

Review

# Salicylate or Phthalate: The Main Intermediates in the Bacterial Degradation of Naphthalene

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**Abstract:** Polycyclic aromatic hydrocarbons (PAHs) are widely presented in the environment and pose a serious environmental threat due to their toxicity. Among PAHs, naphthalene is the simplest compound. Nevertheless, due to its high toxicity and presence in the waste of chemical and oil processing industries, naphthalene is one of the most critical pollutants. Similar to other PAHs, naphthalene is released into the environment via the incomplete combustion of organic compounds, pyrolysis, oil spills, oil processing, household waste disposal, and use of fumigants and deodorants. One of the main ways to detoxify such compounds in the natural environment is through their microbial degradation. For the first time, the pathway of naphthalene degradation was investigated in pseudomonades. The salicylate was found to be a key intermediate. For some time, this pathway was considered the main, if not the only one, in the bacterial destruction of naphthalene. However, later, data emerged which indicated that gram-positive bacteria in the overwhelming majority of cases are not capable of the formation/destruction of salicylate. The obtained data made it possible to reveal that protocatechoate, phthalate, and cinnamic acids are predominant intermediates in the destruction of naphthalene by rhodococci. Pathways of naphthalene degradation, the key enzymes, and genetic regulation are the main subjects of the present review, representing an attempt to summarize the current knowledge about the mechanism of the microbial degradation of PAHs. Modern molecular methods are also discussed in the context of the development of “omics” approaches, namely genomic, metabolomic, and proteomic, used as tools for studying the mechanisms of microbial biodegradation. Lastly, a comprehensive understanding of the mechanisms of the formation of specific ecosystems is also provided.



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## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic substances containing two or more benzene rings. They are an important constituent of environmental pollutants. PAHs are widely distributed contaminants in soils, waters, and air because of their common association with many anthropogenic activities such as oil refining, incomplete combustion of fossil fuels, household waste processing, and others [1,2]. The range of polluting concentrations of polyaromatic compounds in contaminated sites is quite wide and ranges from 1 µg/kg to over 300 g/kg, as has been reported [3]. Possessing a carcinogenic and mutagenic effect, PAHs are poorly soluble in water and are well deposited on the mineral matrix of soil. PAHs can be significantly accumulated in the environment due to their long-term resistance to oxidation and limited biodegradability. In total, more than one hundred polycyclic aromatic compounds are known, most of which are stable in the ecosystem for a long time. Thus, the half-life of a three-ring phenanthrene molecule in soil can be from 16 to 126 days, while the half-life of a five-ring benz(a)pyrene is 229–1400 days [4]. The most dangerous toxicity of PAHs concerns their carcinogenicity. In short, PAHs enter

cells due to their hydrophobicity and induce the expression of genes encoding enzymes of the cytochrome P450 group (CYP). CYPs, by virtue of their non-specific action, convert PAHs into toxic metabolites. Some of the resulting metabolites can bind to DNA and have a mutagenic/carcinogenic effect. In addition to carcinogenicity, PAHs are characterized by developmental toxicity, genotoxicity, immunotoxicity, oxidative stress, and endocrine disruption [5,6].

PAHs are resistant to nucleophilic attack due to the presence of the  $\pi$ -electron density in the aromatic ring [7]. The transformation of PAHs in the environment leads to the formation of oxygen (O-PAH), nitrogen (N-PAH) and azarenes (AZA), or sulfur (PASH) derivatives. These derivatives enhance the toxicity of the parent PAHs. The classification of such derivatives, their characteristics, and the methods of analysis are given in a wonderful review [8]. A huge number of people around the world, especially in developed countries, suffer from chronic respiratory diseases, including asthma, chronic obstructive pulmonary disease, lung cancer, and so on. The review shows the potential contribution of surfactants to the etiology of this class of diseases. This paper reviews epidemiological studies and assesses the association between the gas phase, the particle-associated PAHs in the ambient air, and non-malignant respiratory diseases or closely related physiological processes [9].

The significance of the problem is indicated by the data of bibliometric analysis, which demonstrates that in recent years, “PM2.5 (suspended solid microparticles and tiny liquid droplets (10 nm–2.5 microns in diameter), health risk, impact, source identification” have become hot spots. The keywords with the strongest citation bursts include toxicity, spatial distribution, health risk, and pm 2.5. Among them, toxicity and spatial distribution have increased sharply since 2015; health risk and PM2.5 contained in the air have increased sharply since 2019; and there is no sharp decrease [10].

Due to their ubiquity in the natural environment and various harmful effects on organisms, PAHs are among the most concerning organic pollutants [11]. Naphthalene is the simplest polycyclic aromatic hydrocarbon and its structure consists of a fused pair of benzene rings. The primary use for naphthalene is in the production of phthalic anhydride. Other uses of naphthalene include carbamate insecticides, surface active agents, and resins; utilization as a dye intermediate, synthetic tanning agent, and moth repellent; and utilization in miscellaneous organic chemicals. In addition, naphthalene and phenanthrene are components of petroleum. Similar to other PAHs, naphthalene is a fairly toxic substance. Inhalation, ingestion, and skin contact cause hemolytic anemia, liver damage, and neurological damage. Contact with naphthalene can cause cataracts and damage to the retina. The Environmental Protection Agency (EPA) has classified naphthalene as a Group C probable human carcinogen [12].

Direct releases of crude oil, including emissions to the ocean, are one of the most important pathways for naphthalene to enter the environment. In 2010, the largest oil spill in U.S. history occurred as a result of the Macondo wellhead blowout following the sinking of British Petroleum’s Deepwater Horizon drilling platform in the Gulf of Mexico. The incident released about 4.9 million barrels of light crude oil, which contained a high proportion of low molecular weight hydrocarbons and consisted of about 3.9% PAHs [13,14]. In numerical terms, with an average conversion factor of 1 barrel = 0.1364 tons of oil, it was equal to 26.066 thousand tons of PAHs. Although PAHs account for a small constituent percentage of crude oil, they are considered the most toxic component mainly because of their metabolites [15]. In oil collected directly from the Macondo well during the incident, of all the PAHs, the proportion of two low-molecular weight PAHs, namely naphthalene and phenanthrene, and their homologues, was approximately 74% and 22%, respectively [16]. The toxicity of PAHs, in general and especially because of the waters impacted by Deepwater Horizon, is well documented in fish and has resulted in numerous adverse acute and chronic effects, including skin lesions, cardiotoxicity, liver abnormalities, respiratory changes, decreased fertility, histopathological changes, and mortality [17–22]. The two most common PAHs identified in Deepwater Horizon crude oil, namely naphthalene and phenanthrene, and their associated homologues, have been shown to be very toxic

to fish. Although fish have a relatively high capacity to metabolize PAHs, hydroxylated PAH derivatives produced during the initial metabolic response can adversely affect fish health [15]. The acute toxicity values of naphthalene and phenanthrene for fish vary from 0.51 to 7.9 mg/L and from 0.23 to 1.15 mg/L, respectively [23–28]. The data obtained have repeatedly confirmed the previous statement that PAHs in oil, including phenanthrene and naphthalene, are one of the most toxic components [23,29].

The consequences of this accident were truly dramatic. It was revealed that the impacts of the spill are ongoing and significant. As an illustration, one can cite the example of a study on the dolphin population in the accident area. Based on studies conducted from 2010 to 2015, bottlenose dolphins in the Barataria Bay suffered increased mortality (35% greater than expected based on studies of other bottlenose dolphin populations) and increased likelihood of having adverse health effects (37% greater than expected). Scientists estimated that the Barataria Bay stock of dolphins would take 39 years to recover in the absence of active restoration [[www.fisheries.noaa.gov/national/marine-life-distress/sea-turtles-dolphins-and-whales-10-years-after-deepwater-horizon-oil](http://www.fisheries.noaa.gov/national/marine-life-distress/sea-turtles-dolphins-and-whales-10-years-after-deepwater-horizon-oil)]. Accessed on 3 October 2021].

The restoration of the microbial population also requires a certain amount of time. Microbiome studies at the site of the oil spill accident showed that 3 years after the release of 2000 tons of oil because of the “Qingdao pipeline explosion” in 2013, the bacterial diversity in heavily polluted and slightly polluted sediments was completely different [30]. The main oil degraders were bacteria of the genera *Alcanivorax* and *Lutibacter*. Biotoxicity analysis by luminescence showed large differences between the contaminated sites, the control sites in Jiaozhou Bay, and the non-contaminated area outside the Jiaozhou Bay. Biotoxicity also peaked in the vicinity of the oil spill. These results indicate that the oil spill which occurred 3 years ago still has a negative impact on the environment and bacterial communities in the sediment. The long-term impact of oil spills on macro and microbiocenoses, including the BP Deepwater Horizon (DWH) accident, is being studied quite intensively and can serve as a topic for a separate review [18].

Researchers are practically unanimous in stating that the use of microorganism-destroyers for the bioremediation of soils and water bodies, including the World Ocean, is the most effective, practical, and economically beneficial approach. Since crude oil and its refined products are complex and contain different compounds, biochemical studies are aimed at studying the pathways of the microbial metabolism of its individual aliphatic and aromatic components [31–35]. Studies are also concerned with the investigation of individual genetic determinants of natural bacterial degraders. Since many genetic determinants of the biodegradation of aromatic compounds are localized within extra-chromosomal genetic elements, the plasmid composition of bacterial degraders and the effect of bacteria containing biodegradation plasmids on the utilization of oil and its products are continuously studied [36–43].

Generally, microorganisms that decompose PAHs, including naphthalene, have been studied for quite a long time and a large amount of data on this topic has been accumulated. About 80,000 articles have been published on the microbial decomposition of naphthalene. In the last decade, the genetic regulation of the pathways involved in the degradation of naphthalene by various gram-negative and gram-positive bacteria has been sufficiently studied. By example of this compound and based on both genomic and proteomic data, a deeper understanding of the functioning and evolution of the degradation pathways of high-molecular-weight PAHs in bacteria has been gained, for example in [44].

## 2. Some Representatives of Naphthalene Destructors

Microorganisms that can degrade naphthalene are ubiquitous in soils, in fresh and saline waters, etc. The phylogenetic diversity of the bacteria that degrade PAHs, including naphthalene, is very broad. Among them are bacteria belonging to the genus *Arthrobacter* [45], *Pseudomonas* [46,47], *Rhodococcus* [48], and *Sphingomonas* [49].

Interest in marine microbial-degrading organisms is increasing because, as mentioned above, in recent decades, petroleum products and consequently naphthalene have been

spread intensively in the marine ecosystem. The high hydrophobicity of petroleum products impedes their bioavailability. Thus, it has been shown that marine bacteria play a significant role in the decomposition of PAHs in seawater and marine bottom sediments. Two cultures of *Neptunomonas naphthovorans* strains, namely NAG-2N-126 and NAG-2N-113, capable of using naphthalene as the only carbon and energy source were isolated. Each strain also could degrade 2-methylnaphthalene and 1-methylnaphthalene, and one strain, NAG-2N-113, degraded 2,6-dimethylnaphthalene and phenanthrene during incubation of these strains with PAH in artificial seawater. Acenaphthene was not degraded when used as the sole carbon source but was degraded by both strains when incubated with a mixture of other PAHs [50].

Degenerate primers and PCR were used to isolate a portion of the naphthalene dioxygenase iron-sulfur protein (ISP) gene from each of the strains. Phylogenetic analysis of the amino acid sequences deduced by the PAH dioxygenase ISP showed that the genes isolated in this study were distantly related to the genes encoding naphthalene dioxygenases from *Pseudomonas* and *Burkholderia* strains. Despite the differences in the PAH degradation phenotype between the new strains, the amino acid fragments of these organisms excreted by the ISP dioxygenase were 97.6% identical. Phylogenetic analysis based on 16S ribosomal DNA placed these bacteria in the gamma-3 Proteobacteria subgroup, which is closest to the representatives of the genus *Oceanospirillum*. However, morphological, physiological, and genotypic differences between the new strains and oceanospirilla justify the creation of a new genus and species of *Neptunomonas naphthovorans*. The type strain of *N. naphthovorans* is NAG-2N-126 [50].

A strain identified as *Rhodococcus opacus* M213 with a unique set of genes and a naphthalene metabolic pathway different from those previously described was isolated from soil contaminated with fuel oil [51].

The research on the isolation of a thermophilic bacterium from a compost consisting of wooden ties treated with lignite tar is interesting. *Bacillus thermoleovorans* was able to utilize naphthalene as a sole source of carbon and energy [52]. The authors of this study found the simultaneous presence of 2,3-dihydroxynaphthalene, 2-carboxycinnamic acid, and phthalic acid, along with cinnamic acid and salicylic acid, among the metabolic products. *B. thermoleovorans*, as it was concluded from this observation, produces enzymes of various pathways for the degradation of naphthalene. The formation of 2,3-dihydroxynaphthalene indicates the initial oxidation of naphthalene by 2,3-dioxygenase, whereas the presence of 2-hydroxybenzene derivatives indicates the simultaneous activity of 1,2-dioxygenase (Figure 1).

Two naphthalene-degrading strains were isolated from soil samples, from fertilizer industries, and from different motor-markets in the Chandigarh region. They are *Staphylococcus aureus* and *Pseudomonas fluorescens* strains with a naphthalene degradation efficiency of 63.7% and 50.17%, respectively, after seven days incubation with 150 ppm of naphthalene [53].

The wide variety of bacteria capable of utilizing naphthalene is also illustrated by the study of [54], in which the authors describe the isolation and characterization of seven naphthalene-degrading isolates from oil-contaminated bottom sediments. The isolates were characterized as different strains of the genus *Bacillus*. An interesting point in this study was the isolation of a probable new species of *Paenibacillus* (isolate 5), which could degrade naphthalene to a higher extent (up to 1%) and hence could play an important role in bioremediation. The strain has been patented as an active naphthalene degrader [55].

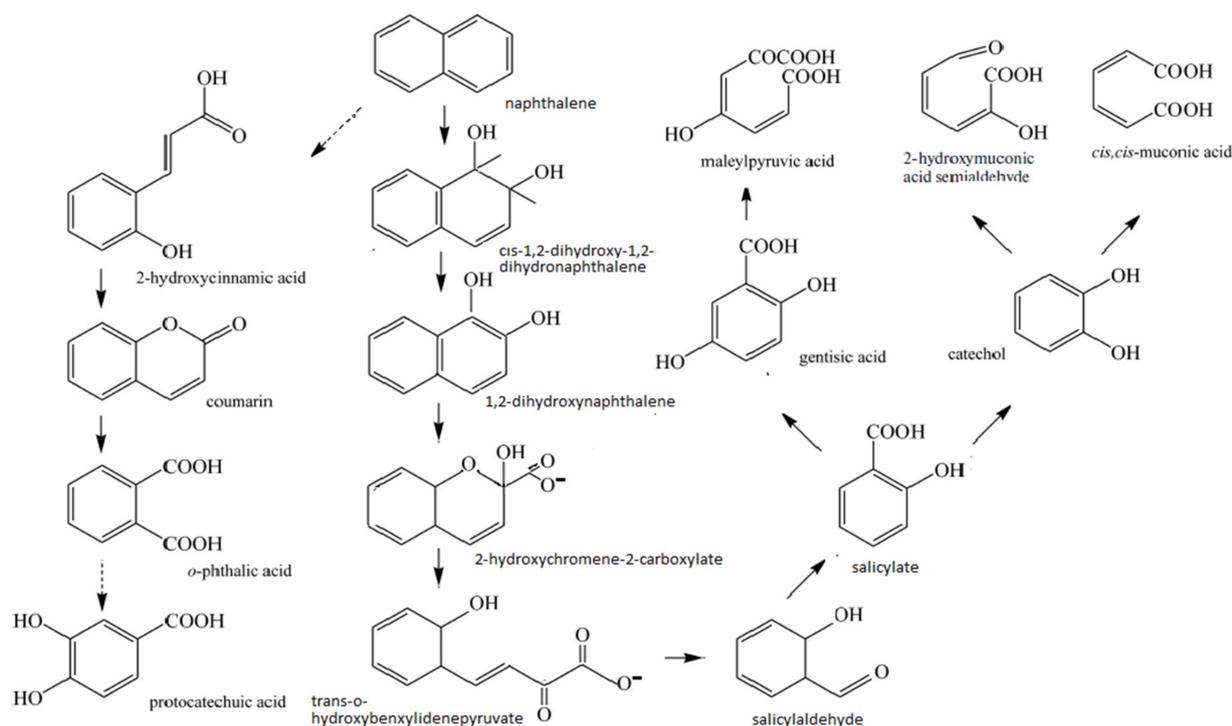


Figure 1. Possible pathways of the bacterial degradation of naphthalene.

### 3. Degradation of Naphthalene by Gram-Negative Bacteria

Naphthalene degradation pathways and their enzymes have been primarily studied in gram-negative bacteria, including *Pseudomonas* species. As a rule, their degradation genes are organized into three operons. The first one encodes enzymes involved in the conversion of naphthalene to salicylate (upper pathway of naphthalene degradation); the second operon encodes enzymes for the conversion of salicylate to intermediate products of the tricarboxylic acid cycle (pyruvate and acetyl-CoA) via the *meta*-cleavage pathway (lower pathway of naphthalene degradation); and the third operon encodes a positive transcription regulator (NahR) [56–63].

The metabolic reactions leading to the degradation of naphthalene were first established in 1964 by Davis and Evans [64]. It has been shown that the bacterial degradation of PAHs, including naphthalene, begins with the monooxygenase or dioxygenase attack of the aromatic ring. The formed dihydroxylated PAHs then undergo cleavage by breaking the aromatic ring to form carboxylated compounds which, if further oxidation enzymes are present in the strain, can be channeled to Krebs cycle intermediates [65–67] (Figure 1).

This typical series of reactions begins with the hydroxylation of the benzoic ring with the help of multicomponent enzymes, which are the non-heme iron-bearing Rieske-type cluster-containing oxygenases (Rieske oxygenases (ORs)). ORs catalyze the oxidative decarboxylation reaction, which is unique to enzymes of this family, resulting in the formation of the corresponding phenolic derivatives. Naphthalene dioxygenase, being a (Rieske-type two-iron two-sulphur center-containing) naphthalene dioxygenase (NOD; encoded by *nahAaAbAcAd*), introduces two oxygen atoms into the aromatic ring of a wide range of aromatic hydrocarbons, such as naphthalene, phenanthrene, and anthracene, converting them to the corresponding dihydrodiols, such as the *cis*-naphthalene dihydrodiol. *cis*-Dihydrodiol dehydrogenase (encoded by *nahB*) then dehydrogenates the dihydrodiol to form 1,2-dihydroxynaphthalene, which undergoes *meta*-cleavage by 1,2-dihydroxynaphthalenedioxygenase (*nahC*) to form 2-hydroxychromene-2-carboxylic acid. Enzymatic *cis-trans*-isomerization (isomerase encoded by *nahD*) produces the product *trans*-*o*-hydroxybenzylidenepyruvate; the side chain at the *trans*-unsaturated bond of the

product is cleaved by hydratase-aldolase (encoded by *nahE*) to form salicylic aldehyde. Salicylate is further oxidized in two ways: through catechol (with the participation of salicylate-1-monooxygenase) or through gentisic acid (with the participation of salicylate-5-hydroxylase).

Bacteria of the genus *Pseudomonas* have been the subject of great scientific interest. This group is presented in large numbers in various natural and contaminated environments, and the interest in these bacteria is due to both their high degree of physiological and genetic adaptability as well as their ability to effectively aerobically decompose a wide range of aromatic compounds. This very group of microorganisms served as the initial model for the study of naphthalene degradation pathways [64,67–72].

The genes responsible for naphthalene degradation in *Pseudomonas* are most often localized on plasmids [73,74]. The mechanism of naphthalene degradation through salicylic acid can be illustrated by the example of the NAH7 naphthalene plasmid isolated from the *Pseudomonas putida* G7 strain. The genes for the degradation of naphthalene and salicylate in this plasmid are organized into two operons [75,76]. *nahABCDEF* is a set of genes (operon) encoding enzymes' decomposition of naphthalene to salicylate; *nahGHINLJK* is an operon encoding enzymes that degrade salicylate to elements of the Krebs cycle; and *nahR* is a gene-coding protein regulator of these two operons. The activity of two degradation operons of naphthalene (*nahABCDEF*) and salicylate (*nahGHINLJK*) is regulated by a protein whose gene is located next to the second operon. This regulatory protein has two equilibrium forms: inactive (NahRi) and active (NahRa). In the absence of salicylate in the medium, this regulatory protein is inactive (NahRi) and has no affinity for DNA. RNA polymerase does not attach to the promoter region considering that without an active regulator protein, it cannot recognize this region as a locus of attachment. An interesting property of the NAH7 plasmid is the presence of the *nahY* gene, which encodes a naphthalene chemoreceptor. This gene is located next to the naphthalene degradation operons [77].

An analysis of the amino acid sequence of dioxygenases from *Beijerinckia* sp. strain B1, capable of growing on biphenyl or *m*-xylene as the only carbon source, showed that 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) belongs to the class of *meta*-cleavage dioxygenases, acting on dihydroxylated polycyclic aromatic hydrocarbons, and differs from the main group of *meta*-cleavage dioxygenases, acting on 2,3-dihydroxybiphenyl. Similarly, catechol 2,3-dioxygenase (XylE) belongs to the class of *meta*-splitting enzymes, acting on dihydroxylated monocyclic aromatic hydrocarbons, but bears little resemblance to the canonical catechol-2,3-dioxygenase encoded by the TOL plasmid [78].

The study of naphthalene metabolism in pseudomonads, including the use of the resident plasmid NAH7, has played an important role in understanding the metabolism of aromatic hydrocarbons and the evolutionary relationships between different strains-destroyers. At the same time an interesting biodegradative potential with respect to naphthalene and other polyaromatic compounds was also shown in gram-positive bacteria.

#### 4. Degradation of Naphthalene by Gram-Positive Bacteria

*Rhodococci* are considered to be perhaps the most metabolically versatile microorganisms. *Rhodococci* inhabit a wide variety of niches of soil and aquatic ecosystems and are capable of decomposing a huge range of aromatic compounds including naphthalene [79–81]. *Rhodococcus* metabolism plasticity and the presence of a large number of peripheral metabolic pathways gives them a strategic advantage over *Pseudomonas* [82,83].

Earlier studies on the analysis of the genetic determinants of the metabolism of polyaromatic compounds in *Rhodococcus* strains showed that they differ from those encoded by the archetypal system of *P. putida* G7 [62], which suggests either a lack of a relationship between them or early divergence from a common ancestor, as was shown by the dioxygenases involved in the degradation of biphenyl [78]. The fact that the genes responsible for the first steps of naphthalene decomposition are not organized into a single cluster, as in pseudomonads, probably provides a wider variety of mechanisms for utilization of this compound and its adaptive plasticity.

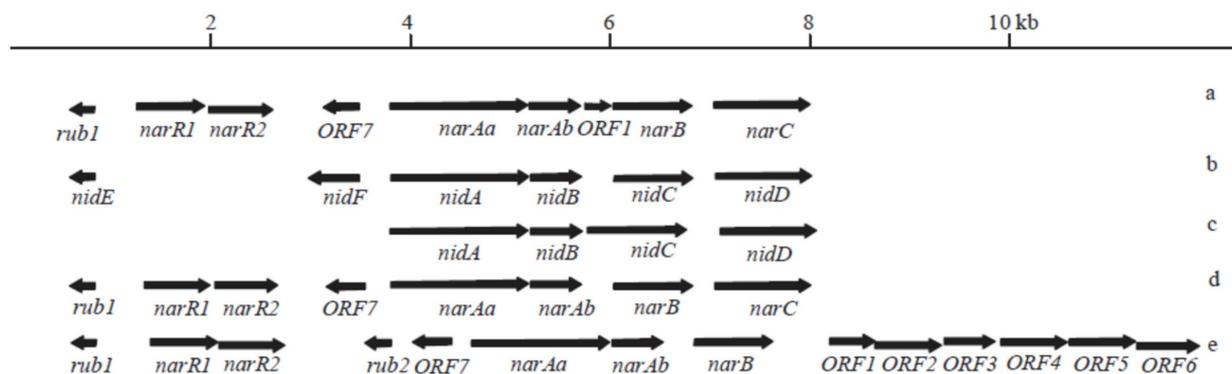
Referring to the example of the *R. opacus* M213 strain isolated from soil contaminated with fuel oil, the naphthalene degradation pathway was shown [51]. It was revealed that the strain did not grow on salicylate and did not induce salicylate hydroxylase. At the same time, induction of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase was shown to be related to catechol 1,2-dioxygenase from *R. opacus* 1CP and catechol 2,3-dioxygenase from *Rhodococcus* sp. I1, respectively. Plasmid analysis revealed the presence of two plasmids (pNUO1 and pNUO2), estimated to be of 750 Kb and 350 Kb in size, respectively.

Naphthalene metabolism by *Rhodococcus opacus* M213 was characterized genetically and biochemically. Pathak et al., in analyzing the genome sequencing of *Rhodococcus opacus* M213, showed genes similar to those involved in the oxidation of both salicylate and *o*-phthalate. This observation indicates the possibility of dual pathways for NAP degradation in the strain M213 [84]. Later, these authors indicated that the metabolism of naphthalene is encoded by a linear megaplasmid (750 kb) as well as by gene clusters localized on gene islands (GEI), and proceeds along a new pathway that does not pass through the salicylate [85]. An analysis of the metabolites produced when the strain grew on naphthalene showed that the metabolism proceeds via *ortho*-phthalate, which is then decomposed via the more standard protocatechuate pathway. *R. opacus* M213 does not grow on anthracene, phenanthrene, in contrast to bacteria such as *Aeromonas*, *Alcaligenes*, and *Micrococcus* spp., which are capable of decomposing anthracene and phenanthrene with the formation of *ortho*-phthalate and protocatechuate [51]. The noted uniqueness of the metabolic system of *R. opacus* M213 is presumably due to horizontal gene transfer and, accordingly, to genomic rearrangements both in the chromosome and in the transmissible plasmids. Nitrosoguanidine-generated mutants deficient in growth on naphthalene, *o*-phthalate, and/or protocatechuate were obtained for the strain, and the mutations were previously mapped to a large linear plasmid contained in M213. It was also shown that some of the genes involved in naphthalene degradation via a specific way are localized in GEIs, which were identified in strain M213 using a combination of bioinformatics, metabolic analyses, and evaluations of catabolic gene expression using RT-PCR. It is suggested that GEIs provide the bacteria with a “quantum jump evolution”, dramatically altering the host phenotype, including providing biodegradative potential. This study once again pays attention to both genome plasticity in bacteria, in general, and to the understanding of the ecological competitiveness of strain M213 due to genome shuffling caused by horizontal gene transfer. These aspects are still not well studied and poorly understood, particularly for soil *rhodococci*. In this regard, *R. opacus* M213 can be an illustration of the mechanisms by which a bacterial cell is able to adapt to xenobiotics.

There were also fundamental differences in the mechanism of naphthalene decomposition by the thermophilic strain *Geobacillus* sp. JF8 [67]. By analyzing the amino acid sequences of the NahB of this organism, it was shown that the encoded enzyme does not belong to the *cis*-dihydrodiol dehydrogenase group, which includes those of the classical naphthalene decomposition pathways.

The considerable diversity of the genetic organization of naphthalene decomposition pathways in the *Rhodococcus* group is confirmed by the study of the *R. ruber* OA1 strain. The naphthalene degradation via a new pathway and a new naphthalene catabolic gene cluster *nar* from *R. ruber* OA1 was identified. It was demonstrated for strain *Rhodococcus ruber* OA1 that phthalate is an intermediate in naphthalene degradation and that the protocatechuate pathway is realized by that strain for naphthalene degradation [86]. The complete gene cluster, *pcaJIGHBARC*, responsible for protocatechuate degradation was identified. Based on this gene cluster, the gene *pcaGH* encoding the protocatechuate 3,4-dioxygenase (3,4-PCD) was found.

Sun and co-authors (2017) described the naphthalene catabolic gene cluster from *Rhodococcus ruber* OA1 and indicated that the catalytic mechanism involved in naphthalene degradation in strain OA1 showed a high similarity to those present in *Rhodococcus* and *Gordonia*, but might be different from that described in *R. opacus* TKN14 (Figure 2) [87].



**Figure 2.** Organization of the naphthalene degradation gene clusters in (a) *Rhodococcus ruber* OA1 (GenBank accession number KY072804), (b) *Rhodococcus opacus* TKN14 (GenBank accession number AB206671.1), (c) *Rhodococcus* sp. I24 (GenBank accession number AF121905.1), (d) *Gordonia* sp. CC-NAPH129-6 (GenBank accession number GQ848233.3), and (e) *Rhodococcus* sp. NCIMB12038 (GenBank accession number AF082663.3) [Reproduced with permission from Sun et al. [87], as an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited, <https://docsdrive.com/pdfs/ansinet/biotech/2017/165-173.pdf>, accessed on 11 September 2021].

Additionally, there are very interesting data from Maruyama et al., who studied *o*-xylene oxygenase genes from *Rhodococcus opacus* TKN14 [88]. The authors demonstrated that a gene (named *nidE*) for rubredoxin (Rd) and a novel gene (named *nidF*) encoding an auxiliary protein, which had no overall homology with any other proteins, were indispensable for the methyl oxidation reaction of *o*-xylene, in addition to the dioxygenase iron–sulfur protein genes (*nidAB*). It has been suggested that these genes, excepted *nidF*, may be involved in the degradation of a wide range of aromatic hydrocarbons by the *Rhodococcus* species as the first key enzyme. This assumption is based on experiments showing that protein NidABE catalyzes the conversion of naphthalene and (di) methyl naphthalenes to their corresponding *cis*-dihydrodiols, i.e., acts as a typical naphthalene dioxygenase.

An alternative pathway of naphthalene utilization is shown for strain *R. opacus* 3D, isolated from the activated sludge of wastewater treatment plants [89]. The strain was unable to grow with salicylate but grew with coumarin, gentisate, *o*-phthalate, 2-hydroxycinnamic acid, and protocatechuate. Other *rhodococci* not capable of metabolizing salicylate are also known. For example, derivatives of *R. rhodochrous* strain NCIMB 13064, which utilize naphthalene, were not able to grow in mineral medium with salicylate and this compound was not detected in the culture media [90].

The previously mentioned *R. opacus* M213 is also unable to assimilate salicylate, but the growth substrates for this strain are gentisate, carboxybenzaldehyde, *o*-phthalate, hydroxyphthalate, and protocatechuate. Metabolites of the *o*-phthalate pathway were detected for this strain, namely cinnamic acid, 2-carboxy and 2-hydroxycinnamic acids, coumarin, hydrocoumarin, and phthalic aldehyde. These metabolites indicate that enzymes and pathways of aromatic compound degradation in *R. opacus* strains 3D and M213 are homologous.

*Rhodococcus* sp. strain B4, isolated from a soil sample contaminated with polycyclic aromatic hydrocarbons, uses naphthalene as the sole source of carbon and energy. Salicylate and gentisate have been identified as intermediate products of naphthalene catabolism. However, unlike the well-studied catabolic pathway encoded by the NAH7 plasmid of *P. putida*, salicylate does not induce the genes of the naphthalene degradation pathway in *Rhodococcus* sp. strain B4. The study shows an unusual requirement for the cofactors for key enzymes. The activity of 1,2-dihydroxynaphthalene oxygenase depends on NADH, whereas salicylate 5-hydroxylase requires NADPH, ATP, and coenzyme A [80].

Thus, it can be concluded that the metabolic mechanisms of gram-positive bacteria are more mobile, diverse, and play an important role in their adaptation to a wide range

of environmental pollutants, including naphthalene. This determines a more profitable environmental strategy, which, among other things, allows them to occupy a wide range of environmental niches [91–93].

### 5. Peculiarities of Econiches for Naphthalene-Degrading Bacteria

Mechanical and chemical methods commonly used to remove hydrocarbons from contaminated sites have limited effectiveness and are expensive. Bioremediation is a promising technology for restoring these contaminated sites because it is cost-effective and can lead to the complete mineralization of toxicants. Bioremediation is fundamentally based on biodegradation, which results in either the complete mineralization of organic pollutants with the formation of carbon dioxide, water, inorganic compounds, and cellular proteins, or the transformation of complex organic pollutants into other simpler organic compounds under the action of microorganisms.

A few bioremediation strategies can be highlighted.

1. Bioaugmentation is a bioremediation strategy that involves introducing indigenous microorganisms to the contaminated site to detoxify and degrade environmental contaminants. This strategy is used in both anaerobic [94] and aerobic conditions. Several successful bioaugmentation cases have been documented. Decomposing naphthalene *Streptomyces* sp. strain QWE-35, isolated from activated sludge, was introduced into a membrane bioreactor, which significantly increased the efficiency of naphthalene decomposition [95]. The effect of microbial inoculation on naphthalene mineralization in biosolid treatment was evaluated in soil manure microcosms. Inoculation by *Pseudomonas putida* G7 carrying the naphthalene dioxygenase (*nahA*) gene resulted in rapid mineralization of naphthalene, whereas indigenous microorganisms in the PAH-contaminated soil required a 28 h adaptation period before significant mineralization occurred [96].
2. Biostimulation concerns the activation of native oil-oxidizing microflora by creating optimal conditions for its development. This strategy also has many successful examples. For example, this is demonstrated in [97]. Thus, in the research of [98], it was shown that under laboratory conditions, the introduction of organic fertilizers increases the decomposition rate of naphthalene in soil samples by more than two times within 28 days.
3. The use of bacterial cultures in ex situ conditions in bioreactors of various configurations. Thus, a good example of the biodegradation of naphthalene using the bacterium *Pseudomonas putida* M8, isolated from soil in the suspension phase, shows the advantage of increased availability of pollutants for bacteria. The experiments were carried out in a reactor with a stirrer and oxygen. The results obtained confirmed the success of the selected bioremediation technology in the treatment of contaminated soils [99]. The use of a Batch Bioreactor with a Moving Bed (MBBR) with mixed microbial consortia operating under anaerobic, anoxic, and aerobic conditions resulted in the degradation of 90–94.8% of naphthalene at a concentration of 10 to 100 mg/L in 24 h (RT) [100]. Highly active strains degrading resistant compounds isolated from contaminated sites can be effectively used in bioreactors. Additionally, strains adapted to the decomposition of pollutants in bioreactors are a source of microflora activity for remediation by bioaugmentation [101].

The most promising cases relate to biotechnologies involving a combination of the two approaches described above, particularly the introduction into the soil of biopreparations that include active bacterial associations and mineral fertilizers. For example, the preparations of the “Lenoil” series are designed to clean various types of soil from oil contamination and have the ability to stimulate plant growth. These preparations include a consortium of oil-oxidizing microorganisms such as *Bacillus brevis* and *Arthrobacter* species IB DT-5; aerobic nitrogen-fixing microorganisms, namely *Azotobacter vinelandii* IB 4; and the biomass of aerobic spore-forming microorganisms, namely *Bacillus* species 739, which allows to simultaneously increase the efficiency of oil products’ biodegradation in soil and to activate the microbiological activity of soil [102,103].

As stated, PAH contamination is a global problem and a number of microbial species have been investigated for the efficient degradation of this class of compounds [83,104].

In situ bioremediation can be more effective under appropriate conditions for microbial growth, such as an adequate supply of nutrients, surfactants, water, and oxygen. It is one of the most significant approaches for the enhancement of bioremediation efficiency at the PAH-contaminated site [105]. The naturally occurring microbial species have been effectively used at contaminated fields and the effects of nutrient addition to stimulate bioremediation on the field scale have been extensively studied.

In Table 1, as an example, some data on the efficiency of the decomposition of naphthalene by microorganisms are presented and the optimal temperature conditions in which these organisms were studied are given, according [106].

As already mentioned, diaromatic compounds—naphthalene and its homologues—are part of diesel fuels. Problems of the bioremediation of the environment from diesel fuel are particularly acute for areas of the far north. This is because in remote areas, such as the Canadian Arctic, on-site bioremediation is the only possible option for cleaning hydrocarbon spills. Several studies have shown that microorganisms, particularly bacteria, are capable of decomposing hydrocarbons at the extreme temperatures common in polar and alpine zones.

Considering that oil production is mostly carried out in zones that are not optimal for the life of microorganisms (high or low temperatures, salinity, arid climate), several author teams working with microorganism inhabitants of extreme regions should be noted.

**Table 1.** Characteristics of the microbial process for naphthalene degradation.

| Microbial Species                            | Source  | Condition; Temperature (°C); Period (Days) | PAH Compound (Ci—ppm)  | Degradation Efficiency (%)          | Genes Coding Enzymes   | Peculiarities  | Reference |
|--|---|--|--|-------------------------------------|--|--|-----------|
| <i>Pseudomonas putida</i> IR1                | Hydrocarbon-contaminated soil                     | Aerobic; 30; 7                             | NAP (200)<br>PHE (200)<br>PYR (200)                            | 72 ± 2<br>60 ± 4<br>69 ± 3          | <i>nahA</i> (naphthalene dioxygenase) and <i>nahE</i> ( <i>trans</i> - <i>o</i> -hydroxybenzylidene pyruvate hydratase-aldolase)   | Biosurfactants were synthesized. The surface tension decreased from 54.9 dN cm <sup>-1</sup> to 35.4 dN cm <sup>-1</sup> . An emulsifying activity of 74% with diesel oil, when grown on dextrose, was present.                              | [107]     |
| <i>Pseudomonas</i> sp.                       | Plants growing at PAH-contaminated sites          | Aerobic; 30; 7                             | NAP (100)<br>FLU (100)<br>PHE (100)<br>PYR (100)               | 95.3<br>87.9<br>90.4<br>6.9         | No information   | Strain P3 has more potential for use in the removal of PAHs from plant tissues.  | [108]     |
| <i>Stenotrophomonas</i> sp.                  | Plants growing at PAH-contaminated sites          | Aerobic; 30; 7                             | NAP (100)<br>FLU (100)<br>PHE (100)<br>PYR (100)<br>B(a)P (10) | 98.0<br>83.1<br>87.8<br>14.4<br>1.6 | No information   |  | [108]     |
| <i>Neptunomonas naphthovorans</i> NAG-2N-126 | Creosote-contaminated sediment                    | Aerobic; 20; 7                             | NAP (5)<br>PHE (1)   | 100<br>100                          | Naphthalene dioxygenase iron-sulfur protein (ISP) gene fragments   | A naphthalene dioxygenase iron-sulfur protein (ISP) gene deduced amino acid sequences and showed that the genes were distantly related to the genes encoding naphthalene dioxygenases of <i>Pseudomonas</i> and <i>Burkholderia</i> strains. | [50]      |
| <i>Sphingomonas koreensis</i> ASU-06         | Oil-contaminated soil                             | Aerobic; 30; 15                            | NAP (100)<br>PHE (100)<br>ANT (100)                            | 100<br>99<br>98                     | Catabolic genes <i>alkB</i> , <i>alkB1</i> , <i>nahAc</i> , C12O, and C23O   | Degradation of 15 various PAH and production of the extracellular biosurfactant.   | [109]     |
| <i>Burkholderia cepacia</i> 2A-12            | Oil-contaminated soil                             | Aerobic; 30; 2                             | NAP (215)<br>PHE (215)<br>PYR (215)                            | 100<br>100<br>0                     | No information   | The PAH degradation rate of the strain was enhanced by the addition of other organic materials such as YE, peptone, glucose, and sucrose.  | [110]     |
| <i>Rhodococcus ruber</i> OA1                 | Pharmaceutical wastewater treatment plant         | Aerobic; 6                                 | NAP (500 mg)   | 100                                 | The genes encoding NDO large and small subunits ( <i>narAaAb/nidAB</i> ), <i>cis</i> -naphthalene dihydrodioldehydrogenase ( <i>narB/nidC</i> ), and putative aldolase ( <i>narC/nidD</i> )<br>Regulated <i>narR1</i> and <i>narR2</i> genes | Heterologous expression of <i>narAaAb</i> and <i>rub1</i> genes.   | [86,87]   |
| <i>Rhodococcus opacus</i> R7                 | Polycyclic aromatic hydrocarbon-contaminated soil | Aerobic; 30; 15                            | NAP (1 g)  | 100                                 | High-quality draft genome sequence of strain R7, consisting of 10, 118, and 052 bp   | 1,2-Dihydro-1,2-dihydroxynaphthalene as well as salicylic and gentisic acids were identified as metabolites.   | [111–113] |
| <i>Rhodococcus</i> sp. strain B4             | Polycyclic aromatic hydrocarbon-contaminated soil | Aerobic, 1                                 | NAP (0.5 g)  | 100                                 |  | Unusual cofactor requirements: 1,2-dihydroxynaphthalene oxygenase activity depends on NADH and the salicylate 5-hydroxylase requires NADPH, ATP, and coenzyme A.   | [80]      |

One of the main world leaders in this field is the group of Professor Rosa Margesin (University of Innsbruck, Austria), who has been working in this area for the past 16 years. Margesin and colleagues have demonstrated the ability of cold-adapted strains of the genus *Rhodococcus* to degrade alkanes (hexadecane) and aromatic hydrocarbons (phenol and anthracene) at low temperatures. The potential of 89 cold-adapted isolates from yeasts and bacteria capable of growth and production of various enzymes (catechol 1,2-dioxygenase, amylase,  $\beta$ -lactamases,  $\beta$ -galactosidase, lipase, and protease) at 10 °C was studied. The effect of temperature on the biodegradation of diesel fuel by yeasts in mineral medium and soil (4–30 °C) was studied. Prof. R. Margesin and colleagues have written several reviews on the applied aspects of enzymes from cold-adapted microorganisms, including a wide range of the metabolic activities of psychrotrophic and psychrophilic microorganisms in alpine cold ecosystems polluted by oil hydrocarbons [114–121].

Several Spanish authors [122–124] and researchers from China [125,126] demonstrated the ability of psychrotrophic hydrocarbon-oxidizing bacteria to degrade oil or diesel fuel under laboratory conditions at low temperatures of 4–15 °C. Additionally, studies on the analysis and mechanisms of the adaptation of psychrophilic and psychrotrophic microorganisms oxidizing hydrocarbons of oil are actively conducted. In these articles, it is demonstrated that with a decrease in temperature, processes such as the changes in membrane fluidity due to fatty acids composition, changes in protein and lipid composition of the cell membrane, synthesis of carotenoids, and synthesis of cold-shock proteins take place. It has been shown that psychrophilic enzymes have high catalytic efficiency and low conformational stability, while the synthesis of exopolysaccharides as cryoprotectors during cold shock is activated.

A team of Australian researchers is investigating both the impact of hydrocarbon (diesel fuel) pollution on various Antarctic ecosystems, as well as the phytoremediation of Antarctic soils contaminated by diesel fuel [127–131]. Another team of Australian scientists, specifically Josie van Dorst and Belinda Ferrari at the University of New South Wales (Kensington), are studying the microorganisms and microbial community dynamics of Antarctic soils exposed to diesel spills [132,133]. A Canadian Research Group (McGill University) is investigating the bioremediation of oil-contaminated Arctic soils in northern Canada by stimulating native microorganisms and changing soil microbiocenoses (composition and abundance of microorganisms) [134–137]. An American/Canadian Arctic team (Terrence Bell at Cornwall University; Etienne Yergeau and David Juck of the National Research Council of Canada; and Lyle White and Charles Greer at McGill University) is studying the effects of diesel fuel on native microbial communities of Canadian Arctic soils and its degradation by autochthonous soil microorganisms [138,139]. Bioremediation methods have a few incomparable advantages, such as the safety, speed, low cost, and high efficiency of the removal of pollutants from the environment.

## 6. Conclusions

Thus, based on the data of the cited literature, several conclusions can be drawn regarding the importance of the research being carried out and their directions. First, the data obtained allow us to develop effective systems for cleaning contaminated sites using biopreparations. However, interest in the processes of the biodegradation of hydrocarbons is not limited to practical purposes only. All over the world, research is being carried out to study the ecological, biochemical, and genetic aspects of the microbial degradation of oil and its components.

Under conditions of increasing environmental pollution by compounds of natural and anthropogenic origin, the metabolic capabilities of existing natural microbial communities are changing and new microbiocenoses are being formed, including those due to introduced bacteria-destructors. Understanding these processes will allow us not only to restore disturbed ecosystems but also their intelligent management.

In this regard, research at the metagenome level comes to the forefront. The problem is that a large number of cultivation-independent methods have shown that contaminant-

degrading organisms obtained by accumulation culture in the laboratory are often not sufficiently efficient in the *in situ* biodegradation of contaminants. Many pollutant-degrading organisms in environmental samples have been shown to be very abundant but are represented by non-culturable forms [140–142]. In this context, many relationships, including interspecies relationships between organisms, are very often left unexplored when studying the physiology of degrading bacteria. According to this scenario, it is appropriate to use molecular microbial tools to identify key catabolic players in polluted sites, to predict contaminant degradation networks in the environment, and to propose methods for rational interventions associated with bioremediation implementation. A good illustration is the study of Guazzaroni et al. (2013), in which the authors, using a metagenomic approach, performed a thorough and holistic (or ecosystem approach) phylogenetic, functional, and proteomic analysis of bacteria in naphthalene-contaminated soil [143]. They examined samples with an enriched microbial community obtained from the soil and samples of the same soil biostimulated with biogenic elements. In all cases, the biodegradation networks of the respective whole communities were reconstructed. This study elucidated the genomic and proteomic basis for understanding microbial biodiversity, ecology, and function in response to PAHs (represented by naphthalene) and biostimulation. Based on a comparison of protein expression profiles and metagenome datasets, hypotheses about the interactions between members of microbial communities were hypothesized. For the first time, the authors used databases to reconstruct “putative” degradation networks of complex microbial communities.

Based on metaproteomic and taxonomic data, the authors proposed a meta-network approach in which they used expression levels and taxonomic definitions of proteins as the most relevant clues to infer an active set of reactions in the naphthalene-degrading microbial community [144]. The approach was applied to develop context-specific metabolic networks of two different naphthalene-enriched communities originating from anthropogenically contaminated soil. The authors were able to detect common functional differences between the two states of microbial communities (under biostimulation and not under biostimulation) at the metabolic level. In addition to the population level, the organization of different pathways at the organismal level has been established, which is relevant to the division of the role of each member in communities.

To summarize, we can say that the microbial bioremediation of soils, bottom sediments, and waters polluted with toxicants of anthropogenic origin is quite an effective tool. The ability of microorganisms to degrade oil components is the result of genetic adaptation, thus a *in-depth* understanding of all aspects of the degradation mechanism at the molecular-genetic level is necessary.

High-throughput technologies such as genomics, proteomics, transcriptomics, and metabolomics are needed to elucidate the genetic pathways involving catabolic genes and their regulation mechanisms [145]. These studies will contribute to understanding the mechanisms of destruction not only at the cellular level but also within the context of the microbial community, understanding the relationships of all organisms in the ecosystem and therefore making it possible to both establish predictions and manage the biocenosis.

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