well-known genera, *Pseudomonas* is believed to be the most effective aromatic hydrocarbon degrader that is widely studied. (Ridgway *et al.*, 1990; Brusa *et al.*, 2001; Yu et al. 2001; Jahn et al. 2005). *Pseudomonas* spp. have a high degree of physiological adaptability since they can grow on both simple and complex organic carbon compounds at 4–42 °C and pH 4.0–8.0 (Moore *et al.*, 2006). Owing to diverse metabolism, members of this genus can survive in a wide range of niches (Gupta *et al.*, 2008), including soils containing high levels of pollutants, such as xenobiotics (Manickam *et al.*, 2008) e.g. haloaromatic-compounds (Stolz et al. 2007), alkylphenols, and petroleum hydrocarbons (Hirota *et al.*, 2011). Strains are known which are able to degrade almost the entire palette of BTEX compounds under aerobic conditions (Alvarez and Vogel, 1991; Haigler et al., 1992; Johnson and Olsen, 1995; Lee and Gibson, 1996; Attaway and Schmidt, 2002). Among indigenous bacteria commonly found in contaminated environments, members of the genus *Sphingobium* are well-known inhabitants of such ecosystems due to their diverse environmental adaptations and biodegradation capabilities (Zhao *et al.*, 2017). Many members of this genus are known to degrade a broad range of mono- and polycyclic aromatic compounds (Bünz et al. 1993; Arfmann et al. 1997; Baraniecki et al.2002; Kertesz and Kawasaki, 2010; Gai *et al.*, 2011; Singh *et al.*, 2013; Mukherjee *et al.*, 2013) and many other methyl-, chloro-, hydroxyl-, and nitroaromatic compounds (Pinyakong *et al.*, 2003; Leys *et al.*, 2004; Chen *et al.*, 2014). Several strains belonging to the genus reported to have the ability to utilize BTEX compounds like *Sphingobium aquiterrae* SKLS-A10^{T} (Revesz *et al.*, 2018), *S. yanoikuyae* B1 (Zylstra and Kim, 1997), *Sphingobium xenophagum* (Hilaire *et al.*, 2021), *Sphingobium terrigena* (Park *et al.*, 2019), *Sphingobium japonicum* (Nagata *et al.*, 2010) *and Sphingobium hydrophobicum* (Chen *et al.*, 2016). Members of the genus *Hydrogenophaga* were also found to be present in noticeable abundance in BTEX contaminated environments (Táncsics *et al.*, 2010, 2012, 2013; Benedek *et al.*, 2016). Among them, some of them have been reported to be able to utilize aromatic hydrocarbon as a sole source of carbon, like *Hydrogenophaga taeniospiralis* and *Hydrogenophaga flava* (Willems *et al.*, 1989). The major group of isolated anaerobic BTEX degraders are *Azoarcus, Clostridium, Thauera, Dechloromonas and Aromatoleum spp.* (Coates *et al.*, 2001; Kasai *et al.*, 2006; Widdel *et al.*, 2010; van der Zaan *et al.*, 2012; Luo *et al.*, 2014). *Geobacter* spp. are known to degrade toluene or xylenes (Kunapuli *et al.*, 2009) and benzene under iron-reducing conditions (Zhang *et al.*, 2012). Under sulphate-reducing conditions strains belonging to the *Desulfosarcina, Desulfobacula, Desulfotignum, Desulforhabdus, Desulfocapsa, Desulfosporosinus* (Heider *et al.*, 1998; Liu *et al.*, 2004; Morasch *et al.*, 2004; Kunapuli *et al.*, 2009; Weelink *et al.*, 2010; Widdel et al. 2010) were found to be able to

degrade BTEX compounds. During methanogenic oxidation, when no other electron acceptor is available, BTEX degradation takes place by syntropy between methanogenic archaea and fermenting degraders (Vogt *et al.*, 2011), members of genus of different hydrogenotrophic (*Methanospirillum, Methanobacterium* spp.) or acetotrophic (*Methanosaeta* spp.) methanogens found to be present in such environments (Ficker *et al.*, 1999; Ulrich and Edwards, 2003; Fowler *et al.*, 2011)

2.5 Catabolic process and key enzyems for BTEX degradation

Bacterial decomposition of hydrocarbons is generally classified into two categories based on the catabolic process involved: oxygen dependent (aerobic degradation) and oxygen-independent (anaerobic degradation). Aerobic degradation of BTEX compounds is initiated by the oxidation process, with the incorporation of molecular oxygen catalyzed by oxygenase. Hydroxylated aromatic rings are subsequently catabolized step by step with the help of a complex enzyme system and transformed into the intermediate like acetyl-CoA, succinate, and pyruvate that are further funnelled into the central catabolic pathways. Mainly, monooxygenases and dioxygenases, these two types of oxygenases play a critical role in the initial oxidation. Briefly, monooxygenase targets the methyl or ethyl substituents on the aromatic ring by incorporating one oxygen atom into the substrate and the other remaining oxygen atom is reduced into water (Tsao et al. 1998). Whereas dioxygenases catalyse by adding both the atoms of the oxygen molecule into the substrate. Thereby introducing two hydroxyl groups to form cis-dihydrodiol. Later, dehydrogenation transforms the cis-dihydrodiol intermediate into a dehydroxylated aromatic compound. Dihydroxylated aromatic rings (majorly catechols) are further catabolized by an extradiol (*meta*-cleavage, C23O) or intradiol (*ortho*-cleavage.C12O) dioxygenase, causing the formation of 2-hydroxymuconic acid aldehyde and cis, cis-muconic acid, respectively, This product further funnelled into central degradation pathways (**Fig. 4**). Under microaerobic conditions, some microorganisms use oxygen only to introduce hydroxyl groups into the aromatic ring as in classical aerobic pathways, whereas their cleavage occurs through anaerobic metabolic pathways (Chakraborty and Coates, 2004; Fuchs, 2008). Additionally, low oxygen levels also inhibit the enzyme activity of dioxygenases, blocking their breakdown through aerobic respiration since there is not enough oxygen to serve as an electron acceptor. (Yerushalmi et al. 2002).

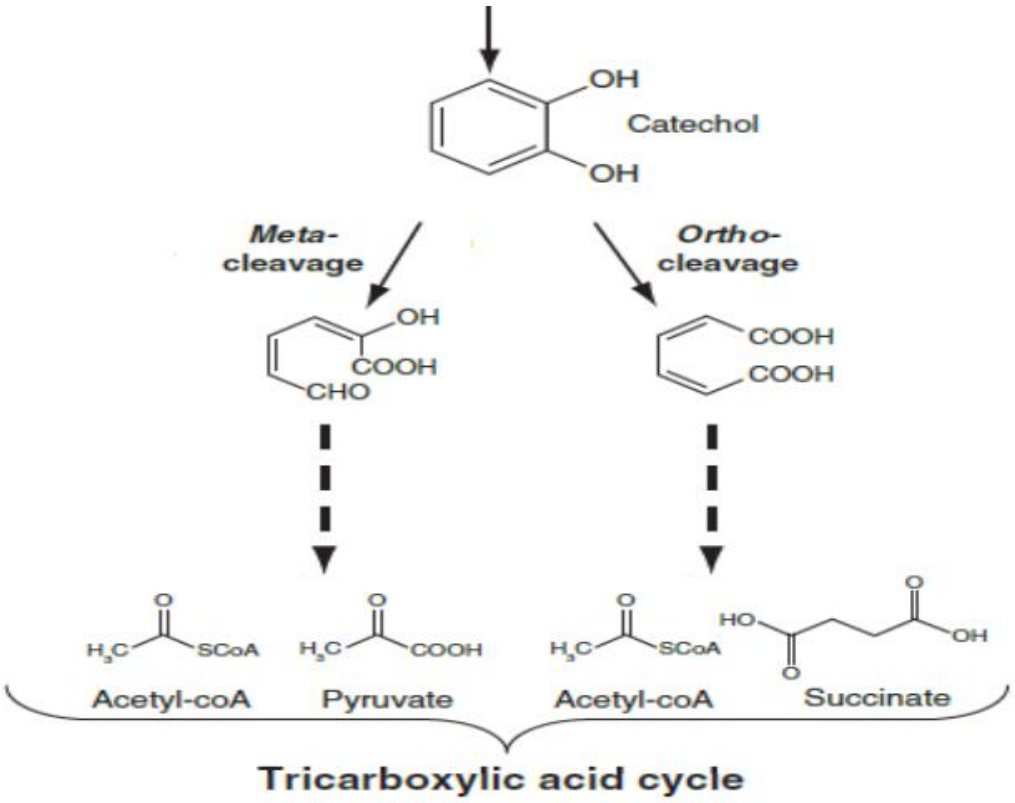


Figure 4: Example of aerobic aromatic hydrocarbon degradation (adapted from George and Hay, 2011).

Generally, anaerobic degradation of BTEX compounds occurs slower than aerobic degradation. Anaerobic catabolism uses different terminal electron acceptors (e.g., sulfate, nitrate, Fe (III) or methane). In anaerobic oxygen limited conditions, aromatic hydrocarbons serve as an electron donator by oxidizing these compounds in the presence of a terminal electron. Anaerobic degradation of aromatic hydrocarbons was first described by Kuhn et al. in 1985. Several studies have been performed to understand the anaerobic degradation of aromatic hydrocarbons (Heider *et al*, 1998; Heider *et al*, 2007; Foght, 2008;). Anaerobic degradation pathways of toluene and ethylbenzene by denitrifying and sulfate-reducing microorganisms are well documented. Moreover, these studies also indicated that anaerobic benzene degradation is the most challenging and anaerobic toluene degradation is the easiest metabolic process. In addition, it was also found that all three xylene isomers are biodegradable anaerobically but with different susceptibilities (Foght, 2008).

Microbial degradation of BTEX compound is a complex enzymatic process involving several enzymes like microbial oxidoreductases, oxygenases, laccases, peroxidases, lipases, esterase etc.

Among them, oxygenase is the most important key enzyme for degradation. Oxygenases are the oxidoreductase group of enzymes. Their mode of action involves the oxidation of reduced substrates by integration of transferring oxygen using FAD/NADH/NADPH as a cosubstrate. Based on the oxygen atoms used in oxygenation, oxygenases are categorised into monooxygenases and dioxygenases. Oxygenase catabolizes hydrocarbons by cleavage of the aromatic ring using the introduction of O_2 atoms into the organic molecule. Bacterial mono- or dioxygenases are the most studied enzymes in bioremediation (Fetzner and Lingens, 1994; Arora *et al.*, 2009). Monooxygenases add only a single atom of the oxygen molecule into the substrate. Based on the presence of cofactor, monooxygenases are categorised into two subclasses: flavin-dependent monooxygenases and P450 monooxygenases. Flavin-dependent monooxygenases harbour flavin as a prosthetic group and require NADP or NADPH as a coenzyme. P450 monooxygenases exist in both eukaryotic and prokaryotic organisms that contain heme. The majority of the monooxygenases studied so far have a cofactor. However, there are still certain monooxygenases that function independently of a cofactor and use only molecular oxygen for their activities and utilize the substrate as a reducing agent (Cirino and Arnold 2002; Arora *et al.* 2010). Methane monooxygenase enzyme is the best-characterized monooxygenase so far. Methane monooxygenase involves in the degradation of hydrocarbons such as substituted methanes, alkanes, cycloalkanes, alkenes, haloalkenes, ethers, and aromatic and heterocyclic hydrocarbons (Grosse *et al.*, 1999). Dioxygenases play a role in environmental remediation by oxidizing aromatic compounds. Dioxygenases are multicomponent enzyme systems belonging to a large family of Rieske nonheme iron oxygenases. All members belonging to this family contain one or two electron transport protein components along with oxygenase components.The crystal structure of naphthalene dioxygenase reveals the presence of a Rieske (2Fe_2S) cluster and mononuclear iron in each alpha subunit (Dua *et al.*, 2002). The catechol dioxygenases (intra-, and extradiol dioxygenases) play a key role in the degradation of aromatic hydrocarbons in the environment. They are present in indigenous soil bacteria that are involved in the transformation of aromatic compounds. The intradiol enzymes utilize Fe(III), and extradiol enzymes utilize Fe(II) and Mn(II) in a few cases (Que and Ho, 1996). Intradiol and extradiol dioxygenases show different amino acid sequence and thus belong to two distinct phylogenetic groups. Intradiol dioxygenases belong to a monophyletic group, but extradiol dioxygenases can be further classified into two large families: I.2 and I.3. named by Eltis and Bolin (1996). Among I.2 family, the enzymes involved in degradation of BTEX are found in the I.2.A, I.2.B, I.2.C and within the I.3 subfamily I.3.A (Vaillancourt *et al.*, 2006). Subfamily I.2.A contains the C23O gene sequences of predominantly fluorescent

Pseudomonas species, and subfamily I.2.B consists mainly of the C23O gene sequences of species belonging to the genus *Sphingomonas*. I.2.C type C23O gene sequences could be connceted to mainly with *Burkholderia* spp., *Ralstonia* spp. , *Commonas* spp. Monitoring of sites polluted with petroleum byproducts frequently involves the monitoring of catechol 2,3-dioxygenase genes. (Junca and Pieper, 2004; Higashioka *et al.*, 2009; Táncsics *et al.*, 2010; Benedek *et al.*, 2018). Because of the extremely high diversity of these genes, their study is only possible with the help of specific primers. Táncsics et al. (2010, 2013) were the first to design PCR primers with which a broad spectrum of C23O genes belonging to the I.2.C subfamily could be detected. Kukor and Olsen in 1996, observed that the extradiol dioxygenase enzymes encoded by the C23O genes of type I.2.C had increased oxygen affinity, suggesting that bacteria possessing these genes may play an essential role in the oxygen-restricted BTEX breakdown. Further research has also demonstrated that these genes are transcribed even under microaerobic conditions (Táncsics *et al.*, 2012).

2.6 Microbiology of xylene degradation

There are several studies conducted that found the role of various microorganisms in the effective degradation of xylene, including *Alcaligenes xylosoxidans, Pseudomonas putida* (Reardon et al. 2000; Yeom and Daugulis, 2001; Robledo-Ortíz *et al.*, 2011), *Pandoraea* sp. (Wang *et al.*, 2015), *Rhodococcus* sp. (Di Canito *et al.*, 2018) etc. A few isolates were reported to grow with *m*-xylene or *o*-xylene (Dolfing *et al.*, 1990; Fries *et al.*, 1994; Rabus and Widdel, 1995; Harms *et al.*, 1999; Morasch *et al.*, 2004), whereas, until now only one study has reported on a pure culture, which can degrade *p*-xylene anaerobically (Higashioka *et al*. 2012). It is widely known that subfamily I.2.C-type catechol 2,3-dioxygenase (C23O) genes are often found in high diversity in polluted subsurface habitats. Most of them are harboured by members of the Burkholderiales (Táncsics *et al.*, 2013), and some of them are involved in the microaerobic degradation of aromatics, e.g. toluene (Táncsics *et al.*, 2020). It is yet unclear how these bacteria and the I.2.C-type C23O genes contribute to the breakdown of xylene. Under clear aerobic conditions, xylene isomers are readily degradable by a plethora of microorganisms. Still, *p*-xylene is classified as a persistent pollutant, while *o*-xylene is also considered as a recalcitrant molecule. While *m*-xylene and *p*-xylene-degrading bacteria are relatively frequently reported, those, which can use the *ortho* isomer of xylene as the sole source of carbon and energy, are seemingly much rarer. Members of the genus *Rhodococcus* are often reported as abundant *o*-xylene degraders (Maruyama *et al.*, 2005), and their *o*-xylene metabolism is deeply studied (Zampolli *et al.*, 2020). Nevertheless, results of a recent enrichment study suggested that members of the genus

Rhodococcus prefer clear aerobic conditions for the degradation of petroleum hydrocarbons and are marginalized under microaerobic conditions (Révész *et al.,* 2020). Microbial degradation of BTEX compounds commonly occurs through two metabolic pathways, the *tod* pathway and *tol* pathways involving two enzymatic systems, dioxygenases and monooxygenases, respectively. In the *tol* pathway, monooxygenase attacks methyl or ethyl substituents of the aromatic ring (Tsao et al. 1998; Khan *et al.*, 2001).This step is followed by several oxidations transformed into pyrocatechols or phenyl glyoxal substituted compounds. In the *tod* pathway, dioxygenase catalyzes the aromatic ring and produces hydroxy-substituted compounds (Jindrova *et al.*, 2002). All types of xylene isomers are transformed into methylated catechols. For example, *m*-xylene degrades to 3-methylcatechol, and *p*-xylene is converted to 3,6 dimethylcatechol (Oh *et al.*, 1994). Later on, these catechol intermediates are catalyzed by any of the two enzymes: catechol 1,2-dioxygenase (C120) (*ortho*- or intradiol cleavage) or enzyme catechol 2,3-dioxygenase (C230) (*meta*- or extadiol-cleavage) (Smith, 1990; Jindrova *et al.*, 2002; Andreoni and Gianfreda, 2007).With the help of these enzymes, the ring is opened and becomes available for degradation (An *et al.*, 2001; Al-Khalid and El-Naas, 2012). Subsequently, the production of low molecular weight compounds (pyruvate and acetaldehyde) takes place that is further oxidized via the TCA cycle (Cozzarelli and Baehr, 2003). Research findings of Tsao et al. 1998 showed that microbes could use either the *tod* or *tol* pathway to oxidise xylene. Though, in case of *p*-xylene, bacteria use the *tod* pathway for degradation and form 3,6-dimethylcatechol as an intermediate. In the case of metabolism of *p*-xylene and *o*-xylene by *Pseudomonas putida* PPO1 similar observation was made. Several works of the literature indicated that xylene isomers could be degraded in the absence of oxygen as well under nitrate-reducing, sulfate-reducing, iron-reducing conditions (Kuhn *et al.*, 1985). Edwards *et al.*1992, showed that *o*-xylene could also be degraded under denitrifying and sulfate-reducing conditions. Even there are numerous pure cultures have been reported to utilize *m*-xylene for growth under nitrate- (Dolfing *et al.*, 1990; Fries *et al.*, 1994; Rabus and Widdel, 1995; Hess *et al.*, 1997) and sulfate-reducing condition. Though a handful of pure cultures of sulfate reducers have been reported to mineralize *o*-xylene anaerobically, among them *Desulfosarcina ovata* (Kuever *et al.*, 2006), and *Desulfotomaculum* sp. strain OX39 (Morasch *et al.*, 2004) are worth mentioning. Herrmann et al. (Herrmann *et al.*, 2009) reported sulfate-reducing enrichment cultures that are able to degrade *m*-, *o*- and *p*-xylenes. An iron-reducing enrichment also demonstrated the ability to oxidize *o*-xylene (Jahn et al. 2005) and *p*-xylene (Botton and Parsons., 2006). Numerous studies suggested that *m*-xylene is the most readily degradable isomer of xylene under anaerobic conditions. (Beller *et al.*, 1995).

3 MATERIALS AND METHODS

To answer the research questions mentioned earlier in the introduction, we have performed two enrichment experiments during the tenure of my doctoral study. In the first experiment, we established aerobic enrichment using *m*-, *p*- and *o*-xylene individually as sources of carbon and energy. For our second enrichment experiment, aerobic and microaerobic enrichments were set up, in which a mixture of xylene isomers (*m*-, *p*-, *o*-xylene) was added as the sole source of carbon and energy. Therefore, from these enrichments, we have examined how the difference in xylene isomers as substrate and oxygen limitation affect the selective enrichment of bacterial communities and the diversity of functional genes that play a vital role in the breakdown of hydrocarbons.

The following diagram **(Fig. 5)** is a simplified schematic representation of the material and methods used in general for the whole study for ease of understanding.

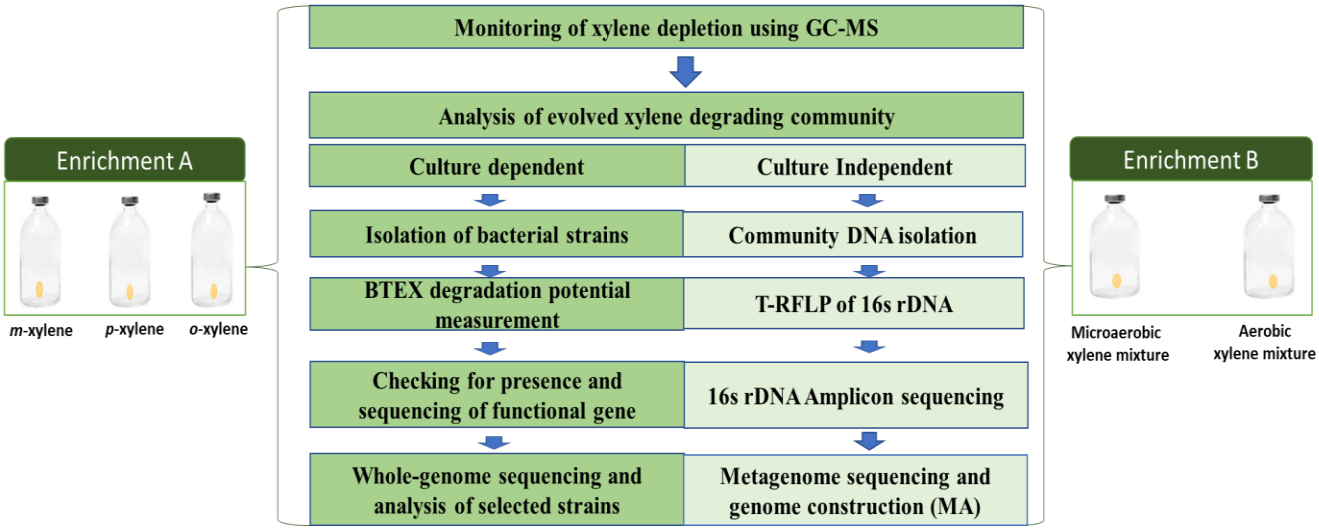


Figure 5: Schematic diagram of the overall processes flow: Enrichment A: aerobic enrichment with individual xylene isomer as carbon source, Enrichment B: Aerobic and microaerobic enrichment with xylene isomers mixture as carbon source.

3.1 Description of the Siklos sampling site and sampling techniques

BTEX contaminated groundwater sample used as inoculum for both the enrichments (Enrichment A and Enrichment B) were obtained from a gasoline contaminated site of Siklós, a town located in the south-west region of Hungary (**Fig. 6)** (Táncsics *et al.*, 2013) during February 2019 to establish Enrichment A and in Oct 2019 to be used for Enrichment B. This site was contaminated by petroleum hydrocarbons due to accidental leakage of the underground storage tank of a former petrol station during the end of the '80s, resulting in hydrocarbon contamination of the surrounding soil and groundwater reserve. Due to the comparatively soluble nature of BTEX compounds, this contamination was alarming for the authorities as they could mix with the drinking water reserve as a consequence. Physicochemical properties and BTEX concentration of contaminated groundwater are monitored orderly by an accredited laboratory (Wessling Hungary Ltd.) according to Hungarian Standard (MSZ 1484-4:1998; MSZ EN ISO 15680:2004) analytical techniques to enable the authorities to plan for strategic measures. The site has been thoroughly studied and described by Táncsics *et al*. (2012, 2013) earlier. The groundwater sample was collected from the monitoring well No. ST2 is located at the centre of the contaminated zone from a depth of ~ 2.5-4m. Due to metabolic activity of the autochthonous microbial community the concentration of contaminants decreased significantly over time compared to the beginning (around 25 years ago) of the contamination. But still concentration of some of the contaminants are beyond the permissible limit according to Hungarian standards. Nowadays, the contaminated aquifer is over dominated by the contamination of xylene. Since the groundwater in the contaminated area is located under a thick layer of clay, the oxygen supply of the contaminated groundwater is limited. Dissolved oxygen concentration values (0.6-2.3mg/L) indicated the same hypothesis.

Moreover, the data suggest that the reducing condition is prevalent in the contaminated zone. This aforementioned fact makes this site a perfect sampling point to answer our set research questions. The dissolved oxygen concentration and redox potential values were measured on-site using a WTW hand-held Meter Multi 350i (WTW, Germany). The sample was collected into sterile 1-L bottles, keeping no headspace, and transported to the laboratory, preserved at 4 °C in the dark for further investigations. Physicochemical characteristics of the collected samples at different times are summarized in **Table 1**.

Table 1: Physicochemical properties and contaminant concentration of groundwater sample of well ST2

Year	BTEX compounds(ug/L)					Physicochemical parameters					
	Well code	Xylene	Benzene	Toluene	Ethyl-benzene	DO(mg/L)	NO_3^- (mg/L)	$Fe2^+$(mg/L)	SO_4^{2-}(mg/L)	CH_4(mg/L)	redox potential(mV)†
2011*	SKV	<2	<0.2	<1	<1	3.3	61	<0.02	71	<0.04	369
	ST2	**6700**	**340**	**64**	**966**	**0.6**	**2**	**4**	**24**	**1.9**	**148**
2019 Feb	SKV	<2	<0.2	<1	<1	2.1	40	0.13	60	<0.04	14
	ST2	**2760**	**227**	**15**	**862**	**1.2**	**<5**	**8.2**	**<30**	**2.19**	-42
2019 Oct	SKV	<5	<0.2	<1	<1	4.4	44	**<0.03**	60	<0.04	171
	ST2	**992**	**49.9**	**2**	**4**	**2.3**	**<0.5**	**10.5**	**<30**	**1.32**	-42

*Data source: Táncsics et al. 2012; †measured with a standard hydrogen reference electrode.

Figure 6: Sampling at Siklos petroleum contaminated sample, A: location of the site, B: field sampling

3.2 Microbial enrichment experiments

In order to investigate the effect of different xylene isomers as carbon and energy sources and the availability of oxygen on the composition and metabolic abilities of bacterial communities involved in the degradation of xylene isomers, two enrichment experiments were performed. In one experiment, different xylene isomers under aerobic (7-8 mg/L dissolved oxygen concentration) conditions and in the other experiment, xylene isomer (*m*-, *p*-, *o*-xylene) mixture under microaerobic

conditions (≤ 0.5 mg/L dissolved O_2) were established. Details of the enrichment set up explained using a simplified diagram in **Fig. 7**.

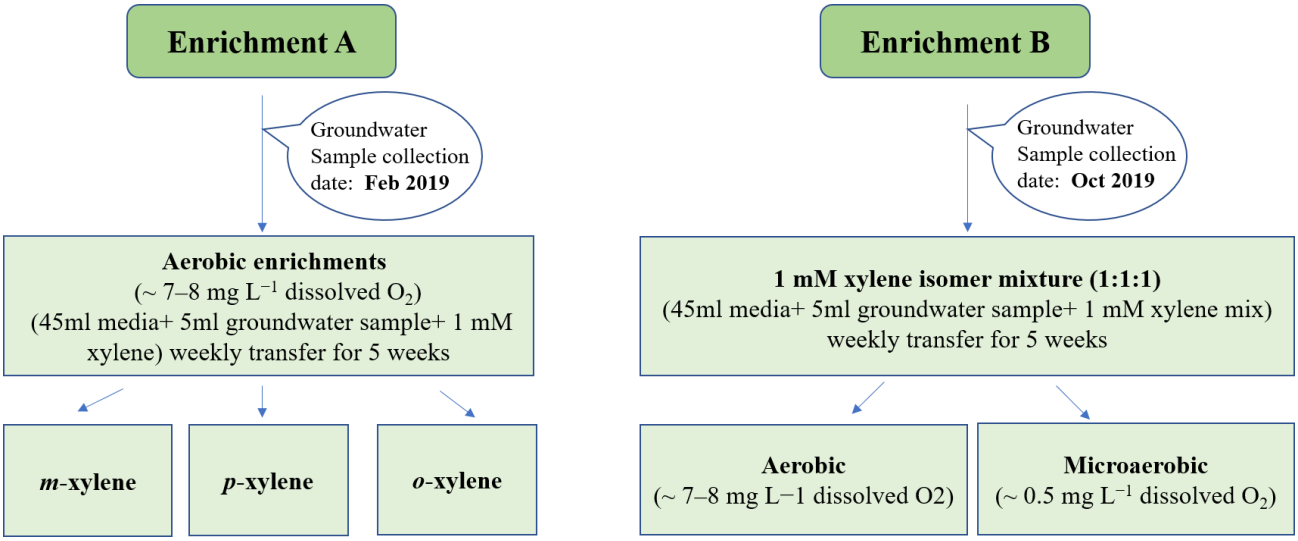


Figure 7: Schematic diagram of enrichment experiments

For enrichment A, we worked with two parallel samples each, and for Enrichment B, three parallel samples were made. As a culture medium, modified minimal mineral salts (MS) medium developed by Fahy et al. (2006) was used. The composition of the medium was as follows:

Fehy Medium (1lt)		
Solution A	**Solution B**	**Solution C**
100ml	**900ml**	**1ml**
5 g $MgSO_4$ x $7H_2O$ 1 g $CaCl_2$ x $2H_2O$ 1000 mL distilled water filter sterile with 0.2 µm	11.1 g Na_2HPO_4 2.5 g KH_2PO_4 10 g NH_4NO_3 1000 mL distilled water sterilized in an autoclave	10 g $FeSO_4$ x $7H_2O$ 0.64 g Na_2EDTA x $3H_2O$ 0.1 g $ZnCl_2$ 0.015 g H_3BO_3 0.175 g $CoCl_2$ x $6H_2O$ 0.15 g Na_2MoO_4 x $2H_2O$ 0.02 g $MnCl_2$ x 4 H_2O 0.01 g $NiCl_2$ x $6H_2O$ 1000 mL distilled water filter sterile with 0.2 µm

The resulting solutions were weighed under sterile conditions and supplemented with 1 mg / L vitamin B1, 15 µ / L biotin, and 20 µ / L vitamin B12 (Thermo Fisher Scientific Inc.USA). 45 mL of the prepared media were then dispensed into 100 mL serum bottles, which were sealed with a butyl rubber

stopper and crimped sealed with an aluminium cap. Subsequently, *m*-, *p*- or *o*-xylene was added (purity> 99%, Sigma-Aldrich Ltd.USA) individually in enrichment A and as a xylene mixture (1:1:1) in enrichment B as sole carbon and energy source at a final concentration of 1mM followed by inoculation with 5mL of the contaminated groundwater sample. Prior to the inoculation, microaerobic enrichments microcosms were sparged with N_2/CO_2 (80:20, v/v) for 10 minutes (**Fig. 8**). Then the desired concentration of dissolved oxygen was achieved by injecting sterile air (0.2 µm pore size filtered) through the butyl-rubber septa. In aerobic and microaerobic conditions, the bottles were monitored non-invasively by Fibox 3 trace v3 fibre optic oxygen meter with PSt3 sensor spots (PreSens, Germany) and OxyViewPST3-V7.01 software at regular intervals to ensure microaerobic and aerobic conditions. The amount of oxygen consumed during biological activity was continuously replenished in the enrichments. The oxygen concentration was restored whenever needed by injecting sterile air. The microcosms were incubated for 7 days in a shaking thermostat at 28°C, 150 rpm. Then 5 mL inoculum from each microcosm was transferred into a fresh medium. Similar transfers were made for consecutive five weeks. To monitor the consumption of xylene as the sole carbon source, the concentration of *m*-, *p*- and *o*-xylene was measured for 5^{th}-week enrichment microcosms at 24 h intervals by headspace analysis on an ISQ Single Quadrupole gas chromatography-mass spectrometer (GC–MS) (Thermo Fisher Scientific Inc.USA) via a SLB-5ms fused silica capillary column (Supelco Analytical.USA).

A **B**

Figure 8: Enrichment setup , A: Sensor spot used to monitor dissolved oxygen concentration; B: sparging of gas (N2/CO2) to remove oxygen

3.3 Isolation and identification of bacterial strains

Cultivable bacterial strains were isolated from fifth week samples of xylene induced enrichment cultures using conventional methods. To do so, 1 ml homogeneous inoculum from the xylene degrading enrichment samples was serially diluted with physiological salt solution (0.9 % NaCl). Then 0.1 ml of serially diluted samples were spread onto solidified R2A agar plates (protease peptone 0.5 g, casamino acids 0.5 g, yeast extract 0.5 g, dextrose 0.5 g, soluble starch 0.5 g, dipotassium phosphate 0.3 g, $MgSO_4$ x $7H_2O$ 0.05 g, sodium pyruvate 0.3 g, agar 15 g, pH 7 ± 0.2 (Sigma-Aldrich, Germany) plates. After one week of incubation at 28 °C, colonies of different morphology were isolated and maintained on new R2A plates. The strains were purified using the streak plate technique and maintained on R2A agar slants at 28 °C. In order to identify the strains, genomic DNA was isolated from pure cultures grown on R2A agar. Followed by 16S rDNA PCR and sequencing. Details of the identification of the strains and the identification of their functional genes are described in the later part of the document.

3.4 DNA isolation

To get an overview of the selectively enriched xylene degrading microbial community in the enrichments, first of all, the genomic DNA of the community was isolated. That was performed with the fifth week samples after five consecutive transfers. As a step forward, microbial biomasses were harvested from 50 mL of the enrichment by centrifuging at 2360 g at 4°C for 10 min using a Rotanta 460 R (Hettich. Germany). Total community DNA was extracted from the pellet using the DNeasy Ultraclean Microbial Kit (Qiagen. Germany) according to the manufacturer's instructions. Likewise, the genomic DNA of isolates was extracted from pure cultures grown on R2A agar using the UltraClean Microbial DNA Kit (Qiagen. Germany), following the manufacturer's instructions. The concentration of extracted DNA was measured using a NanoDrop One Microvolume UV–Vis spectrophotometer (Thermo Fisher Scientific Inc.USA).

3.5 PCR: 16S rRNA and functional gene C23O

The 16S rRNA and C23O functional genes were PCR amplified using previously isolated DNA samples. The PCR amplification for the 16s rRNA gene region of the genomic DNA was performed

using primers 27f 5'-AGA GTT TGA TCM TGG CTC AG-3 ' (Weisburg *et al.*, 1991) and 1492r 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Polz and Cavanaugh, 1998). The conditions and the used temperature profile for the PCR are described in **Table 2**. PCR amplification of subfamily I.2.C-type C23O genes was performed using primers XYLE3f (5′-TGY TGG GAY GAR TGG GAY AA-3′) and XYLE3r (5′- TCA SGT RTA SAC ITC SGT RAA-3′) (Táncsics *et al.*, 2012, 2013). PCR reaction mix contained the following components per sample (50 µL final volume): 5µL 10x Dream Taq buffer (Thermo Fisher Scientific Inc.USA.); 0.2 mM from all four dNTPs (Thermo Fisher Scientific Inc.USA.), 0.3 µL from the forward and reverse primers (Thermo Fisher Scientific Inc.USA), 1.25 U *Taq* polymerase (Thermo Fisher Scientific Inc.USA.), 2 µL of template DNA and then the mixture was made up to a final volume of 50 µL with MQ water. All PCR reactions were performed on a ProFlex PCR System (Life Technologies.USA) based on the thermal profiles given in **Table 2** below. The quality of amplified products was then checked by agarose gel electrophoresis, as described below.

Table 2: Conditions and temperature profile used for PCR

Thermal profile used for 16S rDNA PCR				**Thermal profile used for C23O type I.2.C PCR**			
98 ° C	5 minutes	Initial denaturation		95 ° C	3 minutes	Initial denaturation	
94 ° C	30 sec	Denaturation	32 cycles	94 ° C	30 sec	Denaturation	40 cycles
52 ° C	30 sec	Annealing		50 ° C	30 sec	Annealing	
72 ° C	1 minute	Elongation		72 ° C	1 minute	Elongation	
72 ° C	10 minutes	Extension		72 ° C	10 minutes	Extension	
4 ° C	∞	Cooling		4 ° C	∞	Cooling	

3.6 Agarose gel electrophoresis

The quality and success of DNA isolation and PCR amplification were assessed by agarose gel electrophoresis. Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. The agarose gel was made as follows: 1 g agarose was added with 100 mL of 1xTBE buffer (10.8 g Tris; 10.5 g boric acid; 0.93 g EDTA-Na; and the volume was adjusted to 1 Liter with distilled water), then dissolved by heating. After cooling, 5 µL of 1% ethidium bromide was added to the solution because ethidium bromide can be incorporated into the DNA strand and help to visualise DNA under UV light. Eventually, the agarose solution was

then poured into the casting tray with well combs. Upon solidification of the gel, the well combs were removed and placed into a gel box filled with buffer. Then the 5 µL of the product to be tested (e.g., DNA product) mixed with 3 µL of loading buffer (18.6 g EDTA; 20 g sarcosyl; 600 mL glycerol; 0.5 g bromophenol blue; 1000 mL of distilled water) and loaded together. To analyse the PCR products, loading buffer was not added as the Dream Taq Buffer (Thermo Fisher Scientific Inc.USA.) used during the PCR reaction contained loading dye in it. Along with the test samples, 4 µL of DNA marker (ThermoScientific ™ Gene Ruler Mix DNA Ladder, concentration: 0.5 µg / µL) was added as a standard. Therefore all the samples with the marker, were run at 120 V for 20 minutes, and then under UV light, DNA bands were analysed.

3.7 Community T-RFLP analysis of 16S rRNA

T-RFLP is a nucleic-acid based technique used to assess the diversity of a microbial population (Liu *et al.*, 1997). In the first step, the gene region to be examined for T-RFLP was amplified by PCR. For the PCR, fluorescently labelled (VIC label, Life Technologies ™, USA) forward primers specific for this reaction were used for amplification. The chemicals used for the PCRs and the thermal profile were the same as described in Table 2.

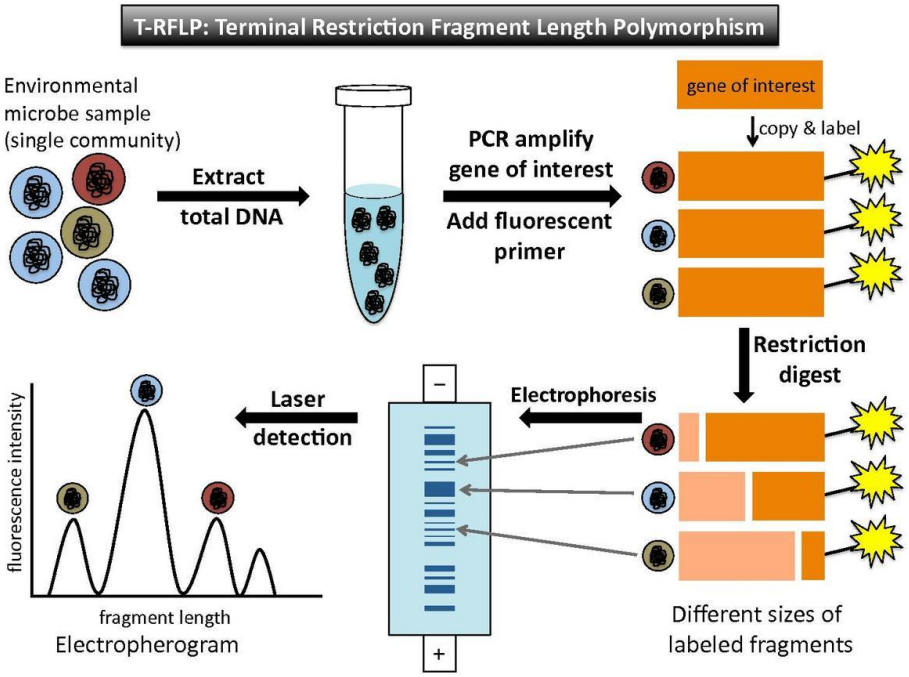


Figure 9: Schematic diagram of the T-RFLP process (based on http1)

The process of T-RFLP is explained in **Fig. 9**. In brief, for T-RFLP analysis, the VIC labelled PCR products were digested with a restriction endonuclease enzyme that recognises and cleave specific nucleotide sequences in DNA. The cleavage sites are specific to certain genera or species. The resulting PCR products that were labelled at their 5 'end and afterwords digested were then visualized by capillary gel electrophoresis using a fluorescent detector. This produces electropherograms in which the peaks represent the length of digested pieces, and the area represents their quantity. Through investigation of these peaks, it is possible to obtain an overview of the microbial diversity. In our examination, the VIC-labelled 16S rDNA were digested with 1U *Rsa*I (GT↓AC) (Thermo Fischer Scientific Inc. USA). For the digestion with these endonucleases, the reaction mixture was made accordingly: 2 µL of restriction enzyme buffer; 3 U restriction enzyme; 10 µL of template, which was made up to a final volume of 20 µL with MQ water. The reaction was performed at 37 ° C for 1,5 hours in a ProFlex PCR System (Life Technologies ™.USA). Then generated, fluorescently labelled terminal restriction fragments (T-RFs) were purified by the ethanol precipitation method. For this, the product was mixed properly with 3 µL of 3M Na acetate solution, 14.5 µL of MQ water and 62.5 µL of 95% ethanol and then incubated for 10 minutes at room temperature. Alcohol in the solution precipitates the DNA, while sodium acetate prevents the remaining dNTPs from precipitating out of the mixture. Then the samples were centrifuged at 3300 g for 20 minutes at 4 ° C after incubation. Subsequently, the supernatant was thrown away; next, 180 µL of 70% ethanol was added. After centrifugation at 3300 g for 15 minutes, the supernatant was discarded again. Furthermore, as the last step of the process, the pellet was suspended in 30 µL of sterile distilled water. From there, 0.3–1 µL of the purified digested DNA was added to HI-DI formamid (Promega, USA) and size standard (Genescan LIZ 500, Applied Biosystems, USA). The fragments were then separated by capillary gel electrophoresis on a Model 3130 Genetic Analyzer (Applied Biosystems.USA). The resulting electropherograms were evaluated using GeneMapper 4.0 software (Applied Biosystem. USA). T-RFs with relative abundances below 1% or less than 50 base pairs were not considered for further analysis. To achieve harmonised data profiles, parallel electropherograms of each sample were aligned with each other with the help of the T-Align program (Smith *et al.*, 2005) with a 0.5-bp confidence interval. Statistical analysis, such as cluster analysis (Bray-Curtis method) of the T-RFLP electropherograms, was performed using the PAST 3.26 software package (Hammer et al.2001).

3.8 Sequencing and sequence analysis

Sequencing was performed following Sanger sequencing or "chain termination" method. For that, at first, the sequencing reaction mixture was prepared that contains 1 µL BigDye, 1.5 µL BigDye Buffer, 0.5 µL forward primer; 1-7 µL of template DNA, MQ water supplemented to a final volume of 10 µL for a single sample. The thermal profile of the reaction is as follows: DNA denaturation for 10 seconds at 94 °C, then cooling at 50 °C for 5 seconds, the primer hybridising to the templates, and finally, at 60 °C for 4 minutes, the DNA strand synthesis. This three-stage cycle was repeated 28 times,then the cooling of samples at 4 °C. Later, the sequencing product was purified by ethanol precipitation for further use, as described earlier in chapter 3.7.The separation of DNA fragments of different lengths was performed on a NanoPOP-7 TM polimer (NimaGen B.V., Netherlands) using an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). The results of the sequencing were investigated using MEGA X software (Kumar *et al.*, 2018), and the 16S rDNA and C23O sequences were identified with the help of the GenBank database using the BLAST search program. Similar sequence comparison was also performed for the 16S rDNA gene in the EzTaxon database (https://www.ezbiocloud.net/). Phylogenetic trees were constructed with MEGA X software using distance-based "neighbor-joining" (Naruya and Masatoshi , 1987) and "maximum-likelihood" (Felsenstein, 1981) algorithm. Tree topologies and distances were evaluated by bootstrap analysis based on 1000 replicates.

3.9 Illumina 16S rRNA gene amplicon sequencing

To acquire in depth knowledge about the microbial community composition, 16S rRNA gene amplicon sequencing was performed for the enrichment sample using the isolated community DNA sample.V3 and V4 variable regions of the 16S rRNA gene were amplified by Bact 341F_Overhang 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3 'and Bact 785R_Overhang 5'-GTC TCG TGG GCT CGG AGA TGT GTA GTA CTA CHV GGG TAT CTA ATC C-3 'primers (Klindworth *et al.*, 2013) to get 16S rDNA amplicons. PCR was done according to the 16S metagenomic sequencing library preparation guide of Illumina using KAPA HiFi HotStart Ready Mix (KAPA Biosystems.USA). Paired-end sequencing reads were generated on an Illumina MiSeq sequencer using MiSeq Reagent Kit v3 (600-cycle) by SeqOmics Biotechnology Ltd. (Mórahalom, Hungary). Primary data analysis (base-calling) was conducted with Bbcl2fastq software

(v2.17.1.14, Illumina.USA). Quality check and length trimming of the obtained reads were performed in CLC Genomics Workbench Tool 9.5.1 using an error probability of 0.05 (Q13) and a minimum length of 50 nucleotides as a threshold. Then the trimmed sequences were processed using mothur v1.41.1 (Schloss *et al.*, 2009) as recommended by the MiSeq SOP page (http://www.mothur.org/wiki/MiSeq_SOP) (Kozich *et al.*, 2013). Based on the alignment using the SILVA 132 SSURef NR99 database (Quast *et al.*, 2013), sequences were assorted. Detection of chimeras was done with Mothur's uchime command (Edgar *et al.*, 2011), and the 'split.abund' command was also used to remove singleton reads, according to Kunin *et al.*,2010. 97% similarity threshold level was used to assign Operational Taxonomic Units (OTUs) as suggested by Tindall et al.(2010) for prokaryotic species delineation. Venn diagrams were generated by the mothur's venn command. Rarefaction curves were also prepared to check sequencing coverage for all the samples.

3.10 Whole genome sequencing and genome analysis

Whole genome sequencing of *Hydrogenophaga* sp. strains D2P1^T and D2P3 isolated from enrichment A and *Pseudomonas* sp. MAP12^T , and *Sphingobium* sp. AS12, from enrichment B were performed. For the complete bacterial genome sequencing, bacterial genomic DNA was first used to create a mate-paired library using the Nextera Mate Pair Sample Preparation Kit (Illumina Inc. USA) according to a slightly modified version of the gel-plus protocol provided by the manufacturer. The 13 kbL Mate Pair Tagment Enzyme reagent was used to generate DNA fragments of 7-11 kb. This gel fragment containing the "smear" region was excised and re-isolated using the Zymoclean Large Fragment DNA Recovery Kit (Zymo Research.USA). Circularized DNA was cut using a Covaris S2 ultrasonizer (Covaris.USA). The TapeStation 2200 (Agilent.USA) was used for DNA quality control during the process described above. For the final quantitative check of DNA before sequencing, a Qubit (Thermo Fisher Scientific Inc.USA) instrument was used, followed by sequencing on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 (500 cycles) (SeqOmics Ltd.Hungary). *De novo* assembly and scaffolding were performed with SPAdes version 3.13.0 (Nurk *et al.*, 2013). Automatic annotation of the genome was performed by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP) v4.5 (Tatusova *et al.*, 2016). For further annotation analyses, we used the RAST server (RASTtk) and the SEED database (Aziz *et al.*, 2008; Overbeek *et al.*, 2014; Brettin *et al.*, 2015). SnapGene software (v4.3.4) was used to visualize the examined gene clusters. For the identification of genomic islands, the IslandViewer 4 was used (Bertelli *et al.*, 2017). Digital DNA-

DNA hybridization (dDDH) was performed using Genome-to-Genome Distance Calculator (GGDC, https://ggdc.dsmz.de/, version 2.1) (Meier-Kolthoff *et al.*, 2013). For the calculation of orthologous average nucleotide identity (Ortho ANI) value between strains, the OAT software was used (Lee *et al.*, 2016). To analyse the metabolic potentials of the investigated strains, the genomes were annotated using the Genoscope platform MAGE (Vallenet *et al.*, 2009, 2006) and with the help of PATRIC 3.5. (Wattam *et al.*, 2017) web interfaced pipelines. Subsequently, analysis was performed by combining automated annotation from MAGE and manual curation using information from MetaCyc (Caspi *et al.*, 2014), KEGG (Kanehisa and Goto, 2000) and UniProt (Bateman, 2019). For the alignment of analysed genomes, the CLC Genomics Workbench version 21.0.3 (Qiagen. Germany) was used. A whole genome based phylogenomic tree was constructed on the TYGS database (based on genome signatures) to determine the precise taxonomic position of the strains (Meier-Kolthoff and Göker, 2019).

3.11 Metagenome sequencing and bin-genome construction

For the genome-resolved metagenomics approach metagenome sequencing was performed on the Illumina MiSeq platform by SeqOmics Kft. Using an Agilent 2200 Tapestation system, the quality and integrity of the metagenomic DNA were assessed. On an Illumina MiSeq sequencer, paired-end fragment reads (2x250 nucleotides) were sequenced using the MiSeq Reagent Kit v2 (500 cycles). Illumina-artifacts and adapters were removed from raw reads with bbduk (https://sourceforge.net/projects/bbmap/). Quality controlling and trimming of reads were performed with sickle version 1.33 (Joshi and Fass, 2011). Using MetaSPAdes pipeline version 3.15.0 (Nurk *et al.*, 2017), quality-controlled reads were assembled**.** To create the final set of bins (**Fig. 10**), different binning tools were used. Abawaca (https://github.com/CK7/abawaca) was run twice. First, including reads with a minimal length of 3000 base pairs (bps) and sequences split after 5000 bps, and secondly, with a minimal scaffold length of 5000 bps and sequences split after 10000 bps. MaxBin2 (Wu et al. 2016) was run with both marker sets. Using DAS Tool (Sieber *et al.*, 2018), all bins created in the four runs were optimized and filtered with DAS Tool. Those bins were further hand curated with uBin (Bornemann *et al.*, 2020). With the help of MiGA-Microbial Genomes Atlas (Rodriguez-R *et al.*, 2018) pipeline phylogenetic affiliation of the bins was ascertained. Bin genomes were then deposited at NCBI under the BioProject number: PRJNA818156.

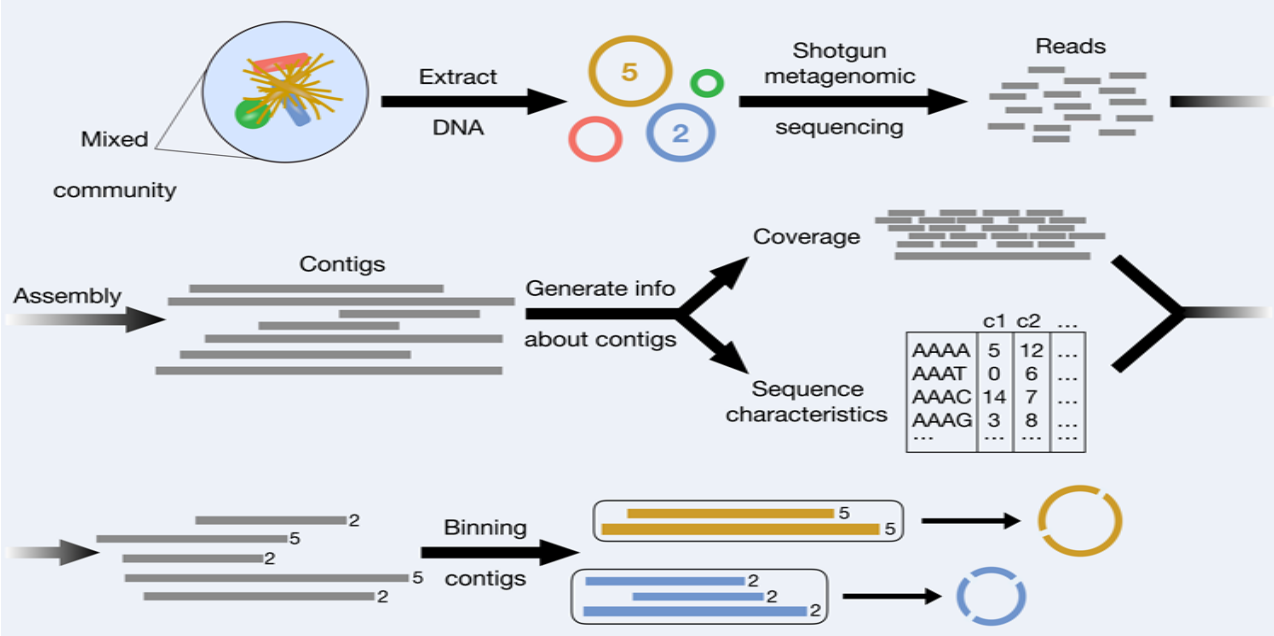


Figure 10: Schematic representation of genome binning technique used (based on http2)

3.12 BTEX degradability measurements by GC-MS

The BTEX degradation potential of the community evolved in the enrichment cultures, and the isolated strains were measured using a GC-MS instrument (Thermo Fisher Scientific Inc.USA.). For the enrichments, the concentration of carbon source was measured in week one and during the fifth week of inoculations. BTEX degradation potential of isolated strains was measured under both aerobic and microaerobic conditions with particular emphasis on xylene degradation as the strains were isolated from a xylene degrading enrichment. Measurements were carried out in triplicates, along with triplicates of negative controls (uninoculated). The experiment was performed in 100 ml crimped serum bottles containing 50 ml MS medium supplemented with BTEX compounds as a carbon source at 5 mg/L concentration and incubated at room temperature for 24h to reach the equilibrium state. Thereafter, 100 µl bacterial inoculum (OD600 0.5) was added to the serum bottles. All of the bottles were then incubated at 28 °C and 150 r.p.m in a rotary shaker incubator. Microaerobic conditions in the microcosms were set as described earlier in chapter 3.2. In short, microcosms were sparged aseptically with N_2: CO_2 (80:20) gas to remove oxygen content and then sterile air was injected into the bottles to achieve desired oxygen concentration. Biodegradation of BTEX compounds was measured at a regular interval of 24h by headspace analysis using an SPME polydimethylsiloxane fiber assembly (Supelco Analytical.USA) for sampling and a Trace 1300 gas chromatograph coupled

to ISQ Single Quadrupole mass spectrometer (Thermo Fisher Scientific Inc.USA) for analysis. For the purpose of analysis, injector and detector temperatures were maintained at 200 °C and 250 °C, respectively. The oven temperature program was set to 40 °C for 3 min, then ramped at a rate of 20 °C /min to 190 °C and held for 1 min. Helium was used as carrier gas at a flow rate of 1.2 ml /min. SLB™-5 ms fused silica capillary column was used for separation (30 m × 0.25 mm × 0.25 μm, Sigma-Aldrich, Supelco). The mass spectrometer was operated in full scan mode.

3.13 Studies conducted to describe new species

During our research, we isolated several strains that showed limited similarity with their closest relatives. Among them, a few of the most interesting strains that had the ability to degrade xylenes were described as new species of the genus, as it was one of our research aims. Finding and exploring the hydrocarbon degradation potential of new species is always crucial because, later, they could be used for bioremediation applications. To assign these strains to a new species, several examinations were performed. (Tindall *et al.*, 2010).

3.13.1 Physiological studies

To perform the physiological analysis, the closest relatives of the presumably new species were obtained from international bacterial strain collections (DSMZ–German Collection of Microorganisms and Cell Cultures and LGM-Belgium Collection of Microorganisms) and studied under the same laboratory conditions. The appearance of colonies was evaluated with the naked eye, and the determination of cell size and morphology was performed with a transmission electron microscope (H-7100; Hitachi.Japan), as described by Ohad et al. (1963), using the shadow-casting technique. Cell size, shape and arrangement of strains were studied by native preparations and Gram-staining according to Claus (1992). The following physiological and biochemical tests were performed according to Barrow and Feltham (1993): reduction of nitrate to nitrite or nitrogen, measurement of urease and phosphatase activity, formation of hydrogen sulfide from cysteine, formation of indole from tryptophan, and hydrolysis of casein, gelatin , Tween 80 and esculin. Catalase activity was determined using a 3% (v / v) hydrogen peroxide solution, and oxidase activity was determined using 1% (w / v) tetramethyl p-phenylenediamine oxalate. Growth at different temperatures (from 4 to 45 °C) was tested by using R2A medium. The optimum pH for growth was estimated using R2A broth. The pH of the medium was adjusted to 4.0–12.0 (at intervals of 1 pH unit)

prior to autoclaving using citrate/ NaH_2PO_4 buffer (0.1 M, for pH range 4.0–5.0), phosphate buffer M, for pH range 6.0–7.0) Tris buffer (0.2 M, for pH range 8.0–10.0) and NaOH (5 M, for pH range 10.0–12.0). Salt tolerance was assessed by inoculating the strain into R2A broth supplemented with 0–12 % (w/v) NaCl at 1 % intervals. During pH and salt tolerance tests growth of strain was determined by measuring optical density (OD) at 600 nm. For additional physiological and biochemical characteristics, API 50CH, API 20NE and API ZYM strips (bioMerieux.France) were used according to the manufacturer's instructions. Growth under anaerobic conditions was determined in R2A broth medium with and without the addition of 0.15 % (w/v) KNO_3 at 28 °C. To ensure anaerobic conditions, 100 ml serum bottles (Glasgeratebau Ochs) with 50 ml R2A broth were crimp sealed and purged with nitrogen under sterile conditions as described earlier by Farkas et al. (Farkas *et al.*, 2015). Gowth of the strain in the presence of different BTEX compounds was also measured in 50 ml MS medium supplemented with individual BTEX compounds as the sole carbon source. The growth was measured by measuring the OD at 600nm.

3.13.2 Chemotaxonomic studies

Whole-cell fatty acid analysis was performed by the Leibniz-Institute DSMZ (Braunschweig, Germany). For this study, test strains along with reference type strains were cultivated on R2A agar at 28 °C. Sufficient amount of cells of comparable physiological age could be harvested from the third quadrant of the plates. Fatty acid methyl esters were recovered from 30 mg of biomass by saponification, methylation, and extraction (Miller, 1982; Kuykendall *et al.*, 1988), followed by gas chromatography with a flame ionization detector according to the Sherlock Microbial Identification System (MIDI Inc.USA). Fatty acids were identified with Sherlock software using the TSBA40 database (v.6.1). Similar to fatty acid analysis, polar lipids and respiratory quinones analyses were also performed by DSMZ. Polar lipids were extracted from 200 mg of lyophilized biomass with a mixture of chloroform methanol: 0.3% NaCl solution, where the polar lipids were concentrated in the chloroform phase (Bligh and Dyer, 1959). The polar lipids were separated from the solution by 2-dimensional silica thin layer chromatography, the first separation being with chloroform: methanol: water and the second separation with chloroform: methanol: acetic acid: water. Total lipid content was determined using phosphomolybdic acid, while each specific lipid group was determined using group-specific reagents (Tindall *et al.*, 2007). Respiratory and lipoquinones were harvested from 200 mg of lyophilized biomass with methanol-hexane following the method described by Tindall (1990).

Then the separation of lipoquinones (menaquinones, ubiquinones, etc.) was performed on silica gel (Macherey-Nagel Art. NO. 805 023) by thin layer chromatography. UV absorbing bands corresponding to different types of quinone types were collected from the thin film plate and analyzed by HPLC. The measurement was performed on a reverse phase column (Macherey-Nagel, 2 mm x 125 mm, 3 mm, RP18) connected to an LDC Analytical (Thermo Separation Products) HPLC using methanol: heptane 9: 1 (v/v) as eluant.

3.13.3 Phylogenetic and phylogenomic studies

The new strains were assigned as new species of their corresponding genus by evaluating their exact phylogenetic position. This was done by blasting the 16S rRNA gene sequences in the public database and constructing a phylogenetic tree with their closest relatives. Moreover, to be more precise in affiliating them in the genus, whole genome based phylogenomic tree was also made with the help of the Type Strain Genome Server (TYGS) (Meier-Kolthoff and Göker, 2019). Furthermore, the phylogenomic tree using a concatenated alignment of 92 core genes was also constructed in UBCGs (Na *et al.*, 2018). 16S rRNA gene sequences and whole genome sequences of the closely related strains were downloaded from the NCBI GenBank database.

4 RESULT AND ANALYSIS

4.1 Microbial community analysis of aerobic *m*-, *p*- and *o*-xylene degrading enrichments

To develop effective bioremediation strategies, it is always important to explore autochthonous microbial community diversity using substrate-specific enrichment. The primary objective of this present study was to reveal the diversity of xylene-degrading bacteria at a legacy BTEX-contaminated site where xylene is the predominant contaminant, as well as to identify potential indigenous strains that could effectively degrade xylenes in order to better understand the underlying facts about xylene degradation using a multi-omics approach. Henceforward, parallel aerobic microcosms were set up using all three different xylene isomers as the sole carbon source to investigate evolved bacterial communities using both culture-dependent and independent methods. The research outcome showed some interesting facts that shed light on the set goals. Those findings are discussed in the following two parts:

4.1.1 Evaluation of the aerobic xylene-degrading potential of the intrinsic microbial community evolved in *m*-, *p*-, *o*-xylene degrading enrichments

This part of the results helps us to understand the changes in the autochthonous microbial communities in xylene isomer induced enrichments and uncover information about key indigenous degraders of a decade-old petroleum-contaminated aquifer. Moreover, this section also furnished information about xylene degradation by isolated *Hydrogenophaga* strains.

4.1.1.1 Microbial community composition of xylene degrading enriched communities revealed by T-RFLP and 16S rRNA gene amplicon sequencing

Using the contaminated groundwater from Siklos (Hungary) as inoculum, aerobic (7-8 mg/L dissolved oxygen) microcosms were set up in duplicates with either *m*-, *p*- or *o*-xylene as a sole source of carbon and energy. Enrichments were then transferred weekly for consecutive five weeks. The xylene

degradation process by enrichments reflected that highly competent aerobic degrading communities evolved in the enrichments by the fifth week. The carbon source in the enrichments was depleted within 48 hours of incubation; (**Fig. 11**). Among the enrichments, *m*-xylene degradation was the fastest, followed by *p*-xylene and *o*-xylene. The complete degradation of the added xylene compounds was confirmed by the change in the initial concentration of 5 mg/L to 0 mg/L.

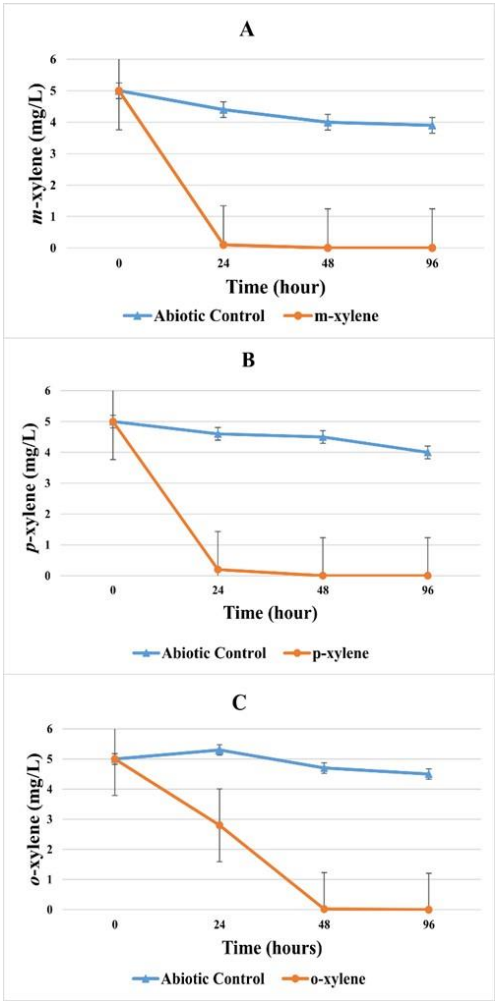


Figure 11**.** Degradation process of xylene isomers in the aerobic enrichments containing (A) *m*-xylene, (B) *p*-xylene, and (C) *o*-xylene. Xylene concentrations were measured by GC–MS analysis at the 5th week of enrichment as described in the main text. Means of duplicate enrichments are given (with standard error)

Since T-RFLP analysis has been successfully applied to study microbial communities of various microbial ecosystems (Scala and Kerkhof, 2000; Schütte *et al.*, 2008), thus as the first step of community composition evaluation, culture-independent 16S rDNA-based T-RFLP analysis was performed using community DNA of the 5th-week enrichments. The primary data of T-RFLP analysis

of enrichments and their duplicates revealed the fact about the similarity of the replicates and their community representation.

The bar plot of T-RFLP fingerprints clearly showed that the composition of the bacterial communities of *m*-, *p*-, and *o*-xylene-degrading enrichments were distinctly different, and the community composition was relatively similar for replicates (**Fig. 12**). The most dominant T-RFs in *m*-xylene degrading enrichments were 841-bp (approx.72%), 418-bp (approx. 5%), in *p*-xylene degrading enrichments 841-bp (approx. 40%), 418-bp (20%), and in *o*-xylene degrading enrichments 117-bp (10%), 416-bp (44%). The most prominent T-RF that was detectable in all three types of enrichments in different fractions was the 841-bp. The major difference creating T-RFs were 411-bp, 435-bp, 460-bp, and 466-bp. These peaks were present in *o*-xylene degrading enrichments and missing in the others enrichments.

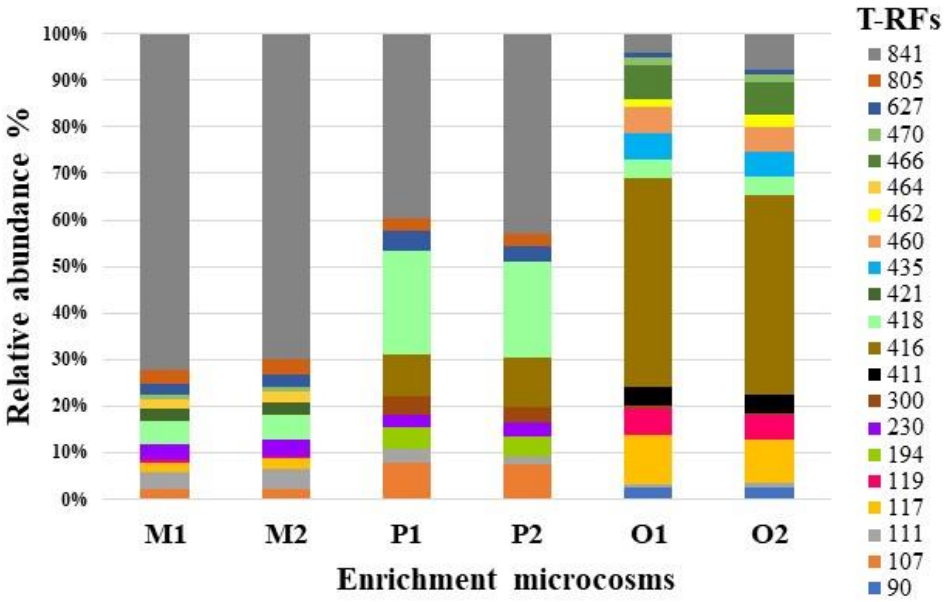


Figure 12. Bacterial community structure of *meta*- (M1, M2), *para*- (P1, P2) and *ortho*-xylene-degrading (O1, O2) enrichment cultures as revealed by 16S rRNA gene based T-RFLP.

The Bray-Curtis similarity-based cluster analysis of the T-RFLP profiles showed intercorrelation-based hierarchical clustering of *beta*-diversity of enrichment samples, considering T-RF fragment abundances. The UniFrac tree showed that the structure of the bacterial community in the *o*-xylene-degrading enrichments was utterly distinct from the other two types of enrichments, where it can be observed that *m*- and *p*- xylene-degrading enrichment samples shared similar microbial communities

by clustering together in different subgroups, unlike the *o*-xylene-degrading enrichment (**Fig. 13, panel A**). Therefore, it is worth mentioning that seemingly *o*-xylene-degrading enrichment harboured the most distinguished community members. Nevertheless, it was also noticeable that the duplicate enrichments had mostly similar community compositions.

PCA analysis based on 16s rDNA T-RFLP profiles revealed the remarkable differences between bacterial community structures of *m*-, *p*- and *o*-xylene-degrading enrichment samples. PCA plot pointed out that the replicate samples clustered together in all the cases and showed visible distance in clustering between *m*-, *p*- and *o*- xylene-degrading enrichment samples from each other. However, PCA showed that the *o*-xylene-degrading enrichment samples were apparently more distinct microbiologically. In the formation of clusters, the following T-RFs played a significant role: for *o*-xylene 117-bp, 416-bp, for *m*-xylene 841-bp long T-RF fragments and *p*-xylene-degrading enrichments 107-bp and 418-bp T-RFs (**Fig. 13, panel B**).

Presumably, these T-RFs denote bacteria capable of aerobic degradation of xylene isomers as carbon sources and became a dominant members of the community. These results confirmed that the replicates are parallel in nature and confer a similar bacterial community. Positive correlations were shown between the first and second components of PC (PC1 =91.23 %, PC2 = 8.68 %, respectively).

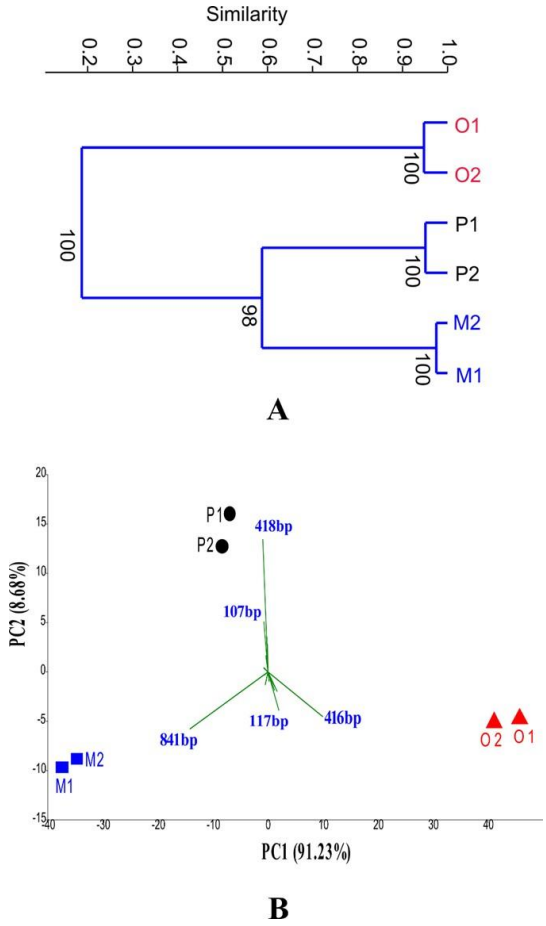


Figure 13**.** Panel A: Cluster analysis of the 16S rRNA gene-based T-RFLP electropherograms of the duplicate enrichment cultures at the 5th week by Bray-Curtis algorithm. Panel B: Principal component analysis 16S rRNA gene-based T-RFLP electropherograms. M1 and M2, *m*-xylene degrading enrichments; P1 and P2, *p*-xylene-degrading enrichments; O1 and O2, *o*-xylene -degrading enrichments.

Based on the T-RFLP results, enrichment samples, namely M1, P1 and O1, were selected for Illumina 16S rRNA gene amplicon sequencing as representative of the community diversity. To evaluate differences in the *alpha*-diversity of OTUs, rarefaction curve was created for each individual sample showing the number of observed OTUs, defined at a 97% sequence similarity cut-off in Mothur (Schloss *et al.*, 2009) (**Fig. 14**). In general, all the enrichments were rich and diverse. The graph demonstrated that enrichments having more or less similar OTU based richness and diversity. All of the samples showed OTU based saturation around 100-120 OTUs.

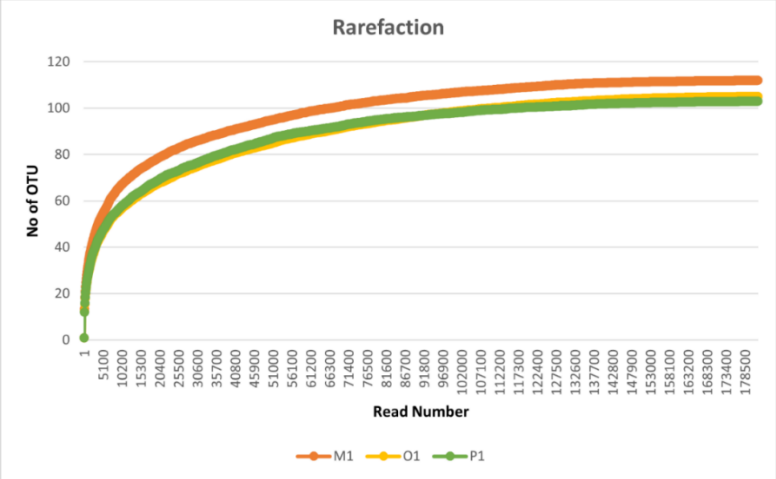


Figure 14: Rarefaction curves were assembled showing the number of OTUs, defined at the 97% sequence similarity cut-off in mothur, relative to the number of total sequences

Finally, a closer, in-depth look into the individual enrichments at class, order and genus levels helped us to understand the community diversity and composition. At the class level, members of the class *Gammaproteobacteria* dominated the bacterial community in most of the enrichments. Enrichment M1 showed the utmost order-based diversity. Both M1 and P1 enrichment communities were dominated by *Gammaproteobacteria* and *Bacteroidia*, and the exception was the O1 enrichment sample, where *Actinomycetia* showed more than 15% relative abundance in the community, which made the community different from the other two enrichments. (**Fig. 15**). Order-based analysis revealed that enrichments harbour primarily genera belonging to the orders *Burkholderiales*, *Pseudomonadales*, *Chitinophagales*, *Corynebacteriales* and *Bacteroidia*.

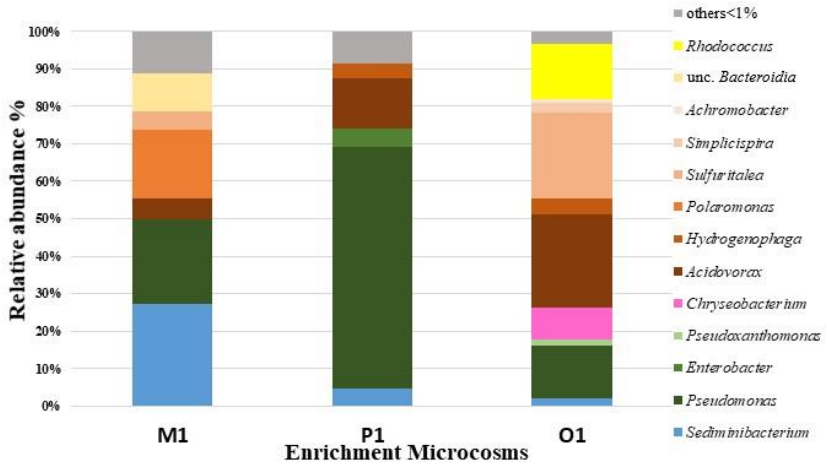


Figure 15**.** Genus level bacterial community structure of enrichments M1, P1 and O1 as revealed by Illumina paired-end 16S rRNA gene amplicon sequencing. Only taxa contributing more than 1% abundance were depicted.

The bacterial community of the *m*-xylene-degrading enrichment (M1) was dominated by members of the genera *Sediminibacterium* (27.1%), *Pseudomonas* (22.8%) and *Polaromonas* (18.4%). Whereas in *p*-xylene-degrading enrichment (P1), members of the genus *Pseudomonas* overwhelmingly dominated the community by showing 64% relative abundance (**Fig. 15**). Besides, members of some other genera like *Acidovorax* (13.2%), *Enterobacter* (5.1%) *Sediminibacterium* (4.6%) and *Hydrogenophaga* (3.9%) were detectable with prominent abundance. However, the *o*-xylene-degrading enrichment (O1) showed an altogether different community structure. *Pseudomonas*-related bacteria were present in the community but only with 14% abundance. *Acidovorax* (24.9%) was the most dominant genus, along with *Sulfuritalea* (22.8%), *Rhodococcus* (14.6%), *Chryseobacterium* (8.4%) and *Hydrogenophaga* (4.4%), making it the most different enrichment based on community diversity in comparison to the two enrichments. Several among these bacterial genera, such as *Acidovorax*, *Enterobacter*, *Pseudomonas*, *Rhodococcus*, *Sulfuritalea*, *Simplicispira*, *Hydrogenophaga*, and *Polaromonas*, have been either found to be present in petroleum-contaminated environments or known to be effective aromatic hydrocarbon degraders. (Nishino *et al.* 2000; Margesin *et al.*, 2003; Jeon *et al.*, 2004; Mattes *et al.*, 2008; Seo et al. 2009; Varjani and Upasani, 2016; Sarkar *et al.*, 2017; Sperfeld et al. 2018; Xu *et al.*, 2020). Analysis of the Illumina data revealed that *m*-and *p*-xylene-degrading enrichments showed the presence of members belonging to the genus *Pseudomonas*. The dominant role of *Pseudomonas*-related bacteria in aromatic hydrocarbon degradation under strictly aerobic conditions is well known. Thus, these organisms were widely investigated as model organisms to study aerobic BTEX degradation. The *p*-xylene-degrading enrichment, which had a high abundance of sequence reads affiliated with genera *Pseudomonas* and *Acidovorax*, enabled us to assume that these bacteria had played a role in the aerobic degradation of *p*-xylene. Members of the genera *Enterobacter* and *Sediminibacterium* were frequently observed in petroleum hydrocarbon contaminated environments, but their role in hydrocarbon degradation is yet unclear (Kaplan and Kitts, 2004; Aburto *et al.*, 2009; Singleton *et al.*, 2018). Another major group was *Hydrogenophaga* present in all of the enrichments, which have been selected to study further to reveal their role in the enrichment community. Likewise, *m*-xylene degrading enrichment was also dominated by *Pseudomonas*, *Sediminibacterium* and *Acidovorax*, making the diversity of the enrichment similar to the *p*-xylene degrading enrichment. The difference was the introduction of the genus *Polaromonas*, which is reported to be a genus with potential BTEX degraders (Mattes *et al.*, 2008). Contrarily, the *o*-xylene-degrading bacterial community was considerably different due to the presence of the genera *Sulfuritalea* and *Rhodococcus*, along with *Pseudomonas* and *Acidovorax* as the

major players in the community. Moreover, the presence of *Chryseobacterium, Simplicispira, Pseudoxanthomonas, Achromobacter* in noticeable fractions makes it an interesting and quite different community compared to the other enrichments. Members of the genera *Sulfuritalea* (Sperfeld et al. 2018), *Chryseobacterium* (Guo *et al.*, 2008), *Simplicispira* (Sperfeld *et al.*, 2018), *Pseudoxanthomonas* (Choi *et al.*, 2013) *Achromobacter* (Guo *et al.*, 2008) reported to be present in BTEX enrichments and probably took part in the degradation of BTEX compounds as a carbon source under aerobic conditions. In addition to this, *Rhodococcus* was the only dominant genus that was not witnessed in other enrichments, assuming it as the leading player for *o*-xylene degradation (Di Canito *et al.*, 2018). Subsequently, this hypothesis was proven in the BTEX degradation experiment as the isolated *Rhodococcus* strain could effectively degrade *o*-xylene (detailed result is presented below). Simultaneously, OTU based bacterial community composition comparison of enrichments using Venn diagrams (**Fig. 16**) demonstrated that the *m*-xylene degrading enrichment was the most diverse community consisting of 57 OTUs while *p*- and *o*-xylene degrading enrichments had 46 and 45 OTUs, respectively.

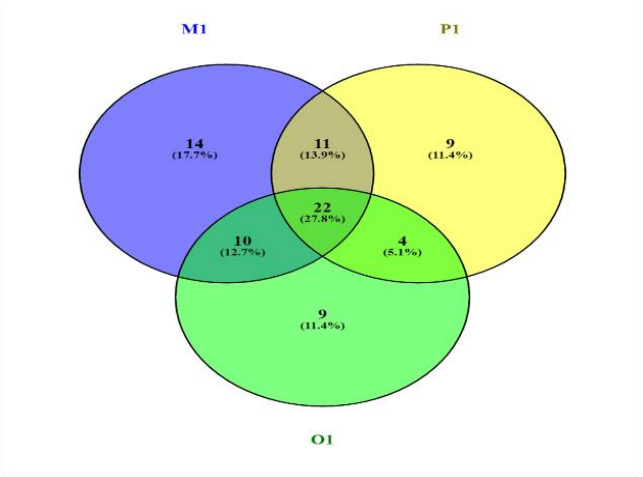


Figure 16**.** A Venn diagram : Venn diagram showing shared and unique OTUs at 97% identity among the three groups M1(*m*-xylene degrading enrichment),P1(*p*-xylene degrading enrichment)and O1(*o*-xylene degrading enrichment). Based on genera with relative abundancy more than 1%.

The data also showed that the *m*- and *p*- xylene-degrading enrichment shared the most OTUs in contrast, *o*- and *p*-xylene-degrading enrichments shared the least number of OTUs . Interestingly, these shared OTUs were among the least abundant population of sample O1. Additionally, OTUs representing *Rhodococcus* and *Simplicispira* were the exclusive abundant genera in *o*-xylene-degrading enrichment. These results might explain the *o*-xylene degrading enrichment community as a unique community. Furthermore, *Hydrogenophaga* was one of the abundant overlapping genera present in all of the enrichments in different proportions.

4.1.1.2 Details of strains isolated from enrichments and their BTEX degradation capability

Following classical microbiological techniques, bacterial strains were isolated on R2A agar plates from enrichments that have also been analysed by Illumina 16S rRNA gene amplicon sequencing. Based on different colony morphology and growth pattern total of 21 strains have been isolated, among which six isolates from *m*-xylene-degrading enrichment, eight isolates from *p*-xylene-degrading enrichment and seven isolates from *o*-xylene-degrading enrichment (**Table 3**). Strains

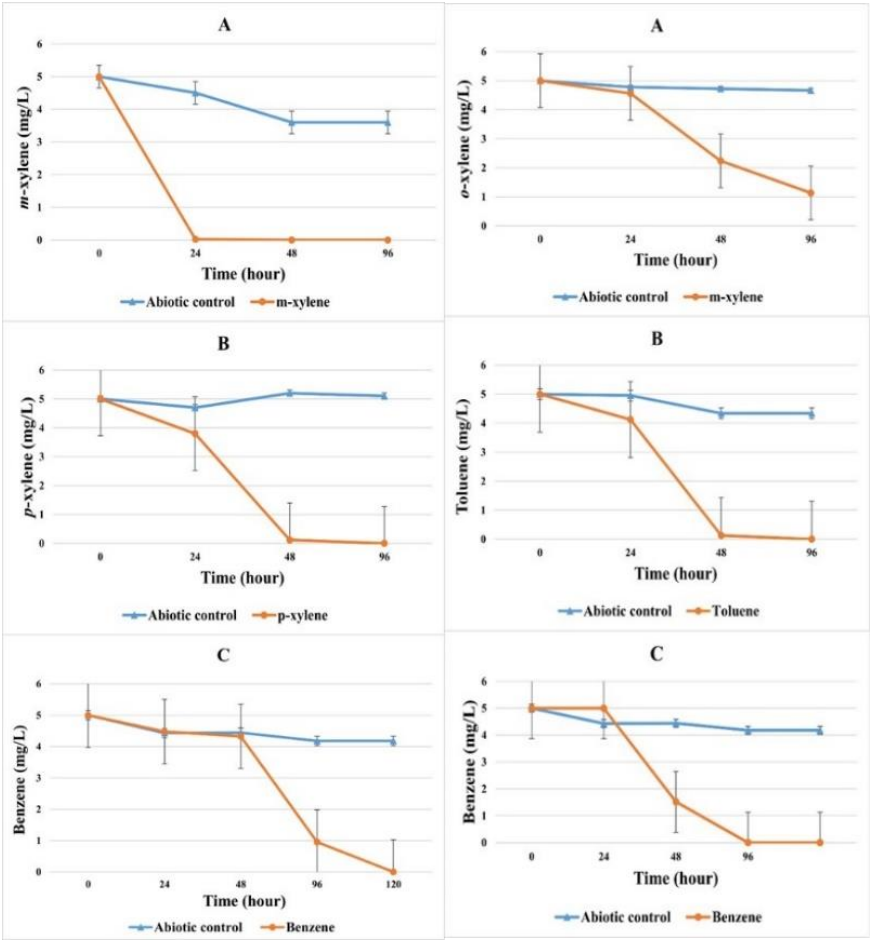
Table 3. Identification of bacterial strains isolated from the enrichments

Strain No.	Closest relative (type strain)	length of 16S rDNA analyzed (bp)	similarity (%)	subfamily I.2.C C23O gene
***m*- xylene degrading enrichment M1**				
D2M1	*Acidovorax delafieldii ATCC 17505*	1376	100	+
MT3	*Pseudacidovorax intermedius DSM 21352*	1420	99.93	+
D3M1	*Polaromonas eurypsychrophila CGMCC:1.15322*	1425	99.93	+
D3M2	*Lysobacter sediminicola JCM 18205*	1445	98.47	-
MW1	*Pseudomonas fluorescens* ATCC 13525	1432	99.93	+
MT4	*Achromobacter xylosoxidans ATCC:27061*	1416	99.93	-
***p*-xylene degrading enrichment P1**				
D2P1^{T}	*Hydrogenophaga taeniospiralis CCUG 15921*	1402	99.05	+*
D2P3	*Hydrogenophaga taeniospiralis CCUG 15921*	1418	99.15	+
D2P5	*Acidovorax delafieldii ATCC 17505*	1428	99.93	+
D3P2	*Mycolicibacterium vanbaalenii DSM:7251*	1410	100	-
P1W2	*Pseudomonas putida ATCC 12633*	1428	99.86	+
P1W3	*Pseudomonas veronii ATCC:700272*	1427	100	-
PT4	*Pseudomonas putida ATCC 12633*	1441	99.86	+
PW1	*Enterobacter roggenkampii DSM:16690*	1438	99.93	-
***o*-xylene degrading enrichment O1**				
D201	*Pseudomonas chlororaphis subsp. piscium DSM:21509*	1402	100	-
D202	*Achromobacter xylosoxidans ATCC:27061*	1419	99.93	-
D203	*Microbacterium paraoxydans DSM:15019*	1412	99.93	-
D204	*Rhodococcus imtechensis DSM:45091*	1409	99.5	-
0W2	*Pseudomonas chlororaphis subsp. Piscium DSM:21509*	1432	100	-
OW3	*Pseudomonas chlororaphis subsp. Piscium DSM:21509*	1402	100	-

	Pseudomonas chlororaphis subsp. Aureofaciens			
OY1	*ATCC:13985*	1419	99.93	-

*Mixed sequence electropherogram was obtained after Sanger sequencing of the I.2.C C23O amplicon, assuming the presence of more than one I.2.C C23O genotype in the genome.

isolated from the *m*-xylene-degrading enrichment (M1) belonged to the genera of *Acidovorax, Pseudacidovorax, Polaromonas, Lysobacter, Pseudomonas* and *Achromobacter*. Amid those strains, *Acidovorax, Pseudacidovorax, Polaromonas, and Pseudomonas* possessed subfamily I.2.C-type *C23O* gene, which was sequenced further. Among those strains, *Polaromonas and Pseudacidovorax* were selected for further degradation study because of limited available knowledge on their role as potential xylene degraders. Unfortunately, the *Polaromonas* strain was lost during subculturing. Hence, the BTEX degradation ability of *Pseudacidovorax* was only checked and observed that it could degrade 5 mg/L concentration of benzene and ethylbenzene in approx. 48hrs (data not shown). In contrast, it was unable to degrade toluene and any isomer of xylene. Overall, eight strains were isolated from the *p*-xylene-degrading enrichment (P1), which were members of the genera *Hydrogenophaga, Acidovorax, Mycolicibacterium, Pseudomonas* and *Enterobacter*. The screening of subfamily I.2.C-type *C23O* gene showed that *Hydrogenophaga, Acidovorax and Pseudomonas* strains harboured such a gene. Among these strains, two *Hydrogenophaga* strains (sharing identical 16S rRNA genes), both showed 99.1% similarity with type strain *Hydrogenophaga taeniospiralis* CCUG 15921T showed limited similarity with their closest relative. The presence of subfamily I.2.C-type *C23O* gene made it a potential candidate of interest as information about their biodegradation potential is not so widely available and also not yet considered as a credible candidate for bioremediation. Hence, the biodegradation ability of both *Hydrogenophaga* strains D2P1^T, and D2P3 were studied extensively. Surprisingly, although these two strains belonged to the same species but showed different degradation potentials towards different BTEX compounds. Strain D2P1^T was capable of degrading 5mg/L concentration of *m*-xylene within 24hrs and *p*-xylene within approx. 48hrs of incubation at 28 °C and degradation of the same concentration of benzene within around 120hrs under similar conditions (**Fig. 17a**). In contrast, *Hydrogenophaga* sp. strain D2P3 was able to degrade 5mg/L concentration of toluene around 48hrs, benzene within 96hrs and *o*-xylene in more than 96hrs incubation at 28 °C. (**Fig. 17b**).



a **b**

Figure 17. Panel a: Aerobic degradation of (A) *m*-xylene, (B) *p*-xylene and (C) benzene by *Hydrogenophaga* sp. strain D2P1^{T}. Panel b: Aerobic degradation of (A) o-xylene, (B) toluene and (C) benzene by *Hydrogenophaga* sp. strain D2P3.Concentrations were determined by GC-MS analysis as described in the main text. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

The lowest diversity of isolates was observable in the case of the *o*-xylene-degrading enrichment O1. The seven isolates obtained from this enrichment belonged to four genera. Besides, none of them harboured subfamily I.2.C-type *C23O* gene. Although the *o*-xylene degradation ability of *Rhodococcus* (Charniauskaya *et al.*, 2018) is well known, still the degradation potential of the isolated *Rhodococcus imtechensis* strain, D2O4 was assessed, which led us to the observation that it could degrade *o*-xylene, toluene and benzene (data not shown), which explains its presence in the *o*-xylene-degrading enrichment community as a key player of *o*-xylene utilisation. Along with that, two strains belonging to the genus *Pseudomonas*: *Pseudomonas chlororaphis subsp. piscium* and *Pseudomonas chlororaphis subsp. aureofaciens* were also investigated to assign their role in the *o*-xylene-degrading community, but unfortunately, they showed the inability to degrade xylenes as a carbon source.

4.1.1.3 Comparative whole-genome analysis of two BTEX degrading strains *Hydrogenophaga* sp. D2P1^T and D2P3, isolated from xylene degrading enrichment culture

Among the isolates, two *Hydrogenophaga* strains, D2P1^T and D2P3, were the most interesting for us since they were able to degrade at least one of the xylene isomers and possessed subfamily I.2.C-type *C23O* genes. The two strains shared identical 16S rRNA genes, but Sanger-sequencing revealed that they encode entirely different subfamily I.2.C-type *C23O* genotypes. Moreover, based on the Sanger-sequencing result, it could be assumed that strain D2P1^T harbours more than one genotype of the corresponding *C23O* gene since mixed sequencing electrophoretogram was obtained. Based on the 16S rRNA gene similarity, strains D2P1^T and D2P3 were closely related to *H. taeniospiralis* (~99.1% homology). They shared an identical 16S rRNA gene sequence with *Hydrogenophaga* sp. strain Rs71, which was isolated by Fahy *et al.* (2006) earlier as a benzene-degrading bacterium. Moreover, it was shown that strain Rs71 was able to degrade toluene, *m*-, and *p*-xylene as well (Fahy *et al.*, 2008).In the later section of the document, strain D2P1^T is described as the type strain of the new species *Hydrogenophaga aromaticivorans*, and the analysis of the whole genome of strain D2P1^T revealed that it has three different subfamily I.2.C-type *C23O* genes. Besides, a large gene cluster (~28 kbp) was identified, which encoded all of the genes (e.g. xylene monooxygenase and benzoate 1,2-dioxygenase) required for the transformation of *p*-, and *m*-xylene to 3-, and 4-methylcatechol, respectively. This cluster contained one of the subfamily I.2.C-type *C23O* genes (locus tag F3K02_21385), and it was identified during this study as part of a genomic island, based on the SIGI-HMM algorithm of IslandViewer 4. Since strain D2P3 was not able to utilize *p*-, and *m*-xylene, but could use *o*-xylene as sole source of carbon and energy, we have sequenced its whole genome as well. Subsequently, the two genomes were aligned to each other and analyzed. The dDDH value between strain D2P1^T and D2P3 was 79.7%, while the OrthoANI value was 97.6%, which clearly indicated that they belong to the same genomic species. Besides, they had highly similar genome size (5.63 and 5.80 Mb, respectively). Alignment of the genome sequences revealed that strain D2P3 lacks the xylene-degradation gene cluster, which was observable in the case of strain D2P1^T. On the other hand, both strains harbour a phenol degradation gene cluster, encoding a multicomponent phenol hydroxylase (mPH) together with a complete *meta*-cleavage pathway. However, these gene clusters are different in structure, and the corresponding genes show only ~80-90% sequence similarity to each other (**Fig. 18**). This difference can be the key to understand the different xylene-degrading ability of

strains D2P1^{T} and D2P3. It was observed in case of *Pseudomonas stutzeri* strain OX1, which is prominent toluene and *o*-xylene-degrading bacterium, that a phenol degradation gene cluster plays a crucial role in its *o*-xylene-degrading ability. The structure of this phenol degradation operon shows similarity to that was observed in strain D2P3.

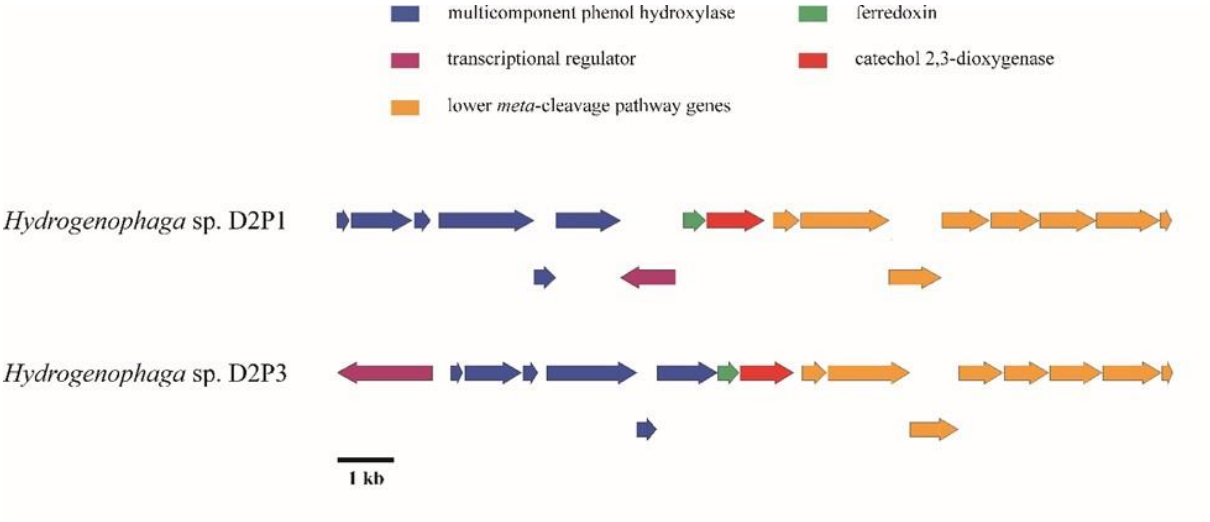


Figure 18. Physical map of the phenol degradation gene clusters of Hydrogenophaga sp. strains D2P1^{T} and D2P3.

Both operons are regulated by a σ^{54}-interacting transcriptional regulator, and the organization of the mPH and lower *meta*-cleavage genes is similar (at least at the known parts of the operon in the case of *P. stutzeri* strain OX1). The C23O enzyme coded in this operon of *P. stutzeri* strain OX1 can cleave 3,4-dimethylcatechol, but cannot cleave 3,5- and 3,6-dimethylcatechols. Consequently, strain OX1 can degrade *o*-xylene but cannot grow on *m*- and *p*-xylenes (Arenghi *et al.*, 2001). A similar scenario can be assumed in the case of *Hydrogenophaga* sp. strain D2P3. Due to the presence of a single transcriptional regulator it can also be speculated that the whole phenol-degradation gene cluster of this strain is transcribed as an extraordinarily large operon, similarly to *Pseudomonas* sp. strain CF600 (Shingler V, 1996). However, it is still a question how the *o*-xylene is converted into 3,4-dimethylphenol, since toluene-*o*-xylene monooxygenase was not found in the genome of strain D2P3. One possible explanation is that the mPH is responsible for both the hydroxylation of *o*-xylene and the subsequent hydroxylation of 3,4-dimethylphenol to 3,4-dimethylcatechol. Nevertheless, transcriptomic analysis will be necessary to answer this question. In the case of strain D2P1^{T}, a LysR-type transcriptional regulator gene was found wedged between the mPH and the ferredoxin gene (**Fig. 18**). Besides, no σ^{54}-interacting transcriptional regulator gene was found upstream of the mPH. Instead, an *orf* encoding an IS5 family transposase was found in the corresponding position, hinting

at the possibility that this gene cluster was acquired through horizontal gene transfer (HGT) by strain D2P1^T. Moreover, it can also be speculated that this gene cluster functions only partially since the mPH lacks its own transcriptional regulator.

The results of this chapter have been published in the following publication:

Banerjee S, Bedics A, Harkai P, Kriszt B, Alpula N, Táncsics A. (2022) Evaluating the aerobic xylene-degrading potential of the intrinsic microbial community of a legacy BTEX-contaminated aquifer by enrichment culturing coupled with multi-omics analysis: uncovering the role of *Hydrogenophaga* strains in xylene degradation. Environmental Science and Pollution Research, 29:28431-28445. DOI: 10.1007/s11356-021-18300-w

4.1.2 Description of a novel aerobic xylene degrading bacterial species isolated from *para*-xylene degrading enrichment (*Hydrogenophaga aromaticivorans* sp. nov)

In this part of the results, we discuss about the novel species of *Hydrogenophaga* sp. D2P1^T that was isolated from *p*-xylene degrading enrichment. Based on our preliminary 16S rRNA gene sequencing results, this strain was identified as a new species of the genus *Hydrogenophaga*. The following part contains results that clarifies the taxonomic position of this novel bacterium using a polyphasic taxonomic approach, including whole genome sequencing and physiological, biochemical and chemotaxonomic analyses.

4.1.2.1 Characterization of the genus *Hydrogenophaga*

The genus *Hydrogenophaga* belongs to the family *Comamonadaceae,* and was first proposed by Willems et al. (Willems *et al.*, 1989). Members of the genus *Hydrogenophaga* are aerobic or facultative anaerobic, Gram-stain-negative, straight to slightly curved rods and motile via flagella (Chung *et al.*, 2007). Many members of this genus are chemoautotrophs, able to oxidize hydrogen as an energy source. The generic name Hydrogeno-phaga (Hy.dro.ge.no'pha.ga. Gr. n. hydoor, water; Gr. n. gennao, to create; M.L. hydrogenum, hydrogen, that which produces water; Gr. v. phagein, to eat; M.L. fern. n. Hydrogenophaga, eater of hydrogen). refers to the metabolic characteristic. Species of *Hydrogenophaga* are positive for oxidase activity and show variable activity for catalase

(Willems *et al.*, 1989). The main cellular fatty acids are C16 : 0 and C16 : 1 ω7c and/or C16 : 1 ω6c (Du *et al.*, 2015; Baek *et al.*, 2017; Lin *et al.*, 2017). The DNA G+C content ranges from 61.3 to 69.9 mol% (Baek *et al.*, 2017; Choi *et al.*, 2020), and the predominant quinone system is ubiquinone-8 (Q-8) (Willems *et al.*, 1989). At the time of writing, fifteen species of the genus *Hydrogenophaga* have been validly described, which were isolated from diverse habitats, such as wastewater (Kampfer, 2005; Yoon *et al.*, 2008), activated sludge (Choi *et al.*, 2020), soil (Yang *et al.*, 2017), freshwater ecosystems (Du *et al.*, 2015; Mantri *et al.* 2016) or even pacific oyster (Baek *et al.*, 2017). Additionally, members of the genus *Hydrogenophaga* can be key benzene-degrading bacteria in petroleum hydrocarbon contaminated subsurface environments (Fahy *et al.*, 2008).

4.1.2.2 Phenotypic and chemotaxonomic characterization of D2P1^T

Strain D2P1^T produced yellow-pigmented, convex, circular colonies after 2– 3 days of incubation on R2A agar at 25ºC. Strain D2P1^T is Gram-stain-negative and catalase- and oxidase-positive aerobic in nature. Cells were rods with rounded edges, approximate width of 0.6 to 0.7 μm and a length of 1.5 to 1.6 μm and motile by means of a monotrichous polar flagellum (**Fig. 19**). The comparative evaluation of chemotaxonomic, biochemical and physiological properties of D2P1^T with reference strains resulted that D2P1^T had a wider temperature (4-45°C) and pH range (6-10) for growth. Although D2P1^T could tolerate up to 2% NaCl (w/v) concentration but grew optimally without adding NaCl at 25 °C and pH 7.

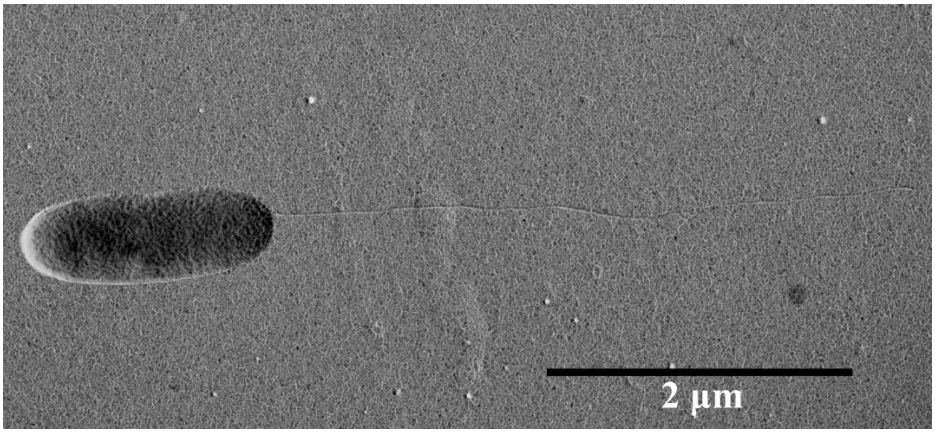


Figure 19**.** Transmission electron microscopic image of D2P1^T, grown in R2A agar at 25ºC for 2 days. Scale Bar: 2 μm.

D2P1^T was unable to hydrolyse Tween 80 (**Table 4**). In API ZYM tests, D2P1^T showed positive enzymatic activity for esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase whereas for API 20NE, strain D2P1^T showed positive results for urease activity and reduction of nitrates to nitrites but was unable to reduce nitrates to nitrogen. Moreover, exhibited assimilation of D-glucose, D-mannose, D-mannitol, D-maltose, potassium gluconate and malic acid. In the case of API50 CH tests, D2P1^T showed a wider substrate range, unlike *H. crassostreae* JCM 31188^T and *H. palleronii* DSM 63^T. Strain D2P1^T could metabolize glycerol, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, D-arabitol. Detailed comparative biochemical characteristics of D2P1^T are presented in **Table 4**. Anaerobic growth of strain D2P1^T was not observed, showing that nitrate does not support its growth in the absence of oxygen. Strain D2P1^T could not oxidize thiosulfate to sulfate and did not grow autotrophically with hydrogen gas. The yellow pigments extracted from strain D2P1^T and the reference strains showed similar absorption spectra, with a peak at 415 nm.

Table 4. Comparative biochemical , physiological characteristics of the strain D2P1^T and the type strains.1. D2P1^T, 2. *Hydrogenophaga taeniospiralis* DSM 2082^T, 3. *Hydrogenophaga crassostreae* JCM 31188^T, 4.*Hydrogenophaga palleronii* DSM 63^T; (+)- Positive , (-) negative; (ng)- no growth. All data were obtained in this study.

Characteristics	**1**	**2**	**3**	**4**
Enzyme activities (API ZYM):				
esterase (C4)	+	+	+	-
β-galactosidase	-	-	-	+
Acid production from (API 50CH):				
Glycerol	+	+	-	-
D-arabinose	-	+	-	-
L-arabinose	-	+	+	-
D-galactose	-	+	+	-
D-glucose	+	+	-	-
D-fructose	+	+	+	-
D-mannose	+	+	+	-
L-rhamnose	-	+	-	-
Inositol	-	-	+	-
D-mannitol	+	+	-	-
D-sorbitol	+	+	-	-
Esculin ferric citrate	-	+	-	+
L-fucose	-	+	-	-
D-arabitol	+	+	-	-
Assimilation of (API 20NE):				
nitrates to nitrites	+	+	-	+
nitrates to nitrogen	-	+	+	-
4-nitrophenyl-β-D-galactopyranoside	-	+	-	+
D-glucose	+	+	-	-
L-arabinose	-	+	-	-
D-mannose	+	+	-	-
D-mannitol	+	+	-	+
D-maltose	+	+	-	+
potassium gluconate	+	+	+	-
adipic acid	-	+	-	-
malic acid	+	+	+	-

BTEX compounds (benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylenes) degradation by D2P1^T under aerobic conditions was also investigated, considering that strain D2P1^T was isolated from a BTEX-contaminated environment. The utilization of BTEX compounds was also investigated by a growth test in which OD600 was measured by a spectrophotometer. The growth test was performed similarly as described above, but applying higher aromatic hydrocarbon concentrations (0.5 mM) to better support biomass formation. Bottles were incubated in a rotary shaker at 25°C and 150 r.p.m. for up to four days. Concentrations of the carbon sources were monitored for every 24h by GC-MS headspace analysis as described above and were replenished upon depletion. OD600 values were monitored in every 24h by spectrophotometer. The data obtained with the GC-MS have shown that strain D2P1^T was able to utilize benzene, *m*- and *p*-xylenes as sole sources of carbon and energy, while toluene, ethylbenzene and *o*-xylene were not degraded (data not shown). In the growth test, the rapid growth of strain D2P1^T on *p*-, and *m*-xylenes was recorded, reaching an OD600 value of 0.2 by the fourth day of incubation, while slower but also clear growth was observed in the presence of benzene. In this latter case, the OD600 value reached 0.1 by the fourth day of incubation.

The major components of the whole-cell fatty acids (>5% of total fatty acids) of strain D2P1^T were summed feature 3 (comprising C16:1 ω7c and/or C16:1 ω6c), C16:0 and summed feature 8 (comprising C18:1 ω7c and/or C18:1 ω6c) similarly to other species of the genus *Hydrogenophaga* (**Table 5**). However, in the case of strain D2P1^T, the hydroxy fatty acid C10:0 3-OH was detected in notable amount (3.3%), while it was missing in the case of *H. taeniospiralis* DSM 2082^T and *H. palleronii* DSM 63^T.

Analysis of respiratory quinones showed that Ubiquinone-8 (Q-8) (100%) was the major respiratory quinone and the polar lipid profile of strain D2P1^T contained phospholipid, phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol (**Fig. S1**). Overall, the chemotaxonomic data were in accordance with those of members of the genus *Hydrogenophaga* (Willems *et al.*, 1989).

Table 5. Cellular fatty acid compositions of strain D2P1^T and related species:Taxa: 1, strain D2P1^T; 2, *Hydrogenophaga taeniospiralis* DSM 2082^T; 3, *Hydrogenophaga palleronii* DSM 63^T; 4, *Hydrogenophaga crassostreae* JCM 31188^T. Data are expressed as percentages of total fatty acids. -, Not detected. Fatty acids which were lower than 1.0% in all strains are not shown. All data are from the present study.

Fatty acid	**1**	**2**	**3**	**4**
Saturated				
$C_{12:0}$	3.7	2.5	-	5.7
$C_{14:0}$	3.3	3.5	0.3	0.5
$C_{16:0}$	31.8	32.4	30.7	23.8
$C_{17:0}$	-	0.5	0.3	1.0
$C_{18:0}$	-	-	-	0.3
Unsaturated				
$C_{17:1}$ ω8c	-	0.4	-	1.8
$C_{17:1}$ ω6c	-	-	-	2.1
Hydroxy				
$C_{10:0}$ 3-OH	3.3	-	-	3.1
Cyclic				
$C_{17:0}$ cyclo	1.2	6.1	13.9	-
Summed feature*				
3	49.5	41.4	29.5	54.5
7	-	-	-	1.9
8	6.4	11.6	23.5	3.0

*Summed features represent groups of two or three fatty acids that could not be separated by gas–liquid chromatography with the MIDI system. Summed features: 3, C16:1 ω7c/C16:1 ω6c; 7, unidentified fatty acid 18.846/C19:1 ω6c/C19:0 cyclo ω10c; 8, C18:1 ω7c/C18:1 ω6c

4.1.2.3 Phylogenetic affiliation of strain D2P1^T

Phylogenetic analysis of 16S rRNA gene sequences showed that strain D2P1^T formed a distinct phyletic lineage within the genus *Hydrogenophaga* (**Fig. 20**). Comparative 16S rRNA gene sequence analysis revealed that strain D2P1^T was most closely related to *H. taeniospiralis* NBRC 102512^T (99.2%) followed by *H. palleronii* NBRC 102513^T (98.14%), *H. laconesensis* HWB-10^T (97.85%), *H. atypica* BSB 41.8^T (97.63%), *H. defluvii* BSB 9.5^T (97.56%) and *H. crassostreae* LPB0072^T (97.43%).

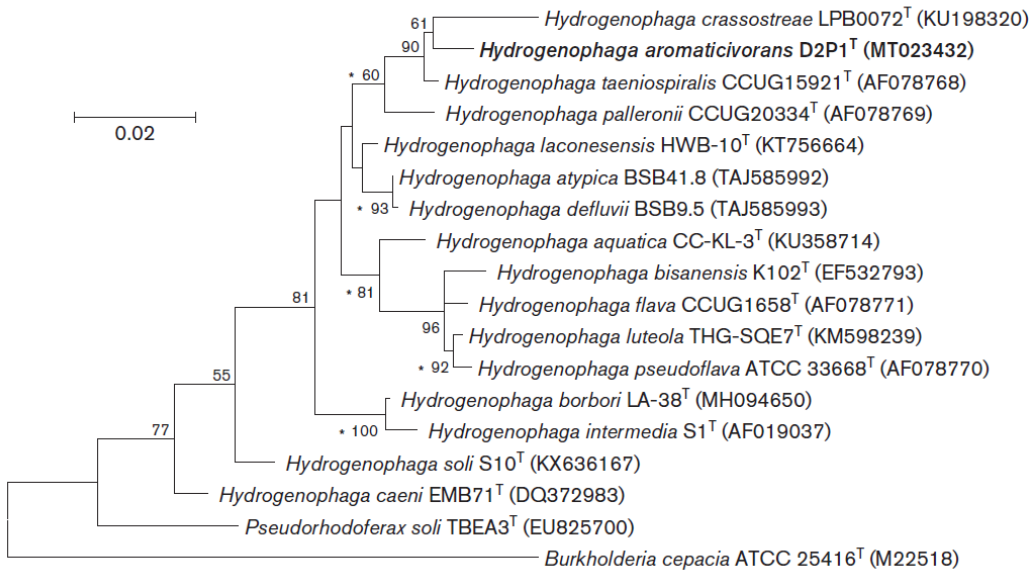


Figure 20. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain D2P1^T T and related taxa. Bootstrap values are shown as percentages of 1000 replicates. Branches signed with an asterisk occurred with every tree making algorithm used in the study. *Burkholderia cepacia* ATCC 25416^T and *Pseudorhodoferax soli* TBEA3^T were used to root the tree. Bar, 0.02 substitution per nucleotide position.

The whole genome shotgun project of strain D2P1^T has been deposited at DDBJ/ENA/GenBank under the accession number VYGV00000000. The version described in this paper is version VYGV01000000. The high-quality draft genome of the strain has been assembled into 28 scaffolds with 5 638 831 bps, with 5505 total genes and 5370 putative coding sequences. Furthermore, the genome encodes three different subfamily I.2.C-type C23O enzymes for the degradation of aromatic compounds. Genome comparison of D2P1^T with other five type strains, including the closest relatives (*Hydrogenophaga taeniospiralis* NBRC102512^T, *Hydrogenophaga palleronii* NBRC102513^T, *Hydrogenophaga crassostreae* LPB0072^T, *Hydrogenophaga flava* NBRC102514^T and *Hydrogenophaga pseudoflava* NBRC102511^T) was carried out using the Genome-to-Genome Distance Calculator and the OAT software. This analysis yielded digital DNA–DNA hybridization values ranging from 21.4% to 29.8% and OrthoANI values ranging from 77.2% to 85.4% (**Fig. 21**). Considering the 70% DNA–DNA relatedness and the 95-96% OrthoANI threshold values accepted for species delineation, strain D2P1^T represents a novel species (Moore *et al.*, 1987; Lee *et al.*, 2016). The DNA G+C content of the strain D2P1^T was 65.5%, according to the draft genome sequence.

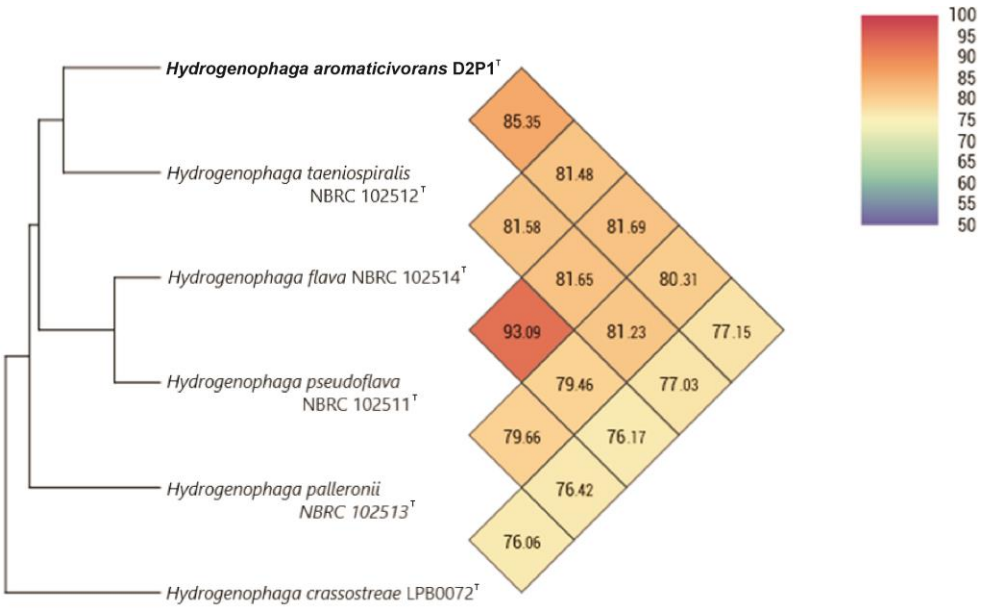


Figure 21**.** Heatmap generated with OrthoANI values between strain D2P1T [T] and other type strains of the genus *Hydrogenophaga*, including the closest relatives *H. taeniospiralis* and *H. palleronii*.

From the results of the phenotypic, genetic and chemotaxonomic analyses, it is obvious that strain D2P1T represents a novel species within the genus *Hydrogenophaga.* For this species, we proposed the name *Hydrogenophaga aromatica* sp. nov..

The results of this chapter have been published in the following publication:

Banerjee S, Tancsics A, Toth E, Revesz F, Boka K, Kriszt B. (2021) *Hydrogenophaga aromaticivorans* sp. nov., isolated from a *para*-xylene-degrading enrichment culture, capable of degrading benzene, *meta*-and *para*-xylene. International Journal of Systematic and Evolutionary Microbiology, 71:004743. DOI: 10.1099/ijsem.0.004743

4.2 Comparative analysis of aerobic and microaerobic xylene degrading bacterial enrichments

This part explains the results of the second enrichment (Enrichment B); here, we have investigated the evolving bacterial community composition under both aerobic and microaerobic conditions using

a mixture of xylene isomers as the carbon and energy source. Dissolved oxygen concentration values of the contaminated groundwater sample showed that the contaminated groundwater reserve has a microaerobic environment as it is present under a thick layer of clay. In such a hypoxic environment, xylene and benzenes are the most persistent molecule. Hence, the study of the indigenous microbial population of this contaminated groundwater (primarily with xylenes) was performed by selective culturing. Moreover, we have also tried to isolate strains of the most dominant aerobic and microaerobic xylene degraders with the hope of studying them further to explore their metabolic potential as bioremediation candidates.

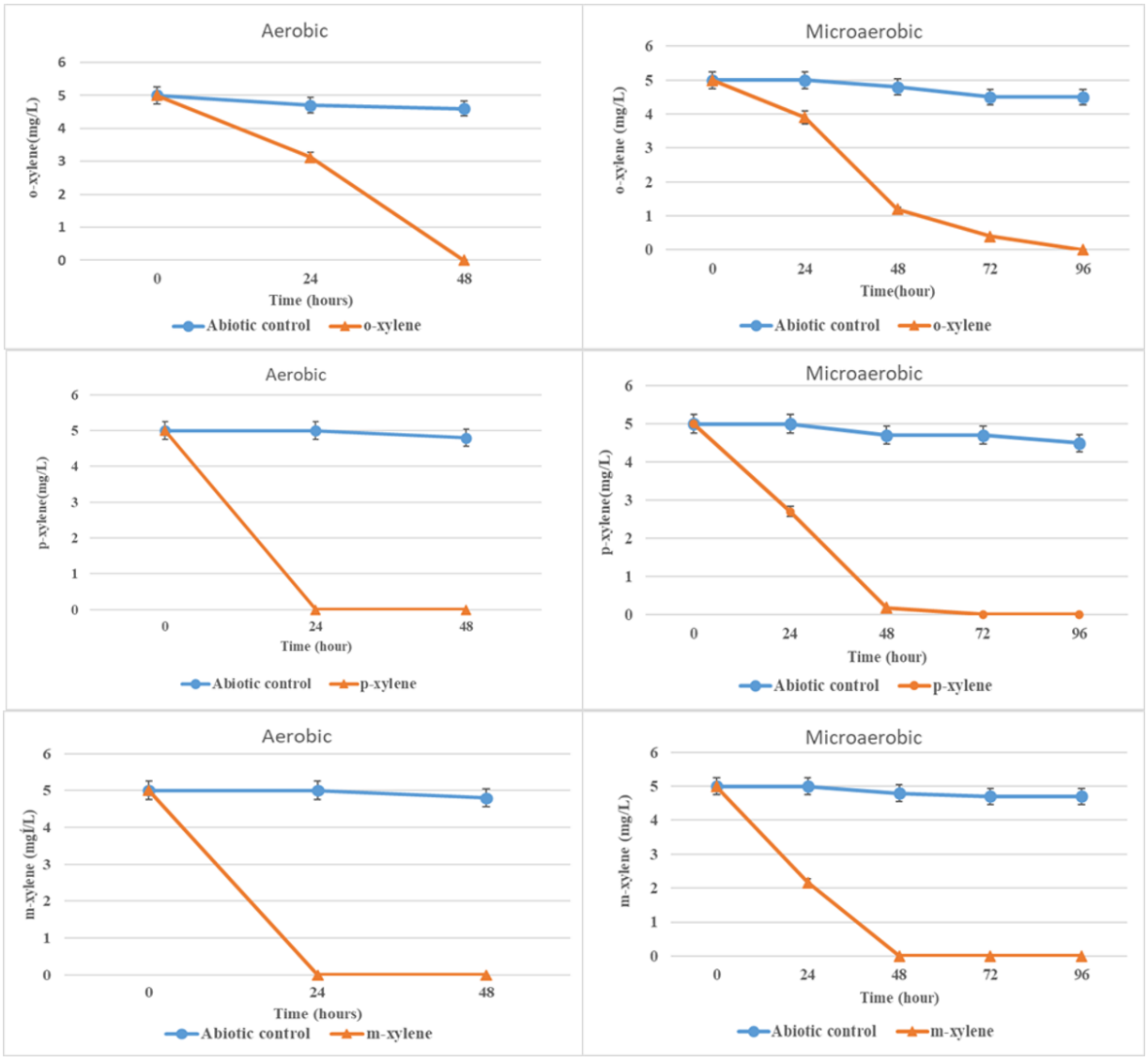
4.2.1 Evaluating oxygen-mediated changes in aerobic and microaerobic xylene degrading microbial communities

In this section, we aimed to investigate the difference in the composition and metabolic abilities of the bacterial community evolved as an effect of the availability of low dissolved oxygen concentration (0-0.5 mg L^{-1}). Same as our previous enrichment experiment, a contaminated groundwater sample from the Siklós contaminated site was used as a starting inoculum as described earlier. To study the functional diversity aspect of the microbial population, an assessment of *C23O* genes was also performed. Moreover, metagenomic studies were also conducted to have in-depth knowledge about the presence and function of the uncultured community members. The genome binning of the dominant uncultured bacterial community members provides us with the artificially constructed genome of some uncultured strains. Genome analyses were also conducted to explain the details of the metabolic potential of those xylene degrading strains. The following result discussed in this section supports our aim and hypotheses.

4.2.1.1 Bacterial community composition of aerobic and microaerobic xylene-degrading enrichments revealed by 16S rDNA amplicon sequencing

The result of xylene degradation measured by GC-MS, of both aerobic and microaerobic enrichments reflected that by the fifth week, the enrichments had developed extremely competent aerobic and microaerobic xylene degrading microbial communities that could degrade xylene effectively. In the presence of oxygen *m*-, *p*-xylene took 24h of incubation, and *o*-xylene took 48h of incubation for the complete degradation (**Fig. 22 Panel A**). Likewise, under hypoxic conditions, degradation was a bit

slower, where it took 48h for *m*- and *p*-xylene degradation and 96h for complete elimination of *o*-xylene (**Fig. 22 Panel B**).



A B

Figure 22. Panel A: xylene degradation in aerobic enrichments. Panel B: xylene degradation in microaerobic enrichments

To have a complete insight into the community composition and diversity at class, order and genus level of individual enrichments, 16S rRNA gene amplicon sequencing was performed. Results revealed that the aerobic and microaerobic xylene degrading enrichments harboured noticeably different bacterial communities. The aerobic xylene degrading enrichments were predominantly dominated by the members of the genus *Pseudomonas* with relative abundance values between 47-59%, followed by *Sphingobium* (20-30%) and *Acidovorax* (4-6%). Noticeable minor community

players were: *Flavobacterium*(6%) and *Roseimicrobium* (4%). On the other hand, in the case of microaerobic enrichments, members of the genus *Pseudomonas* were overwhelmingly dominant in the community by showing ~50% abundance value. But interestingly, *Sphingobium* was not detectable in any of the microaerobic enrichments. **(Fig. 23)**. Members of the genus *Sphingobium* were completely replaced with either members of the genus *Azovibrio* (25% relative abundance in enrichment MIC1), or *Rhodoferax* (20 and 31% relative abundances in enrichments MIC2 and MIC3, respectively)

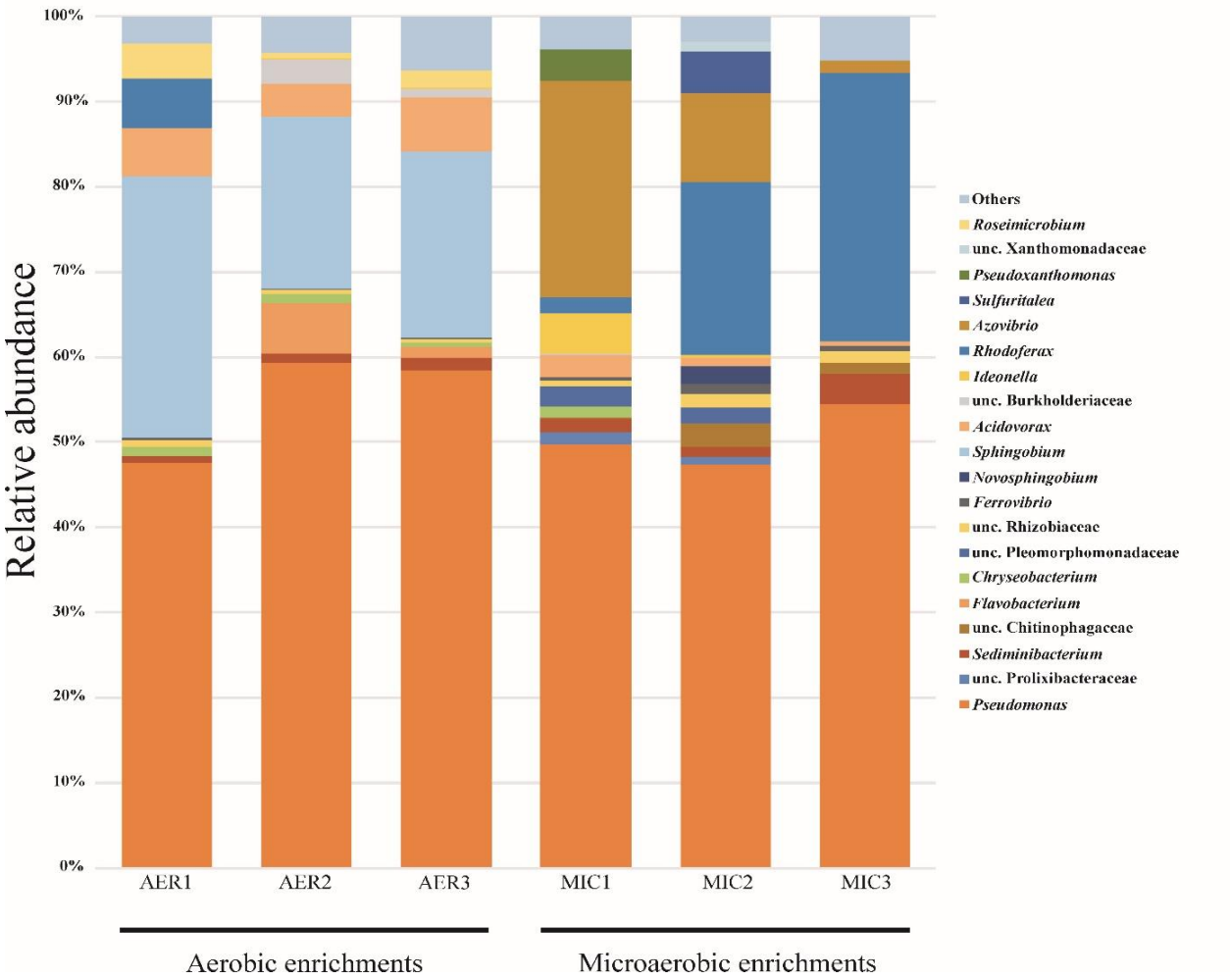


Figure 23. Genus level bacterial community structure of aerobic and microaerobic xylene-degrading enrichments as revealed by Illumina paired-end 16S rRNA gene amplicon sequencing. Only taxa contributing more than 1% abundance were depicted.

Besides these dominant groups, there were several other genera also present in relative abundance of more than 0.5% in aerobic and microaerobic enrichments like *Sediminibacterium, Flavobacterium, Chryseobacterium, Ferrovibrio, Ideonella, Sulfuritalea, Pseudoxanthomonas*. Minor community players of the microaerobic enrichments (e.g. *Pseudoxanthomonas, Sulfuritalea, Ideonella*) were

typically present only in one of the triplicate enrichments. Other than *Pseudomonas*, members of the genus *Sediminibacterium* were the only ones that could be found in all of the enrichments, both in aerobic and microaerobic settings. It is not surprising to register the overwhelming dominance of members of the genus *Pseudomonas* in all the enrichments as *pseudomonads* are extensively studied and well-known bacteria capable of utilising and transforming hydrocarbons, including xylenes as carbon and energy sources (Das and Chandran, 2011) and are often selected for bioremediation purposes (Di Martino *et al.,* 2012). There are a number of well-known petroleum hydrocarbon degraders among *sphingomonads* as well (e.g. *Sphingobium, Sphingomonas, Novosphingobium and Sphingopyxis*). *Sphingomonads* that have the ability to utilize monoaromatic hydrocarbons as the sole source of carbon can usually degrade *m*-, and *p*-xylene, such as *Sphingobium aquiterrae*, which was isolated earlier from the groundwater sample of the Siklos site (Révész *et al.,* 2018). The absence of *Sphingobium*-related sequence reads in the microaerobic enrichments indicates that members of the genus *Sphingobium* only contribute to xylene degradation under strict aerobic conditions. This statement could be supported by the fact that the *Sphingobium* strains isolated from the aerobic xylene degrading enrichment could effectively degrade all three xylene isomers aerobically. Overall, the composition of the bacterial community was distinctly different from microaerobic enrichments in general. The three parallel microaerobic enrichment cultures show similarity in microbial composition but with one enrichment (MIC1) bit different from the others in the percentage of *Azovibrio* and *Rhodoferax*. Interestingly a competition among these two groups could be observed where the proportion of one increases with the decrease of another. Hence, a compitition between them has occurred to be the second most dominant genus of the community. The genus *Azovibrio* contains mostly nitrogen-fixing bacteria. The only described species in the genus *Azovibrio* is *A. restrictus* (Reinhold-Hurek and Hurek, 2000). *Azovibrio* sp. is often found in hydrocarbon-contaminated facultative anaerobic and anaerobic populations. (Sarkar *et al.*, 2017). It is well known that a certain phyletic lineage of the genus *Rhodoferax* is associated with aromatic hydrocarbon-contaminated subsurface environments (Táncsics *et al.*, 2013). Furthermore, DNA stable-isotope probing studies suggested their role in toluene and phenanthrene degradation (Martin *et al.*, 2012; Táncsics *et al.*, 2018). However, so far, no cultivable representative belonging to this lineage has been isolated from a petroleum hydrocarbon-contaminated environment. A genome resolved metagenomics study of enrichment MIC3 was conducted in order to elucidate the genome of the *Rhodoferax*-related bacteria abundant in the microaerobic enrichments.

4.2.1.2 Metagenome assembled genome analysis

From metagenome data of enrichment MIC3, in total, twelve high-quality metagenome-assembled genomes (MAG) were reconstructed; among them, one *Comamonadaceae*-related MAG was found (bin1, **Table 6**).

Table.6. Phylogenomic affiliation of MAGs according to the MiGA pipeline

Bins	**Phylogenomic affiliation**	**Size (Mbp)**	**Completeness**
Bin1	*Rhodoferax* sp. (*Gammaproteobacteria/Burkholderiales/Comamonadaceae*)	4.70	97.2%
Bin2	*Azovibrio* sp. (*Gammaproteobacteria/Burkholderiales/Rhodocyclaceae*)	2.93	99.1%
Bin3	*Chitinophagaceae* bacterium (*Chitinophagia/Chitiniphagales*)	3.27	95.3%
Bin4	*Sediminibacterium* sp. (*Chitinophagia/Chitinophagales/Chitinophagaceae*)	3.29	95.3%
Bin5	*Sediminibacterium* sp. (*Chitinophagia/Chitinophagales/Chitinophagaceae*)	3.50	92.5%
Bin6	*Rhodospirillales* bacterium (*Alphaproteobacteria*)	4.11	90.6%
Bin7	*Pseudolabrys* sp. (*Alphaproteobacteria/Rhizobiales/Xanthobacteraceae*)	4.21	83.0%
Bin8	*Pseudomonas sagittaria* (*Gammaproteobacteria/Pseudomonadales/Pseudomonadaceae*)	1.93	67%
Bin9	*Pseudomonas umsongensis* (*Gammaproteobacteria/Pseudomonadales/Pseudomonadaceae*)	5.83	95.3%
Bin10	*Siculibacillus* sp. (*Alphaproteobacteria/Rhizobiales/Ancalomicrobiaceae*)	4.55	97.2%
Bin11	*Rugosibacter aromaticivorans* (Gammaproteobacteria/Burkholderiales/Rhodocyclaceae)	2.48	84.9%

Bin12	*Variovorax* sp. (Gammaproteobacteria/Burkholderiales/Comamonadaceae)	4.86	98.1%

*Significance at p-value below 0.5.

The phylogenomic analyses revealed that this MAG Bin1 represented a *Rhodoferax* genus-related bacterial lineage. The MAG was 4.70 Mb in size with a G+C content of 56.45% but did not contain SSU sequences, although its completeness was >97%. The recovered genome was found to be a member of the genus *Rhodoferax* as indicated by both MiGA and the UBCG pipelines (**Fig. 24**), and showed the highest phylogenetic relatedness with *Rhodoferax* sp. strain MIZ03, *R. fermentans and R. ferrireducens*, respectively.

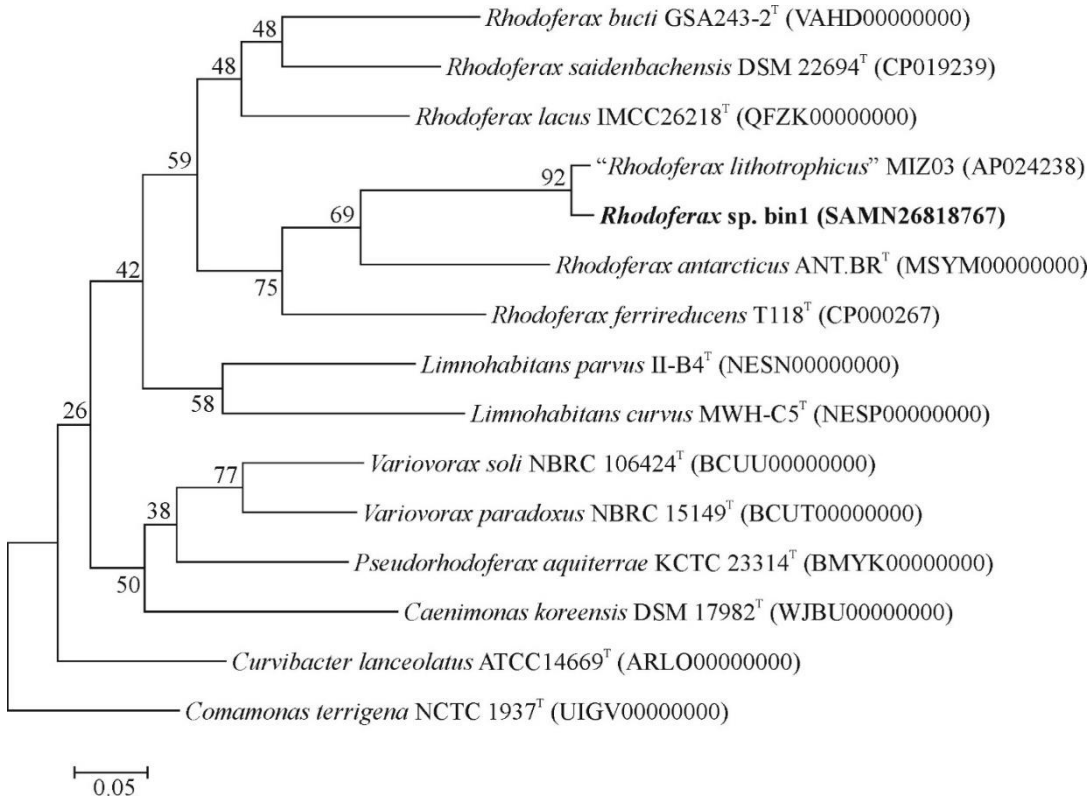


Figure 24**.** Phylogenomic tree constructed using UBCGs (concatenated alignment of 92 core genes) showing the phylogenetic position of *Rhodoferax* sp. bin1. Bar, 0.05 substitution per nucleotide position.

During the genome analysis, the presence of a gene cluster encoding a toluene-4-monooxygenase, by genes *tmoABCDEF* was uncovered. The mentioned gene cluster harboured a gene that encodes an outer membrane transport protein which is most likely involved in the movement of monoaromatic hydrocarbons from the extracellular environment to the periplasm (Hearn *et al*., 2008). Another gene cluster coding for a multicomponent phenol hydroxylase (mPH) and a full *meta*-cleavage pathway

was detected around 20 kilobases downstream of the gene cluster coding for Tmo (mPH1 cluster). In addition to that, another contig was also found to encode a second mPH and *meta*-cleavage enzymes (mPH2 cluster). Important to mention that these two mPH clusters showed completely different gene organizations (**Fig. 25**)

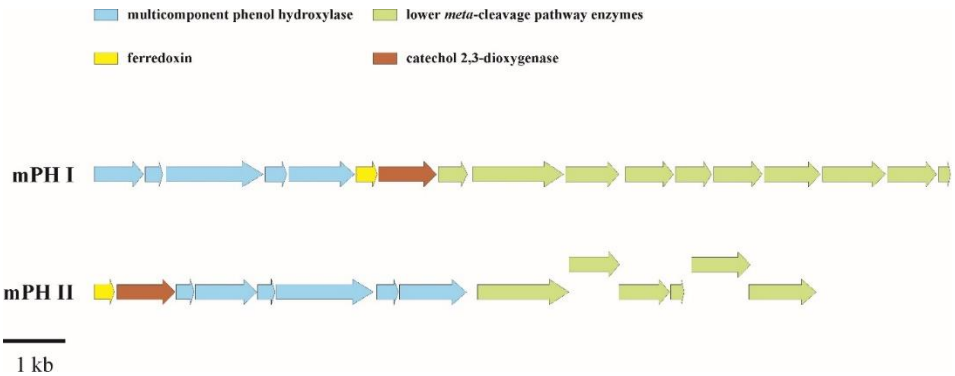


Figure 25 Physical maps of the multicomponent phenol hydroxylase (mPH) gene clusters in the MAG of *Rhodoferax* sp. bin1. The map was created by using CLC Genomics Workbench Tool version 2.1. Arrows represent predicted ORFs, while the direction of the arrows represents the direction of transcription. The different colours denote different functional groups of genes involved in aromatic hydrocarbon degradation.

Interesting to note that neither of the mPH clusters coded for subfamily I.2.C-type extradiol ring-cleavage dioxygenase (EDO) enzymes. On the phylogenetic tree, they actually formed a distinct but as-yet-undescribed subfamily of EDO enzymes. (**Fig. 26**). Based on the evolutionary classification of extradiol dioxygenases (Eltis and Bolin, 1996; Suenaga *et al.*, 2014; Terrón-González *et al.*, 2016), this new subfamily was assigned as I.2.I. (**Fig. 26**).

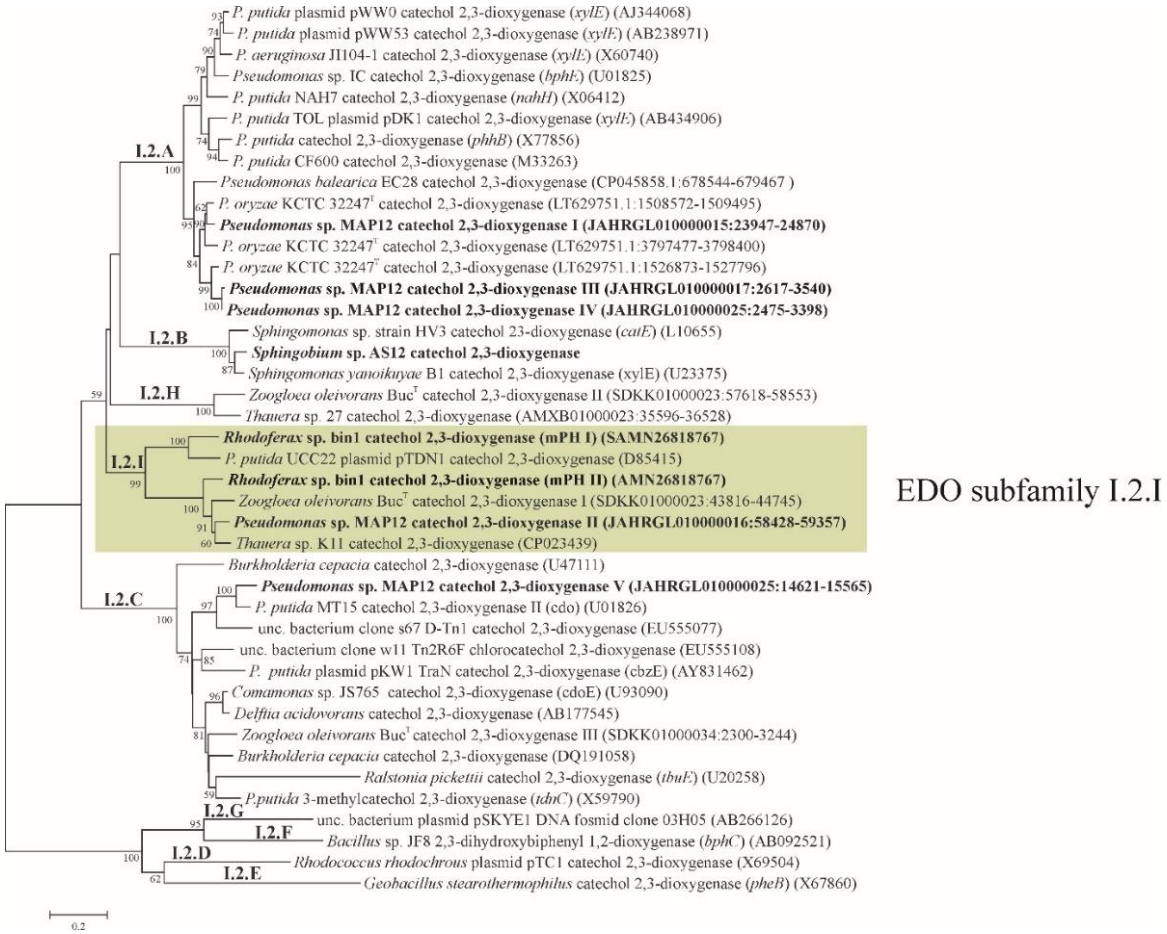


Figure 26. Neighbor-joining phylogenetic tree of deduced amino acid sequences of catechol 2,3-dioxygenase (*C23O*) genes encoding class I EDO enzymes. The tree shows the phylogenetic position of the newly defined class I EDO subfamily I.2.I. The C23Os revealed by the present study are highlighted with boldface type. Class I EDO subfamilies are indicated at the main branches of the tree. Only bootstrap values >50 are indicated. Scale bar, 0.2 substitutions per amino acid position.

The genome of *Rhodoferax* sp. strain MIZ03 (tentatively but not validly designated as "*R. lithotrophicus*") showed the presence of all of the aromatic hydrocarbon-degrading gene clusters that were found in *Rhodoferax* sp. bin1. This strain was described as the first single bacterium capable of both Fe(II) oxidation and Fe(III) reduction at circumneutral pH, isolated from a wetland in Japan (Kato and Ohkuma, 2021). The genomic relatedness value between *Rhodoferax* sp. bin1 and *Rhodoferax* sp. strain MIZ03 obtained by OrthoANI (97.5%) indicated that they belong to the same genomic species. Hence, it may be assumed that this species has had the capacity to degrade aromatic hydrocarbons, and this property is not acquired recently. In addition, it can also be concluded that *Rhodoferax* lineage is strongly associated with aromatic hydrocarbon-contaminated subsurface environments (Táncsics et al., 2013). An in-depth analysis of the *Rhodoferax* sp. bin1 genome

revealed the presence of a complete *narGHIJ* operon, encoding the respiratory nitrate reductase and upstream of this operon gene encoding a nitric oxide reductase complex. The existence of these genes may imply that this bacterium can respire with nitrate to convert it to nitrite. Since the MAG lacked nitrite and nitrous-oxide reductase genes, thus, its ability to carry out full denitrification remains unclear.

4.2.1.3 Description of strains isolated from aerobic and microaerobic enrichment cultures

As a part of the culture-dependent examination strategy, culturable bacterial strains from the fifth-week enrichment samples were isolated. Several strains were isolated, among them strains isolated from aerobic enrichment belonging to 6 genera and strains isolated from microaerobic enrichments belonging to 9 genera (**Table 7**). Among aerobic and microaerobic strains belonging to common genera are *Pseudomonas, Variovorax,* and *Rhizobium*.

Table 7. Result of bacterial strains isolated from the enrichments

No. of isolate	Affiliation*	length of 16S rRNA gene analysed (bp)	similarity (%)	Presence of I.2.C-type C23O gene
Aerobic enrichments				
AV1	*Variovorax ginsengisoli*	1431	99.3	+
AF2	*Flavobacterium terriphilum*	1410	97.9	-
AR5	*Rhizobium daejeonense*	1378	97.6	-
AF6	*Flavobacterium terriphilum*	1410	97.9	-
AP7	*Pseudomonas moorei*	1427	99.9	+
AP8	*Pseudomonas moorei*	1427	99.9	+
AF9	*Flavobacterium terriphilum*	1410	99.3	-
AS10	*Sphingobium terrigena*	1328	99.4	-
AV11	*Variovorax ginsengisoli*	1431	99.3	+
AS12	*Sphingobium terrigena*	1328	99.4	-
AV14	*Variovorax ginsengisoli*	1431	99.3	+
AV15	*Variovorax ginsengisoli*	1431	99.3	+
AF17	*Flavobacterium terriphilum*	1410	97.9	-
AV20	*Variovorax ginsengisoli*	1432	99.9	+
AS21	*Sphingobium terrigena*	1328	99.4	-
AS22	*Variovorax ginsengisoli*	1431	99.3	+
Microaerobic enrichments				
MAP1	*Pseudomonas linyingensis*	1005	98.0	+
MAP2	*Pseudomonas moorei*	1366	99.9	+
MAP3	*Pseudomonas moorei*	1366	99.9	+
MAP5	*Pseudomonas moorei*	1366	99.9	+

MAP6	*Pseudomonas moorei*	1366	99.9	+
MAH7	*Herbaspirillum huttiense* subsp. *Putei*	1432	99.6	-
MAA8	*Acidovorax delafieldii*	1365	100	+
MAP9	*Pseudomonas songnenensis*	1427	98.1	-
MAB10	*Bosea eneae*	1257	99.6	-
MAP11	*Pseudomonas moorei*	1366	99.9	+
MAP12^{T}	*Pseudomonas linyingensis*	1436	98.4	+
MAP13	*Pseudomonas songnenensis*	1427	98.1	-
MAP14	*Pseudomonas linyingensis*	1436	98.4	+
MAP15	*Pinisolibacter ravus*	1304	97.3	-
MAP16	*Pseudomonas linyingensis*	1436	98.4	+
MAP17	*Pseudomonas linyingensis*	1436	98.4	+
MAV19	*Variovorax ginsengisoli*	1429	99.3	+
MAR20	*Rhizobium deajeonense*	1379	98.5	-
MAP21	*Pseudomonas linyingensis*	1436	98.4	+
MA2-2	*Pinisolibacter ravus*	1362	97.1	+
MAV25	*Variovorax ginsengisoli*	1394	99.9	+

The majority of the isolated strains from aerobic enrichments belonged to genus *Variovorax* (~40% of the isolates) along with representatives of genera *Flavobacterium*, *Sphingobium* and *Pseudomonas*. But in contrast, strains isolated from microaerobic enrichments were majorly found to be members of the genus *Pseudomonas* (~60% of the isolates). Based on the highest 16S rRNA gene similarity, the isolated *Pseudomonas* isolates could be divided into two groups *P. moorei* and P. *sagittaria/linyingensis* lineages, respectively. Results of genome-resolved metagenomics also supported this fact. Noticeably, the strains belonging to *Pseudomonas sagittaria/linyingensis* lineages were only found in microaerobic enrichments. Apart from *Pseudomonas*, strains belong to the genera *Pinisolibacter* and *Variovorax* were also isolated from microaerobic enrichments. Strain belonged to the genus *Pinisolibacter* found to represent a new lineage of the genus. Hence, this isolated strain named as MA2-2 was described as a new species of the genus and designated as *Pinisolibacter aquiterrae* has the ability to degrade aromatic hydrocarbons (Bedics *et al.*, 2022). The whole genome analysis of *Pi. aquiterrae* also indicated towards its genetic ability to break down monoaromatic hydrocarbons due to the presence of catechol 2,3-dioxygenase (C23O) gene in a gene cluster, encoding a partial *meta*-cleavage pathway (Bedics *et al.*, 2022). As *Pinisolibacter* is the genus that shows the closest relatedness with the genus *Siculibacillus*, thus, the genome relatedness between these two was analysed. To do so, Bin10 MAG, which was identified as *Siculibacillus* sp. by the MiGA pipeline and *Pinisolibacter* strain MA2-2 were used. The OrthoANI results revealed more than 99.9% relatedness between those two genomes, proving their degree of similarity. This result also

authenticates the preciseness and biological reality of our MAG recovery process. The recovered Bin 10 MAG could be assigned as SMAG (Setubal, 2021). Among the isolated strains, we have identified some strains that represent a new lineage of genus *Pseudomonas* and only present in microaerobic enrichments. Besides, strains that presumably belong to a new lineage of genus *Sphingobium* and only present in aerobic enrichments as a representative of the most dominant community members were also isolated. These groups of organisms create the main difference between aerobic and microaerobic enrichment community structures. Therefore, it is interesting and important to analyse genomes of *Sphingobium* sp. strain AS12 and *Pseudomonas* sp. strain MAP12^T. Phylogenetic analysis based on 1406 bp long partial 16S rRNA gene sequence of AS12 revealed that the strain forms a distinct phylogenetic lineage as a member of the genus *Sphingobium* (**Fig. S3**). Strain AS12 showed the closest 16S rRNA gene sequence similarity with *Sphingobium terrigena* strain EO9^T (99.2%) followed by *Sphingobium xenophagum* NBRC 107872^T (98.3%). Since, the 16S rRNA gene similarity of strain AS12 with its closest relative was more than 99%. a whole-genome-based phylogenomic tree was constructed by the TYGS pipeline (based on genome signatures) (Meier-Kolthoff and Göker, 2019) to determine the precise taxonomic position of *Sphingobium* strain AS12. The result of GBDP-based phylogenomic tree showed the presence of an independent branch supported by a bootstrap value of 100% that can differentiate strain AS12 from the other previously described *Sphingobium* species (**Fig. S4**) and uncovered that strain AS12 clustered closely with *Sphingobium terrigena*. OrthoANI analysis confirmed low genomic relatedness to its closest relatives. Genome-relatedness analysis of AS12 with the closest type strain, *S. terrigena* EO9^T showed an ANI value of 92% and a dDDH value of 48.8%. These facts supported the hypothesis that strain AS12 represents a yet undescribed lineage of the genus *Sphingobium* (Family: Sphingomonadaceae and Class: Alphaproteobacteria). The genome annotation showed that the genome of strain AS12 is 4,862,982 bp in size with a GC content of 62.5 % (NCBI accession number: JAHRGM000000000.1).The total number of putative coding sequences (CDSs) in the genome was 4,727, among which 3821 CDSs were assigned to functional classes. The draft genome of AS12 contained 49 tRNA and 5 rRNA genes (including 5S, 16S and 23S rRNA). Genes responsible for aromatic hydrocarbon degradation were found on a ~62 kB large scaffold of the genome. This includes a C23O gene, that encodes a subfamily I.2.B-type extradiol dioxygenase (EDO) enzyme. The central, ~39 kb-long portions of this genomic segment displayed strong similarities to those seen in *Sphingobium yanoikuyea* B1 (**Fig. 27 panel A**). The *Sphingobium yanoikuyea* strain B1 is known to degrade a wide range of mono- and polycyclic aromatic hydrocarbons (Zylstra and Kim, 1997) (PAHs) and biphenyls. This gene cluster contains the majority

of the catabolic genes required for the degradation of the above-mentioned compounds in an interesting, "disheveled" arrangement (Chadhain *et al.*, 2007). Similarly to *S. yanoikuyae* B1, the dioxygenase genes bphA1fA2f, playing key role in the initial oxidation of biphenyls and naphthalene were found on a distinct, ~8.5 kb large genomic fragment (**Fig. 27 panel B**). Despite the highly similar gene arrangement of these clusters in strains B1 and AS12, the corresponding genes shared only ~75% identity. This low similarity may explain why strain AS12 is able to use all three isomers of xylene as sole carbon sources, while *S. yanoikuyae* B1 cannot use *o*-xylene. Either one of the aromatic ring-hydroxylating enzymes coded on the above-mentioned gene clusters has the ability to convert *o*-xylene to 3,4-methylcatechol, or degradation of *o*-xylene is initiated by oxidation of the methyl substituent to form 2-methylbenzyl alcohol. Nevertheless, a transcriptomic analysis will be necessary to answer this question.

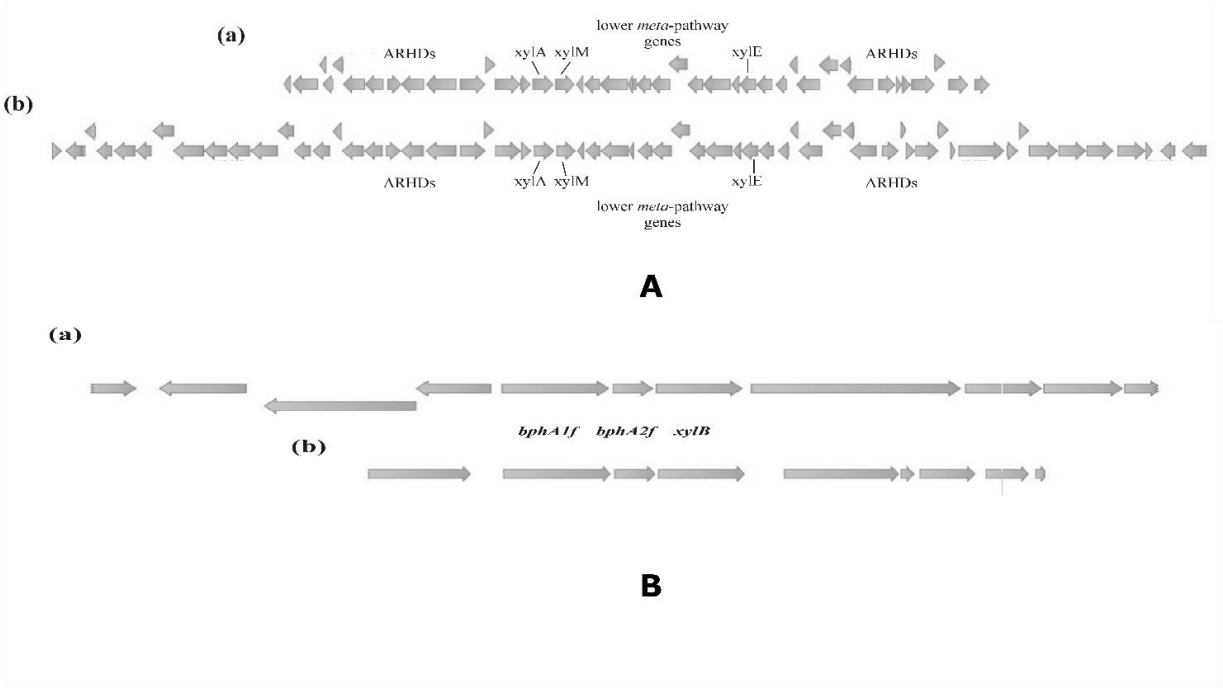


Figure 27**.** Physical map of the genomic fragment encoding most of the aromatic hydrocarbon degradation genes in (A) *Sphingobium yanoikuyae* B1 and (B) *Sphingobium* sp. strain AS12. ARHD: aromatic ring-hydroxylating dioxygenase.

Strain AS12 is capable of degrading all isomers of xylene, unlike its closest relatives. A rather rapid xylene degradation was observed under strict aerobic conditions, whereas in comparison, the degradation process was much slower under microaerobic (0.5 mg/L DO) conditions (**Fig. 28**). This finding might help to explain why *Sphingobium* species were only found in aerobic enrichments. Another crucial point is that all *Sphingobium* species have the inability to reduce nitrate as a common

trait (Pal et al. 2006). Therefore, in oxygen-limited situations, they are less effective at degrading aromatic compounds than other bacteria, such as *Rhodoferax*, which may utilise both oxygen and nitrate as electron acceptors simultaneously (Wilson and Bouwer., 1997). In addition, the whole-genome sequencing suggested that the aromatic ring hydroxylating dioxygenases (ARHDs) in *Sphingobium* sp. strain AS12 are predominantly responsible for the first activation of the aromatic ring. The genomic fragment that encodes the enzymes that take part in aromatic hydrocarbon degradation, contained numerous ARHD genes, while toluene or xylene-monooxygenase genes were absent in any other parts of the genome. However, ring monooxygenation is the predominant mechanism for the aerobic degradation of toluene under hypoxic conditions, according to Martínez-Lavanchy *et al.* (2015). This aids in the understanding of why members belonging to the genus *Sphingobium* were restricted to the aerobic enrichment cultures only. Strain AS12 was deposited at the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Budapest, Hungary) with the accession number NCAIM B.02669

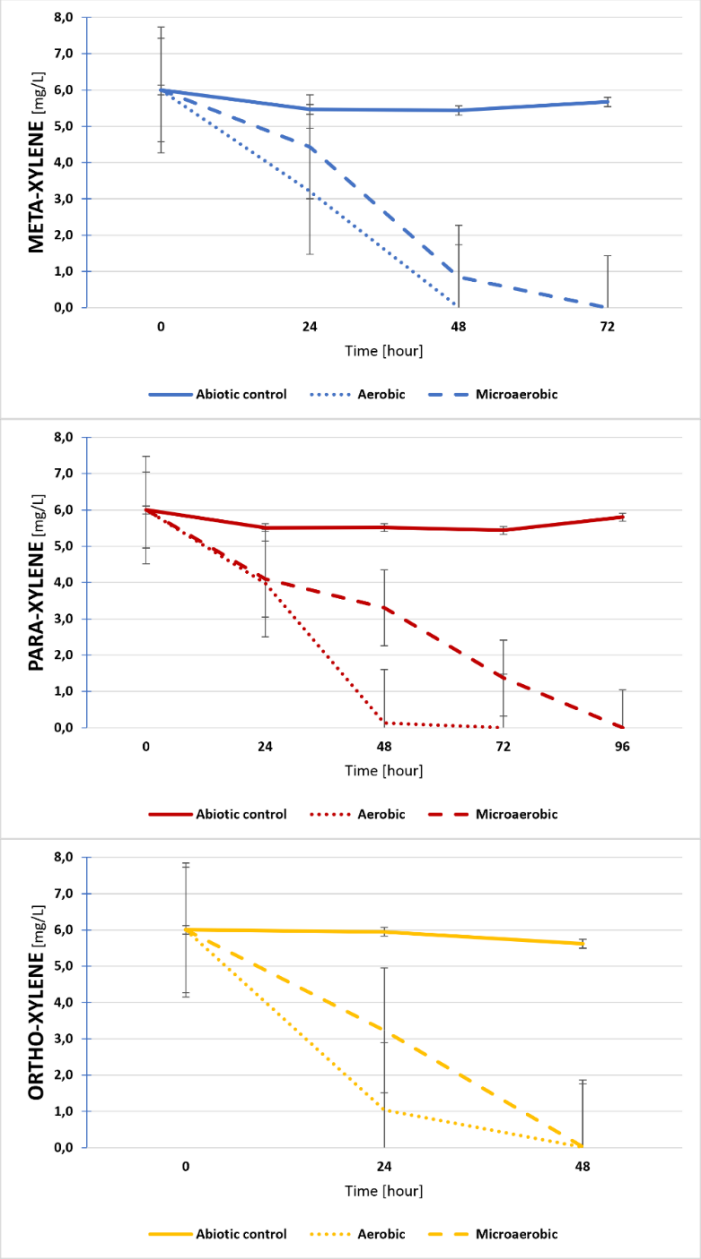


Figure 28. Aerobic and microaerobic degradation of xylene isomers by *Sphingobium sp.* strain AS12. The concentration of xylene isomers was measured by GC-MS analysis for every 24 hours. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

Though from microaerobic enrichments, we were unable to cultivate strains belonging to *Rhodoferax* (31.7% dominant in the community) and *Sediminibacterium* (3.6% dominant in the community) but we have isolated several strains of the genus *Pseudomonas*, maybe because *Pseudomonas* over dominated the enrichment with 54.4% of relative abundance. One of such strains, MAP12^{T} showed the highest 16S rRNA gene sequence similarity to *Pseudomonas linyingensis* LYBRD3-7^{T} (98.4%) and *Pseudomonas sagittaria* JCM 18195^{T} (98.2% similarity). The assembled whole genome of strain

MAP12^{T} was 4.39 Mbp large with G+C content of 65.7%. The ortho ANI value of <89% and dDDH values of <53% with its closest relatives indicated the fact that MAP12^{T} represents a novel species in the genus *Pseudomonas*. The strain harboured a subfamily I.2.C-type *C23O* gene in its genome, as it showed a positive result in *xylE*3 PCR. This fact was supported by the presence of this gene in the annotated genome as a part of a partial toluene/xylene degradation gene cluster (containing upper TOL pathway genes but lacking lower *meta*-cleavage genes) (**Fig. 29**).

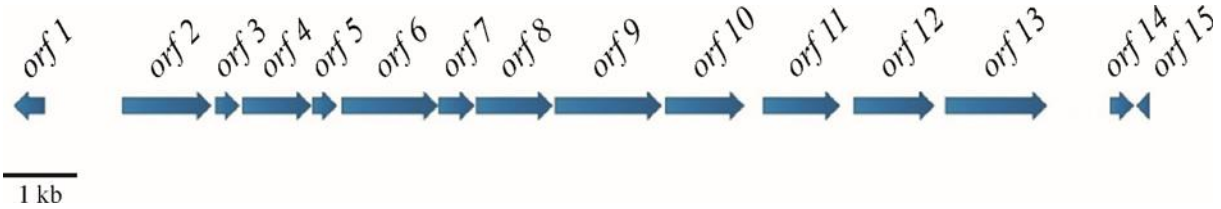


Figure 29**.** Physical map of the partial toluene/xylene degradation gene cluster of *Pseudomonas* sp. strain MAP12^{T} encoding upper TOL pathway enzymes. ORF1: IS3 family transposase; ORF2: ferredoxin reductase; ORF3: ferredoxin; ORF4: catechol 2,3-dioxygenase (XylE); ORF5: ferredoxin; ORF6: benzoate 1,2-dioxygenase large subunit (XylX); ORF7: benzoate 1,2-dioxygenase small subunit (XylY); ORF8: benzyl alcohol dehydrogenase (xylW); ORF9: benzaldehyde dehydrogenase (XylC); ORF10: xylene monooxygenase (xylM); ORF11: xylene monooxygenase electron transfer subunit (XylA); ORF12: benzyl alcohol dehydrogenase (XylB); ORF13: outer membrane protein transport protein (XylN); ORF14: hypothetical protein; ORF15: IS3 family transposase

This gene was sequenced and revealed that it showed a sequence similarity of only ~82% with the *C23O II* gene of *Pseudomonas putida* MT15, which is an archetype of subfamily I.2.C-type *C23O* genes. It was also found that *Pseudomonas* sp. strain MAP12^{T} is harbouring at least five different *C23O* genes (one I.2.C-type *C23O*, three subfamily I.2.A-type *C23O* genes, and surprisingly a subfamily I.2.I-type *C23O*) in its genome. The I.2.I-type *C23O* gene was found to be part of a phenol-degradation gene cluster, the same as it was observed for the *C23O*s of *Rhodoferax* sp. bin1. The result of xylene degradation measured using GC-MS revealed that MAP12^{T} was unable to utilize *o*-xylene as the sole source of carbon and energy but could only use *p*- and *m*-xylene. Furthermore, it could be concluded that oxygen availability does not affect the degradation efficiency of *m*-xylene as the degradation rate was similar for both aerobic and microaerobic experiments. **(Fig. 30)**. However, in the case of *p*-xylene degradation, microaerobic degradation was slower after 24hrs compared to aerobic degradation, though complete degradation occurred after 48hrs in both cases.

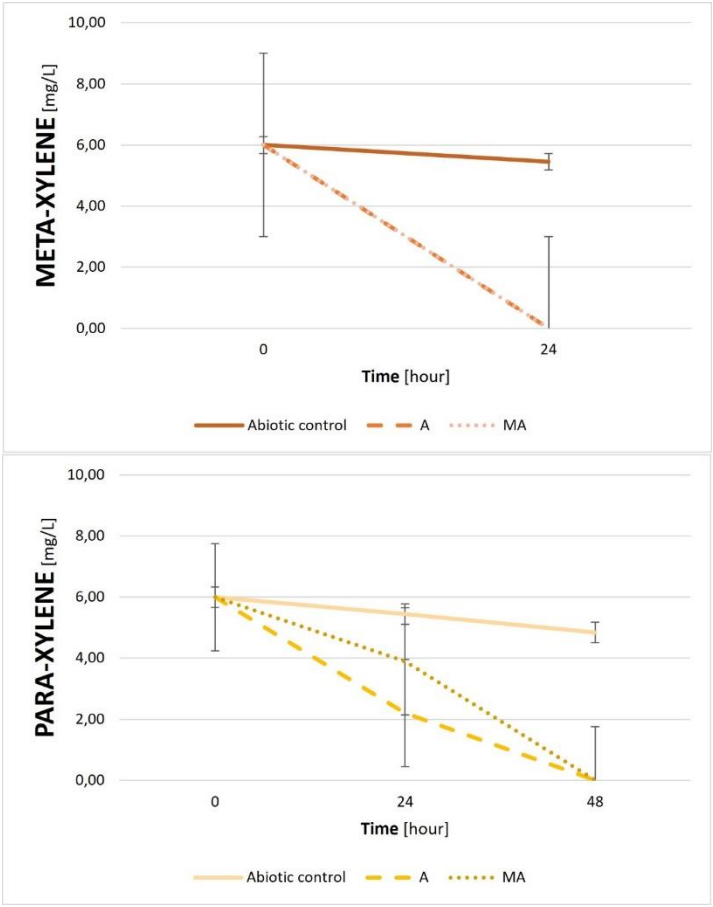


Figure 30**.** Aerobic and microaerobic degradation of meta- and para-xylene by *Pseudomonas sp*. strain MAP12^{T}. The concentration of xylene isomers was measured by GC-MS analysis for every 24 hours. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

The results of this chapter will be published in the following publication:

Táncsics A, **Banerjee S**, Soares A, Bedics A, Kriszt. (2023) Combined Omics Approach Reveals Key Differences between Aerobic and Microaerobic Xylene-Degrading Enrichment Bacterial Communities: *Rhodoferax*─ A Hitherto Unknown Player Emerges from the Microbial Dark Matter. Environmental Science & Technology, 57, 7, 2846–2855. doi:10.1021/acs.est.2c09283.

4.2.2 Description of novel microaerobic xylene degrader *Pseudomonas aromaticivorans* sp. nov.

As the contaminated groundwater ecosystem is often microaerobic in nature, it was one of our major priorities to isolate indigenous strains belonging to the most dominant community that could potentially degrade xylene under both aerobic and, most importantly, microaerobic conditions. Strains that can degrade aromatic hydrocarbons microaerobically make them a lucrative candidate for

bioremediation application because contaminated subsurface environments are generally hypoxic in nature. Moreover, the study of such organisms helps us to understand the microaerobic xylene degradation in depth. This strain MAP12^T was isolated from microaerobic xylene degrading enrichment. Primary 16s rDNA-based studies indicated that this strain represents a yet undescribed lineage of the genus *Pseudomonas*, which was the most dominant bacterial group in the xylene degrading microaerobic enrichments.

The aim of the study was to clarify the taxonomic status of this presumably novel member of the genus *Pseudomonas* using a polyphasic taxonomic approach, including whole genome sequencing, physiological, biochemical, and chemotaxonomic analyses and evaluation of its microaerobic xylene degradation capability.

4.2.2.1 Characterization of the genus *Pseudomonas*:

Indigenous bacteria naturally degrade pollutants in the contaminated environment by using those pollutants as a source of carbon and energy (Xu *et al.*, 2018). In polluted subsurface environments, rapid aerobic degradation by the native bacterial population causes an oxygen deficit that eventually results in hypoxic and anaerobic environments. Removal of contaminants from such an environment is facilitated by autochthonous microorganisms that are able to degrade them microaerobically. Several such bacteria have been reported to degrade BTEX compounds. Among them, according to literature, *Pseudomonas* species can dominate hydrocarbon polluted ecosystems and contribute significantly to the breakdown of BTEX. (Ridgway *et al.*, 1990; Brusa *et al.*, 2001; Yu et al. 2001; Jahn *et al.*, 2005). Despite several reports describing the bioremediation capacity of the genus *Pseudomonas*, there is yet no proof that any *Pseudomonas* strain pure culture is capable of benzene and xylene degradation in microaerobic environments. Furthermore, as they are the least degradable and most persistent BTEX chemicals in subsurface ecosystems, only a limited number of different bacterial strains are known to break down benzene and *para*-xylene in oxygen-limited conditions (Margesin *et al.*, 2003; Wartell *et al.*, 2021). Migula (1894) was the first to describe the genus *Pseudomonas*, which is a member of the family *Pseudomonadaceae* within the *Gammaproteobacteria*. The genus *Pseudomonas* is a quite species-rich genus. At the time of writing, this genus had more than 200 species with recognized names (LPSN; https://www.bacterio.net/index.html) (Parte *et al.*, 2020). Members belonging to this genus are generally aerobic, Gram-stain-negative, non-spore-forming, catalase and oxidase test positive (Lin *et al.*, 2013) rod-shaped bacteria containing one or several polar flagella or peritrichous flagella. The

main respiratory quinone present in the genus *Pseudomonas* is ubiquinone 9. (Jia *et al.*, 2020). The genome G+C content values vary between 58–69 mol% (Lang *et al.*, 2010). Since they can thrive on both simple and complex organic carbon molecules, *Pseudomonas spp*. has a high degree of physiological flexibility. They can grow at temperatures of 4–42 °C and pH 4.0–8.0 (Moore *et al.*, 2006). Members of this genus may survive in a variety of niches, including those for plants, animals, soil, water, and air (Madigan M, 2005; Gupta *et al.*, 2008), including soils containing high levels of pollutants, such as xenobiotics (Manickam *et al.*, 2008) thanks to their broad metabolic potential.

4.2.2.2 Phylogenetic and phylogenomic characterization of MAP12^{T}

The annotated genome of 4,392,784 bp in length was deposited to NCBI with accession number JAHRGL000000000. The MAP12^{T} genome has an average G+C content of 65.67 % and contains 4199 protein-coding sequences.. In addition, the genome harbours the genes that encode at least five different C23O enzymes that break down aromatic molecules, among which one of them encoded a subfamily I.2.C-type extradiol dioxygenase, the ring–cleavage enzymes are known to play a critical role under hypoxic degradation (Kukor and Olsen, 1996).

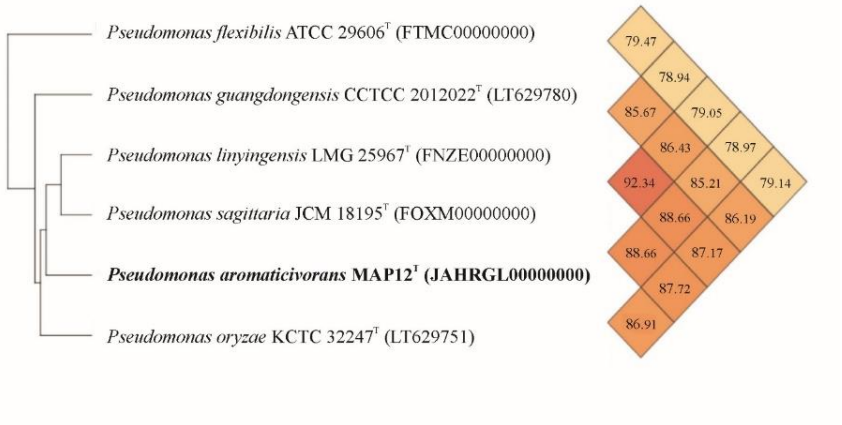


Figure 31**.** Heatmap generated with OrthoANI values between strain MAP12^{T} and other closely related type strains of the genus *Pseudomonas*.

To evaluate genome similarity of strain MAP12^{T} with its closely related type strains using OrthoANI and digital DDH values, the publicly available genome sequences of five closely related type strains, namely *Pseudomonas sagittaria* JCM 18195^{T} (FOXM01000000), *Pseudomonas linyingensis* LMG 25967^{T} (FNZE01000000), *Pseudomonas oryzae* KCTC 32247^{T} (NZ_LT629751.1), *Pseudomonas*

guangdongensis CCTCC AB 2012022^T (NZ_LT629780.1) and *Pseudomonas flexibilis* ATCC 29606^T (FTMC01000000) were downloaded. The OrthoANI values between strain MAP12^T and phylogenetically closest neighbours were between 78-88% (**Fig. 31**), which is much lower than the threshold value of 95-96%, recommended for species level delineation (Meier-Kolthoff *et al.*, 2013).

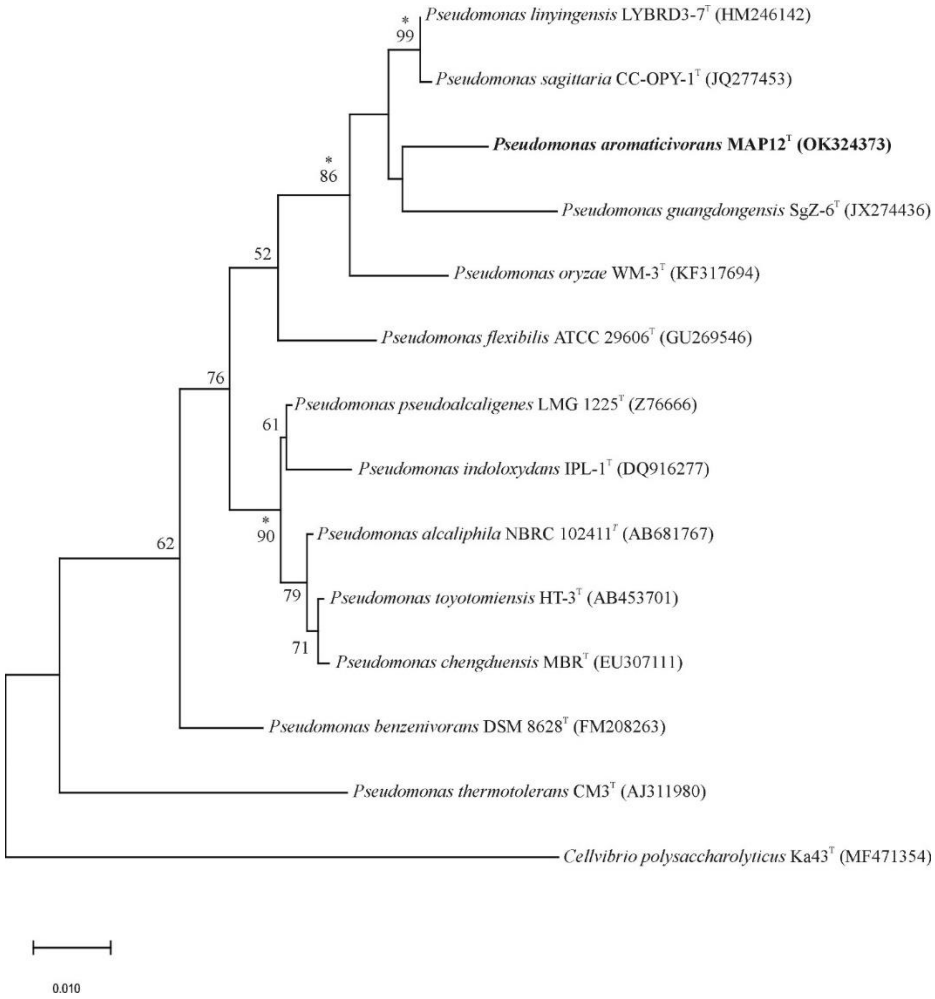


Figure 32**.** Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain MAP12^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates. Branches signed with an asterisk occurred with every tree-making algorithm used in the study. *Cellvibrio polysaccharolyticus* Ka43^T was used to root the tree. Bar, 0.01 substitution per nucleotide position**.**

The dDDH values ranging from 22–52% were also less than the specified threshold (70%) recommended for species demarcation. These outcomes confirmed that the MAP12^T strain represented a novel species within the genus *Pseudomonas*. Additionally, the phylogenomic tree constructed using UBCGs (concatenated alignment of 92 core genes) also proved that strain MAP12^T was a novel member of the genus *Pseudomonas* (**Fig. 33**).

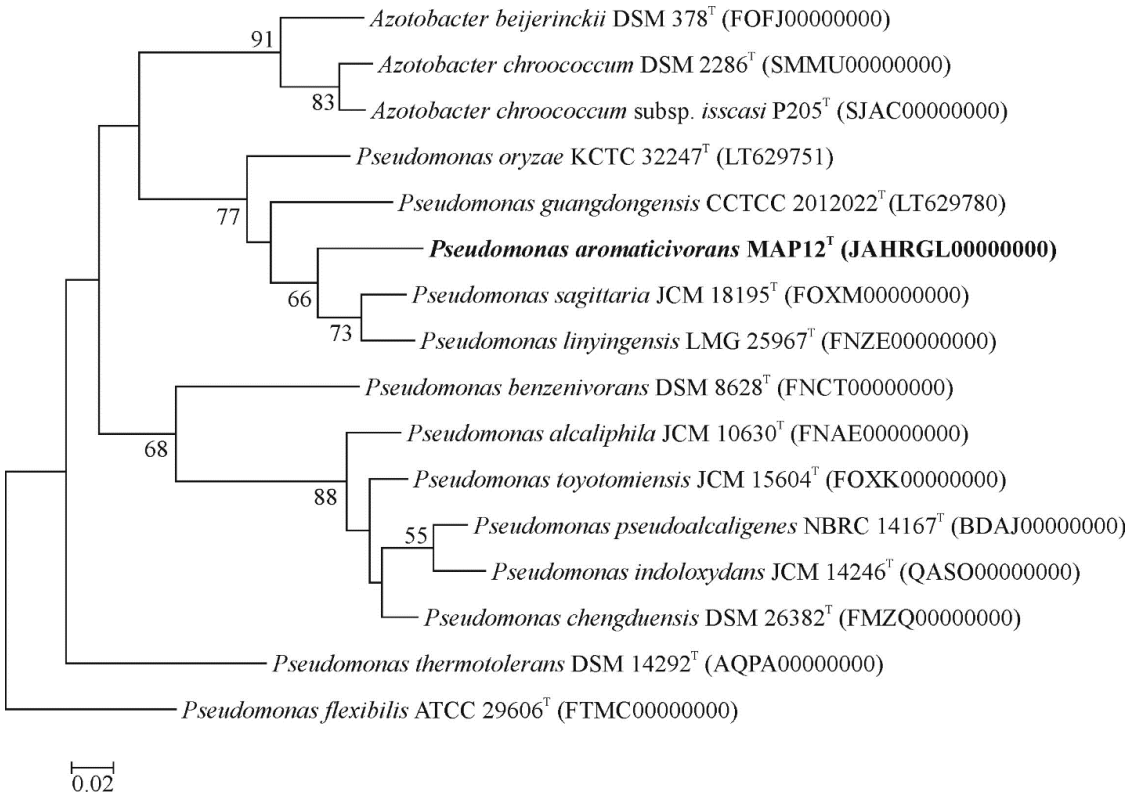


Figure 33. Phylogenomic tree constructed using UBCGs (concatenated alignment of 92 core genes). For inferring the tree the FastTree algorithm was used. Bar, 0.05 substitution per nucleotide position.

Genome-resolved metagenomics investigation of enrichment MIC3 showed that strain MAP12^T was present in notable abundance in the enrichment community. Twelve high-quality metagenome-assembled genomes (MAGs) were obtained as an outcome of genome binning, and two of them could be affiliated with the genus *Pseudomonas*. The *P. sagittaria/linyingensis* lineage might be represented by one of the *Pseudomonas* MAGs, known as XYLBin8 (NCBI BioSample accession number SAMN26818775), despite the fact that it only had a partial genome (1.9 Mb large sequence, 23 contigs). OrthoANI relatedness comparison between this partial MAG and the strain MAP12^T whole genome sequence generated a value of 99.94 percent on the overlapped regions, suggesting that the genome of strain MAP12^T could also be partially rebuilt from the metagenome sequence data. By mapping sequence data of the XYLBin8 MAG to trimmed, quality-controlled reads, the relative abundance of XYLBin8 was found to be 8.66% in the metagenome (mean coverage of 95.39x). Therefore, it may be hypothesized that strain MAP12^T was a prominent member of the enrichment community and may have been a significant key microaerobic xylene degrader.

4.2.2.3 Phenotypic and chemotaxonomic characterization of MAP12^T

The MAP12^T cells are rod-shaped, Gram-stain-negative, aerobic and motile with a polar flagellum. The cells were approximately 2.2-2.5 μm in length and 0.6–0.8 μm in diameter (**Fig. 34**). Strain MAP12^T formed white, glossy, opaque, and circular colonies after 24 hrs of incubation on R2A at 28 °C. The strain showed a negative result for the indole test. MAP12^T grew well at 4 °C, unlike reference strains. MAP12^T can grow at a wide temperature range of 4–45 °C, at a pH range from 5.0 to 12.0, and can tolerate up to 6 % NaCl. In addition, strain MAP12^T grew better in saline (1-4% NaCl) than in the salt-free control.

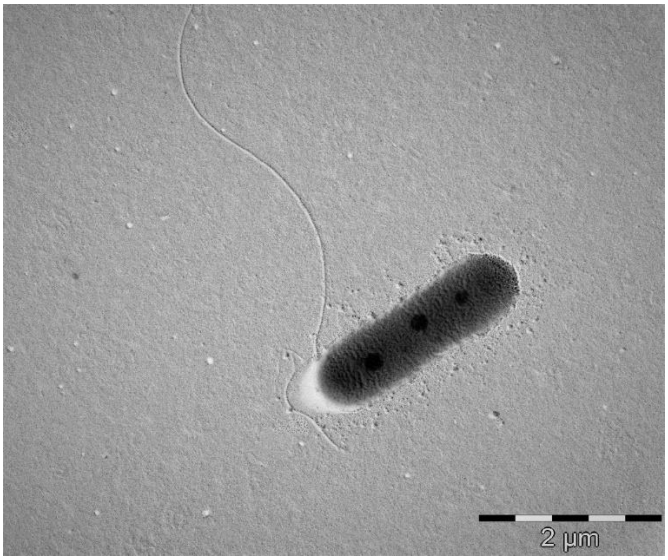


Figure 34**.** Transmission electron microscopic photograph showing cell morphology and presence of flagella in strain MAP12^T. Bar = 2 μm.

MAP12^T was unable to hydrolyse Tween 80 and showed a positive result for the catalase and oxidase test. In API ZYM tests, MAP12^T presented positive enzymatic activity for esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and weakly positive results were detected for leucine arylamidase and trypsin. For API 20NE, strain MAP12^T showed positive results for urease activity and esculin ferric citrate (β-glucosidase) hydrolysis. Whereas it showed negative reactions for NO_3^- reduction. MAP12^T exhibited assimilation of D-glucose, D-mannose, adipic acid and malic acid. The results of API50 CH tests showed that strain D2P1^T could metabolise substrates like glycerol, D-fructose, inositol, esculin ferric citrate and D-arabitol. In the biochemical tests, the strain showed positive results for the NO_2^- reduction, H_2S production, indole production and MR test. Nitrate does not support the growth of MAP12^T in the

Table 8.:Major phenotypic and biochemical characteristics of strain MAP12^T and other closely related type strains Strains: 1, MAP12^T ; 2, *Pseudomonas sagittaria* DSM 27945^T; 3, *Pseudomonas linyingensis* LMG 25967^T. +, Positive; w+, weak positive −, negative. All data were obtained in this study.

Characteristics	**1**	**2**	**3**
Temperature range for growth (°C)	4 -45	4 – 45	4 - 45
pH range for growth	5 - 12	5.5 - 12	5 - 12
NaCl tolearnce (%, w/v)	1 - 5	1 – 4	1 – 4
Enzyme activities (API ZYM):			
Alkaline phosphatase	-	+	+
Esterase (C 4)	+	+	+
Esterase lipase (C 8)	+	+	+
Leucine arylamidase	+	+	+
Valine arylamidase	w+	+	-
Cystine arylamidase	-	+	-
Trypsin	w+	+	+
Acid phosphatase	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+
α- galactosidase	-	-	+
β- galactosidase	-	-	+
β- glucunoridase	-	-	+
α- glucosidase	-	+	+
β- glucosidase	-	+	+
N-acetyl-β-glucosaminidase	-	w+	+
α- mannosidase	-	w+	+
α- fucosidase	-	w+	+
Assimilation of (API 20NE):			
Urea (urease)	+	+	+
Esculin ferric citrate (β-glucosidase) hydrolysis	+	+	+
Gelatin (protease) hydrolisys	-	-	-
4-nitrophenyl-βD-galactopyranoside (β-galactosidase)	+	+	+
D-glucose (assimiliation)	+	-	+
D-mannose	+	-	-
Adipic acid	+	-	-
Maltic acid	+	+	+
Trisodium citrate	-	+	-
Acid production from (API 50 CHB/E)			
Glycerol	+	-	-
D-fructose	+	+	-
Inositol	+	-	+
D-mannitol	-	-	+
D-sorbitol	-	+	-
Esculin ferric citrate	+	-	+
D-arabitol	+	-	-

absence of oxygen, as demonstrated by the anaerobic growth monitoring experiment, which showed no growth of MAP12^T after incubation. Detailed comparative characteristics are given in **Table 8** with phylogenetically closest species of the genus *Pseudomonas***.**The major fatty acids (>5%) of strain MAP12^T were summed feature 3 (C16:1ω6c and/or C16:1ω7c), C16:0, summed feature 8 (C18:1ω6c and/or C18:1ω7c) and C12:0, which are often seen in species of *Pseudomonas*. Despite minor quantitative variations from the reference strains, the major fatty acid content of MAP12^T confirmed its genus *Pseudomonas* affiliation. The details of fatty acid profiles of MAP12^T and other closely related species are given in **Table 9.**

Table 9. Cellular fatty acid compositions of strain MAP12^T and related species**.** Taxa: 1, strain MAP12^T; 2, *Peudomonas sagittaria* DSM 27945^T; 3, *Pseudomonas linyingensis* LMG 25967^T; Data are expressed as percentages of total fatty acids.. Fatty acids which were lower than 1.0% in all strains are not shown. All data are from the present study.

Fatty acid	**1**	**2**	**3**
Saturated			
$C_{12:0}$	7.6	7.3	7.3
$C_{16:0}$	27.6	22.8	22.0
Hydroxy			
$C_{10:0}$ 3-OH	4.0	4.0	3.9
$C_{12:0}$ 3-OH	3.6	3.5	3.7
Cyclic			
$C_{17:0}$ cyclo	3.7	3.5	3.6
$C_{19:0}$ cyclo *ω8*c	1.3	1.2	1.9
Summed feature*			
3	34.7	33.7	33.9
8	15.9	22.9	22.0

* Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed features: 3, $C_{16:1}$ω6c and/or $C_{16:1}$ω7c; 8, $C_{18:1}$ω6c and/or $C_{18:1}$ω7c.

The major respiratory quinones were ubiquinone Q9 (86.7%) and Q8 (13.3%), which is compatible with other species of the genus *Pseudomonas*. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine (**Fig. 35**), which is in agreement with data published

earlier for species of the genus *Pseudomonas* (Cámara *et al*., 2007; Stolz *et al*, 2007; Romanenko *et al*., 2008).

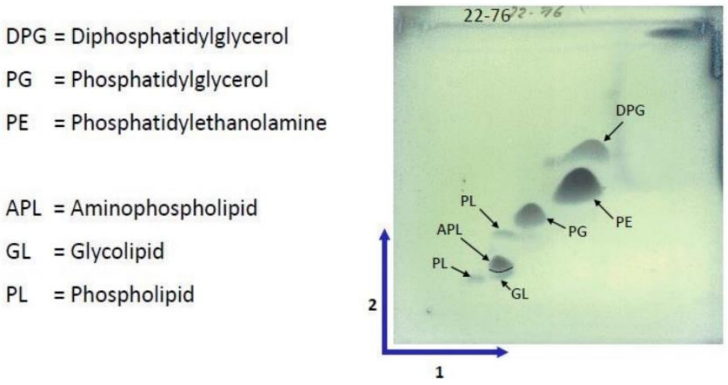


Figure 35: Polar lipid profile of strain MAP12^{T}

4.2.2.4 Genomic overview of strain MAP12^{T} with emphasis on aromatic hydrocarbon degradation

The detailed profiling of clusters of orthologous groups (COG) provides in-depth information on the genomic competence of this isolate about its ability to degrade and survive in complicated habitats polluted with hydrocarbons. Metabolism forms the major function like carbohydrate, amino acid, cofactors and vitamins, nucleotides and energy (**Fig. 36**). Considering the context of our study, it was also observed that functions of particular importance linked to the biodegradation of xenobiotics were present. Functions of special interest related to the biodegradation of xenobiotics were also found to be present. The presence of five catechol 2,3-dioxygenase (*C23O*) genes (Sequence Locus Tag KRX52_06295, KRX52_06950, KRX52_07180, KRX52_11010, KRX52_11075) in the genome is most probably the reason behind the effective BTEX degradation capability of this strain. As mentioned

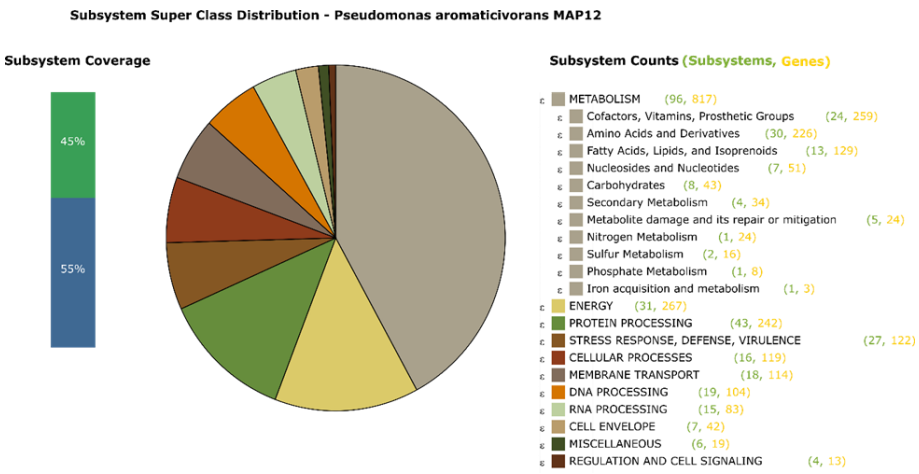


Figure 36**.** Subsystem coverage and category distribution of whole genome. The pie chart indicates the counts of each subsystem feature and the subsystem coverage. The number of genes in each subsystem category was shown in brackets.

earlier, one of the *C23O* genes (locus tag KRX52_11075) encoded a subfamily I.2.C-type extradiol dioxygenase, which is known to function under hypoxic conditions, thus enabling aerobic ring-cleavage under microaerobic conditions as well (Kukor and Olsen , 1996). Deep dive into the genome sequence also indicated the existence of multiple genes for the complete lower *meta*-cleavage pathway, essential for the entry of cleaved BTEX compounds to the TCA cycle. Automatic annotation by MaGe, identified the presence of genes encoding enzymes involved in the utilization of various aromatic compounds in strain MAP12^{T} as a source of carbon and energy (**Fig. 37**).

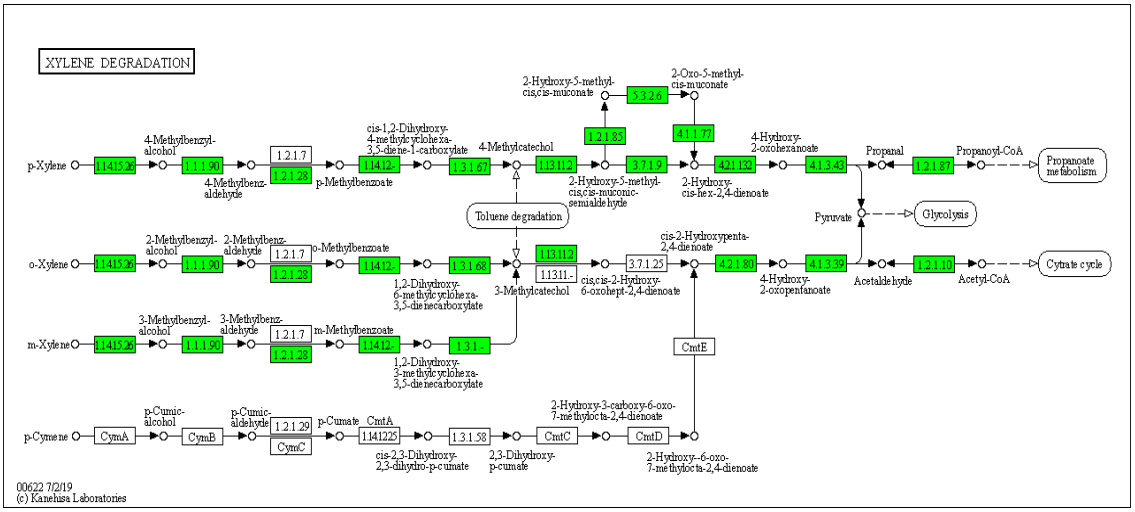


Figure 37. Automated annotation of xylene degradation pathway of strain MAP12^{T} by MaGe Microscope platform

In general, the oxidation of a methyl substituent to form methylbenzyl alcohol by xylene monooxygenase (XylM) often initiates the biodegradation of xylenes (Yu *et al*., 2001; Jung *et al*.,

2005). In this xylene monooxygenase pathway, the methylbenzyl alcohol is subsequently converted to methylbenzaldehyde, methylbenzoate and 1,2-dihydroxy-methylcyclohexane-3,5-dienecarboxylate by benzylalcohol dehydrogenase (XylB), benzaldehyde dehydrogenase (XylC) and benzoate 1,2-dioxygenase (XylX), respectively and later with the help of catechol 2,3-dioxygenase (XylE) as a ring cleavage enzyme, and lower *meta*-cleavage pathway enzymes converted to pyruvate through a series of enzymatic reactions. The *xylECMABN* genes were present as part of a partial (only upper TOL pathway genes containing) gene cluster flanked by mobile genetic elements (IS3 family transposases) in the genome of MAP12^T, and the subfamily I.2.C C23O gene (encoding the XylE enzyme) was part of this cluster. Its inability to utilize ethylbenzene as a source of carbon is explained by the absence of genes like ethylbenzene dioxygenase or naphthalene dioxygenase. Additionally, the reason behind the benzene-degrading potential of strain MAP12^T might be owing to the presence of a phenol-degradation gene cluster in its genome encoding for a multicomponent phenol-hydroxylase system together with a complete *meta*-cleavage pathway. Furthermore, the presence of cold-shock genes *cspA* and *cspC* in the genome of strain MAP12^T perhaps clarifies its ability to grow at low temperatures. These aforementioned genome features make this strain a promising bioremediation candidate for contaminated subsurface, especially for groundwater reserves where the temperature is low and oxygen availability is limited.

4.2.2.5 Microaerobic and aerobic BTEX degradation analysis of strain MAP12^T

To comprehend the capability of MAP12^T to grow and degrade BTEX compounds under both aerobic and microaerobic conditions, biodegradation experiments were performed using individual BTEX compounds as the sole source of carbon and energy to understand the difference in BTEX biodegradation patterns influenced by oxygen availability. It was found that the strain was capable of efficiently degrading benzene, toluene, *m*- and *p*-xylene both aerobically and microaerobically. Which is confirmed by the formation of CO_2 as an end product of complete degradation and transformation of the added 5mg/L concentration of BTEX compound to 0 mg/L concentration as a sign of total consumption of BTEX compound (**Fig. 38**), compared to abiotic controls**.** Besides, it was found that MAP12^T was unable to degrade *o*-xylene and ethylbenzene. Complete microaerobic degradation of toluene and *m*-xylene took place within 24hr and benzene within 24hr to 48hr, whereas the slowest degradation was noticed for *p*-xylene, which took 48hr. Results of the aerobic degradation experiment

unveiled that MAP12^T can degrade the same BTEX compounds within a similar timeline. These facts indicate that the biodegradation efficiency of MAP12^T doesn't get affected by low oxygen availability.

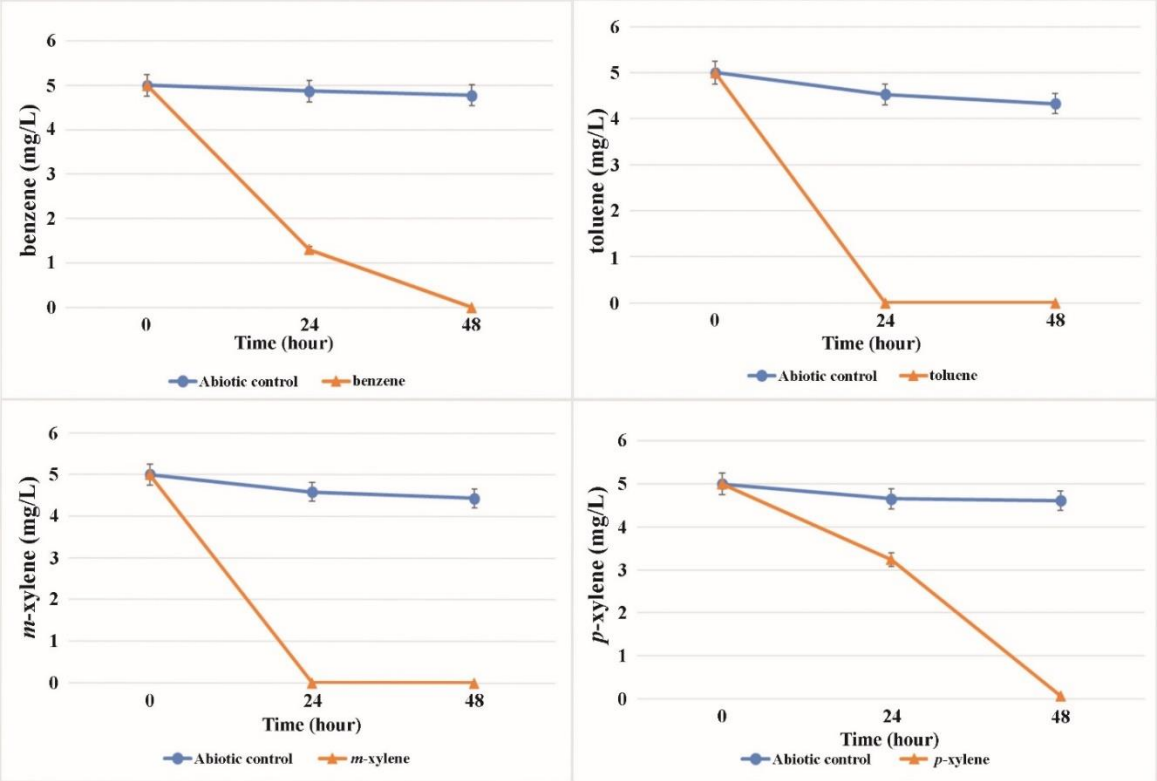


Figure 38. Microaerobic degradation of BTEX compounds (benzene, toluene, *m*-xylene, *p*-xylene) by strain MAP12^T. Concentrations were determined by GC–MS analysis as described in the main text. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

It could be concluded from the results of phylogenetic, genomic and chemotaxonomic studies that the strain MAP12^T represents a new species within the genus *Pseudomonas*, which was named *Pseudomonas aromaticivorans* sp. nov. that can degrade benzene, toluene, and *meta*, *para*-xylene microaerobically.

The results of this chapter have been published in the following publication:

Banerjee S, Bedics A, Tóth E, Kriszt B, Soares AR, Bóka K, Táncsics A. (2022) Isolation of *Pseudomonas aromaticivorans* sp. nov. from a hydrocarbon-contaminated groundwater capable of degrading benzene-, toluene-, *m*-and *p*-xylene under microaerobic condition. Frontiers in Microbiology ,13:929128, doi:10.3389/fmicb.2022.929128

5 CONCLUSION

Groundwater contamination by monoaromatic hydrocarbons is a global environmental concern. Removal of these contaminants hence always attracts the interest of the scientific community to improvise the bioremediation process. It is well documented that degradation of aromatic hydrocarbons occurs naturally by the indigenous bacterial community of the contaminated ecosystem. In the presence of adequate amount of dissolved oxygen, the degradation is quite faster and more effective, but the main concern is the degradation under microaerobic conditions, which results due to the rapid aerobic degradation of contaminants. In the complete lack of oxygen, a reduced zone is formed where iron-reducing and methanogenic organisms grow optimally. However, they don't prove to be very effective hydrocarbon degraders. Several research by our research group in the past, demonstrated the fact that environmental adaptation helped several bacterial genera to be transformed into an effective microaerobic degraders by acquiring the genes that, under microaerobic conditions, transcribed into enzymes that play an effective role in microaerobic hydrocarbon degradation. But till now, there is not much information available regarding xylene degrading bacterial communities and the effect of oxygen availability on the bacterial community and functional diversity. Moreover, it is important to find out which functional genes are majorly linked to the xylene degrading community. Whether only I.2.C type *C23O* gene plays a major role in the microaerobic xylene degradation. Additionally, it would be interesting to link such genes with the bacterial species.

A detailed study of the first enrichment clearly indicated that among the xylene isomers, *o*-xylene is the least degradable. Under strict aerobic conditions, it took a longer time to degrade *o*-xylene compared to *m*-and *p*-xylene by the enriched bacterial community. It was also found that different isomers of the same compound can significantly contribute to the diversity of the enriched bacterial communities. Though bacteria belonging to the class *Gammaproteobacteria* and order *Burkholderiales* were the most dominant community members in the majority of the enrichments, at genus level, *o*-xylene-degrading enrichment showed distinctly different community structure than that of *m*-, *p*-xylene degrading enrichments. Overall, it could be concluded that members of the genus *Pseudomonas* were the major *m*-and *p*-xylene degraders, but for *o*-xylene degrading enrichments,

members of genus *Rhodococcus* dominated the community and presumably played an effective role in aerobic degradation of *o*-xylene. Another important observation was the presence of the genus *Hydrogenophaga* as a noticeable group in all the enrichments. In-depth genome analysis of two *Hydrogenophaga* strains showed that despite the fact that they shared similar 16s rRNA gene sequence identity and were isolated from the same environment (*p*-xylene degrading enrichment) but interestingly encoded an entirely different set of subfamily I.2.C-type *C23O* genotypes and were able to degrade different BTEX compounds. Besides, the strain D2P1^T was found to be having three different subfamiliy I.2.C-type *C23O* genes in its genome. These two strains had a highly similar genome size (5.63 and 5.80 Mb, respectively), but the strain D2P3 lacked the xylene-degradation gene cluster, unlike strain D2P1^T. This observation hinting toward the environmental adaptation in a contaminated environment in the form of horizontal gene transfer. Moreover, it could also be assumed that members of the genus *Hydrogenophaga* that contain the I.2.C *C23O* gene could be prominent xylene-degraders, and some of them have the ability to degrade *o*-xylene effectively under aerobic conditions.

In our second enrichment, which was established to identify the oxygen-mediated key changes in aerobic and microaerobic xylene degrading microbial community, as hypothesized, remarkable differences were found. In general, it was noticed that xylene degradation was slower in microaerobic xylene degrading enrichments compared to aerobic enrichments. Proving that xylene degradation in the presence of adequate oxygen is much faster. The evolved bacterial communities in aerobic and microaerobic enrichments were also profoundly different. The dominance of *Gammaproteobacteria* and *Alphaproteobacteria* was observed in aerobic enrichments, whereas the owerwhelming dominance of *Gammaproteobacteria* was found in microaerobic enrichments indicating a clear community shift. Although in both enrichments, members of the genus *Pseudomonas* dominated the community, yet a difference in the second most dominant group was noticed. In aerobic enrichments, *Sphingobium* was found to be the most abundant xylene degrader after *Pseudomonas,* but due to oxygen limitation, *Sphingobium* was completely replaced by genus *Rhodoferax or Azovibrio* in the case of microaerobic enrichments.

Members of a novel bacterial species of *Pseudomonas* was isolated from microaerobic enrichment in several copies, described by us as *Pseudomonas aromaticivorans* which was only present in microaerobic enrichments. The metagenomic analysis supported the fact that this lineage of *Pseudomonas* was abundant in the microaerobic xylene-degrading enrichment. A whole-genome analysis of one of the members of this species, MAP12^T , revealed that this strain contains five

different *C23O* genotypes including a I.2.C-type *C23O* gene. Hence, the supremacy of the bacterial community harbouring I.2.C-type *C23O* genes in the microaerobic enrichments was clearly observable. This justifies the fact that the presence of I.2.C-type *C23O* genes is not restricted to the "fluorescens" lineage of the genus *Pseudomonas*. Genome analysis of a hitherto unknown and uncultivated bacterial species belonging to the genus *Rhodoferax*, was obtained by metagenome sequencing and genome binning and showed that *Rhodoferax* is might be one of the most effective xylene degraders under microaerobic conditions besides *Pseudomonas*. Furthermore, this *Rhodoferax* species doesn't encode subfamily I.2.C-type EDOs. Instead of that, it encodes a newly defined EDO subfamily, designated as I.2.I. Most interestingly, MAP12^T also contains the gene that encodes this newly defined EDO subfamily. Henceforward, these provoke us to presume that I.2.I. type EDO, might play a key role in the microaerobic degradation of xylene. However, in-depth transcriptomics study will be needed to establish the presumption.

Throughout the investigation, we have isolated several strains that can effectively degrade BTEX compounds. Only a handful of them was found to be degrading at least one xylene isomer. Three of them were investigated with special attention as they showed a yet undescribed lineage of their respective genus. The first one was strain D2P1^T, described as *Hydrogenophaga aromaticivorans*, it can degrade *m*- and *p*-xylene aerobically. Second is strain MAP12^T, described as *Pseudomonas aromaticivorans* able to degrade *m*-, *p*- xylene both aerobically and microaerobically. And the third is AS12, which presumably belongs to a novel species of *Sphingobium*, can degrade all three xylene isomers. The strain MAP12^T could be a prospective candidate for use in bioremediation of subsurface contamination because its degradation rate does not get affected by oxygen limitations. The information revealed during our research might provide useful insights in the future regarding the bioremediation of hydrocarbon-contaminated environments.

6 NEW SCIENTIFIC RESULTS

- Study of *m*-, *p*- and *o*-xylene degrading aerobic enrichment cultures revealed that though members of the genera *Pseudomonas* and *Acidovorax* are abundant in all kinds of xylene isomer degrading enrichments but genera *Rhodococcus* and *Chryseobacterium* are the major players in the aerobic *o*-xylene degrading community. Moreover, members of the genus *Hydrogenophaga,* containing I.2.C *C23O* gene, can be prominent xylene-degraders.

- From the aerobic *p*-xylene degrading enrichment, a new bacterial strain designated as D2P1^T was isolated, belonging to yet undescribed species of the genus *Hydrogenophaga*. This strain showed the ability to degrade benzene, *m*- and *p*- xylene aerobically. According to international requirements, it was investigated to assign a taxonomic position, and the species was named *Hydrogenophaga aromaticivorans*.

- Whole-genome analysis of two *H.aromaticivorans* strains (D2P1^T and D2P3) isolated from the *p*-xylene degrading enrichments showed that different subpopulations of the same species might coexist in the same environment with different xylene degrading potential. Moreover, it was also found that three or more subfamily I.2.C-type *C23O* genotypes can be linked to one single hydrocarbon degrading strain. Accordingly, the high diversity of subfamily I.2.C-type *C23O* genes does not always guarantee a high diversity of degraders in a contaminated environment.

- A bacterial strain, designated as MAP12^T, has been isolated from microaerobic xylene degrading enrichment and identified as a novel bacterial species of the genus *Pseudomonas*. It was analysed using polyphasic taxonomic approach and named *Pseudomonas*

aromaticivorans. This newly described strain has the ability to degrade benzene-, toluene-, *m*- and *p*-xylene under both aerobically and microaerobically; in addition, it harbours five catechol 2,3-dioxygenase (*C23O*) genes including a newly defined subfamily of extradiol dioxygenases, designated as I.2.I.

- A new bacterial strain belonging to the genus *Sphingobium* was isolated from aerobic xylene degrading enrichment. Phylogenomic analysis of the strain disclosed that the strain presumably represents a yet undescribed lineage of the genus *Sphingobium.* The strain was able to degrade all three xylene isomers (*m*-, *p*- and *o*-xylene). It's the first report of any *Sphingobium* strain that can degrade all three xylene isomers along with toluene and ethylbenzene as a pure culture.

- With the help of metagenome sequencing and metagenome-associated genome analysis, a hitherto unknown and uncultivated bacterial species belonging to the genus *Rhodoferax* was identified, which was only predominant in microaerobic xylene degrading enrichments. The genome of this bacterium coded two catechol 2,3-dioxygenase enzymes which belonged to a newly defined subfamily of extradiol dioxygenases, designated as I.2.I. that might play a key role in the microaerobic degradation of xylene.

7 SUMMARY

Xylene is considered as one of the most common organic volatile toxic environmental contaminant found in the world, as well as in Hungary. Because of their relatively high-water solubility and least degradability, these compounds are often considered as a threat to the environment. It was observed that indigenous bacterial populations could naturally degrade such contaminants rapidly under strict aerobic conditions. But this rapid aerobic degradation leads to the formation of hypoxic conditions in the contaminated subsurface environment. Certain groups of bacteria that could adapt to these hypoxic environments via their special enzyme system play a role in the degradation of monoaromatic hydrocarbons. The main purpose of our study was to reveal how oxygen availability affects the development of xylene degrading bacterial communities and the functional genes involved in xylene degradation. Moreover, to find potential strains that belong to the most abundant communities and harbour subfamily I.2.C *C23O* genes, which are believed to have a key role in the hypoxic degradation of aromatic hydrocarbons. To achieve our research goal, two enrichments were established and investigated accordingly.

For the first experiment, aerobic enrichments were set up with xylene-contaminated groundwater supplemented with *m*-, *p*-, and *o*-xylene as the sole carbon and energy source, respectively. Results revealed that distinctly different bacterial communities played role in the degradation of the different xylene isomers. *Pseudomonas* and *Acidovorax* were found to be the among abundant members present in all kinds of xylene degrading enrichments, but genera *Rhodococcus* and *Chryseobacterium* were the noticeable players in the aerobic *o*-xylene degrading community. Members of the genus *Hydrogenophaga* were also found to be present in abundance in *p*-and *o*-xylene degrading enrichments. Two representative strains of genus *Hydrogenophaga* were isolated from *p*-xylene-degrading enrichments and turned out that they were capable to degrade different isomers of xylene and harboured I.2.C *C23O* genes. The whole-genome analysis of these two *Hydrogenophaga* strains led us to the opinion that strains belonging to the same species with different xylene degrading potentials may coexist in the same environment. Moreover, it was also found that three or more subfamily I.2.C-type *C23O* genotypes can be linked to one single strain of hydrocarbon degrading bacterium. This observation allowed us to conclude that a high diversity of subfamily I.2.C-type *C23O* genes does not always mean a high diversity of degraders in a contaminated environment.

To understand the oxygen-mediated changes in the xylene degrading bacterial community composition, strictly aerobic and microaerobic enrichment microcosms were set up using a xylene mixture as a sole carbon source. The results showed that there was a significant difference in the microbial community structure. Though members of the genus *Pseudomonas* were the most dominant bacterial community present in both type of the enrichments but the rest of the community composition was noticeably different. In the case of aerobic enrichment, the genus *Sphingobium* was the second most abundant bacterial population involved in aerobic xylene degradation, whereas in microaerobic enrichment, members of the genera *Rhodoferax* or *Azovibrio* were the second most dominant community members and possibly participated in the microaerobic degradation of xylene. To support this statement, with the help of metagenomics and genome binning analysis, a yet unknown and uncultivated species of genus *Rhodoferax* was identified from the microaerobic xylene degrading enrichment harbouring a unique type of *C230* gene (subfamily I.2.I.) which might play a crucial role in the microaerobic breakdown of xylene.

As a part of our research goal, we have successfully isolated several bacterial strains that could be useful for bioremediation purposes in future. From the first experiment, two strains of xylene degrading strains represented a novel species of the genus *Hydrogenophaga* and were described as *Hydrogenophaga aromaticivorans* sp. nov. In the second enrichment, we have isolated a microaerobic xylene degrading strain belonging to a yet undescribed species of the genus *Pseudomonas*. This strain was taxonomically positioned and named as *Pseudomonas aromaticivorans* sp.nov. This strain harbours five type of C23O genes including I.2.I.type C23O gene . From the aerobic enrichment, a strain of *Sphingobium* was isolated, which showed ability to degrade all three xylene isomers effectively. Phylogenomic analysis revealed that, presumably, this strain represents novel species of the genus *Sphingobium*.

8 ACKNOWLEDGMENTS

First and foremost, I would like to express my heartfelt gratitude and thanks to my supervisor, Dr András Táncsics, for accepting me as his PhD student and allowing me to work under his supervision for my doctoral research. Thank you so much for all his support, guidance, motivation, personal and professional help and advice over the years.

I would like to thank my present and past colleagues from the Institute of Aquaculture and Environmental Sciences, especially the Department of Molecular Ecology, Department of Environmental Toxicology and Department of Environmental Safety, for their help and kindness, they showed during my PhD studies.

I am grateful to Professor Erika Micheli and Dr Balázs Kriszt, for providing me with the opportunity to work at the Doctoral School of Environmental Science. I am thankful to Tempus Public Foundation and the Hungarian Government for funding my studies.

Last but not the least, it was the immense support, sacrifices, patience, help, and motivation of my wife, without which, it would not have been possible for me to pursue and complete my PhD studies. I would like to thank my family: my parents, brother and my friend Dr. Tanmoy Chakraborty for their continuous encouragement and motivational words to overcome any odds along the way. Thank you so much for always being there for me.

9 APPENDICES

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Online resources used for figures:

http1:

https://en.wikipedia.org/wiki/File:Step-by-step_procedure_of_using_T-RFLP_analysis_in_microbiology.pdf

http2:

astrobiomike.github.io.

9.2 Data availability

The GenBank/EMBL/DDBJ accession number for *Hydrogenophaga* sp. D2P1^T 16S rRNA gene sequence is MT023432.Whole genome sequence data are available under the BioPoject accession number PRJNA565673.The accession number for the whole genome sequence of strain D2P1^T is VYGV00000000 and. D2P3 is JAGPWB000000000.16S rRNA gene amplicon sequencing data are available under the BioProject accession number PRJNA704261. The 16S rRNA and *C23O* gene sequences of the isolates were deposited to GenBank under the accession numbers MW647763-MW647782, MZ127192 and MW691988-MW691994 subsequently.

The Whole Genome Shotgun project of strain AS12 has been deposited at DDBJ/ENA/GenBank With the accession JAHRGM000000000. and BioProject accession number PRJNA224116 .The 16S rRNA sequences of the strain were submitted under the accession numbers OL444946.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains MAP12^T is OK324373. The whole-genome shotgun project of strain MAP12^T has been deposited at DDBJ/ENA/GenBank under the accession number JAHRGL010000000. Amplicon sequence data were deposited at NCBI SRA under the BioProject number PRJNA745543, while bin genomes are available under number PRJNA818156. The metagenome assembled genome accessible at NCBI under the BioSample accession number SAMN26818775.

The type strain D2P1^T (=LMG 31780 , =NCAIM B 02655), AS12 =NCAIM B.02669, MAP12^T (=LMG 32466, =NCAIM B.02668) are publicly available.

9.3 Supplementary Data

Fig. S1. Polar lipid profile of strain D2P1^{T} .

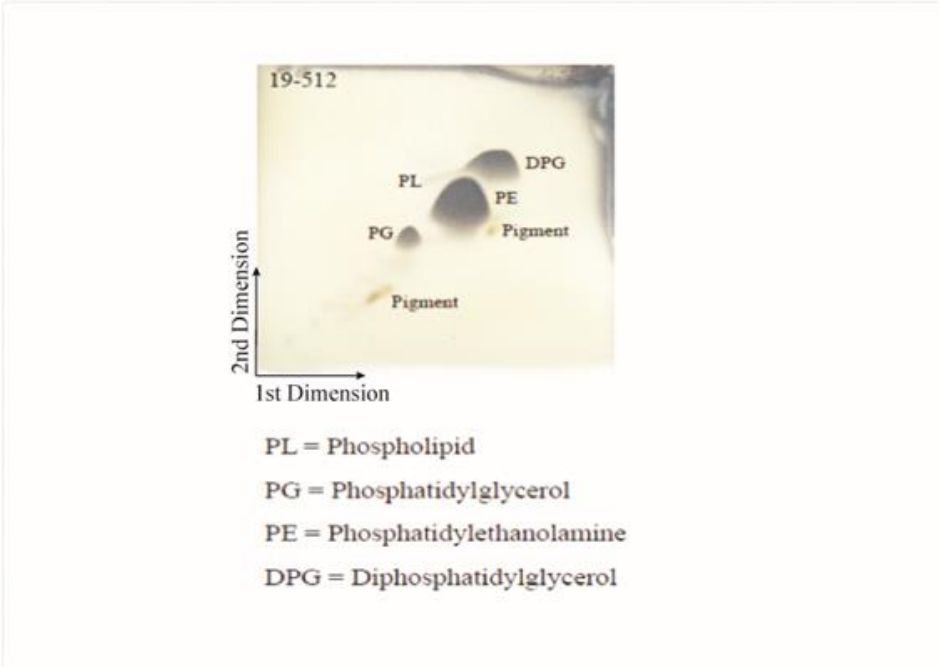

Fig. S2. Physical map of the gene cluster encoding the upper pathway genes of xylene degradation in the genome of strain D2P1^{T}. The corresponding ORFs are highlighted with red colour. ORF 7: xylene monooxygenase; ORF 8: xylene monooxygenase electron transport component; ORF 9: oxidoreductase (possibly aryl-alcohol dehydrogenase); ORF 10: benzaldehyde dehydrogenase; ORF 25: benzoate 1,2-dioxygenase large subunit; ORF 26: benzoate 1,2-dioxygenase small subunit; OF 27: 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase

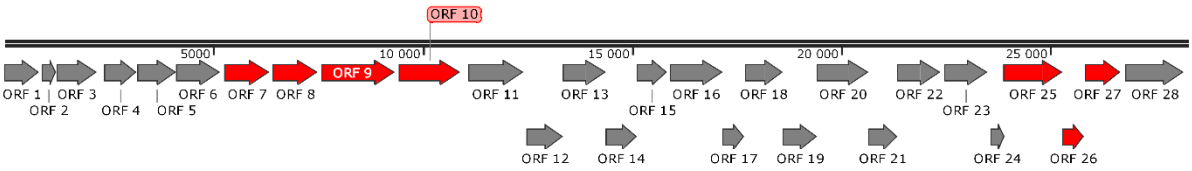

Fig. S3. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain AS12 and related taxa. Bootstrap values are shown as percentages of 1000 replicates. *Flavobacterium hydrocarbonoxydans* GA093^{T} was used as the root for the tree. Bar 0.050 substitutions per nucleotide position.

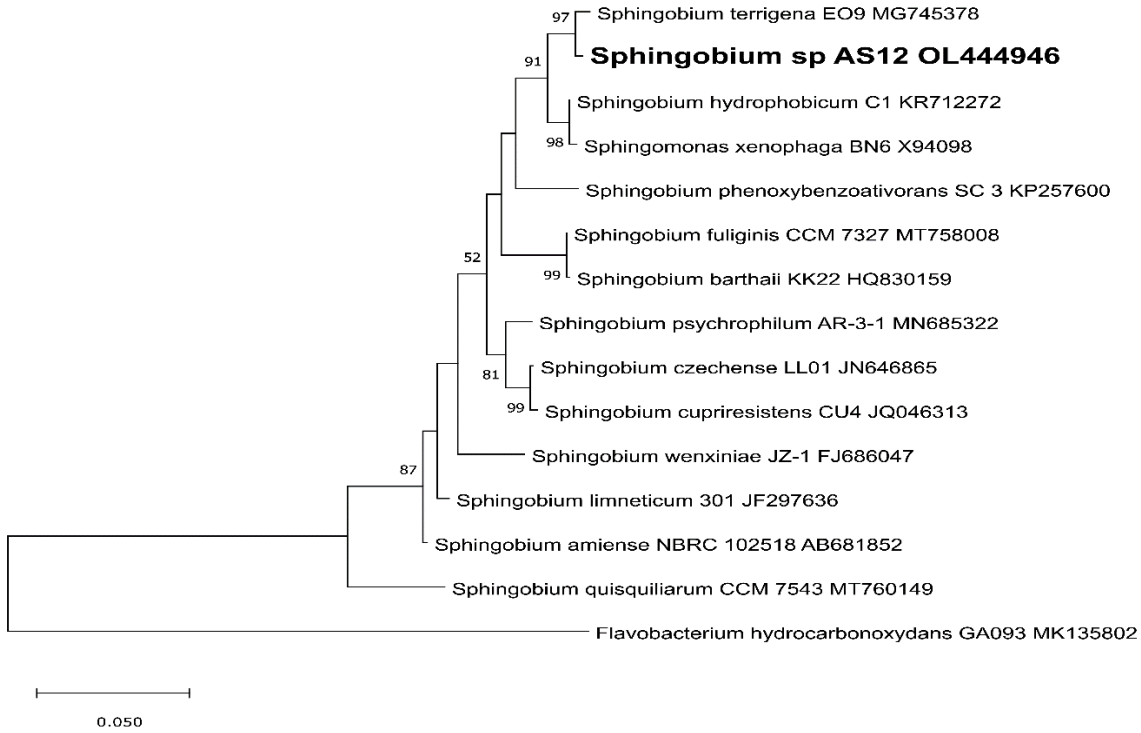

Fig. S4. Phylogenomic tree showing the taxonomic position of Sphingobium sp. strain AS12, predicted on TYGS database.