Article title: 2,2-Diphenic Acid: A Reliable Biomarker Of Phenanthrene Biodegradation

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2,2'-DIPHENIC ACID: A RELIABLE BIOMARKER OF PHENANTHRENE BIODEGRADATION

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Abstract

Phenanthrene is among the 16 priority pollutant and its mitigation in the environment has been a global concern. It serves as a model compound when it comes to biodegradation study of polyaromatic hydrocarbons (PAHs) because it has both the Bay- and K-region found in most PAH pollutants. Like other PAH pollutants, different means are available for its remediation in the environment, including microbial biodegradation. Diverse species of bacteria and fungi metabolize phenanthrenes as their sole source of carbon and energy. However, bacteria are more diverse in comparison to fungi. This has been shown in published pathways of phenanthrene biodegradation implicating various intermediary metabolites, including 2,2'-diphenic acid, which is a downstream metabolite of 9,10-dihydroxyphenanthrene. Though the 2,2'-diphenic acid has been widely demonstrated to produce carbon (iv) oxide and linked to phthalate, only few has traced salicylic acid as its downstream molecule. 2,2'-diphenic acid mounts equivalent position to 1-hydroxy-2-naphthoic acid, metabolite that ends the phenanthrene metabolic pathway. This is because they both produce phthalic acid and salicylic acid. As a product of bacteria and fungi during phenanthrene degradation, 2,2'-diphenic acid can serve as a dependable biomarker of phenanthrene metabolism in a polluted habitat, where microbial community exist freely.

Keywords: 1-hydroxy-2-naphthoic acid; 2,2'-diphenic acid; Bacteria; Intermediary metabolites; Phenanthrene;

Introduction

Phenanthrenes are polyaromatic hydrocarbons with three-fused benzene rings in angular configuration. They are isomers of anthracenes but are more stable due to the efficient π -bonding (Pradhan *et al.*, 2020). Their physical properties makes them to be insoluble and form hydrophobic films that block sunlight and air penetration. In addition to their chemical properties, they cause reduction of microbial biomass, negatively affect plant growths, compromise ecological services and are toxic to fish and algae (Zindler *et al.*, 2016; Xin *et al.*, 2020). Consequently, they are included among the 16 priority pollutants (Bhyyan and Giri, 2020). Though phenanthrenes are man-made, they also occur naturally in higher plants, crude oil and coal in different derivatives (Kusz *et al.*, 2021). Besides, they are used in making drugs, dyes and pesticides (Mangwani *et al.*, 2014).

Phenanthrenes as environmental pollutants requires mitigation from the environment. One trending method of achieving this is through microbial degradation, the science behind bioremediation technology. Biodegradation of phenanthrenes follows diverse pathways but the most dominant one starts with deoxygenation at the 3,4 position to form *cis*-phenanthrene dihydrodiol then to the formation of 3,4-dihydroxyphenanthrene (Pagnout *et al.*, 2007). Through series of reactions, including ring cleavage reactions, 1-hydroxy-2-naphthoic acid is formed which in turn lead to protocatechuic acid and salicylic acid (Gao *et al.*, 2013). The formation of 1-hydroxy-2-naphthoic acid terminates the upper catabolic pathway of phenanthrene degradation (Habe and Omori, 2003).

A link has been shown between 9,10-dihydroxyphenanthrene, 2,2-diphenic acid and phthalic acid but downstream metabolites of 2,2-diphenic acid has not been widely publicized. Both bacteria and fungi induced biodegradation of phenanthrene has shown to produce 2,2-diphenic acid, hence can serve as a reliable biomarker of phenanthrene biodegradation driven by unrestricted microbial community.

Phenanthrenes: definition, physical properties and sources

Phenanthrenes are low molecular weight species of polyaromatic hydrocarbons (PAHs) that are composed of three fused benzene rings in angular arrangement. They appear as yellow/white crystalline compounds. It is important to note that anthracene is also a three-fused benzene rings but in planar structure. This makes anthracenes and phenanthrenes to be structural isomers. Beside, phenanthrenes are more stable due to the π -bonding efficiency (Poater *et al.*, 2018). Their physical properties are listed below:

Appearance:	Yellow/white crystals
Molecular formula:	C ₁₄ H ₁₀
Molecular mass:	178.23 g/mol
Density:	1.18 g/mL
Boiling point:	340 °C
Melting point:	101 °C
Flash point:	171 °C
Aqueous solubility:	1.1 mg/L (at 25 °C)
Octanol/water partition coefficient ratio:	2.9 x 10 ⁴
Carcinogenicity:	3 (not classifiable)

Their insolubility make them to form nonaqueous films, which hinder penetration of sunlight, block air penetration thereby creating anoxic/toxic environment. Consequently, they cause drastic changes of microbial diversity, affect healthy growth of plants and compromise ecological services. Due to their stability feature, they accumulate in living cells and through food chain they biomagnify in higher organisms. Phenanthrenes are amongst the 16 priority pollutants that are toxic to algae and fish (Waigi *et al.*, 2015). Phenanthrenes are both natural and synthetic; and can exist in different derivatives.

Phenanthrenes occur in higher plants including but not limited to species in the Orchidaceae, Jugaceae, Dioscoreaceae, Hepaticaceae, Betulaceae and Combretaceae families. Some principal examples are *Bletilla*, *Cymbidium*, *Eria*, *Dendrobium*, *Coelogyna* and *Bulbophyllum* used as traditional medicinal plants (Kovacs *et al.*, 2008; Shi *et al.*, 2017; Bus *et al.*, 2018; Kusz *et al.*, 2021). More than 450 compounds has been described, involving monomeric, dimeric and trimeric derivatives of phenanthrenes (Toth *et al.*, 2017). Other sources of phenanthrenes are crude oil, coal derivatives, plant (for wood treatment, asphalt production, incineration) sites, combustion of woods/forests. Phenanthrene is produced in cigarette smokes and are used in making dyes, manufacturing pesticides, plastics, drugs, steroids and cholesterol (Wang *et al.*, 2012).

Phenanthrene biodegradation

Biodegradation is the process through which biological agents and biological entities reduce metabolic compounds into smaller compounds (Ukiwe *et al.*, 2013). According to Okpokwasili and Nkweke (2005) the ecological scenarios that condition a pollutant to be biodegraded include (1) as carbon and energy source (2) as an electron acceptor (3) been a source of other cell compound and (4) been susceptible to non-specific enzyme of growing and non-growing cells. Biodegradation of phenanthrenes are much easier in comparison to other PAHs, which have high molecular weight (Seo *et al.*, 2009).

Synopsis of PAH biodegradation

Polyaromatic hydrocarbon microbial degradation follows a rather unique order, though the higher the number of aromatic rings the more complex the biochemical reaction becomes. The first reaction is catalyzed by dioxygenase to form *cis*-PAH dihydrodiol. For example, naphthalene is catalyzed by naphthalene dioxygenase (NDO) to produce *cis*-naphthalene dihydrodiol (Raissa *et al.*, 2012). *Cis*-PAH dihydrodiol is then transformed to dihydroxyPAH by dehydrogenase. For instance *cis*-3,4-phenanthrene dihydrodiol is transformed into 3,4-dihydroxyphenanthrene (Pagnout *et al.*, 2007). Similarly *cis*-7,8-(or 1,2-)fluoranthene dihydrodiol is converted to 7,8-(or 1,2-)dihydroxyfluoranthene (Sei *et al.*, 2003).

By series of biochemical reactions, central metabolic compounds are formed from dihydroxyPAHs, which represent the end-point of upper catabolic pathway of PAHs and serve as the beginning of PAH lower catabolic pathway. Salicylic acid (from naphthalene), 1-hydroxy-2-naphthoic acid (from phenanthrene) and naphthalene-1,8-dicarboxylic acid (from fluoranthene) are common central metabolic compounds (Sihag and Pathak, 2014). Salicylate is catabolized into gentisate and catechol; 1-hydroxy-2-naphthoic acid is biodegraded into protocatechuic acid and salicylic acid while naphthalene-1,8-dicarboxylic acid is transformed into 2-hydroxyisophthalate (which produces salicylate) and benzene-1,2,3-tricarboxylic acid (which produces protocatechuate). Protocatechuate, salicylate and catechol are critical metabolites that are channeled into the tricarboxylic acid (TCA) cycle to yield energy for the degraders (Kumar *et al.*, 2011). Catechol biodegradation has two established pathways-*ortho* and *meta* catabolic pathways. These two pathways are finally channeled into the central TCA cycle with the evolution of carbondioxide.

Metabolic pathways of phenanthrene

Phenanthrene is majorly catalyzed by dioxygenase (Gran-Scheuch *et al.*, 2017; Ahmad *et al.*, 2019; Jia *et al.*, 2019) but the involvement of monooxygenases has been reported also. Bacterial degradation of phenanthrenes starts with the formation of isomers (9,10-; 1,2-; and 3,4-) but the *cis*-3,4-dihydrophenanthrene-3,4-diol isomer (Mishra *et al.*, 2019; Yin *et al.*, 2020) has proven to be the dominant (Fig. 1.). *Cis*-3,4-dihydrophenanthrene-3,4-diol is consequently transformed to 3,4-dihydroxyphenanthrene. The latter undergo series of reaction including ring cleavage to produce 1-hydroxy-2-naphthoic acid, which terminates the upper catabolic pathway of phenanthrene (Zeb *et al.*, 2020). The hydroxynaphthoic acid takes two routes: phthalic acid and naphthalene routes. The phthalic acid pathway leads to protocatechuic acid while the naphthalene pathway leads to salicylic acid which in turn yields gentisic acid and catechol. The catechol is driven by two ring cleavage pathway: *ortho* cleavage pathway (with catechol-1,2-dioxygenase) and *meta* cleavage (directed by catechol-2,3-dioxygenase) (Mawad *et al.*, 2020). The *ortho* pathway yields succinyl CoA and Acetyl CoA while the *meta* pathway produces acetaldehyde and pyruvic acid. The gentisic degradation produces fumaric acid and pyruvic acid. The products of the salicylic degradation enters the tricarboxylic acid cycle, which ultimately produces carbondioxide (Bai *et al.*, 2021; Zhou *et al.*, 2021).

Fungi also degrade phenanthrene but not as diverse as bacteria. The most documented phenanthrene degradation by fungi is the 9,10-dioxygenase pathway to produce 2,2-diphenic acid (Gupta *et al.*, 2021). Initially it was thought that only fungi can produce 2,2-diphenic acid from phenanthrene degradation. But subsequent study has shown that some species of bacteria also produce 2,2-diphenic acid. The diphenic acid is a downstream product of 9,10-dihydroxyphenanthrene (Mishra *et al.*, 2019). Overall, more than 33 metabolites from phenanthrene degradation has been reported (Seo *et al.*, 2009).

Fig. 1. A simplified phenanthrene biodegradation pathway, with each arrow representing multiple reactions (Adapted from Seo *et al.*, 2009; Huang *et al.*, 2016; Pandey *et al.*, 2016)

Factors affecting biodegradation of phenanthrene hydrocarbon

Phenanthrene degradation, like other PAHs, are affected by diverse factors that rely on three basic categories: chemical nature of pollutants, environmental factors and biological factors.

Nature of chemical compound

Factors associated with the chemical nature of phenanthrene are molecular structure, concentration, bioavailability and toxicity of intermediary metabolites. Phenanthrene has both Bay- and K-region, thus can be used as a model to study biodegradation PAHs with higher molecular weight possessing the Bay- and K-region. Besides, phenanthrene as low molecular weight (corresponding with lower electrochemical stability; Semple *et al.*, 2003) PAH is easy to be metabolized by microorganisms (Esadeha *et al.*, 2015). In comparison to its linear isomer, phenanthrene is more stable because of its angular structure (Atagana *et al.*, 2003). Concentration of phenanthrene also affects its degradation. The higher the concentration, the more it affects the degraders with the result of reduced removal (Strand *et al.*, 2007). Bioavailability enhances biodegradation of phenanthrene (Becher *et al.*, 2000). Thus, synthetic surfactants or biosurfactants can lead to higher rate of phenanthrene degradation. Some microorganisms are adapted to producing biosurfactants real-time, making them to be expert degraders of phenanthrenes. Oxy-compounds (ketones, quinones and coumarins (7,8-benzocoumarine)) from phenanthrene degradation often leads to poor phenanthrene degradation because they are more toxic than the phenanthrene (Lundstedt *et al.*, 2003).

Biological factors

The ability of microbial community to degrade PAHs rests on the number of competent microorganisms. Bacterial number of less than 1×10^3 CFU/g will cause the persistence of phenanthrenes, like other PAHs (Petrovic *et al.*, 2008). Table 1 displays examples of bacteria that degrade phenanthrenes. A range of 10^4 to 10^7 CFU/g represents a hopeful number of microbial populations for biodegradation of phenanthrenes and other hydrocarbons. Communities of competent microorganisms constituted by different phenotypes degrade phenanthrenes much faster and efficiently (Vinas *et al.*, 2005). For instance, a community of microorganism consisting of fungi, algae and bacteria according to Rajaei *et al.* (2011) degrade pollutants faster than a community of bacteria only. This is due to the release of enzymes that degrade the pollutants through metabolic pathways as a carbon and energy source (Igwo-Ezikpe *et al.*, 2010), and enzymes produced from co-metabolism phenomenon (Leys *et al.*, 2008). Table 1 itemizes bacteria species known to degrade phenanthrene. Production of biosurfactants which increases the surface area of the hydrophobic pollutants, desorbs the pollutants from surface and makes hydrophobic pollutants to become soluble (Alrumman *et al.*, 2015). The cumulative effect of biosurfactant production is to overcome mass transfer limitation for metabolizable phenanthrenes (Bustamante *et al.*, 2012; Gutierrez *et al.*, 2013; Fenibo *et al.*, 2019; Khajavi-Shojaei *et al.*, 2020). Another, mechanisms which microbes employ to increase bioavailability of pollutants is by modifying their cell surface properties such that their cell membranes becomes more hydrophobic (Delille *et al.*, 2002). The effect of this microbial strategy is evidenced by the elicitation of cell bound formation of lypopolysaccharides, fatty acids, glycolipids, lipoteichoic acids, lipoglycans and neutral lipids (Van Hamme *et al.*, 2003). Altered cell surface can lead to biofilm formation and enhance cell colonization and spread (Isola *et al.*, 2008). Biofilms serves as a protection state against toxicity of pollutants and chemicals and beyond this engender metabolite exchange, nutrient accession and lateral gene transfer (Kostka *et al.*, 2011). According to Johnsen and Karison (2004), biofilm formation on PAHs' crystals may cause mass transfer of PAHs crystals directly into the bacterial cells thus favouring PAH availability. This confirms that biofilm formation greatly influences substrate (PAH) availability (Johnsen and Karison 2004).

Table 1. Reported phenanthrene-degrading bacteria

Species	Note	Reference
<i>Acidovorax delafieldii</i> P4-1	Phenanthrene degradation was enhanced by the addition of Triton X-100. RKJ4 displays cometabolic degradation of phenanthrene	Samanta <i>et al.</i> (1999)
<i>Arthrobacter sulphureus</i> RKJ4		
<i>Ochrobactrum</i> spp.	Found in petroleum contaminated soil with phenanthrene degrading ability	Liang <i>et al.</i> (2021)
<i>Pseudomonas</i> sp. Lphe-2	A bacterium strain, isolated from coking plant with the express ability to degrade phenanthrene	JI <i>et al.</i> (2020)
<i>Burkholderia cocovenenans</i> BU-3	Isolated from petroleum polluted soil and degraded phenanthrene by 95% at a rate of 4.2 mg/h	Wong <i>et al.</i> (2002)
<i>Diaphorobacter</i> sp. YM-6	Bacteria strain found in PAH-contaminated sediment with phenanthrene assimilation competence	Wang <i>et al.</i> (2020)
<i>Janibacter</i> sp. YY-1	A dibenzofuran-degrading bacterium that has the ability to cometabolize phenanthrene in soil	Yamazoe <i>et al.</i> (2004)
<i>Pseudomonas</i> sp. strain MP6-0207	Bacterium found in engine oil-contaminated mangrove swamp	Chantarasiri and Campus (2020)
<i>Bacillus thuringiensis</i>	Collected from crude oil contaminated soil with proven competence of degrading phenanthrene	Abdel-Razeka <i>et al.</i> (2020)
<i>Mycobacterium</i> <i>vanbaalenii</i> PYR-1	A multi-PAH-degrading bacterium with functional genes that degrade phenanthrene	Stingley <i>et al.</i> (2004)
<i>Sphingobium yanoikuyae</i> SJTF8	Isolated and characterized as utilizer of phenanthrene as sole carbon source.	Yin <i>et al.</i> (2020)
<i>Pseudomonas putida</i> OUS82	A model bacterium isolated from oil contaminated soil and used for the study of phenanthrene and naphthalene assimilation	Tay <i>et al.</i> (2014)
<i>Paeniglutamicibacter</i> <i>terrestris</i> sp. ANT13_2 ^T	A constituent of phenanthrene-degrading consortium from Antarctic soil	Sakdapetsiri <i>et al.</i> (2021)
<i>Pseudomonas</i> <i>aeruginosa</i> ATAI19	Demonstrates dioxygenase activity with the ability of producing biosurfactants and degrading phenanthrenes	Amini <i>et al.</i> (2017)
<i>Sphingomonas</i> <i>paucimobilis</i> EPA505	Isolated from creosote and grow effectively on phenanthrene	Story <i>et al.</i> (2004)

<i>Pseudomonas fluorescens</i> AH-40	Isolated from crude oil polluted soil which consumed phenanthrene as a source of carbon and energy	Mawad <i>et al.</i> (2020)
<i>Stenotrophomonas maltophilia</i> C6	Initiate phenanthrene degradation via 1,2-; 3,4- and 9,10-dioxygenations	Gao <i>et al.</i> (2013)
<i>Streptomyces</i> sp. MC1	Isolated from fuel contaminated soil and genetically confirmed in degrading phenanthrene	Sineli <i>et al.</i> (2021)

Environmental factors

Key environmental factors that may influence PAH biodegradation are nutrient availability, presence of toxicants, oxygen content, temperature, pH, moisture and soil particle characteristics. Nutrient availability reduces the high C:N ratio in phenanthrene contaminated environment (Marisnescus *et al.*, 2011) thereby addressing the rate limiting factor, hence facilitating degradation rate. Optimal resource ratio and balance is very critical because excess of nutrient can be inhibitory since some microbes, such as fungi are known nutrient recyclers, specifically nitrogen (Sihag and Pathak, 2014). Toxicant presence, such as heavy metals, slows down metabolic activities and growth (Umrana, 2006). The type of metals, concentration and kind of microbes exposed to such toxicants influences the degree and mechanisms of toxicity. Oxygen as an electron acceptor influences phenanthrene degradation more than any other electron acceptor (Erdogan and Karaca, 2011). So, when oxygen becomes rate limiting, anaerobic respiration ensues starting with denitrification process according to the thermodynamic feasibility order: oxygen > nitrogen > manganese > iron > sulphate > carbondioxide (Coates *et al.*, 1997). Temperature influences phenanthrene degradation rate by controlling the rate of enzyme reaction, increases the solubility of pollutants but decreases oxygen solubility (Tomei and Daugulis, 2013). Study on the effect of temperature has shown that the rate of biodegradation doubles per 10 °C rise of temperature up to a maximum of about 65°C (Thapa *et al.*, 2012). Though most biodegradation studies tend to focus on mesophilic temperatures, phenanthrene degradation had shown to occur at extreme temperatures (Simarro *et al.*, 2012; Al-Mur *et al.*, 2021).

Biodegradation of phenanthrenes and other hydrocarbons is being executed under a wide range of pH though optimal at the border of between of 6.5-8.5. However, fungi and sulphate reducing bacteria thrive best in acidic medium (Das *et al.*, 2009). Studies have shown that pH affect nutrient (phosphorus) and metal mobility in soil and adsorption to soil apart from effect on microbial degradation (Bamforth and Singleton, 2005). Soil moisture influences the rate of phenanthrene degradation like other petroleum hydrocarbons by influencing solubility of concerned pollutants including osmotic pressure and soil pH (Ezeonu *et al.*, 2012). The presence of water in pore spaces of soil affects exchange of oxygen (Nkeng *et al.*, 2012). Oxygen is usually consumed faster in saturated condition when compared to when it is replenished in the soil vapour space thus leading to anaerobic condition. On the contrary, moisture content between 25-85% of water holding capacity is optimal for biodegradation in soil (Mrozik *et al.*, 2003). The water holding capacity is the percentage of water remaining in soil after it has been saturated and gravitational drainage has ended (Salleh *et al.*, 2003). Soil moisture largely affects nutrient availability to microorganisms through its impact on diffusion, solubility and biological process (uptake) affecting ion flow in soil (Shukla *et al.*, 2010). At low moisture content, nutrient supply rate to microbes is slow due to increased tortuousity of ion movement, soil texture, organic matter content and biological activity (Bulmer and Simpson, 2005).

For enhanced degradation of phenanthrenes interaction of optimal environmental and biological factors is necessary. An environment characterized with nutrient availability (C:N:P ratio of around 100:10:1), temperature that will ensure phenanthrene solubility without affecting oxygen availability (20-30 °C), a redox potential of > 50 millivolts, pH of 6.5-8.0 and concentration of phenanthrene not more than 10%, relative to the weight of the environmental medium would lead to fast reduction of phenanthrene reduction.

Conclusion

Global concern for the 16 priority PAHs, including phenanthrene calls for a sustainable approach in remediating them. One such approach is microbial biodegradation, involving bacteria and fungi, which use phenanthrene as their sole source of carbon and energy. The metabolites derived from phenanthrene metabolism has been shown to be more than thirty three. A central molecule from 1,2- and 3,4-dihydroxyphenanthrene is 1-hydroxy-2-naphthoic acid and marks the beginning of the lower metabolic pathway of phenanthrene. The equivalent metabolite from 9,10-

dihydroxyphenanthrene is 2,2'-diphenic acid which produces intermediary compound as that of 1-hydroxy-2-naaphthoic acid. Both of them yield phthalic acid and salicylic acid. Thus, 2,2-diphenic can serve as a reliable biomarker in an environment characterized with phenanthrene pollution.

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