Metabolism of Nitroaromatic Compounds by Microbes and Study of Chemotaxis Toward These Compounds

12

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Abstract

Nitroaromatic compounds are mainly man-made compounds having diverse functions in industry and otherwise. These are toxic compounds, and their complete mineralization by natural or engineered microbes is desirable via aerobic, anaerobic, or dual pathways. Bacterial chemotaxis has been shown to improve degradation rates and also result in biofilm formation, which in turn assists breakdown of the toxic compounds. These properties may be harnessed for engineering bugs for enhanced and varied degradation of NACs. The microbial diversity of unculturable microbes may be tapped for discovering "new" genes for mineralization of xenobiotic and persistent/recalcitrant compounds.

Keywords

Nitroaromatic · Xenobiotic · Chemotaxis · Bioavailability · Breakdown

Abbreviations

HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine
NACs	Nitroaromatic compounds
RDX	Cyclotrimethylenetrinitramine
TNB	1,3,5-Trinitrobenzene
TNT	2,4,6-Trinitrotoluene

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12.1 Nitroaromatic Compounds: Synthesis and Applications

Nitroaromatic compounds (NACs) are mainly man-made and applied for the manufacture of explosives (TNT, RDX, etc.), as pesticides/insecticides (Ju and Parales 2010), and in industries, e.g., tannery, polyurethane foams, rubber photographic chemicals, azo dyes, varnishes, and pharmaceuticals (derivatives of phenothiazines, substituted nitrobenzenes, chloromycetin). The natural formation of nitroaromatic compounds may occur in both air and water conditions. In cities or small towns, hydrocarbons are released due to complete burning and incomplete burning of fossil fuels and thereafter become substrates for generating nitrobenzene(s), nitrotoluene(s), and nitro-polyaromatic hydrocarbons (nitro-PAHs) after nitration with nitrogen dioxide (Ju and Parales 2010). In aquatic environment, nitration and halogenations are sunlight catalyzed with the formation of 2- and 4-nitrophenol, chlorophenol, and bromophenol (Ju and Parales 2010).

Currently, two mechanisms are known for the production of biogenic nitroaromatic compounds (NACs). Oxygenases and haloperoxidases (under unnatural/stress) are known to catalyze the addition of nitro moieties to aromatic compounds. The other mechanism of formation of biogenic nitroaromatic compounds is via an electrophilic interaction of a nitronium cation that may directly help in attaching a nitro group to the aromatic ring (Ju and Parales 2010). NACs are also biologically active metabolites existing in plants and fungal species (e.g., alkaloids) although the reason for their presence is largely unknown. Table 12.1 includes some of the naturally and artificially produced NACs and their applications in various areas.

Man-made or synthetic NACs include picric acid, TNT, lidocaine, and dinoseb and are primarily produced by nitration at *para*, *meta*, and/or *ortho* positions of the aromatic ring. The Zincke nitration is where sodium nitrite reacts with phenols to replace Br with a NO_2 group (Raiford and LeRosen 1944), and Wolfenstein-Böters reaction is where benzene can be converted to 1,3,5-trinitrobenzene (Davis et al. 1921). TNT was generally synthesized via sequential nitrification of toluene (Ju and Parales 2010). Aromatic amines, e.g., anilines used by the chemical industry, are manufactured by catalytically reducing NACs (Ju and Parales 2010).

12.2 Toxicity and Health Issues of Nitroaromatic Compounds

Several of the NACs are considered as “priority pollutants” and listed by the US Environmental Protection Agency (USEPA), and most of them are toxic and even mutagenic and capable of causing cancers on long exposures. The properties of NACs that are preferable for use as pesticides or other industrial application make them dangerous to mankind and animals. Ames test using *Salmonella* and *E. coli* tester strains has been popularly used to detect the potential of NACs to cause mutation that consequently leads to DNA damage through deletions, transversions, and transitions (Purohit and Basu 2000).

Several nitroaromatic compounds pose a threat to the environment and to all living beings, e.g., benzene, naphthalene, polycyclic aromatic hydrocarbons, and biphenyls (Kovacic and Somanathan 2014). The toxicity is mainly due to formation

Table 12.1 List of synthetic or naturally occurring nitroaromatic and chloro-nitroaromatic compounds, their applications, and effects on human beings

Name of compound	Application	Natural/ synthetic	Effects
TNT, DNT (tri-, dinitrotoluene)	Explosive	Synthetic	Adverse
RDX (cyclotrimethylenetrinitramine)	Explosive	Synthetic	Adverse
HMX (cyclotetramethylenetetranitramine)	Explosive	Synthetic	Adverse
p-Nitrophenol	Tannery	Natural	Mineralized by microbes but otherwise adverse effect
4-Nitrocatechol	Useful metabolic marker for the presence of functional cytochrome P450 2E1 in mammalian cell microsomes	Natural	Mineralized by microbes but otherwise adverse effect
Nitrobenzoates	Dye industry	Synthetic	Allergic skin reaction, eye damage
3-Methyl-4-nitrophenol, *m*-nitrophenol	Diesel exhaust particles, pesticides	Synthetic	Adverse
Nitrobenzene or halonitrobenzenes	Pharmaceuticals	Synthetic	Cancer inducing
Chloronitrobenzenes	Precursor for useful compounds	Synthetic	Hazardous
Lidocaine	Local anesthetic	Synthetic	Useful
Anilines	Drugs, rubber, polyurethane foams, azo dyes, photographic chemicals, varnishes	Synthetic	Disorientation, dizziness
4-Nitropyrene	Diesel and gasoline engine exhausts	Natural	Carcinogenicity
p-Nitrochlorobenzene	Pesticides and dyes	Synthetic	Coughing and wheezing
1-Nitronaphthalene	Industrially important chemical	Synthetic	Not significant
Chloramphenicol	Antibiotic	Natural	Beneficial to human

of electron transfer, reactive oxygen species, and oxidative stress. Different classes of nitroaromatic compounds are known to affect human population via various mechanisms, and this has been briefly described below.

12.2.1 Nitrobenzenes

Nitrobenzenes, commonly used as pesticides, drugs, ammunition, or explosives, intermediates of chemical synthesis of industrial products, are known to be potential

carcinogens due to nitro group reduction via two or more mechanisms leading to formation of reactive oxygen species or causing oxidative stress. When mice were exposed for a long time (2 years) to *o*-nitrotoluene, alterations in ras, p53, and *β*-catenin genes were observed in hemangiosarcomas leading to mutagenesis (Hong et al. 2003). The toxicity of 2,4,6-trinitrotoluene (TNT), a well-known explosive, covalently binds proteins and DNA in its reduced forms and also perturbs enzymatic redox cycling and/or serves as redox-cycling substrates for single ET as shown experimentally (Šarlauskas et al. 2004). Upon chronic exposure to TNT, there has been DNA damage in testes in rats and reduced semen secretion in Chinese workers (Homma-Takeda et al. 2002; Li et al. 1993)

12.2.2 Nitrobenzanthrones (NBA)

Nitrobenzanthrones (NBA), consisting of four fused aromatic rings, e.g., 3-nitrobenzanthrone (3-NBA), occur in diesel exhausts and in airborne particles that exhibit significant mutagenic activity and serve as potent carcinogens, which cause tumors in the lungs of rodents and cause damage via H_2O_2 formation in human cells (Murata et al. 2006).

4-Nitrobiphenyl (NBP) is a bladder carcinogen of dogs and has mutagenic properties. When enzymatically reduced, the intermediate *N*-hydroxylaminobiphenyl is also reported as a mutagen, and the major product 4-aminobiphenyl (ABP) is a bladder carcinogen (Culp et al. 1997; Kovacic and Somanathan 2014).

Nitrated derivatives of polyaromatic hydrocarbons (PAHs) are reported as airborne pollutants (e.g., 1-nitropyrene and 1,3- and 1,8-dinitropyrene) that mainly arise from combustion of diesel in engines. Diesel engine emission poses as an important contaminant and the exhaust releases particles that are potent air pollutants. Oxidative attack of some nitroaromatic molecules on DNA and formation of DNA adduct molecules are important in cancer formation, for example, 3-NBA and nitropyrenes (Kovacic and Somanathan 2014).

12.3 Degradation of Nitroaromatics

Enormous use of NACs in explosives, dyes, agricides, etc. and their release in the environment via groundwater, soil, and streams/water bodies have flamed up strong criticism and aroused concerns due to their potential health hazards. The costs for conventional cleanup have been estimated to be enormous and might not be sustainable and environment friendly either, and therefore, biological means of cleanup are being considered and researched upon. Nitroaromatic compounds are comparatively more recalcitrant to biodegradation than their analogs, which are not nitrated (Alexander and Lustigman 1966). To understand biodegradation, a few concepts are very important, i.e., **mineralization**, **co-metabolism,** and **transformation**. *Mineralization* (complete degradation) refers to catabolism of the pollutant/substrate to its elements and is the preferred over the other types of degradation for developing bioremediation technologies. Once a compound is mineralized, it yields energy in the biological system and

may be incorporated into various biomolecules to increase cellular biomass (Alexander 1981). Since energy is generated during the catabolism of NACs, the reaction is a self-sustainable process and proceeds continuously when the contaminant exists in proper concentration, also providing a selective pressure to promote the proliferation and growth of the degrading organism over others. In contrast to mineralization, there is another process called *co-metabolism*, where enzymes involved in the breakdown of some growth-inducing substrate (primary substrate) nonspecifically transform another contaminant/substrate (Alexander 1981). Few co-metabolized compounds may provide nutrition and energy, but only as long as the primary substrate exists and therefore, (i) need for some primary contaminant and (ii) lack of selective pressure created by primary substrate,entails co-metabolic bioremediation more expensive and labor intensiveas compared to mineralization. The stark difference between mineralization (complete degradation) and *transformation* is the difference in the products, which in the former case are harmless minerals and biomass, whereas, in the latter case, the products are essentially organic derivatives of the contaminant that may be more toxic than before or nontoxic (preferred for bioremediation). Polynitroaromatic compounds are partially biodegraded or transformed to generate amino-nitro products and are not mineralized further (Kaplan 1992); however, para-nitrophenol is degraded by several bacteria and is easily mineralized (Prakash et al. 1996;

Samanta et al. 2000; Chauhan et al. 2000; Pandey et al. 2002). Table 12.1 lists nitroaromatics that undergo mineralization, co-metabolization, or transformation via biological agents.

12.3.1 Aerobic or Anaerobic Degradation Pathways

Microbes evolved various strategies for dispensing nitro group(s) during conversion of NACs to simplified forms. Nitro group(s) present in the compound may be converted to NO_2^- after dioxygenation of its aromatic ring to an intermediate (dihydroxy compound) and monooxygenation to another intermediate (epoxide and/or hydride-Meisenheimer complex) (Nishino et al. 2000). Partial reduction to hydroxylaminobenzenes generates ammonia. The hydroxylaminobenzenes are processed by mutases and rearranged to *o*-aminophenols by few microbes. Alternately, hydroxylamino intermediate(s) may be transformed to catechol upon the release of the ammonium moiety. Few common interpretations about biodegradation of different types of NACs have been outlined below:

Mononitrophenols, e.g., (2-nitrophenol (2NP), 4-nitrophenol (4NP), 4-chloro-2-nitrophenol (4C2NP)) get hydroxylated to replace the NO_2 group consequently releasing (NO_2^-) resulting in *ortho*−/*para*-dihydroxybenzene (Nishino et al. 2000) (Fig. 12.1). Few organ phosphate pesticides, such as parathion and methyl-parathion, are transformed to *p*-nitrophenol and then hydroxylated. *Para*-nitroanisole is *O*-demethylated to *p*-nitrophenol and then hydroxylated. **Flavoprotein monooxygenases** may be involved in monooxygenation and all the monooxygenation reactions in some cases giving rise toquinone(s) (Kadiyala and Spain 1998).

Sometimes, microbial enzymes catalyze **dioxygenation** reaction to produce dihydroxy intermediates, which further attacked dioxygenases as in 2-nitrotoluene

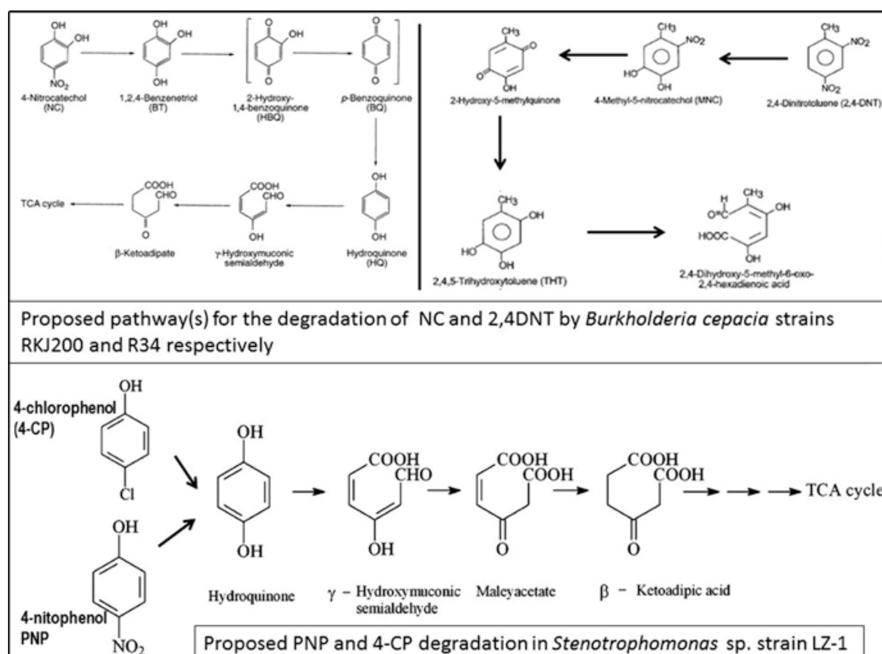

Fig. 12.1 The aerobic degradation of few NACs following various pathways. (Adapted from Chauhan et al. (2000), Johnson et al. (2002), and Liu et al. (2009))

(2NT), NB, 2,6-dinitrotoluene (2,6-DNT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrophenol, 1,3-dinitrobenzene, and 3-nitrobenzoic acid (3-NBA) (Nishino et al. 2000). The **dihydroxy-nitro cyclohexadienes** (intermediates) formed upon dioxygenase activity of nitro groups are unstable and re-aromatize after elimination of nitrites to produce **catechols**. Microbes aerobically convert 2,4-dinitrophenol and 2,4,6-trinitrophenol to unstable hydride-Meisenheimer complex and release the first nitrite (Nishino et al. 2000; Behrend and Heesche-Wagner 1999) (Fig. 12.1).

Anaerobic pathways include microaerobic or partial aerobic conditions. *Rhodobacter capsulatus* converts 2,4-dinitrophenol to *o*-amino-*p*-nitrophenol under anaerobic and/or microaerobic environment in the presence of light using suitable nitro reductases. Subsequently, *o*-amino-*p*-nitrophenol is degraded via a constitutive activity requiring light, O_2, and other sources of carbon and nitrogen. Nitro group is partially reduced to NO_2^- via the hydroxylamino derivative following a well-known chemical reaction.

Hydroxylamino moieties may be transformed by **hydroxylaminolyase** to corresponding **catechols** and dissemination of ammonium moiety. **Mutase**s catalyze the intramolecular rearrangement of hydroxylaminophenol to *o*-aminophenol, e.g., 2-chloro-5-nitrophenol, *p*-chloronitrobenzene, and *m*-nitrophenol (Nishino et al. 2000; Blasco and Castillo 1997; Meulenberg and de Bont 1995).

12.3.2 Anaerobic-Aerobic Dual Systems

Biodegradation of nitrobenzene has been carried out using a dual system consisting of aerobic microbes, followed by anaerobic ones (Dickel et al. 1993). Nitrobenzene may be completely degraded by aerobic microbial processes, but there are problems that may be ameliorated via an anaerobic process by reducing nitrobenzene to aniline and subsequently converting it via aerobic reactions. Anaerobic phase would be using glucose as carbon source (C source) and hydrogen donor. For the intermediates released during TNT biodegradation, a similar two-stage system has been tried (Fig. 12.2). Hydroxytoluenes or amino toluenes may be eliminated quickly under aerobic conditions (Funk et al. 1993; Rieger and Knackmuss 1995).

12.3.3 Fungal Degradation, Phytoremediation, and Composting of NACs

The degradation mechanism of several microorganisms is based on the breakdown of any aromatic nitro group to an amino moiety. However, white-rot fungi produce extracellular ligninolytic enzymes that oxidatively transform and/or mineralize xenobiotics such as TNT, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), chlorinated phenols, and pesticides, e.g., DDT. This process is an

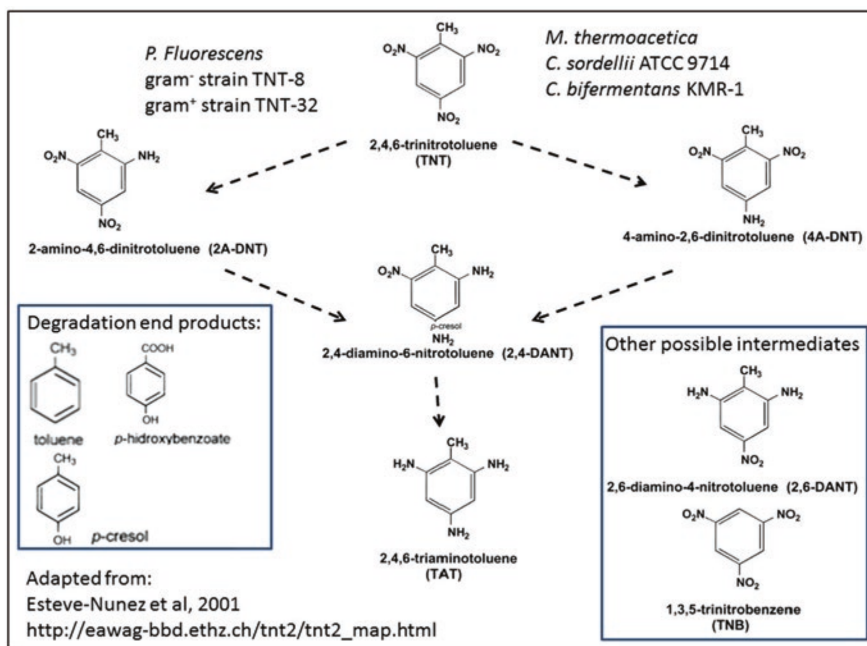

Fig. 12.2 Anaerobic degradation of TNT following various pathways

example of co-metabolism where carbohydrates are utilized as growth-promoting substrates for the fungi. Therefore, white-rot fungi have been fruitfully used as tools for bioremediation of persistent toxicants from contaminated sites. TNT and related compounds have been successfully treated by *Phanerochaete chrysosporium* (syn. *Sporotrichum pulverulentum*) and is a favorite model for research (Alexander and Lustigman 1966; Alexander 1981; An et al. 1994).

TNT is first reduced to mono-amino-dinitrotoluene and then transformed to azoxy, azo, and hydrazo intermediates. Primary metabolites (Hydroxy-Azo-DiNitro Toluenes and Azo DiNitro Toluenes) are rearranged to aminophenols (Bamberger rearrangement). A condensation reaction of nitroso and hydroxylamino intermediates generates azoxy compounds. Other researchers have proposed a combination of fungi with bacteria in bioremediation systems in which the fungi detoxify or modify the xenobiotic compound such that the bacterial population that may be mineralized by bacteria (Barr and Aust 1994).

The rapid disappearance of contaminants, e.g., TNT in aquatic environment dominated by specific plant species, has provoked researchers to consider phytoremediation as an option for removal of NACs using water and terrestrial plant systems. The researchers focused on deciphering plant metabolism and learning about mechanisms for phytoremediation including remediation by microbe in the phyllosphere and rhizosphere and alterations made by the plant body rendering the

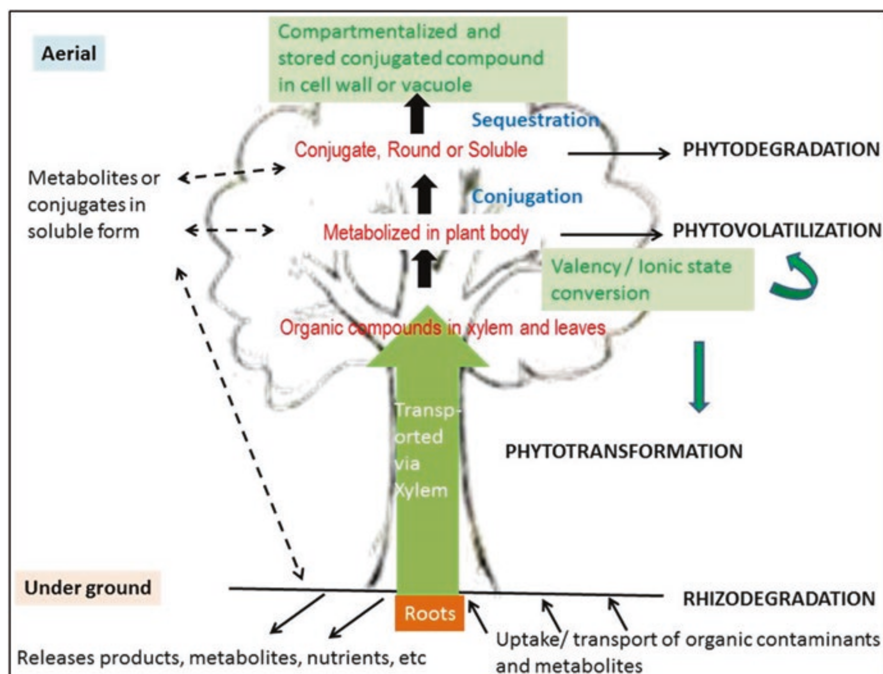

Fig. 12.3 The "green liver" model of **phytoremediation** for removal of xenobiotics in three stages: transformation, conjugation, and sequestration

environment conducive for decontamination (pH, redox changes, etc.). For certain cases, indirect mechanisms are more significant for decontamination, and therefore, one might use "plant-assisted" remediation. The green liver model (Fig. 12.3) is often used to correctly justifies the treatment of xenobiotics as, unlike microbes, plants do not exhibit a vast range of enzymatic pathways to break down given metabolites; instead, it recognizes foreign matter as toxins and degrades or transforms them, and this detoxification mechanism is common to the human liver; hence, the jargon "green liver" prevails (Klein and Scheunert 1982; Sandermann 1994). The theory was introduced because it was observed that plants exposed to herbicides readily metabolized it in three stages: transformation, conjugation, and sequestration (Fig. 12.3).

(a) Transformation: It includes reduction, oxidation, and hydrolysis reactions. Oxygenases or hydroxylases are involved.
(b) Conjugation: Compounds released after transformation are conjugated with plants' organic molecule (glucose, malonate), which leads to reduction in toxicity (Singh and Jain 2003).
(c) Sequestration: The storage of conjugate in plant organelles such as vacuoles, or are "bound" as xenobiotic conjugates and incorporated into biopolymers such as lignin where they are no longer capable of interfering with cell function.

Composting (humification) has been of great interest for environmental waste management as it greatly reduces weight and volume and results in less hazardous and often a useful biofertilizer. In the case of NACs, ex situ physicochemical treatment of incineration has proven to be costly and undesirable although it is accepted in case of TNT contamination. However, the ex situ technique of composting at lab and pilot scale has been successfully applied to various contaminants and xenobiotics (herbicides, chlorophenols, etc.). Synthetic musk fragrances (fragrances in cosmetics, soaps, lotions, washing powder, etc.) contain NACs that have significant environmental consequences and are classified as persistent organic pollutants (POPs), e.g., musk xylene, which is similar to TNT, and musk ketone, which is similar to 2,4-DNT. The presence of such toxicants in human milk, adipose tissue, and food items has been alarmingly recognized especially as musk xylene is suspected to be a carcinogen. They accumulate in agricultural systems via sewage sludge, so controlled composting is believed to be a possible treatment for decontamination. Soil/sludge is mixed with degradable organic matter and sufficient moisture to start composting (Sandermann 1994; Williams et al. 1992). Composting proceeds via the following phases in which the microbial community also changes sequentially: mesophilic phase (up to 45 °C), thermophilic phase (45–60 °C, <80 °C), cooling phase (45–20 °C), and maturation span (~20 °C). A highly diverse microbial population is active during the mesophilic phase, followed by the second phase (shortest), in which spore-forming bacteria, e.g., *Bacillus* sp., and thermophilic fungi dominate and highest amount of degradation occurs during this phase. Finally, fungi emerge as dominant species because spores withstand high

temperatures and fungal enzymes degrade lignin-cellulosic parts to derive energy. Rise in temperature to about 80 °C deactivates microbial processes and inhibits composting (Garg et al. 1991, Griest et al. 1993). Important factors influencing composting include aeration, pH, moisture, and C/N content of the substrate.

Although composting is an aerobic press, in the case of TNT contamination, anaerobic/aerobic composting systems have been shown to be highly efficient. Aerating the compost by injecting air or by turning over the compost pile favors composting. Structure of composting material mainly assists aeration and may sometimes create anaerobic micro-pockets. Light material, such as wood chips and straw, inhibits compression of the compost (Bruns-Nagel et al. 2000).

The compost pH varies from 5.5 to 8.0; however, in case of anaerobic pre-phase, pH drops below 4.0 but returns to optimal range once aerobic treatment commences. The optimal moisture tolerated by bacteria in compost is ~50–60%. Moisture over this limit creates anaerobic conditions. The C/N ratio for an efficient composting process is 26–35.32 Higher N_2 concentrations give rise to ammonia that elevates pH, but lower nitrogen slows cellular growth and increases organic acids to elevate pH, and both conditions impede composting. Composting is of five major types: (1) in-vessel static piles, (2) static piles, (3) mechanically agitated in-vessel (MAIV) composting, (4) windrow composting, and (5) anaerobic/aerobic composting.

Different contaminants show varying rates of decomposition, e.g., some petroleum hydrocarbons can be mineralized, and some co-metabolized to less dangerous forms and are incorporated into humus. Doyle et al. (1986) tried to compost radiolabeled^{14} C-TNT,^{14} C-RDX,^{14} and C-HMX using different conditions and achieved varying degrees of degradation by increasing the amount of soil. Craig et al. (1995) showed better results on windrow composting of TNT, RDX, and HMX, proving that composting is successful for removing dangerous NACs from soil and may be successfully applied in case of significantly polluted soils because it may be used as an ex situ or in situ technique and not very cost-intensive or labor-intensive. On the other hand, phytoremediation may be applied to mildly polluted large areas that are abandoned due to pollution and can be used to reclaim them, although weather conditions and risks due to introduction of non-native species persist. This technique is not cost- or labor-intensive like composting, unlike using bioreactors for treatment.

12.4 Evolution of New Pathways Via Genetic Changes

Although synthetic NACs have been introduced in nature for a short period of time, bacteria capable of breaking down these chemicals have been isolated from polluted sites suggesting that they are capable of adaptation by evolving new enzymes and pathways to endure and survive the selective stress created by the contaminants (Kivisaar 2009). The contaminated sites are therefore good resources to fish out strains with excellent properties for bioremediation.

It has been observed that genes encoding enzymes for catabolizing aromatic compounds may be associated with transposons carried by plasmids showing conjugation and genomic islands, to promote dissemination via horizontal transfer (Nojiri et al. 2004; Juhas et al. 2009). Bacteria oppose stress of starvation, desiccation, and unsuitable pH and/or temperature, where stress and starvation lead to (1) genetic changes and further transposition of mobile elements (Kivisaar 2003; Robleto et al. 2007) and (2) errors in DNA synthesis and mechanism of duplication (Kivisaar 2009; Tark et al. 2005).

Error-prone DNA polymerase or homologues of Pol-V may be coded by naturally occurring conjugative plasmids, and they may also have a contribution for manifestation of bacterial metabolic diversity (Permina et al. 2002; Tark et al. 2005). Few NACs, e.g., nitrobenzene, dinitrotoluenes, and nitrophenols, are powerful carcinogens (Kulkarni and Chaudhari 2007; Kivisaar 2009) and have the potential to induce mutations at higher concentrations (badly contaminated environment).

Bacterial evolution to use NACs as nutrients is exemplified by the 2,4-dinitrotoluene (2,4-DNT) pathway of *Burkholderia cepacia* R34. In this organism, the sequence of genes contained in the 27-kb DNA segment coding for 2,4-DNT pathway indicates that pathway gene(s) depict three points of ancestry (Johnson et al. 2002): (a) initial dioxygenase (DNT dioxygenase), catalyzing the first denitrification of the intermediate, that may have originated from naphthalene catabolic pathway; (b) second denitrification (by 4-methyl-5-nitrocatechol monooxygenase) from chloroaromatic degradation pathway; (c) third gene from amino acid pathway. Open reading frames having unknown function in 2,4-DNT degradation in *Burkholderia cepacia* R34 and the presence of several point mutations and transposon in the region advocate an intermediate phase during evolution of the pathway by incorporating genetic material with divergent ancestry (Johnson et al. 2002; Kivisaar 2009) via horizontal transfer and movement of transposable elements.

In a recently hypothesized concept about evolution depending on the proximity of proteins (O'Loughlin et al. 2006; Tokuriki and Tawfik 2009), it is suggested that protein evolves by directed evolution to perform a new role via "enzyme promiscuity" resulting in unique enzymes that break down newly introduced synthetic compounds (Aharoni et al. 2005; Afriat et al. 2006). The concept of "promiscuity" also plays significant roles during the development of novel regulators that respond to effectors (Cases and de Lorenzo 2005). XylR, a transcriptional activator in the toluene degradation pathway, is encoded by TOL plasmid pWW0 and acquires a new role by responding to both 2,4-DNT and its monosubstituted precursor molecules and to the dissimilar isomeric compound *m*-nitrotoluene and various chlorophenols (Galvão et al. 2007). The mutations leading to such a change were based on amino acid substitutions at the surface of proteins leading to conformational shifts affecting binding of effector and modulating transmission of signal between XylR domains (Galvão et al. 2007).

Ju et al. (2009) demonstrated that the functioning of the regulator NtdR (nitrotoluene responsive) was contributed by five mutations in NagR-like ancestor regulator. NtdR and NagR differed by 5 amino acids, located at position numbers 74, 169,

189, 227, and 232 (Lessner et al. 2003); however, NagR recognized 5 out of the 63 tested compounds, namely, salicylate, gentisate, 4-hydroxybenzoate, 4-isopropylbenzoate, and methyl salicylate, and, especially, does not interact with the NACs. On the other hand, NtdR, could activate 2-nitrotoluene degradation pathway genes in the presence of NACs, and a broad range of aromatic acids and their analogues, that may not be metabolized by *Acidovorax* sp. strain JS42 (exhibiting 2-nitrotoluene pathway).

12.5 Bacterial Metabolism of Chloro-Nitroaromatics

Chlorinated nitroaromatic compounds (CNAs) such as chloronitrobenzenes, chloronitrophenols, and chloronitrobenzoic acids enter our environment via agricultural practices, industrial discharges, or improper waste disposal and are known to be hematotoxic, immunotoxic, splenotoxic, genotoxic, hepatotoxic, nephrotoxic, and carcinogenic (Travlos et al. 1996; Li et al. 1999). 4-Chloronitrobenzene undergoes three types of transformation in mammals: (a) nitro group reduction, (b) glutathione conjugation for chloride displacement, and (c) hydroxylation of ring. The toxic nature renders many of the CNAs as priority pollutants as listed by the USEPA.

There are several methods including advanced oxidation processes (AOPs) to remove CNAs from industrial wastewater, which relies on nonspecific oxidation by hydroxyl radical (OH∗) (Vilhunen and Sillanpää 2010). AOPs also treat wastewater using UV rays, H_2O_2, UV-H_2O_2, photo-Fenton, ozonation, catalytic ozonation (Vilhunen and Sillanpää 2010), and the combination of these techniques. However, the main drawback of physicochemical techniques is their unsuitability for in situ biological application, and they are not cost-effective. CNA degradation is significantly affected by the position of chloro- and nitro-substitution on the benzene ring. The compounds, which have nitro groups at the *ortho* or *meta* positions, are predicted to be more difficult to degrade, as compared to the compounds having nitro groups at *para* positions (Arora et al. 2012). 4C2NP is more difficult to degrade than 2-chloro-4-nitrophenol (2C4NP) (Arora et al. 2012). Bacteria that utilize 2C4NP could not catabolize 4C2NP due to altered position of Cl and NO_2 groups (Pandey et al. 2011). The underlying reason for this phenomenon is that the enzymes, which catalyze reaction at the *para* positions, do not function at the *meta* or *ortho* position and vice versa (Arora et al. 2012). Degradation of CNAs with Cl and NO_2 moieties at different positions is acted upon by specified set of enzymes. The nitro group makes the benzene ring more recalcitrant than chloro group (Arora et al. 2012).

The **enzymes** mediating 2C4NP degradation in strain OCNB-1 were determined to be *aniline dioxygenase*, *nitrobenzene reductase*, and *catechol-1,2-dioxygenase*. A 100-kb plasmid carried gene(s) for degrading 4C2NP in *P. putida* strain ZWL73 and *Comamonas* sp. CNB-1. Some CNAs showed multiple routes of degradation, e.g., pentachloronitrobenzene (PCNB), which gives rise to metabolites with or without sulfur, many of which can be detected in soils contaminated with PCNB. Several reports on bioremediation of CNAs have proven the utility of rhizoremediation (*Comamonas* sp. CNB-1 associated with alfalfa roots), bioaugmentation (*P. putida*

ZWL73, *Rhodococcus imtechensis* RKJ300), and decontamination via membrane bioreactor followed by bacterial growth.

12.6 Bioavailability and Biodegradation via Microbes

Chemotaxis (chemo, chemical; taxis, movement) is the swimming of microbes with the aid of their flagella toward or away from a particular chemical. If microbial cells migrate toward a substance, the progression is called positive chemotaxis, while cellular motion in reverse direction is known as negative chemotaxis (Pandey and Jain 2002). Many microbial isolates have been applied for degrading NPs and CNPs, out of which few are capable of moving and show chemotaxis positive (Arora et al. 2012, 2014). Such strains are exemplified by *Burkholderia* sp. SJ98, which utilizes 4-nitrophenol, 2-chloro-4-nitrophenol, and 3-methyl-4-nitrophenol as the only C source and energy resource, and exhibited positive chemotaxis toward the above-said compounds (Bhushan et al. 2000, Samanta et al. 2000, Pandey et al. 2012). *Bacillus subtilis* PA-2 exhibited chemotaxis away from 4-chloro-2-nitrophenol, 4-nitrophenol, and 2,6-dichloro-4-nitrophenol (Arora et al. 2015).

The optimal environment for a bacterium is one in which energy generation is maximum, such as when there is a balance between the amount of electron donor and electron acceptor available (Taylor and Zhulin 1998). Since the chemoeffectors that attract bacteria (chemo-attractants) are often electron donors bacteria consume, metabolism of the chemo-attractant in the cell produces a gradient of electron acceptors to which the bacteria can also respond.

Positive chemotaxis helps microbes to sense a chemical that it can metabolize (either as nutrient) or co-metabolize by moving up a gradient of concentration of the particular chemical (here, NACs or CNAs) such that it avoids toxic levels and at the same time can continue feeding until the chemical persists in the environment. However, negative chemotaxis is a survival strategy shown when the organism feels engendered by toxic levels of the chemical in question and avoids it by moving away (Pandey et al. 2002, Arora et al. 2015).

The first step in bioremediation is the bioavailability of the substrate to the microbes, expedited by positive chemotaxis. Non-bioavailability or unavailability of organic pollutants for microbial cells significantly limits the efficiency of bioremediation of contaminated areas (Head 1998; Stelmack et al. 1999). Soil from contaminated sites possesses a separate or nonaqueous-phase liquid such as drops or liquid films on the surface of soil. Biodegradation readily occurs when target substrates are soluble in any aqueous phase (Stelmack et al. 1999, Pandey and Jain 2002; Law and Aitken 2003). Several pollutants, specially hydrophobic compounds, are nearly insoluble and persist superficially adsorbed on the nonaqueous-phase liquid (Head 1998; Stelmack et al. 1999; Parales and Haddock 2004). For onset and progress of biodegradation, bacteria must be able to access target compounds, by either dissolving it in liquid phase or by adhering directly to the interface of water and nonaqueous-phase liquid. For gaining access to adsorbed substances, pollutant-degrading bacteria may attach to surfaces possibly by forming biofilms.

12.6.1 Response of Microbes

Molecular mechanisms behind chemotaxis have been explained by three routes: (a) Signaling by chemoreceptors across the cell membrane (transmembrane) is the superior-most method acquired by bacteria where a ligand binds an external domain of a particular receptor that spans the membrane, thereby producing a signal to modulate the activity of kinase inside the cell, which results in chemotaxis (Pandey et al. 2002; Falke and Hazelbauer 2001). This transmembrane signaling mechanism does not depend on catabolism of the pollutant or its non-catabolizable analogues. (b) Chemotaxis therefore only depends on alteration in the cells' energy affected by complete metabolism of substrate (Alexandre and Zhulin 2001). This chemotactic response is referred to as metabolism dependent, and it is observed in many microbes, e.g., *Escherichia coli*, *Rhodobacter sphaeroides*, and *Azospirillum brasilense* (Pandey et al. 2002; Alexandre et al. 2000).

(c) The third mechanism suggests that chemotaxis signals are generated in collaboration with transport of effectors into cells. For example, chemotactic movement of *Bacillus subtilis* and *E. coli* toward carbohydrates and sugars is attached to the transport of the chemo-attractant. In *P. putida*, the gene pcaK codes for an unessential transporter protein for carrying 4-hydroxybenzoate and is essential for chemotaxis. Similarly, a permease protein coded by tfdK enables *Ralstonia eutropha* JMP123 to move toward very low levels of 2,4-dichlorophenoxyacetate (2,4-D); however, in this case, this gene is not responsible for entering the cells (Hawkins and Harwood 2002; Harwood et al. 1994).

12.7 Chemotaxis in Semisolid Medium TNT Chemotaxis Recent

There are several methods to study the chemotaxis of bacteria toward and away from the cells. The most commonly describes ones are (a) drop plate assay, (b) swarm plate assay, and (c) capillary assay.

To study negative chemotaxis, researchers have used various techniques, namely, (1) the chemical-in-plug method, (2) chemical-in-pond method, (3) chemical-in-plate method, (4) test-tube method, and (5) high-throughput micro-well method. To demonstrate negative chemotaxis, the chemical-in-plug method is widely acceptable (Arora et al. 2015; Tso and Adler 1974).

Here, we describe the most prevalent techniques with suitable diagrammatic representations.

For drop plate assay, degradative microbes would be cultured on a suitable medium trypticase soy broth, collected at middle logarithmic phase and resuspended in minimal medium containing 0.3% Bacto agar to prepare petri plates. Some crystals of NACs/CNAs placed in the center of the petri plate would act as chemo-repellant, and the samples should be incubated at 30°C. The response should be observed after 6 h of incubation. Chemotaxis would be suggested by the ring

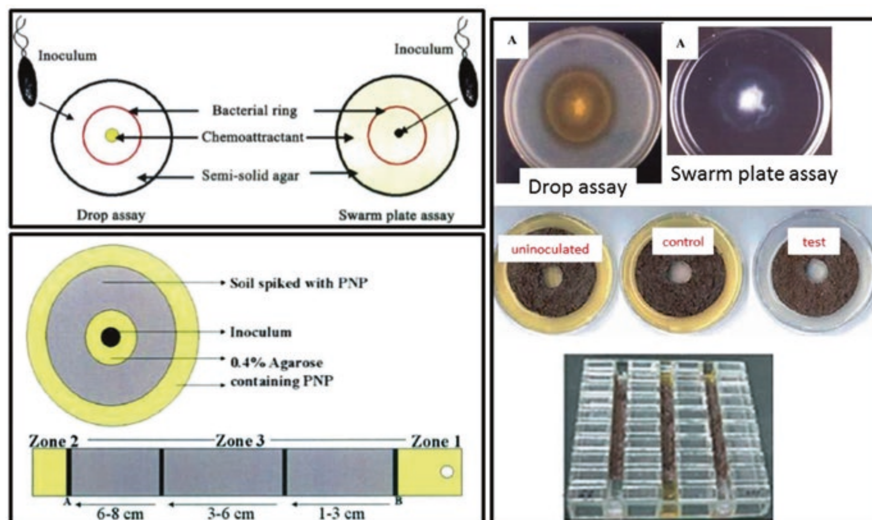

Fig. 12.4 Assays of chemotaxis (diagrammatic and actual experiment) against coloured nitroaromatic compounds in semisolid medium (drop and swarm plate assay) and in soil using plate and tray assays (Adapted from: Pandey et al. 2002; Paul et al. 2006)

formation (clearing zone) around the crystal as cells move away from it; heat-killed cells of the same strain may be used as control.

To perform swarm plate assay, *p*-nitrophenol (conc. 0.3 mM) should be dissolved in swarm plate agar medium (minimal medium having 0.16% Bacto agar) prior to casting plates. Around 75–100 μL induced and washed cells suspended in minimal medium should be gently poured in a petri dish and incubated at around 25 °C. One millimolar glucose may be provided to the cell in suspension for energizing them. Ring development was viewed 12–16 h after incubation (Fig. 12.4).

To perform capillary assays, special capillary tubes (Drummond Scientific, USA) of 1 μL volumetric capacity should be used. The appropriate NAC should be added to chemotaxis buffer consisting of 100 mM pot. Phosphate buffer (pH 7.2) and 20 mM EDTA to attain concentrations of 10, 20, and 200 mM. Aspartate may be used as positive substrate control. Capillaries loaded with the above were inserted in cell suspension (10^6–10^7 cells/mL in buffer for chemotaxis) taken on a shallow cover glass. After 30 min incubation time, cell suspension from the capillaries was diluted serially and plated on nutrient agar (NA). Bacterial colonies obtained on the plates were counted after overnight cultivation at 30 °C. Suitable positive and negative controls were maintained. Chemotaxis index (CI) was calculated as follows:

Number of bacterial cells accumulated in the test capillary containing NAC divided by that of the control.

12.7.1 Negative Chemotaxis Assays

1. Chemical-in-capillary method. Positive chemotaxis is assayed by inserting a capillary that was previously dipped in a solution of chemo-attractant into a suspension (the "pond") of bacterial cells that are motile. Microbes move toward the capillary and enter through it. After a given time, the total number of bacteria entered into the capillary is detected by counting colony-forming units (CFUs). If the chemical is inert, some bacteria nevertheless enter the capillary owing to random motility of the bacteria This is the "background" number and is deducted from reading as noise. If the given compound behaves as a chemo-repellent, fewer than this "background" number will be present inside the capillary. This capillary method is helpful in determining chemo-attractants and/or repellents, although repelling effects may be too insignificant for detection. Inhibition of motility might end up giving the same result as negative chemotaxis; however, this may be cross-checked via "motility assay."
2. Chemical-in-pond method. The repellent is present in the pond of bacteria, and none is put into the capillary. The bacteria then escape into the capillary for "refuge," and the number accumulated in the capillary is determined as before. Without any repellent, some bacteria enter the capillary by random swimming; with repellent, the accumulation is larger, and hence, this assay provides a positive result. (The values are never as strikingly above background as for positive chemotaxis in the chemical-in-capillary method.) There is a threshold value for repulsion. At high concentrations, loss of motility or viability, or saturation of the chemotactic apparatus. The negative chemotactic assay against 4C2NP was reported by Arora et al. (2015) (using *Bacillus subtilis* strain PA2) using chemical-in-plug method. For this bacteria, suspension was prepared as described before (10^6–10^7 cells/mL chemotaxis buffer). The bacterial solution was poured around hard agar plugs composed of minimal media, 2% Bacto agar, and 4C2NP (100 mM) or 4NP (100 mM) or dichloro nitrophenol (DCNP) (100 mM). After solidifying, the plates were incubated at 30∘C for 6 h, at which time they were evaluated for chemotactic response.

A convenient agarose-in-plug bridge method was used to demonstrate chemotaxis in the Archaeon *Halobacterium salinarum* (Yu and Alam 1997). Hot liquid agarose solution with chemo-effectors was placed in the center of a microscope slide with a bridge that is built by using two plastic strips 16 mm apart. A coverslip should be placed on the molten agarose immediately. The agarose plug gets encircled quickly by the bacterial cell suspension. This method has been tried for amino acids, but not for NACs.

Leungsakul et al. (2005) observed the chemotaxis toward 2,4,6-trinitrotoluene (TNT), 2,5-DNT, 2,3-dinitrotoluene (2,3-DNT), 2,6-DNT, 2,4-DNT, 2-nitrotoluene (2NT), 4NT, and 4-methyl-5-nitrocatechol (4-M-5NC). They used drop assay for their studies and found that, although there are cases where the substrate is not the source of C and nitrogen for the organism, they still can be a chemo-attractant. For example, 2,4-DNT in case of *B. cepacia* R34 and *Burkholderia* sp. DNT is the C

and nitrogen source, but not a good chemo-attractant as 2,5-DNT (not a C/N source). Also TNT, 2,3-DNT, 2,5-DNT, 2NT, and 4NT are chemo-attractants, but not carbon and energy sources. This implies that the chemotactic machinery of the above strains works for other NACs as well apart from the compounds that they degrade or serve as intermediates in degradation pathway. The results also suggest that degradation pathway gene(s) are not associated with chemotaxis, so their presence/absence has no impact.

Organophosphates (OP) degrading *Pseudomonas* BUR11 isolated from an agricultural site utilized parathion and chlorpyrifos or their intermediates as sole sources of carbon along with being positively chemotactic toward them (Pailan and Saha 2015).

12.7.2 Chemotaxis Through Soil

Chemotaxis of *Ralstonia* sp. SJ98 toward *p*-nitrophenol was demonstrated in laboratory using various assays in semisolid medium; two assays were designed for demonstrating chemotaxis in soil microcosm, i.e., a small-scale qualitative assay in petri plate and another assay was done using a specially designed tray giving quantifiable results on a larger scale. For such experimental strategies, bacterial suspension was prepared in PO_4 buffer saline (PBS) in order to attain an approximated density of 10^{10} cells/mL.

1. Plate assay: Soil spiked with para-nitrophenol (PNP) was used to prepare concentric zones of soil and Bacto agar in a petri plate such that the organism travels radially out of the center, through the soil and agar zones (Fig. 12.4). Bright-yellow color of PNP changes to colorless indicating depletion of PNP. Positive and negative controls were maintained. Moisture content of soil was maintained at 40–50% of soil's water holding capacity (WHC) by sprinkling water whenever necessary.
2. Tray assay: For quantitative soil chemotaxis, a glass tray with three parallel lanes was fabricated, containing markings at 1 cm interval (Fig. 12.4). Glass stoppers prevented the mixing of agar and soil. As indicated in the diagram, the zones 1 and 2 constituted of 0.5 mM *p*-nitrophenol suspended in 0.4% semisolid Bacto agar and zone 3 had PNP-spiked soil in all three lanes. The first lane was inoculated with cell suspension of chemotaxis-positive strain *Ralstonia* sp. SJ98, second lane was inoculated with non-chemotactic *Burkholderia* sp. *strain* RKJ200, and the third lane was kept uninoculated. Both types of bacteria were capable of degrading PNP. The yellow color of PNP in zone 1 disappeared, indicating that bacteria travelled through agar zone and reached the soil.

CFUs of soil bacteria were determined by spreading proper dilutions of soil taken at different time points from each lane (Fig. 12.4). For determining residual PNP in samples collected from the start and end at various zones of the soil, high-performance liquid chromatography (HPLC) was performed by a method as reported earlier (Labana et al. 2005).

12.7.3 Application of Chemotaxis in Biofilms

Flagella are required for attaching cells to various surfaces and facilitating biofilm development (Pratt and Kolter 1999). In addition, biofilm-forming bacteria show chemotaxis to move along the surface to grow and spread (Stelmack et al. 1999). These biofilms may be useful for degradation of CNAs and NACs. For removal of 2,4-dichlorophenol (DCP) from wastewater, Kargey and Ekker made use of a perforated rotating tube biofilm reactor comprising of a mixed biomass of microbes originating from an activated sludge and was supplemented with DCP-degrading *P. putida*. It was observed that DCP was completely mineralized. Similarly, bacteria capable of adhering to polyaromatic hydrocarbons (PAHs) often expedite breakdown of PAHs (Wolfaardt et al. 1995). This phenomenon is exemplified by the two-ring herbicide called diclofop-methyl, methyl 2-[4-(2,4-dichlorophenoxy)phenoxy] pyruvate, which adsorbs onto biofilms formed by microbial exopolymers. The microbial community of the biofilm catabolized the diclofop-methyl during a period of starvation. Nitroaromatic compounds fall under another group of xenobiotics having multiple uses during the synthesis of pharmaceuticals, foams, pesticides, and explosives. Due to the nitro side group, these compounds are resistant to biodegradation; microbial transformation might lead to harmful metabolic intermediates (Lendenmann et al. 1998). Lendenmann et al. (1998) used a consortium that degraded a mixture of dinitrotoluene (DNT) using fluidized-bed biofilm reactor containing 2,4-DNT (40 mg/L) and 2,6-DNT (10 mg/L). Efficiency of degradation was more than 98% for 2,4-DNT and ~94% for 2,6-DNT. Degradation of 4,6-dinitro-ortho-cresol (an old synthetic pesticides) was reported using batch cultures in a fermenter called fixed-bed column reactor.

12.8 Microbial Diversity, Microbial Evolution, and Bioremediation Strategies

The unexplored "unculturable" microbial wealth holds a tremendous promise for various resources. About 1 g of soil might hold approximately 1000–10,000 unknown species belonging to prokaryotes, and further diversity is expected within each species (Torsvik and Ovreas 2002). Phylogenetically, the "unculturable" microorganisms may show some or 100% similarity to the culturable ones while possessing a unique physiological conformation making them recalcitrant to standard culturing techniques (Rondon et al. 1999). Another probability could be that the unyielding remaining microbial population might represent novel lineages phylogenetically dissimilar in nature and therefore cannot be grown via standard lab techniques (Rondon et al. 1999). Various techniques showed that significant dissimilarities were observed on comparing the community of contaminated to uncontaminated sites, especially with respect to unculturable organisms. In an example, experimental plots where oil was spilt (to mimic contamination) were compared to that of control sites (uncontaminated) via techniques such as phospholipid fatty acid

analysis (PLFA) along with denaturing gradient gel electrophoresis (DDGE) analysis (MacNaughton et al. 1999). These studies suggested that culturing-based method elucidated only small fractions of the entire soil microbial diversity. Therefore, soil metagenome continues to be an unexploited reservoir of novel gene(s) and/or gene cluster(s) for bioremediation and other applications. The biosynthetic diversity of microbes from different environments has been accessed using "metagenomics" where operons or genes responsible for the degradation of pollutants are acquired from the metagenome. Large-scale projects, e.g., http://www.tigr.org/tdb/MBMO/, led by "The Institute for Genomic Research" and "Monterey Bay Coastal Ocean Microbial Observatory" are databases that make metagenomic information available on many unexplored metabolic processes exhibited by microbes. Fang et al. (2014) used metagenomics study using six data sets (16 Gb data) to report diversity, abundance, and potential biodegradation genes (BDGs) and metabolic pathways of recalcitrant pollutants of freshwater and marine sediments, e.g., dichloro-diphenyl-trichloro-ethane (DDT), hexachloro-cyclo-hexane (HCH), and atrazine (ATZ). Nearly complete catabolic pathways for breakdown of DDT and ATZ were found in the sediments.

Such recent approaches help in constructing effective "designer biocatalysts" for various biotechnological applications including bioremediation. Exploring the microbial diversity leads to the elucidation of (a) effective or new pathways for improved catabolism of pollutants, (b) chemotactic or biosurfactant-producing strains for better or faster access and solubilization of sparingly soluble or "aged" chemicals for degradation, and (c) regulatory operons and gene(s) for construction of containment systems for regulated bioremediation. It is the responsibility of biotechnologists to understand the ethical responsibilities before application of novel techniques for biological applications. Also, there should be complete understanding of genetic modifications, and confinement of the introduced constructs should be ascertained before releasing designer bugs for bioremediation of contaminated sites.

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