BIOREMEDIATION OF NITROAROMATIC COMPOUNDS

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DECLARATION

I hereby declare that the work embodied in the dissertation entitled **"BIOREMEDIATION OF NITROAROMATIC COMPOUNDS"** is original piece of work and was conducted in the Center For Fire Explosives And Environment Safety (CFEES), Defence Research & Development Organization, Ministry Of Defence, Government of India(New Delhi) in co-operation with Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. The matter presented in this dissertation has not been submitted in part or full, to this or any other University/Institute for any other degree or diploma.

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CERTIFICATE

This is to certify that the dissertation entitled, "Bioremediation of Nitroaromatic Compounds", is an authentic work carried out by Apurva Goel student of M.Tech.(Environmental Sciences and Technology), Thapar University, Patiala, during the year 2011-2012, at Centre for Fire Explosives and Environment Safety(CFEES), Defence Research & Development Organization, New Delhi in partial fulfillment for the award of the Degree of Master of Technology and that the dissertation has not formed the basis for the award previously of any degree, associate ship, fellowship or any other similar title to any other university or institute.

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CHAPTER 1
INTRODUCTION

1.1 BACKGROUND INFORMATION

Nitro aromatic compounds are used worldwide as explosives, pesticides, and as precursors for the manufacture of many products, including dyes, pharmaceuticals, and plastics. These compounds do not only come from man-made sources; they also are formed by some natural processes, such as photochemical reactions in the atmosphere. These are produced by incomplete combustion of fossil fuel or nitration reactions and are used as chemical feedstock for synthesis of explosives, pesticides, herbicides, dyes, pharmaceuticals, etc. Nitro aromatic compounds are well known as toxins; some are mutagenic and/or carcinogenic, and others are uncouplers of cellular phosphorylation reactions. Because they are widely used, nitro aromatics have become serious environmental contaminants, stimulating considerable research over the years into both their fate in soil and water systems and their catabolism by pure microbial cultures.[Crawford, 1995] The indiscriminate use of nitro-aromatics in the past due to wide applications has resulted in inexorable environmental pollution. Hence, nitro-aromatics are recognized as recalcitrant and given Hazardous Rating-3. Although several conventional pump and treat clean up methods are currently in use for the removal of nitro-aromatics, none has proved to be sustainable.[Kulkarni, Chaudhari;2007]

Explosives and related compounds have become widely recognized as serious environmental contaminants. Among the nitro substituted aromatic compounds causing particular concern are 2,4,6-trinitrotoluene (TNT); Dinitrotoluene(DNT); 2,4,6-trinitrophenol (picric acid), and many nitro- and/or amino-substituted aromatics that result from the manufacture and transformation of explosives. The threat posed by the presence of these compounds in soil and water is the result of their toxicity and is compounded by their recalcitrance to biodegradation. Contamination by nitro aromatic compounds, especially TNT, stems primarily from military activities. During the manufacture of explosives and the disposal of old munitions, large quantities of water became contaminated. This wash water was typically disposed of in unlined lagoons that facilitated the slow release of the explosives from the soil in the lagoons into ground water, lakes, and rivers. The mutagenicity of TNT, as well as its toxic effects on algae and fish, humans, and other vertebrates, make it an environmental hazard. TNT has been listed as a priority pollutant by the U.S. Environmental Protection Agency.[Ju K.S et al.,2010]

Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. It will not always be suitable, however, as the range of contaminants on which it is effective is limited, the time scales involved are

relatively long, and the residual contaminant levels achievable may not always be appropriate. Although the methodologies employed are not technically very complex, considerable experience and expertise may be required to design and implement a successful bioremediation program, due to the need to thoroughly assess a site for suitability and to optimize conditions to achieve a satisfactory result[Vidali; 2001]

The remediation process consists of 4 elements: Identifying and correcting the source of contamination; Controlling the contaminated area to prevent spreading of microbes; Source removal of the microbes, contaminated dust, debris and other unwanted materials within the ventilation system; and Treating the affected areas with an antimicrobial chemical, if necessary.[NADCA; 2004]

Knowledge about the relationship between microbial community structure and hydro geochemistry (e.g., pollution, redox and degradation processes) in a site is required to develop tools for predicting and monitoring natural attenuation through microbes. The naturally occurring microbes secrete enzymes and these enzymes split the contaminant into molecules. Thus, each individual molecule is encircled by a ring of enzymes secreted by the microbes and the isolated molecules then become the source of nutrition for the microbes. As more and more molecules are encapsulated, more and more food is available for microbes, which then consume the contamination molecules.

But for the remediation and consumption of hazardous material by microbes in large quantities, huge volumes of enzymes have to be secreted. And this process is time consuming. This is the reason why the natural remediation process takes years to initiate and decades to end on field scale. Once the contaminants are totally consumed, the available food source diminishing, the microbes will die and their number will come back to original naturally balanced level. In this process, there is no net increase or decrease of naturally occurring microbe count. This process has been proven in numerous field applications containing various kinds of hazardous and other wastes.

Recently, remediation by biological systems(BIOREMEDIATION) has attracted worldwide attention to decontaminate nitro-aromatics polluted sources. The incredible versatility inherited in microbes has rendered these compounds as a part of the biogeochemical cycle. Several microbes catalyze mineralization and/or non-specific transformation of nitro-aromatics either by aerobic or anaerobic processes.

Aerobic degradation of nitro-aromatics applies mainly to mono-, dinitro-derivatives and to some extent to poly-nitro aromatics through oxygenation by: (i) mono oxygenase, (ii) dioxygenase catalyzed reactions, (iii) Meisenheimer complex formation, and (iv) partial reduction of aromatic ring. Under anaerobic conditions,

nitro-aromatics are reduced to amino-aromatics to facilitate complete mineralization. The nitro-aromatic explosives from contaminated sediments are effectively degraded at field scale using in situ bioremediation strategies, while ex situ techniques using whole cell/enzyme(s) immobilized on a suitable matrix/support are gaining acceptance for decontamination of nitro phenolic pesticides from soils at high chemical loading rates.[Kulkarni, Chaudhari;2007]

The optimal remediation strategy for nitro aromatic compounds depends on many site-specific factors. Composting and the use of reactor systems lend themselves to treating soils contaminated with high levels of explosives (e.g. at former ammunition production facilities, where areas with a high contamination level are common). Compared to composting systems, bioreactors have the major advantage of a short treatment time, but the disadvantage of being more labour intensive and more expensive. Studies indicate that biological treatment systems, which are based on the activity of the fungus Phanerochaete chrysosporium or on Pseudomonas sp. ST53, might be used as effective methods for the remediation of highly contaminated soil and water. [Zita et al;2002]

1.2 OBJECTIVES OF STUDY

The basic objectives of present study that is Bioremediation of Nitro aromatic compounds(Trinitrotoluene) :

a. **Physical, Chemical and Biological characterization of given Soil and wastewater samples from nitroaromatics contaminated sites.**

b. **HPLC analysis of wastewater samples from nitroaromatics contaminated sites.**

c. **Isolation and identification of bacteria from soil samples contaminated with nitroaromatic compounds. Potential of the isolated culture on various optimized conditions.**

d. **Bioremediation of TNT contaminated water samples(Laboratory prepared) with the help of the isolated bacterial culture from soil samples and Evaluating Bioremediation Potential of isolated microbe for treating TNT contaminated water samples through HPLC.**

e. **Evaluating growth pattern of isolated microbial colony from soil sample used for bioremediation of TNT contaminated water samples through Spectrophotometric technique.**

CHAPTER 2
REVIEW OF LITERATURE

BIOREMEDIATION

2.1 DEFINITION

Bioremediation refers to the productive use of biodegradative processes to remove or detoxify pollutants that have found their way into the environment and threaten public health, usually as contaminants of soil, water, or sediments. Also it is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. It will not always be suitable, however, as the range of contaminants on which it is effective is limited, the time scales involved are relatively long, and the residual contaminant levels achievable may not always be appropriate. Although the methodologies employed are technically complex, considerable experience and expertise may be required to design and implement a successful bioremediation program, due to the need to thoroughly assess a site for suitability and to optimize conditions to achieve a satisfactory result.

Technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are**:** phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting,bioaugmentation, rhizofiltr ation, and biostimulation.

Bioremediation can occur on its own (natural attenuation or intrinsic bioremediation) or can be spurred on via the addition of fertilizers to increase the bioavailability within the medium (biostimulation). Microorganisms used to perform the function of bioremediation are known as **bioremediators**.

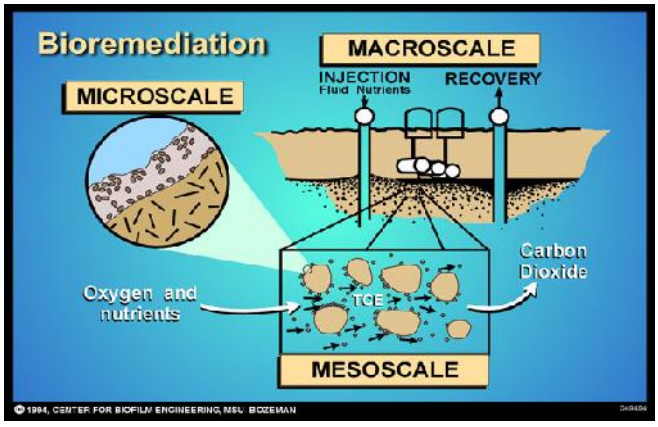


Fig 2.1 : Overview of Bioremediation process

Though biodegradation of wastes is a centuries-old technology, it is only in recent decades that serious attempts have been made to harness nature's biodegradative capabilities with the goal of large-scale

technological applications for effective and affordable environmental restoration. This development has required a combination of basic laboratory research to identify and characterize promising biological processes, pilot-scale development and testing of new bioremediation technologies, their acceptance by regulators and the public, and, ultimately, field application of these processes to confirm that they are effective, safe, and predictable. As shown in fig. 2.1, the micro-scale shows biological processes that is using microbes for remediation, Meso-scale shows pilot scale process and macro-scale shows testing of new bioremediation technologies on-site(field applications).

2.2 FACTORS FOR BIOREMEDIATION :

The control and optimization of bioremediation processes is a complex system of many factors. These factors include:

a. The existence of a microbial population capable of degrading the pollutants;

b. The availability of contaminants to the microbial population;

c. The environment factors (type of soil, temperature, and pH, the presence of oxygen or other electron acceptors, and nutrients). [Vidali, 2001]

2.2.1 Microbial Populations for Bioremediation Processes:

Microorganisms can be isolated from almost any environmental conditions. Microbes will adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen, and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirements are an energy source and a carbon source. Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards.

We can subdivide these microorganisms into the following groups:

***a. Aerobic*:** In the presence of oxygen. Examples of aerobic bacteria recognized for their degradative abilities are *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium*. These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy.

***b. Anaerobic*:** In the absence of oxygen. Anaerobic bacteria are not as frequently used as aerobic bacteria. There is an increasing interest in anaerobic bacteria used for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE), and chloroform.

***c. Ligninolytic fungi*:** Fungi such as the white rot fungus *Phanaerochaete chrysosporium* have the ability to degrade an extremely diverse range of persistent or toxic environmental pollutants. Common substrates used include straw, saw dust, or corn cobs.

d. Methylotrophs: Aerobic bacteria that grow utilizing methane for carbon and energy. The initial enzyme in the pathway for aerobic degradation, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds, including the chlorinated aliphatics trichloroethylene and 1,2-dichloroethane.

2.2.2 Availability of contaminants to the microbial population :

For degradation it is necessary that bacteria and the contaminants be in contact. This is not easily achieved, as neither the microbes nor contaminants are uniformly spread in the soil. Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving toward it. Other microbes such as fungi grow in a filamentous form toward the contaminant. It is possible to enhance the mobilization of the contaminant utilizing some surfactants such as sodium dodecyl sulphate (SDS).[Vidali,2001]

2.2.3 Environmental factors :

a. Nutrients

Although the microorganisms are present in contaminated soil, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated. Biostimulation usually involves the addition of nutrients and oxygen to help indigenous microorganisms. These nutrients are the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants. All of them will need nitrogen, phosphorous, and carbon (e.g., see Table 2.1). Carbon is the most basic element of living forms and is needed in greater quantities than other elements. In addition to hydrogen, oxygen, and nitrogen it constitutes about 95% of the weight of cells.

Element	**Percentage**	**Element**	**Percentage**
Carbon	50	Sodium	1
Nitrogen	14	Calcium	0.5
Oxygen	20	Magnesium	0.5
Hydrogen	8	Chloride	0.5
Phosphorus	3	Iron	0.2

Sulfur	1	All others	0.3
Potassium	1		

Table 2.1 : Composition of a microbial cell

Phosphorous and sulfur contribute with 70% of the remainders. The nutritional requirement of carbon to nitrogen ratio is 10:1, and carbon to phosphorous is 30:1.

b. Environmental requirements

Optimum environmental conditions for the degradation of contaminants are reported in Table 2.2

Parameters	**Conditions required for microbial activity**	**Optimum value for an oil degradation**
Soil moisture	25 – 28 % of water holding capacity	30 – 90 %
Soil pH	5.5 – 8.8	6.5 – 8.0
Oxygen Content	Aerobic , Minimum air – filled pore space of 10 %	10 – 40 %
Nutrient Content	N and P for micronial growth	C : N : P = 100 : 10 : 1
Temperature (in celsius)	15 – 45	20 – 30
Contaminants	Not too toxic	Hydrocarbon 5 – 100% of dry weight of soil
Heavy Metals	Total content 200 ppm	700 ppm
Type of soil	Low clay or silt content	

Table 2.2 : Environmental Conditions affecting degradation

Microbial growth and activity are readily affected by pH, temperature, and moisture. Although microorganisms have been also isolated in extreme conditions, most of them grow optimally over a narrow range, so that it is important to achieve optimal conditions. If the soil has too much acid it is possible to rinse the pH by adding lime.Temperature affects biochemical reactions rates, and the rates of many of them double for each 10 °C rise in temperature. Above a certain temperature, however, the cells die. Plastic covering can be used to enhance solar warming in late spring, summer, and autumn.

Available water is essential for all the living organisms, and irrigation is needed to achieve the optimal moisture level. The amount of available oxygen will determine whether the system is aerobic or anaerobic. Hydrocarbons are readily degraded under aerobic conditions, whereas chlorurate compounds are degraded only in anaerobic ones. To increase the oxygen amount in the soil it is possible to till or sparge air. In some cases, hydrogen peroxide or magnesium peroxide can be introduced in the environment. Soil structure controls the effective delivery of air, water, and nutrients. To improve soil structure, materials such as gypsum or organic matter can be applied. Low soil permeability can impede movement of water, nutrients, and oxygen; hence, soils with low permeability may not be appropriate for *in situ* clean-up techniques.[Vidali,2001]

2.3 FACTORS FOR EVALUATING SITE OF BIOREMEDIATION

There are at least five critical factors that should be considered when evaluating the use of bioremediation for site clean up. These factors are:

a. Magnitude, toxicity, and mobility of contaminants:

It is imperative that the site be properly investigated and characterized to determine the (a) horizontal and vertical extent of contamination; (b) the kinds and concentrations of

contaminants at the site; (c) the likely mobility of contaminants in the future, which depends in part on the geological characteristics of the site.

b. Proximity of human and environmental receptors:

Whether bioremediation is the appropriate cleanup remedy for a site is dependent on whether the rate and extent of contaminant degradation is sufficient to maintain low risks to human or environmental receptors.

c. Degradability of contaminants:

The biodegradability of a compound is generally high if the compound occurs naturally in the environment (e.g., petroleum hydrocarbons).Often, compounds with a high molecular weight, particularly those with complex ring structures and halogen substituents, degrade more slowly than simpler straight chain hydrocarbons or low molecular weight compounds. Whether synthetic compounds are metabolized by microorganisms is largely determined by whether the compound has structural features similar to naturally occurring compounds.

d. Planned site use:

A critical factor in deciding whether bioremediation is the appropriate cleanup remedy for a site is whether the rate and extent of contaminant degradation is sufficient to reduce risks to acceptable levels.

e. Ability to properly monitor:

There are inherent uncertainties in the use of bioremediation for contaminated soils and aquifers due to physical, chemical and biological heterogeneities of the contaminated matrix. It is important to recognize that biological processes are dynamic and, given current knowledge, often lack the predictability of more conventional remediation technologies. Thus, it is important to insure that unacceptable risks do not develop in the future. These risks may include migration of contaminants to previously uncontaminated media and the failure of bioremediation to achieve acceptable contaminant concentrations. The rate and extent of contaminant degradation by microorganisms. These can be broadly grouped into two classes of factors:

(a) Biological factors and

(b) Environmental factors.

The biological factors are primarily concerned with the numbers of specific kinds of microorganisms present and the expression and activity of metabolic enzymes, in other words, the amount of "catalyst" present. The environmental factors include chemical and physical characteristics that influence the bioavailability of contaminants, the availability of other nutrients, the activity of biological processes (temperature and pH, for example), characteristics of the contaminants with respect to how they interact with the site's geochemical and geological characteristics.

2.4 PRINCIPLES OF BIOREMEDIATIONS

As discussed above, factors of Bioremediation act as principle for Bioremediation to work successfully and in accordance with the requirements.

Microorganisms must be active and healthy in order for bioremediation to take place. Bioremediation technologies assist microorganisms' growth and increase microbial populations by creating optimum environmental conditions for them to detoxify the maximum amount of contaminants. The specific bioremediation technology used is determined by several factors, for instance, the type of microorganisms present, the site conditions, and the quantity and toxicity of contaminant chemicals. Different microorganisms degrade different types of compounds and survive under different conditions.[Martin A , 1994]

PRINCIPLES OF BIOREMEDIATION

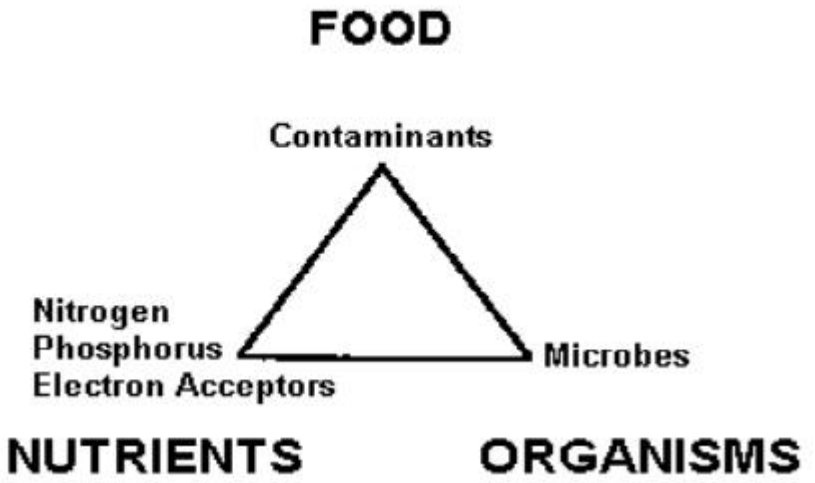


Figure 2.2 : Bioremediation Triangle

There are three essential components(factors) needed for bioremediation. These three components are microorganisms, food, and nutrients. These three main components shown in Figure 2.2 are known as the bioremediation triangle and forms basic principle of Bioremediation.

Microorganisms are found almost everywhere on earth with the exception of active volcanoes. So a lack of food and nutrients are usually the missing ingredients that prevent successful bioremediation. Microorganisms find the food they eat in the soil or water where they live. However, if a contaminant is present it can become an additional food source for the microorganisms.

The contaminant serves two useful purposes for the microbes. First, the contaminant provides a source of carbon needed for growth. Second, the microbes obtain energy by breaking chemical bonds and transferring electrons away from the contaminant. This is known as an oxidation-reduction reaction.

The contaminant that loses electrons is oxidized and the chemical that gains the electrons (electron acceptor) is reduced. The energy gained from the electron transfer is used along with the carbon and some electrons to produce more cells. Microbes generally use oxygen as an electron acceptor but nitrate, sulfate, iron, and CO_2 are also commonly used. The use of oxygen as an electron acceptor is called aerobic respiration. The major by products of aerobic respiration are carbon dioxide, water, and an increase in the microbe population.Anaerobic respiration uses nitrate, sulfate, iron, or CO2 as the electron acceptor instead of oxygen. Anaerobic respiration can occur after the oxygen has been depleted by aerobic respiration or where there is not sufficient oxygen in the first place. Table 2.3 shows several contaminants that can be degraded using this anaerobic respiration process.[Nathan F,1999]

Carbon Tetrachloride	Tetrachloroethylene
Chloroform	Phenols
Vinyl Chloride	Benzoates
DDD	Toluene
DDT	Ethylbenzene
TCE	Xylene
Tetrachlorethane	PCBs

Table 2.3: Compounds Degraded by Anaerobic Conditions

There are also several nutrients that must be accessible to the microorganisms for bioremediation to be successful. These include moisture, nitrogen, phosphorus, and other trace elements. Microorganisms like other organisms need moisture to survive and grow. In addition, microbes depend on the moisture to transport food to them since they do not have mouths. The optimal moisture content for microbes in the vadose zone has been determined to be between 10 and 25%. Besides moisture, nitrogen (ammonia) and phosphorus (orthophosphate) are two major nutrients needed for the microorganisms. The microorganisms also require minor elements such as sulfur, potassium, magnesium, calcium, manganese, iron, cobalt, copper, nickel, and zinc . However, these minor elements are usually available in the environment in sufficient amounts where nitrogen and phosphorus may be lacking and need to be added.[Nathan F,1999]

2.5 TYPES OF BIOREMEDIATION

Bioremediation can be broken into two main types:
Intrinsic and Engineered.

2.5.1 Intrinsic bioremediation: Also known as natural attenuation or passive bioremediation. Intrinsic bioremediation is preferred to engineered bioremediation primarily because the cost is much lower. Intrinsic bioremediation consists of allowing the natural occurring microorganisms to degrade the contaminants without implementing any engineered steps to enhance the process. There are four main requirements that must be met for intrinsic bioremediation to be successful. These four requirements are

i. Sufficient microorganisms that can biodegrade the contaminant.
ii. Required nutrients are available.

iii. Good environmental conditions exist.

iv. The time to allow the natural process to degrade the contaminant.

This method is different from doing nothing because intrinsic bioremediation must prove that the microorganisms are degrading the contamination faster than the plume is migrating. This requires a network of monitoring devices to determine the location and concentration of the contaminant, the number of microbes, and other appropriate parameters.

2.5.2 Engineered bioremediation: Also known as enhanced bioremediation. Engineered bioremediation is a process that adds to or enhances the natural process of degradation. Generally it is used when any one of the four necessary conditions for intrinsic bioremediation is not available or when the process needs to be completed faster. An example of engineered bioremediation is to install wells to circulate fluids and nutrients to stimulate the microorganisms.

Engineered bioremediation often must add an electron acceptor for the microorganisms to be stimulated. Generally, this electron acceptor is oxygen. Research has shown that there is a strong correlation between the amount of oxygen and the number of bacteria that can grow as shown in Table 2.4. This increase in the population of bacteria corresponds to a greater quantity of contamination being degraded. Also a larger population has the ability to degrade the contamination much faster than a smaller population.

Maximum Available Oxygen (ppm)	**Bacterial Population Density [colony forming units (cfu) / g dry soil]**	
	Total Heterotrpohic Bacteria	**Gasoline – Utilizating Bacteria**
8 (air)	5 * 10 ^ 4	1 * 10 ^ 2
40 (O_2)	5.5 * 10 ^ 6	7 * 10 ^ 5
112 (250 ppm H_2O_2)	7.5 * 10 ^ 7	2.7 * 10 ^ 7
200 (500 ppm H_2O_2)	2.1 * 10 ^ 8	3.1 * 10 ^ 7
Correlation Coefficient ,r	0.97	0.93

Table 2.4 : Relationship of Available Oxygen to Bacteria Numbers

There are two basic ways to provide oxygen for bioremediation. These two ways are physically and chemically.The physical method forces air or pure oxygen into the contaminated soil or ground water. The chemical method provides oxygen through the introduction of another substance such as hydrogen peroxide, which then is converted into oxygen. [Flathman,P.E et al.; 1994]

2.6 BIOREMEDIATION STRATEGIES

Different techniques are employed depending on the degree of saturation and aeration of an area.
In situ techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance.
Ex situ techniques are those that are applied to soil and groundwater at the site which has been removed from the site via excavation (soil) or pumping (water). *Bioaugmentation* techniques involve the addition of microorganisms with the ability to degrade pollutants.

2.6.1 In situ bioremediation:

These techniques are generally the most desirable options due to lower cost and less disturbance since they provide the treatment in place avoiding excavation and transport of contaminants. *In situ* treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases.[Vidali,2001]

In situ techniques do not require excavation of the contaminated soils so may be less expensive, create less dust, and cause less release of contaminants than ex situ techniques. Also, it is possible to treat a large volume of soil at once. In situ techniques, however, may be slower than ex situ techniques, may be difficult to manage, and are most effective at sites with *permeable* (sandy or encompassed) soil. The goal of aerobic in situ bioremediation is to supply oxygen and nutrients to the microorganisms in the soil. Aerobic in situ techniques can vary in the way they supply oxygen to the organisms that degrade the contaminants. Two such methods are bioventing and injection of hydrogen peroxide. In situ bioremediation may not work well in clays or in highly layered subsurface environments because oxygen cannot be evenly distributed throughout the treatment area. In situ remediation often requires years to reach cleanup goals, depending mainly on how biodegradable specific contaminants are. Less time may be required with easily degraded contaminants. [US.EPA,1994]

The most important land treatments are:

2.6.1.1 Bioventing: It is the most common *in situ* treatment and involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing

volatilization and release of contaminants to the atmosphere. It works for simple hydrocarbons and can be used where the contamination is deep under the surface. An air blower may be used to push or pull air into the soil through the injection wells. Air flows through the soil and the oxygen in it is used by the microorganisms. Nutrients may be pumped into the soil through the injection wells. Nitrogen and phosphorous may be added to increase the growth rate of the microorganisms.

2.6.1.2 In situ biodegradation: It involves supplying oxygen and nutrients by circulating aqueous solutions through contaminated soils to stimulate naturally occurring bacteria to degrade organic contaminants. It can be used for soil and groundwater. Generally, this technique includes conditions such as the infiltration of water-containing nutrients and oxygen or other electron acceptors for groundwater treatment.

2.6.1.3 Biosparging: Biosparging involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. Biosparging increases the mixing in the saturated zone and thereby increases the contact between soil and groundwater. The ease and low cost of installing small-diameter air injection points allows considerable flexibility in the design and construction of the system.

2.6.1.4 Bioaugmentation: Bioremediation frequently involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit:

1) no indigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and

2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed.[Vidali,2001]

2.6.1.5 Injection of Hydrogen Peroxide: This process delivers oxygen to stimulate the activity of naturally occurring microorganisms by circulating hydrogen peroxide through contaminated soils to speed the bioremediation of organic contaminants. Since it involves putting a chemical (hydrogen peroxide) into the ground (which may eventually seep into the groundwater), this process is used only at sites where the groundwater is already contaminated. A system of pipes or a sprinkler system is typically used to deliver hydrogen peroxide to shallow contaminated soils. Injection wells are used for deeper contaminated soils. Fig 2.3 shows the same concept of using injection wells for providing nutrients plus aeration required.[US.EPA,1996]

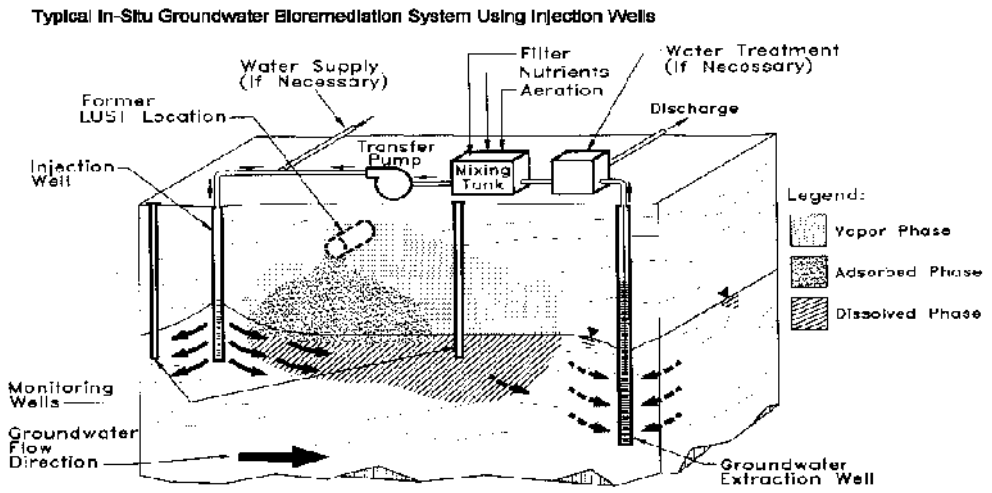


Fig 2.3: In – situ groundwater bioremediation system using injection wells

2.6.2 Ex situ bioremediation: These techniques involve the excavation or removal of contaminated soil from ground. Ex situ techniques can be faster, easier to control, and used to treat a wider range of contaminants and soil types than in situ techniques. The following fig 2.4 shows the same concept of Ex – situ bioremediation in which an excavated soil pile is being treated

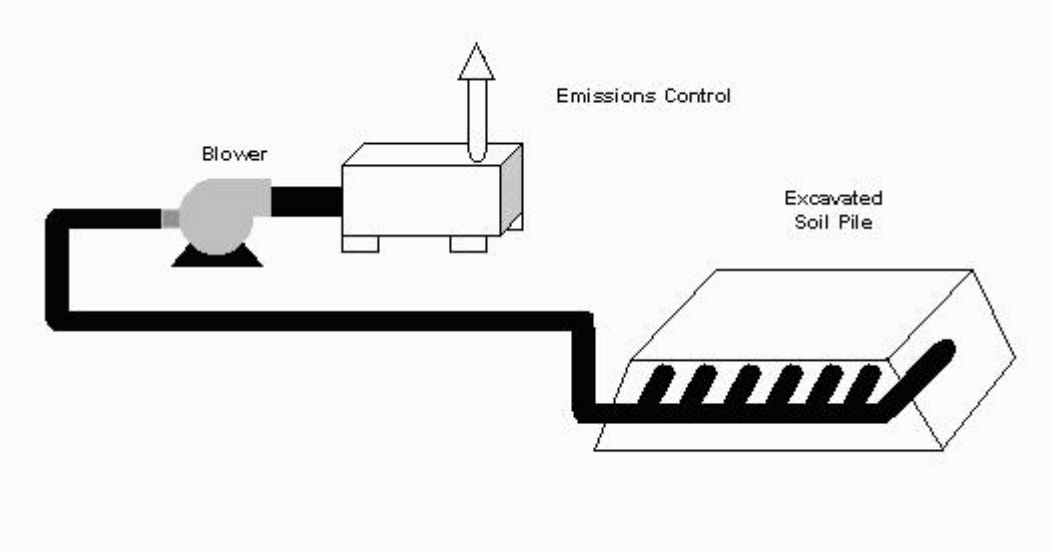


Fig 2.4: Ex – situ Bioremediation Technique

2.6.2.1 Land farming: It is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10–35 cm of soil. Since landfarming has the potential to reduce monitoring and maintenance costs, as well as clean-up liabilities, it has received much attention as a disposal alternative.

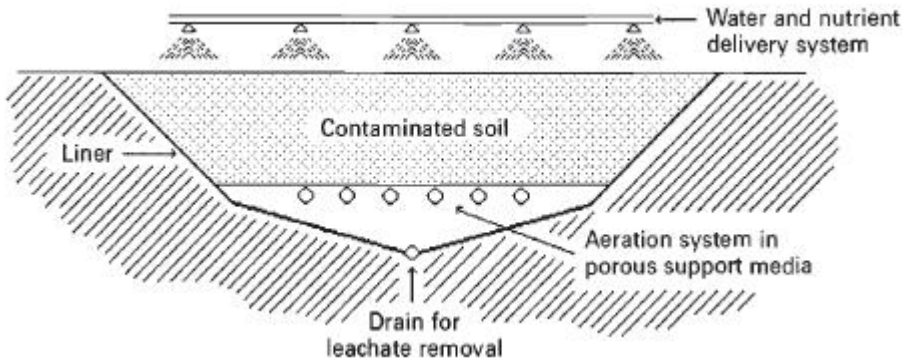


Fig 2.5: Landfarming Technique of Ex – situ Bioremediation

Biological soil treatment by landfarming is a relatively simple and inexpensive method for treating soil contaminated by compounds that are readily degraded aerobically. Contaminated soil is evacuated and usually treated in pits lined with a high-density synthetic or clay liner. Perforated pipes can be placed in a layer of sand between the liner and contaminated soil to collect drainage that can be separately treated or recycled. Alternatively, the treatment area can be graded to a sump where runoff is collected. Aeration can be accomplished by tilling the soil or with forced aeration. With tillage, soil is usually spread to a depth of 15 to 50 cm. For forced aeration, soil is placed above slotted PVC pipes that are manifolded to a blower (Figure 5). Nutrients may be added and pH adjusted. Nutrient requirements are estimated from contamination concentrations or laboratory treatability tests, and water is added or sprayed onto the soil to maintain optimum moisture. Landfarming has been widely implemented at petroleum production and storage sites, and at sites contaminated with polynuclear aromatic residues (PNAs) or pentachlorophenol (PCP). A potential problem in soil treatment is the residual contaminant concentration that is slowly or not noticeably degraded by soil microorganisms.The mixing and surfactant addition that may be necessary to release contaminants increase the cost of landfarming and favor the use of more intensive bioremediation methods such as slurry reactors.

2.6.2.2 Composting: It is a technique that involves combining contaminated soil with nonhazardous organic amendants such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting.

2.6.2.3 Biopiles: These are a hybrid of landfarming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of landfarming that tend to control physical losses of the contaminants by leaching and volatilization. Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganisms.

2.6.2.4 Bioreactors: Slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soilbound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. Whereas a Solid-phase bioremediation is a process that treats soils in above-ground treatment areas equipped with collection systems to prevent any contaminant from escaping the treatment. Moisture, heat, nutrients, or oxygen are controlled to enhance biodegradation for the application of this treatment. Solid-phase soil treatment processes include *landfarming*, *soil biopiles*, and *composting*.[US.EPA,1996]

In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable. Despite the advantages of reactor systems, there are some disadvantages. The contaminated soil requires pre treatment (e.g., excavation) or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction (e.g., vacuum extraction) before being placed in a bioreactor.

Some specific reactor designs, such as the EIMCO soil slurry reactor, require fine milling and classification of the soil prior to introduction into the reactor . Pretreatment methods include operations that enhance desorption by the reduction of soil particle size and addition of surfactant, and operations that concentrate the waste to be treated through concentrating the smaller particle sizes. Fractionation eliminates the heavier particles that are difficult to suspend in the slurry reactor. Contaminants often adsorb preferentially to finer soil particles. Fig 2.6 shows the process of slurry phase bioremediation in which contaminanted soil is fed and after screening and classification it is fed to slurry bioreactor for treatment. [Crawford R.L et al.; 2005]

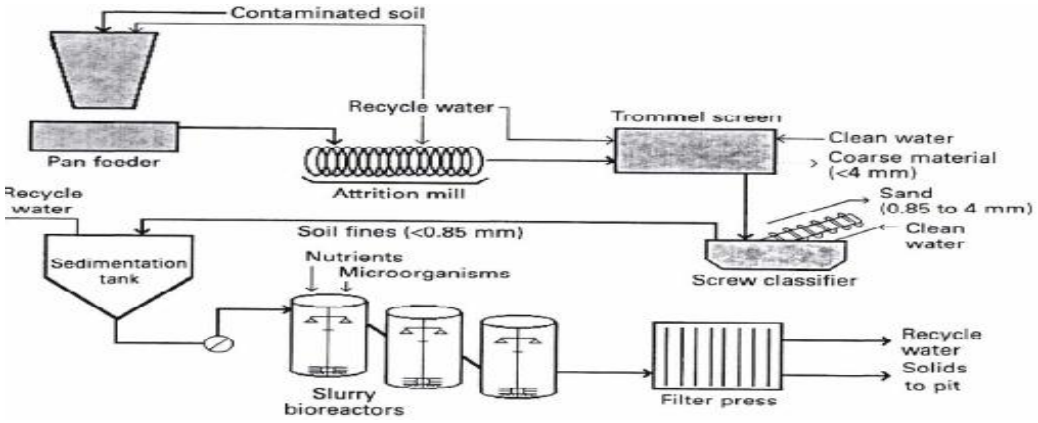


Fig 2.6: Slurry based Bioreactor Technique

2.6.3 Techniques for Ground Water Bioremediation :

There are three main types of bioremediation systems for treating ground water:

- Water circulation ,
- Air injection systems and
- Vadose Zone Bioremediation

2.6.3.1 Water circulation systems:

It work by circulating water that contains nutrients and other substances needed to help the microorganisms grow between the injection and recovery wells. Generally as much of the free product as possible is removed before this process begins. This system injects nutrients such as nitrogen and phosphorus and an electron acceptor (often hydrogen peroxide, H2O2) into the contaminated soil and ground water. The microorganisms biodegrade the contaminants and then the water is removed using a recovery well. The recovered water is then treated with an air stripper to remove any remaining volatile contaminants. In addition, this method has the option of providing an additional above ground treatment facility. The recovered water can be injected into the system again or it can be placed somewhere else and uncontaminated water can be used for injection. [Nathan F,1999]

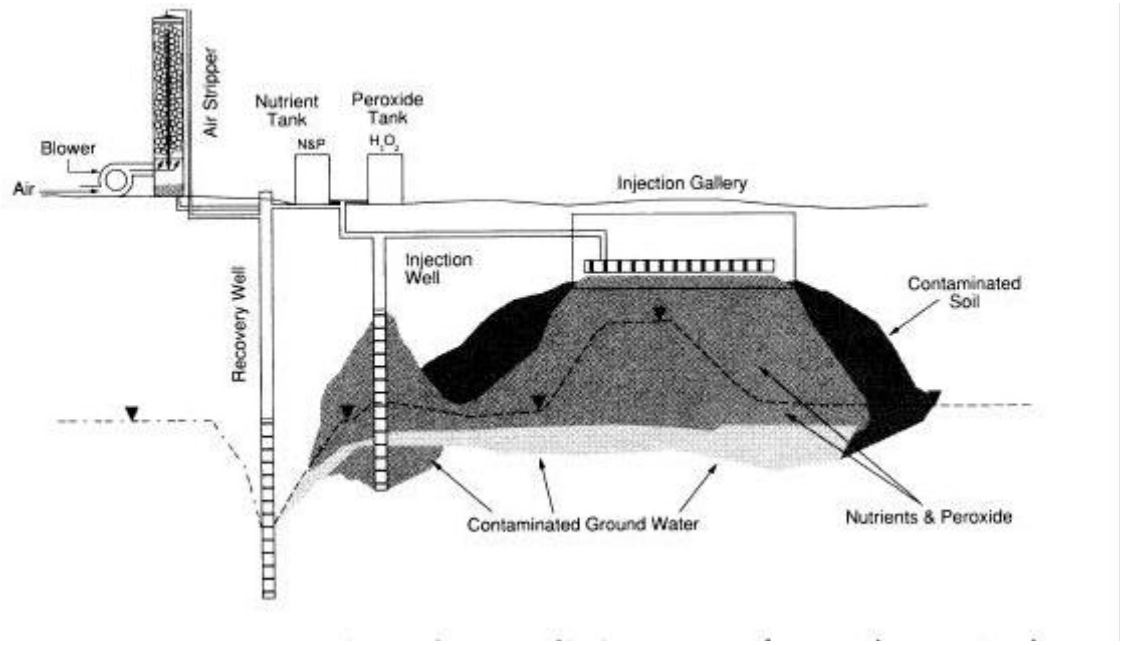


Fig 2.7: Water Circulation system

2.6.3.2 Air injection systems:

Air injection systems or air sparging is another way to treat contaminated ground water. One of the greatest advantages of this ground water treatment technique is that water does not have to be pumped. This process involves the injection of air directly into the ground water below the contaminant plume. The air displaces the water in the ground providing the microorganisms with an electron acceptor needed for bioremediation. The air also helps to remove the volatile contaminants that can be captured by using a soil vapor recovery system. If nutrients or water are not present in sufficient quantities they can be provided using an injection well. This system works because air movement helps to mix and distribute the nutrients to the microorganisms as shown in Figure 2.8

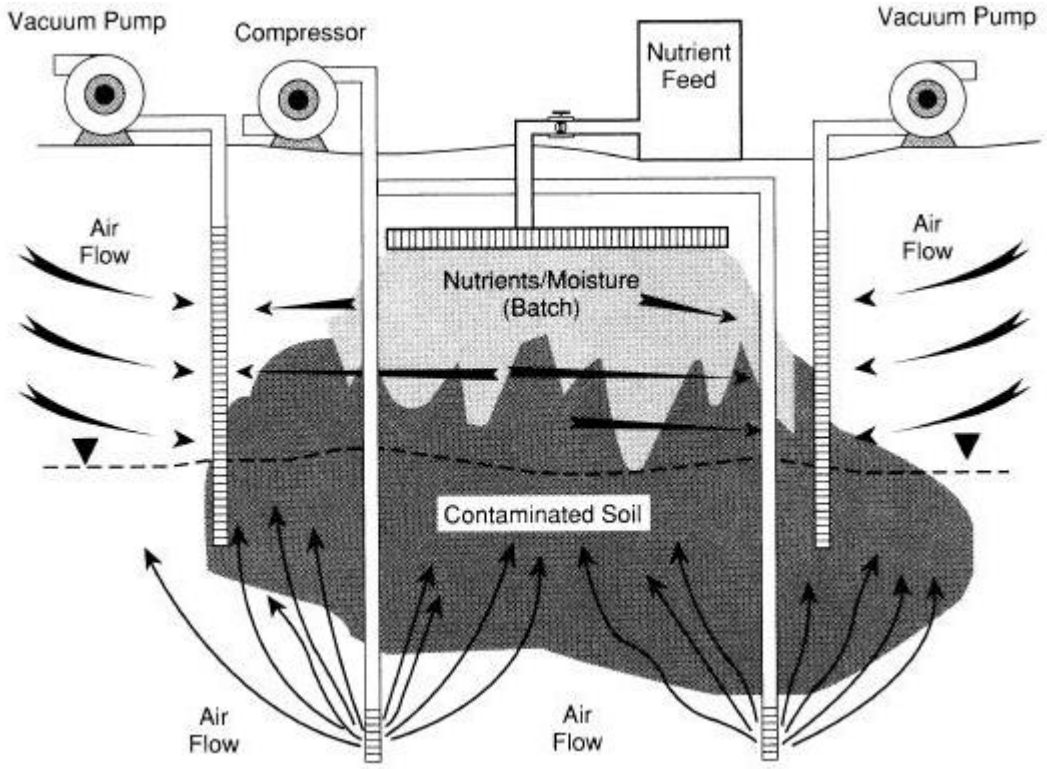


Fig 2.8: Air injection system

2.6.3.3 Vadose Zone Bioremediation:

Vadose zone bioremediation is similar to an air injection system for treating contaminated ground water. Vadose zone bioremediation differs from a ground water air injection system by injecting the air above the water table instead of below the water table and the contaminant plume. The injection of moisture is also more important since the soil can become dry due to the circulating air. However, too much moisture can transport the contaminant deeper into the ground and possibly into the ground water. A diagram of a vadose zone bioremediation system is shown in Figure 2.9[Nathan F; 1999]

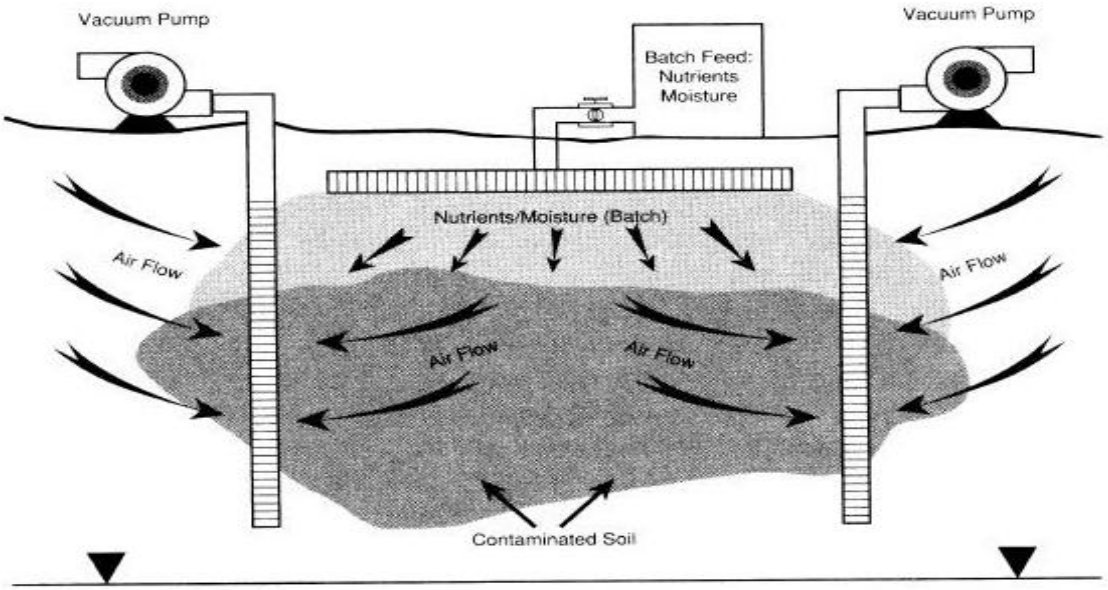


Fig 2.9: Vadose zone bioremediation

2.7 ADVANTAGES OF BIOREMEDIATION

• Bioremediation is a natural process and is therefore perceived by the public as an acceptable waste treatment process for contaminated material such as soil. Microbes able to degrade the contaminant increase in numbers when the contaminant is present; when the contaminant is degraded, the biodegradative population declines. The residues for the treatment are usually harmless products and include carbon dioxide, water, and cell biomass.

• Theoretically, bioremediation is useful for the complete destruction of a wide variety of contaminants. Many compounds that are legally considered to be hazardous can be transformed to harmless products. This eliminates the chance of future liability associated with treatment and disposal of contaminated material.

• Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible.

• Bioremediation can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation.

• Bioremediation can prove less expensive than other technologies that are used for clean-up of hazardous waste. [Vidali,2001]

2.8 DISADVANTAGES OF BIOREMEDIATION

• Bioremediation is limited to those compounds that are biodegradable. Not all compounds are susceptible to rapid and complete degradation.

• There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compound.

• Biological processes are often highly specific. Important site factors required for success include the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants.

• It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations.

• Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids, and gases.

• Bioremediation often takes longer than other treatment options, such as excavation and removal of soil or incineration.

• Regulatory uncertainty remains regarding acceptable performance criteria for bioremediation. There is no accepted definition of “clean”, evaluating performance of bioremediation is difficult, and there are no acceptable endpoints for bioremediation treatments. [Vidali,2001]

NITROAROMATIC COMPOUNDS

2.9 DEFINITION

Nitro compounds are organic compounds that contain one or more nitro functional groups (-NO_2). They are often highly explosive, especially when the compound contains more than one nitro group and is impure. The nitro group is one of the most common explosophores (functional group that makes a compound explosive) used globally. This property of both nitro and nitrate groups is because their thermal decomposition yields molecular nitrogen N_2 gas plus considerable energy, due to the high strength of the bond in molecular nitrogen.[Ju K.S et al.; 2010]

Aromatic nitro compounds are typically synthesized by the action of a mixture of nitric and sulfuric acids on an organic molecule. The one produced on the largest scale, by far, is nitrobenzene. Many explosives are produced by nitration including trinitrophenol (picric acid), trinitrotoluene (TNT), and trinitroresorcinol (styphnic acid).

Nitroaromatic compounds are among the largest and most important groups of industrial chemicals in use today. These compounds are organic molecules that consist of at least one nitro group (-NO_2) attached to an aromatic ring. The vast majority are synthetic, although several biologically produced nitroaromatic compounds have been identified. The strong electronegativity of the nitro group stems from the combined action of the two electron-deficient oxygen atoms bonded to the partially positive nitrogen atom. When attached to a benzene ring, the nitro group is able to delocalize π-electrons of the ring to satisfy its own charge deficiency. This not only provides charge to the molecule but also imparts unique properties that make the nitro group an important functional group in chemical syntheses.When aromatic compounds with multiple nitro groups react with electrophiles, stable complexes can be formed. These characteristics contribute to the stability and recalcitrance to degradation of this class of chemicals.

2.10 TYPES OF NITROAROMATIC COMPOUNDS

On the basis of Occurrence:

2.10.1 Synthetic Nitroaromatic compounds:

Nitration is the main reaction used to synthesize nitroaromatic compounds. Nitronium ions (NO_2^+) are generated in a mixed-acid reaction of sulfuric and nitric acids and then added onto aromatic substrates via electrophilic substitution . In this fashion, benzene, toluene, and phenol are converted into nitrobenzene, nitrotoluenes, and nitrophenols, the simplest of all nitroaromatic compounds. Conditions can be modified to direct nitration to the *ortho*, *meta*, or*para* position. Nitration can also be tailored to multiple substitutions on

a single molecule. The unique chemistry of the nitro group has led to the use of several nitroaromatic compounds in high-energy explosives. In this oxidation state (+III), the nitrogen atom readily accepts electrons and thereby allows nitroarene explosives to act as self-oxidants. As a result, energy is rapidly released from these compounds when an explosive charge is detonated .[Martin A,1994]

In contrast to picric acid, 2,4,6-trinitrotoluene (TNT) is chemically stable and insensitive to impact. Although TNT was widely manufactured by sequential nitration of toluene and was extensively used in both World Wars, it is no longer produced in North America due to problems of environmental contamination and persistence at manufacturing sites. TNT is still found as a major component of many composite explosives that include chemicals such as aluminum, barium nitrate, or other explosives, such as the heterocyclic nitroaromatic compounds cyclotrimethylenetrinitramine (RDX) and cyclotetramethylenetetranitramine (HMX). TNT also serves as the starting point for the synthesis of other nitroaromatic explosive compounds. Elimination of the methyl group of TNT can also be directed to produce 1,3,5-trinitrobenzene (TNB) (Fig. 2.10), which is a higher-energy explosive with decreased shock sensitivity. The explosive properties of TNB can be enhanced further by forming hexanitrobenzene (HNB).[Ju K.Set al.; 2010]

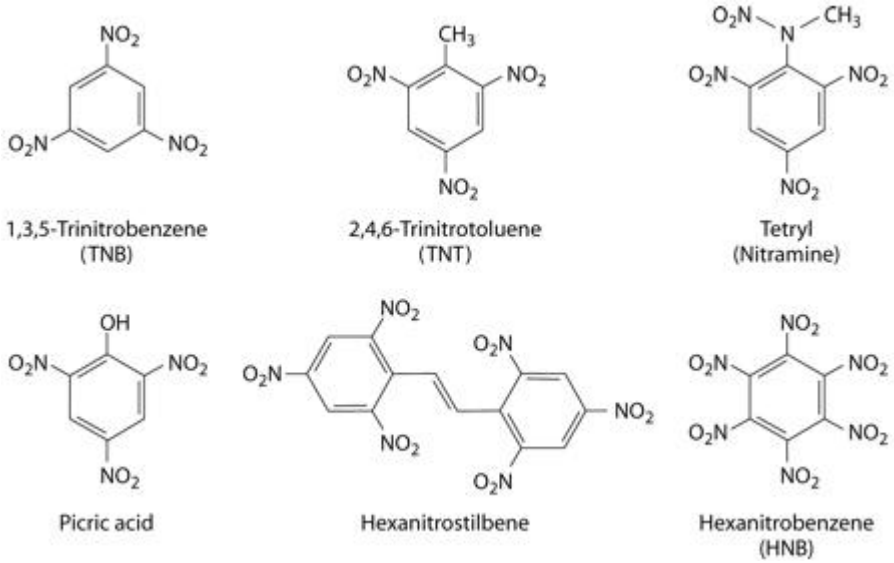


Fig 2.10: Some Nitroaromatic Compounds

In addition to explosives, many commonly used industrial and consumer products are produced using nitroaromatic compounds as starting materials. Nitrobenzene, nitrotoluenes, nitrophenols, and their halogenated derivatives serve as starting compounds in the production of a wide variety of pesticides . Nitrophenols are used in the synthesis of compounds such as carbofuran , parathion , fluorodifen , nitrofen, and bifenox . Dinitrophenols have been used in the production of all categories of pesticides (ovicides,

insecticides, herbicides, fungicides, etc.) and include compounds such as 2,5-dinitro-*o*-cresol, dinoseb, and binapacryl.

Many pharmaceuticals also have their chemical origins in nitroaromatic compounds. Substituted nitrobenzenes and nitropyridines are used to create a diverse collection of indoles, which are bioactive components not only of drugs but also of agrochemicals.

2.10.2 Naturally Occuring Nitroaromatic Compounds:

Nitroaromatic compounds can form naturally in both atmospheric and aqueous environments. In urban settings, hydrocarbons released from natural combustion processes and the incomplete combustion of fossil fuels serve as substrates for nitration with atmospheric nitrogen dioxide.

Although the vast majority of nitroaromatic compounds are manufactured chemicals, they have also been discovered as natural products from a variety of bacteria, fungi, and plants. Members of the genus *Streptomyces* are known to produce a wide variety of antibiotics, including those with a nitroaromatic component. Perhaps the best-known nitroaromatic antibiotic is chloramphenicol (originally named chloromycetin), produced by *Streptomyces venezuelae* . Nitroaromatic compounds are also bioactive metabolites found in plants and fungi. 1-Nitroaknadinine is an alkaloid from *Stephania sutchuenensis*, a traditional Chinese herbal plant used to alleviate arthritis and sore throats[Ju K.S et al.; 2010]

2.11 ENVIRONMENTAL CONTAMINATION BY NITROAROMATIC COMPOUNDS

Nitroaromatic compounds are acutely toxic and mutagenic, and many are suspected or established carcinogens. Several are listed on the U.S. Environmental Protection Agency's list of priority pollutants. The same properties that allow nitroaromatic compounds to be useful in chemical applications also make them hazardous to the health of both humans and wildlife. The interactions of nitroaromatic compounds with DNA and the resulting mutagenicity have been characterized extensively and reviewed for a variety of monocyclic, polycyclic, and heterocyclic nitroaromatic compounds. Oxidation and reduction products of nitroaromatic compounds can damage DNA directly or cause the formation of adducts that induce mutagenesis by misincorporation of nucleotides during DNA synthesis. The position of the nitro group on the aromatic ring and the presence of other functional groups can influence the mutagenicity and carcinogenicity of these chemicals.[Einschlag F.S.G et al.; 2011]

Nitroaromatic compounds are environmental contaminants associated with anthropogenic activities such as production and use of dyes, explosives, pesticides and pharmaceuticals. Many of these substances, such as nitrobenzene and nitrophenols, usually found in wastewaters of these industries are considered potentially toxic. Because nitro-substituted aromatic compounds have strong electron withdrawing groups, they are poorly biodegradable by aerobic treatments. The detoxification of wastewaters containing these hazardous substances is very difficult since, due to their high stability, they are usually refractory to conventional biological treatments.

Some nitroaromatic compounds are intentionally applied to the environment (i.e., pesticides), improper handling and/or storage practices by both producers and users have resulted in their accidental release in the environment in nations throughout the world. The annual tonnage of chemicals released reflects the shear scale of this problem. In 2002, approximately 5.1 metric tons of nitrobenzene and 1.1 metric tons of 2,4-dinitrotoluene were released into soil in the United States alone.

The manufacture, storage, and handling of munitions have left a legacy of environmental contamination by nitroaromatic compounds. As of June 2009, there were 70 Superfund sites throughout the United States (as defined by the 1980 Comprehensive Environmental Response, Compensation, and Liability Act [CERCLA]) that are contaminated with nitroarene explosives or their chemical precursors. Only 14 have been removed from the national priority list as having been completely remediated.

Nitro-PAHs formed from atmospheric radical chemistry of PAHs contribute to air pollution in urban settings. Although the exhaust from all combustion engines contains hydrocarbons that are subject to nitration, the greatest source of atmospheric pollution is diesel engine-powered motor vehicles. Diesel exhaust contains PAHs such as naphthalene, acenaphthene, fluorene, anthracene, and pyrene, which themselves have mutagenic and carcinogenic properties and are on the EPA's list of priority pollutants. Addition of the nitro group further increases the toxicity of these compounds and their threat to human health .[Ju K.S et al.; 2010]

2.11.1 Effects of Nitroaromatic compounds:

a. A number of nitroaromatic compounds are toxic to living beings and adversely affect to human health due to their hematotoxicity, immunotoxicity, splenotoxicity, genotoxicity, hepatotoxicity, nephrotoxicity and carcinogenicity.

b. Nitroaromatics have a direct relation to the effect of stress on plant metabolism , they reduce soil fertility to a degrading extent.[Talmage S.S et al.; 1999]

c. Nitroaromatics are carcinogenic, and mutagenic too and may be the cause of cancers.

d. If these percolates in ground water, makes it highly unfit to drink .

e. Higher intake of nitroaromatics may lead to insensitivity in wildlife creatures.[Arora P.K; 2011]

2.12 BIOREMEDIATION TECHNIQUES FOR NITRO AROMATIC COMPOUNDS

Nitro aromatics form an important group of recalcitrant xenobiotics. Only few aromatic compounds, bearing one nitro group as a substituent of the aromatic ring, are produced as secondary metabolites by microorganisms. The majority of nitro aromatic compounds in the biosphere are industrial chemicals such as explosives, dyes, polyurethane foams, herbicides, insecticides and solvents. These compounds are generally recalcitrant to biological treatment and remain in the biosphere, where they constitute a source of pollution due to both toxic and mutagenic effects on humans, fish, algae and microorganisms. However, few microorganisms have been described as being able to use nitro aromatic compounds as nitrogen and/or carbon and energy source. **The best-known nitro aromatic compound is the explosive TNT (2,4,6-trinitrotoluene).** The highest amount of TNT was produced at the end of WWII, when the world production was estimated on 150 k ton per month. Though most of the munitions waste sites date back from the past, many are still a serious environmental hazard. Sites contaminated with TNT range from ammunition plants and testing facilities to military zones and battlefields. [Kulkarni , Chaudhari ,2007]

2.12.1 Tri nitro toluene (TNT)

TNT is classified as a secondary explosive because it is less susceptible to initiation and requires a primary or initiating explosive to ignite it. TNT can be used as a booster or as a bursting charge for high-explosive shells and bombs. Also, TNT may be mixed with other explosives such as Royal Demolition Explosive (RDX) and High Melting Explosive (HMX) and it is a constituent of many explosives, such as amatol, pentolite, tetrytol, torpex, tritonal, picratol, ednatol. It has been used under such names as Triton, Trotyl, Trilite, Trinol, and Tritolo.

TNT is powerful, brisant, easy to load by casting since its melting point (Grade A) is 80.2 degrees C., stable under all stowage conditions, insensitive enough to stand all normal handling, and even capable of standing bullet impact when cast. The velocity of detonation is 22,300 ft./sec. at a density of 1.55. Its Laboratory Impact Value is 100. Its Bullet Impact Value is 100. Its color is yellow to buff.[U.S. EXPLOSIVE ORDNANCE, 1947]

The advantages of TNT include low cost, safety in handling, fairly high explosive power, good chemical and thermal stability, compatibility with other explosives, a low melting point favorable for melt casting operations and moderate toxicity.

TNT is a crystalline substance. The importance of TNT as a military explosive is based upon its relative safety in manufacture, loading, transportation, and stowage, and upon its explosive properties. Manufacturing yields are high and production relatively economical. TNT is toxic, odorless, comparatively stable, nonhygroscopic, and relatively insensitive. When TNT is pure, it is known as grade A TNT and varies from white to pale yellow. When the proportion of impurities is much greater, the color is darker, often brown, and the chemical is known as grade B TNT. It maybe ignited by impact, friction, spark, shock, or heat. TNT does not form sensitive compounds with most metals. The melting point varies between 80.6° C for grade A (refined TNT) and 76° C for grade B (crude TNT).

TNT does not appear to be affected by acids but is affected by alkalies (lye, washing soda, and so on), becoming pink, red, or brown, and more sensitive. It is practically insoluble in water, but soluble in alcohol, ether, benzene, carbon disulfide, acetone, and certain other solvents.

Fig 2.11 : Trinitrotoluene

S.No	TNT Properties	
1	Molecular Weight	227
2	Molecular Formula	$C_7H_5N_3O_6$
3	Odor	Odorless
4	Boiling Point	240 deg C (explodes)
5	Melting Point	80.1 deg C
6	Density/Specific Gravity	1.654 at 20 deg C/4 deg C
7	Solubility in water	115 mg/L at 23 deg C
8	Vapor Pressure	8.02X10-6 mm Hg at 25 deg C
9	Heat of decomposition	5.1 kJ/g

Table 2.5: Properties of TNT [EPA,2012]

2.12.1.1 Use

2,4,6-Trinitrotoluene has been classified as a high explosive. The compound is used as a military explosive in bombs and grenades. It has been widely used for filling shells and airborne demolition bombs since it is sufficiently insensitive to the shock of ejection from a gun barrel but can be exploded on impact by a detonator mechanism. 2,4,6-Trinitrotoluene has been used either as the pure explosive or in binary mixtures. The most common binary mixtures of 2,4,6-trinitrotoluene are cyclotols (mixtures with RDX), octols (mixtures with HMX), amatols (mixtures with ammonium nitrate), and tritonals (mixtures with aluminum. In addition to military use, small amounts of 2,4,6-trinitrotoluene may be used for industrial explosive applications, such as deep well and underwater blasting. Other industrial uses of 2,4,6-trinitrotoluene include use as a chemical intermediate in the manufacture of dyestuffs and photographic chemicals .[ATSDR,1995]

2.12.1.2 Releases To The Environment

a. **Air** : 2,4,6-Trinitrotoluene is released to the ambient atmosphere as a result of open detonation and open burning techniques used in the demilitarization of munitions.Gases and particulates are released to the atmosphere as a result of these activities and from the disposal of munitions containing 2,4,6-trinitrotoluene in rotary kiln incinerators. 2,4,6-Trinitrotoluene dusts and vapor are released into indoor air atmospheres in military production and processing facilities during manufacturing of 2,4,6-trinitrotoluene and munitions. Fugitive dusts containing the compound are probably generated at sites with contaminated surface soils (e.g., military installation burning grounds.

b. **Water** : 2,4,6-Trinitrotoluene has historically been discharged in large quantities in the aqueous effluents of explosives production/manufacturing facilities and ammunition load, assemble, and pack (LAP) plants, from decommissioning activities, and through field use/disposal. Estimates of the loadings of 2,4,6-trinitrotoluene in these effluents vary. Some investigators have reported concentrations of about 120 mg/L in manufacturing facility waste waters and 25 mg/L in loading plant facility effluents. Others report concentrations of 40-120 mg/L in manufacturing plant effluents, with generally higher concentrations in LAP plant waste waters. Concentrations of 0.1-3.4 mg/L have been detected in about 20% of the samples of sellite manufacturing process condensate wastewater collected from a 2,4,6-trinitrotoluene manufacturing facility . .[ATSDR,1995]

c. Soil : 2,4,6-Trinitrotoluene is released to soils from spills, disposal of solid waste, open incineration and detonation of ordnances, leaching from inadequately sealed impoundments (e.g., pits, ponds, and lagoons), and demilitarization of munitions. Demilitarization of munitions can result in contamination of surface soils by activities such as open burning and open detonation or landfilling of solid wastes generated during rotary kiln incineration and nondestructive reprocessing of munitions containing 2,4,6-trinitrotoluene . .[ATSDR,1995]

2.12.1.3 Environmental Fate

a. Transport and Partitioning

On the basis of the relatively low vapor pressure (1.99×10^{-4} mmHg at 20°C) and relatively high water solubility (130 mg/L at 20°C) of 2,4,6-trinitrotoluene, the compound is not expected to partition from surface waters to the atmosphere. Volatilization half-lives of 10,000 days have been estimated for ponds, streams, and lakes. A volatilization half-life of 119 days has been estimated from a model river at 20°C 1 meter deep flowing at the rate of 1 meter/second, with a wind speed of 3 meters/second. On the basis of the measured and estimated values for the soil organic carbon adsorption coefficient (Koc) of 300-1,100, 2,4,6-trinitrotoluene is not expected to significantly partition from surface waters to sediment or strongly absorb to soil particulates

b. Transformation and Degradation

b.1 Air : No information is available on the transformation of 2,4,6-trinitrotoluene in the atmosphere. However, 2,4,6-trinitrotoluene released to the atmosphere should undergo direct photolysis, as it does in surface water. Estimates of the photolytic half-life of the compound in air range from 3.7 to 11.3 hours; these estimates are based on the rate of photolysis of the compound in distilled water. Estimates of the photooxidation half-life of the compound in the atmosphere range from 18.4 to 184 days. These estimates are based on the estimated rate constant for reaction with hydroxyl radicals in the atmosphere.[ATSDR,1995]

b.2. Water : 2,4,6-Trinitrotoluene does not undergo hydrolysis, as demonstrated by the stability of the compound in sea water after 108 days at room temperature .Photolysis of 2,4,6-trinitrotoluene in aqueous solutions is a well-known phenomenon, which is responsible for the development of “pink water,” and is probably the most important fate process for 2,4,6-trinitrotoluene in aqueous systems. For example, the estimated half-life of 2,4,6-trinitrotoluene in surface waters is 0.16-1.28 hours, based on the rate of photolysis and photooxidation in sunlit natural waters. The rate of photolysis of 2,4,6-

trinitrotoluene in natural surface waters has been found to be much greater than that of the compound in pure water. Phototransformation of 2,4,6-trinitrotoluene in surface waters occurs via direct and indirect photolysis. 2,4,6-Trinitrotoluene is also transformed in surface waters by microbial metabolism, although this process occurs more slowly than photolysis. For example, the estimated biodegradation half-life of 2,4,6-trinitrotoluene in surface water, under both aerobic and anaerobic conditions, is l-6 months. This estimate is based on aerobic river die-away test data with unacclimated microorganisms. The relative slowness of microbial degradation may be due in part to the enhanced toxicity of 2,4,6-trinitrotoluene to aquatic organisms in the presence of the near-ultraviolet component of sunlight. Examples of biotransformation of the compound in aqueous systems include the white rot fungus Phanerochaete chrysosporium**,** which was found to degrade ringlabeled ^{14}C-2,4,6-trinitrotoluene. Within 12 days, 35% of the labeled 2,4,6-trinitrotoluene added to the solution was recovered as $^{14}CO_2$. Pseudomonad bacteria **(**Pseudomonas sp.) have been found to reduce 2,4,6-trinitrotoluene under aerobic conditions in laboratory studies to monoaminodinitrotoluenes and a diaminomononitrotoluene.

b.3. Soil : Solid chunks of 2,4,6-trinitrotoluene buried in soil or exposed on the soil surface can persist for many years .In smaller amounts, 2,4,6-trinitrotoluene may undergo photolysis in surface soils to trinitrobenzene and trinitrobenzaldehyde .The transformation of 2,4,6-trinitrotoluene in soils has been found to be influenced by a number of environmental factors. Composting of 2,4,6-trinitrotoluene in soils has been examined in laboratory scale and large-scale tests. In laboratory tests with ring labeled ^{14}C-2,4,6-trinitrotoluene, rapid biotransformation was found, with initial average activity levels of 93.5% reduced to 46.6% and 16.6% after 3 weeks and 6 weeks, respectively. No degradation products were detected in samples collected after 3 weeks. [ATSDR,1995]

2.12.2 Di nitro toluene(DNT)

More popularly known as DNT, has various isomers. The commercial or technical grade dinitro toluene is a mixture of approximately 76 percent 2,4-DNT, 19 percent 2,6-DNT and 5 percent of the remaining isomers are 2,3-, 2,5-, 3,4- and 2,5-dinitro toluene. DNTs are not known to occur naturally in the environment but have been detected in the soil, surface water, and groundwater of hazardous waste sites that contain buried ammunition wastes and wastes from manufacturing facilities that release DNT[U.S EPA, 2008]. This isomeric mixture is a combustible oily liquid. 2,4- and 2,6-dinitro toluenes are used in a number of industries. The major use of DNT is in the production of toluene di-isocyanate, which is primarily used in

the production of polyurethane foams and polymers. 2,4-DNT is produced along with 2,6-DNT during the manufacture of toluene di-isocyanate. The reaction sequence involves the nitration of toluene, which is hydrogenated to yield 2,4-diamino toluene which is then treated with phosgene to finally yield toluene 2,4-di-isocyanate. The nitration step produces two isomers 2,4-dinitro toluene and 2,6- dinitrotoluene, the former predominating. Reacting toluene di-isocyanate with glycerol then produces polyurethane foams. DNT is used by the munition industry as a modifier of smokeless powders and as an explosive intermediate**.** in moderate and high explosives**,** DNT is used as a plasticizer and is also used as a low melting mixture of six isomers, which is an important component in the manufacture of both gelatinous and commercial explosives. A pure product consisting mainly of the 2,4-isomer is also employed as a component of gunpowder.

Molecular Weight	182.14
Melting point (° C)	71
Boiling point (° C)	300 (decomposes)
Water solubility (mg/L)	300 at 22 ° C
Vapour pressure @ 25° C (mm Hg)	1.4×10^{-4}
Henry's law constant (torr M^{-1})	3.4
Octanol/ water partition coefficient (Log K_{ow})	1.98
Absorption maxima (λ max)	259 nm

Table 2.6: Physical and chemical properties of 2,4-dinitro toluene [U.S EPA, 2008]

2.12.2.1 Environmental Fate

DNT has been found in the soil, surface water, and groundwater of hazardous waste sites that contain buried ammunition wastes and wastes from manufacturing facilities that release DNT . The water solubilities of 2,4-DNT and 2,6-DNT are moderate, and the log Kow and log Koc are low for both isomers. Since the partitioning of organics to the sediment from the aqueous phase does not become a major loss until the log Koc values exceed 3.5, the relatively low log Koc values for 2,4-DNT and 2,6-DNT indicate that these compounds would have only a slight tendency to sorb to sediments, suspended solids, and biota. Therefore, there is potential for transport via surface water or groundwater. The low lipophilicity of this compound predicts it is not expected to bioaccumulate in animal tissues. [U.S EPA, 2008]

2.12.2.2 Environmental Degradation

Microbial biodegradation of DNT in water has been observed under both aerobic and anaerobic conditions. Biotransformation occurs mainly through the reduction of the nitrogroup. Microorganisms isolated from DNT-contaminated sites are capable of growth on 2,4-DNT and 2,6-DNT as their sole carbon and energy source. . Biotransformation of DNT by Pseudomonas aeruginosa isolated from a propellant wastewater treatment plant, was observed under both aerobic and anoxic conditions. Since microorganisms readily metabolize 2,4-DNT and 2,6-DNT to CO2 as the final product, DNT is not expected to persist in the environment. However, studies show persistence is water-body dependent, and the length of time any particular nitroaromatic compound resides in the environment ultimately depends on the compound's unique interaction with the natural organics and biota in its surroundings. [U.S EPA, 2008]

2.13 DEGRADATION MECHANISMS BY MICROORGANISMS

2.13.1 Aerobic conditions :

Nitroaromatic transformations have been observed in aerobic environments. Aerobic composting of explosives has been investigated to address the feasibility of bioremediating soils contaminated with TNT, RDX, and/or HMX . Through this process, TNT and other explosives have been significantly depleted from soil, but the fate of the biotransformed TNT molecule is not completely known. Degradation of nitroaromatic compounds by aerobic bacteria involves mainly mono- and di-nitro-aromatics as a source of carbon and/or nitrogen and energy by complete mineralization. That is :

(i) Isolation of various microbes, which degrade/mineralize nitroaromatics aerobically and

(ii) Deciphering the catabolic pathways and enzymes involved in aerobic degradation processes.

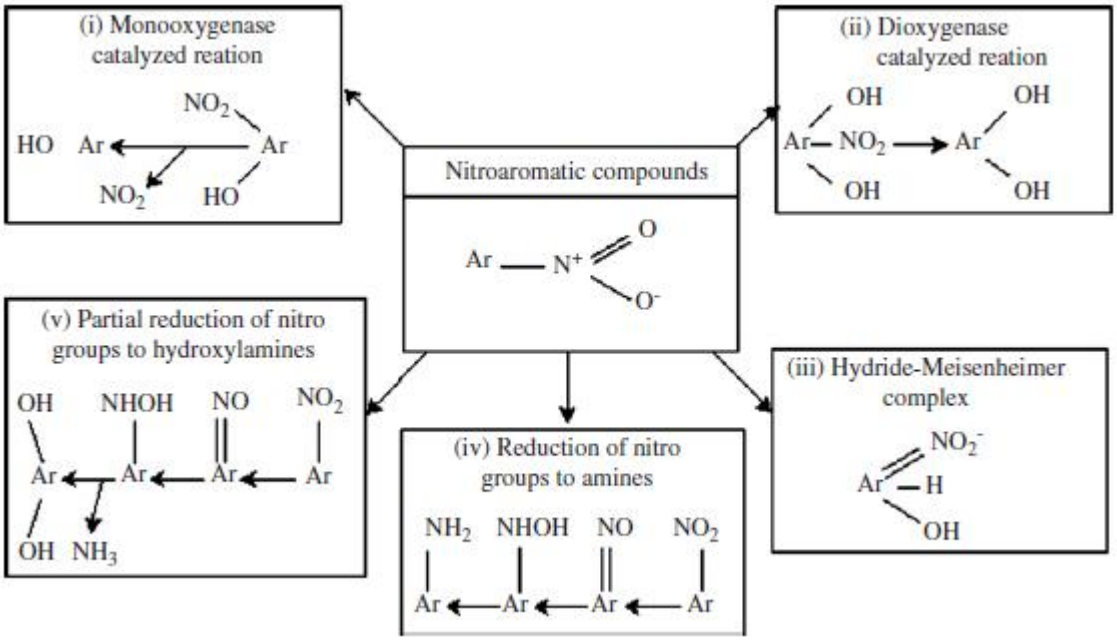


Fig 2.12 : Microbial strategies for remediation of nitro-aromatic compounds by aerobes (i, ii, iii and iv) and anaerobes (v)

2.13.1.1 Monooxygenase catalyzed reactions :

Monooxygenase adds a single oxygen atom and causes elimination of nitro groups from mono-nitrophenols. Simpson and Evans (1953) recognized the role of monooxygenase from Pseudomonas sp., which was capable of converting 4-nitrophenol to hydroquinone, with the release of nitrite. [Kulkarni , Chaudhari ,2007]

2.13.1.2 Dioxygenase catalyzed reactions :

Dioxygenase introduces two hydroxyl groups with the removal of a nitro group as nitrite from the aromatic ring. Evidence for this mechanism was revealed while studying the transformation of 2, 6-dinitrotoluene in Alcaligenes eutrophus

2.13.1.3 Meisenheimer complex formation :

The partial reduction of the aromatic ring of di- or tri- nitrocompounds through addition of hydride ions leads to formation of a hydride-Meisenheimer complex. This complex was also observed in polynitroaromatics such as TNT and picric acid, where oxygenolytic attack under aerobic conditions is difficult due to their electron deficient nature; the electrophilic reaction is easy. The complex re-aromatizes after the release of nitrite anion.

2.13.1.4 Partial reduction of aromatic ring :

The nitro group is partially reduced to corresponding hydroxylamine and upon hydrolysis yields ammonia. Compounds such as 4-nitrobenzoate, 2-nitrobenzoate are commonly metabolized through partial reduction

of the nitro group to its amino derivative. For example, Comamonas acidovorans reduced 4-nitrobenzoate to 4-hydroxyl-aminobenzoate in the presence of NAD(P)H

Organisms	**Nitro-aromatics**	**Pathways**
Moraxella sp.	4- Nitrophenol	Release of nitrite, mineralization to CO2
Pseudomonas	4- Nitrophenol	Mineralization to CO2
Pseudomonas N26-8	2,6 - Dinitrophenol	Use as a sole source on nitrogen
Alcaligenes eutrophus	2,6-Dinitrophenol	Relaese of nitrite
Rhodococcus erythropolis	2,4-Dinitrophenol	Utilizes carbon , nitrogen and energy source, nitrite releases and ring cleavage
Actinomycete	4-Nitrophenol	Release of nitrite , mineralization to CO2
Pseudomonas peckettii	4-Nitrobenzoate	Formation of 4 – hydroxylamino benzoate with release of NH3
Pseudomonas putida	3-Nitrobenzoate	Formation of 3-hydroxylaminophenol to 1,2,4 benzenetriol followed by ring cleavage
Pseudomonas	TNT	Use as a sole N source, elimination of TNT via production of hydride Meisenheimer complex and transform to dinitrotoluene, nitrotoluene and toluene
Rhodococcus erythropolis	TNT	Catalyze ring hydrogenation, forming hydride-and dihydride TNT-Meisenheimer complex and transform to dintrotoluene ,nitrotoluene and toluene
Phanerochaete chrysosporium	TNT	Formation of hydroxylamino dinitrotoluene
Arthrobacter protophormiae RKJ100	p-Nitrophenol	Complete degradation in soil microcosm
Nocardiodes CB22-2	TNT	Dinitration leads to formation Meisenheirmer complex
Candida pulcherrima	M - Dinitrobenzene	Complete mineralization to CO2
Litter decaying fungus	TNT	Concentration decreased to 95% in 300 days with no reduced intermediate formation
Phlebia chrysosporium	TNT	Mineralized under lignolytic

		condition with formation of 9 different acetylated products
Phanerochaete chrysporium	4 - Nitrophenol	Degraded to 1,2 – dimethoxy-4-nitrobenzene via 4-nitroanisole

Table 2.7 : Aerobic degradation of nitro-aromatics by various microorganisms

In a treatability study, Craig & Sisk (1994) composted 30 cubic yards of highly contaminated soil by forced aeration in a two-window configuration. After 40 days of treatment, the concentration of explosives decreased > 99% for TNT and RDX and 96.9% for HMX. The toxicity of the compost leachate was reduced 87% to 92%, as shown by a *Ceriodaphnia dubia* assay, and a 99.3% to 99.6% reduction in mutagenicity was indicated by the Ames assays. *Phanerochaete chrysosporium* is a basidiomycete white-rot fungus capable of transforming and mineralizing TNT.

Aerobic biotransformations of TNT are initially similar regardless of the microorganism. The initial transformation generally involves the reduction or removal of one of the nitro substituents, giving way to an amino derivative or free nitrite.[Kulkarni , Chaudhari ,2007]

2.13.2 Anaerobic conditions :

Anaerobic transformation of nitro-aromatics is receiving increased attention.It involves reduction of nitro groups to aromatic amines through a six-electron transfer mechanism. The reduction of nitro-groups to nitroso derivatives, hydroxylamines or amines is catalyzed by nitroreductases through the successive addition of electron pairs donated by co-substrates. Most of the poly-nitroaromatics are susceptible to degradation, only under anaerobic conditions. Individual species of anaerobes rarely bring out complete conversion of nitro-aromatic substrate to CO_2 or methane. Hence, synergistic participation of a consortium of bacteria is required for partial/complete degradation of several compounds viz. dinitrotoluene, 3,5-dinitrobenzoic acid, 2-, 3- and 4-nitrophenol, TNT.

A large number of nitro groups increase concomitant conversion to amino groups with decreasing reaction velocity. It has been reported that axenic cultures of Desulfovibrio and Clostridium sp. can rapidly degrade TNT .

Microorganisms	Nitro-aromatics	Metabolic path
Haloanaerobicum praevalens	Nitrobenzene	Reduction to corresponding aromatic amines
Desulfovibrio sp. Strain B	TNT(100 mg/L) , DNT, nitrobenzene	TNT as a nitrogen source,100%

		biotransformation by removal of amino groups from ring by reductive deamination
Methanobacterium formicium	3-,4- Nitrophenol	Complete transformation
Clostridium bifermentans	TNT,RDX	TNT converted to TAT to 2,4,6-trihydroxytoluene to p-cresol
Methanogenic bacteria	4-Nitrophenol	Complete degradation
Granular slugde from UASB reactor	2-Nitrophenol 4-Nitrobenzoate	Complete mineralization
Sulphate reducing and methanogenic consortia	TNT	Utilize TNT as nitrogen source
Sphingomonas capsulate	TNT	Amine metabolites
Klebsiella sp.C1	TNT	Reduced to hydroxylamino dinitrotoluenes, aminodinitrotoluenes and to nitrite via denitration
Ralstonia eutropha	3-Nitrophenol Nitrobenzene	Complete mineralization
Clostridium thermoaceticum	TNT	By carbon monoxide dehydrogenase
Anaerobic Consortia	Mono-nitrophenols	Corresponding aminophenol
Methyllobacterium sp. BJ001	TNT	Reduced to amino-dinitrotoluenes with no significant release of CO2

Table 2.8: Anaerobic degradation of nitro-aromatics by various microorganisms

Reduction of aromatic nitro groups is the primary initial activity observed when anaerobes metabolize nitroaromatics , the sequential reduction of the nitro substituents on TNT by a *Desulfovibrio.* This sulfate-reducer was able to use TNT as the sole nitrogen source, while anaerobically respiring pyruvate; sulfate served as an energy source. TNT was completely reduced to triaminotoluene (TAT) and then further degraded to unknown intermediates. 2,4-diamino-6-nitrotoluene (2,4-DANT) was found to be the limiting step in the formation of TAT, a reduction proceeding through 2,4-diamino-6-hydroxylaminotoluene (DAHAT) as an intermediate. The conversion of DAHAT to TAT was inhibited by CO and NH_2OH. Here, the anaerobic cultures reduced aromatic nitro groups to their corresponding amino groups without further conversion of the molecule. The methanogenic bacteria were unable to grow in the presence of these highly oxidized compounds; however, whole cell suspensions as well as crude cell extracts were able to carry out the reductions.

The two nonaromatic but related nitramine explosives, RDX and HMX, are sometimes found with TNT and other nitroaromatics in explosives-contaminated soils. RDX and HMX degraded by means of three aerotolerant bacteria isolated from nitramine-contaminated soil. These were *Providencia rettgeri* B1, *Morganella morganii*, and *Citrobacterfreundii* NS2. Initially, the cultures were grown aerobically in the presence of RDX and HMX without notable degradation of the nitramines. However, upon O_2 starvation, some researchers were able to completely transform RDX.

Using a mixed culture, it have been found that nitrobenzene is completely degraded by sequential anaerobic-aerobic treatments. In the anaerobic stage, nitrobenzene is reduced to aniline, which is then mineralized to CO_2 in the subsequent aerobic phase.[Zita et al.,2002]

2.14 MECHANISM FOR BIOREMEDIATION OF NITRO AROMATIC COMPOUNDS

The nitro-group of the aromatic ring exists as a resonance hybrid, wherein oxygen atoms are more electro-negative than nitrogen atoms, causing polarization of the nitrogen-oxygen bond. The partial positive charge and high electro-negativity of the nitrogen atom imparts to the nitro group a more electrophilic character. Hence, nitro-aromatics are reduced in biological systems. This reduction gives rise to secondary abiotic coupling reactions, which generate biologically inert azo-, azoxy- and polymeric compounds. Reduction occurs either by a one- or two-electron mechanism to either a nitro anion or nitroso group, respectively, which is achieved by nitroreductases (type-I oxygen insensitive and type-II oxygen sensitive). The oxygen sensitive (type-II) enzymes are found not only in Clostridium and E. coli, but also in plants and animals. They yield nitroso anion because of one electron reduction of the nitro group.

Reduction of the nitro-group by a pair of electrons is oxygen-insensitive (type-I), since anion formation does not occur. Instead, nitroreductase (type-I) enzymes reduce the nitro-group in the order of nitroso, hydroxylamino and amino derivatives because of donation of a pair of electrons by reduced pyridines (NADH and NADPH). The reaction mainly occurs under anaerobic conditions and leads to formation of terminal aromatic amines. Its pathway generates highly reactive and unstable nitroso derivatives, which react readily with a variety of biological materials to initiate chemical mutagenesis and carcinogenesis. Their high reactivity contributes to more toxicity and carcinogenicity compared to that of nitroaromatic compounds. Finally, amino-substituted aromatic compounds or dark polymerization products are formed in the presence of oxygen as terminal metabolites. The reduction of the aromatic nitro group depends on the nature of other substituents, and their relative position on the ring.[Ju K.S et.al; 2010]

2.15 BIOREMEDIATION TECHNOLOGIES USED

Bioremediation ultimate objective here is to degrade organic chemicals to concentrations below the permissible limits established by regulatory authorities and preferably to undetectable levels. An inoculation of potent bacteria (natural bioattenuation) enhances the destruction of pollutants in the soil. Initially, such studies have been carried out with 10–30 g of soil at rhizospheric depths. In contaminated soil (5.0 g parathion/kg), more than 90% of parathion was destroyed within 3 weeks because of direct inoculation of Pseudomonas stutzeri and Pseudomonas aeruginosa.[Gatell X.M; 1997]

2.15.1 In situ bioremediation :

An in situ process is applied at the contaminated site, without removal of contaminated material from its original location. In situ processes include :

(i) land farming,

(ii) soil bio-piles,

(iii) composting,

(iv) photo-remediation,

(v) bio-restoration ,and

(vi) bio-stimulation.

Nitro-aromatic explosives from contaminated sediments were effectively degraded at field scale using composting. Complete bioremediation of TNT at laboratory-scale suggested that an initial anaerobic treatment was essential before the aerobic phase The main drawbacks of the composting method are:

(i) lack of full-scale information about biological systems,

(ii) long incubation period,

(iii) lack of effective control on various parameters that may affect the outcome, and

(iv) poor chemical characterization of the outcome of the process.

The merit of the composting method is its moderate cost by virtue of in situ application.

In land farming, contaminated soil is mixed with nutrients and moisture and periodically aerated. This method for the bioremediation of TNT contaminated soil is used by the application of molasses.

2.15.2 Ex situ bioremediation :

Ex situ bioremediation refers to removal of contaminated material from the source (soil/water) and processing it in bioreactors under controlled operating parameters (temperature, pH, and aeration).Therefore, this process is more expensive than the in situ approach. In this process, heavily

contaminated soil in a soil: water ratio of 1:1 (w/w) is stirred in a reactor to form a slurry and treated under aerobic and anaerobic conditions . Initially, the reactor is made anaerobic by addition of co-substrate like starch and metabolizing it by indigenous bacteria to consume the dissolved oxygen. Under these conditions, the TNT concentration was reduced from 3000 mg to 1 mg/kg of soil in 5 months. The incorporation of co-substrate served a dual purpose:

(i) oxygen removal by growing aerobes and

(ii) availability of electrons for reduction of nitro groups of nitro-aromatics.

Ex situ treatment could use free and immobilized cell systems. In free cell systems, live bacteria/fungi or their consortia are used as an inoculum to degrade the organics. However, it suffers from drawbacks:

(i) survival of inoculum gets difficult at threshold concentration of toxic chemicals,

(ii) reduction in chemical load is limited, and

(iii) presence of heavy metals inhibits treatment.

To avoid wash out of biomass at low concentrations of toxic chemicals and increase its rate of degradation, immobilization of degradative bacteria has been proposed by several researchers. Whole cells immobilized using adhesion/crosslinking/ entrapment to various matrices (peat, agarose, clay, alginate, diatomaceous earth, k-carragenan, etc.) or self immobilization on granular sludge provide an alternative to overcome the existing limitations experienced with the free cell system of bioremediation . While free systems provide characteristic advantages, the immobilized one can treat nitro-aromatics by providing a high loading rate. Due to this distinct advantage provided by immobilized cells over free cells, there is a growing interest in the use of the immobilized bacterial cell technique (IBT) for the cost-effective bioremediation of chemical wastes. IBT utilizes highly selected, toxic chemical degrading bacteria isolated from natural sources in a bioreactor, designed to provide optimal conditions for bioremediation. IBT has several benefits for enhancing the bioremediation rate vis-a`-vis conventional clean-up systems. [Zita et al.,2002]

2.16 REACTORS USED FOR BIOREMEDIATION OF NITRO AROMATICS

2.16.1 Anaerobic slurry reactors :

Anaerobic treatment systems have been proposed as a means of avoiding the accumulation of partially reduced intermediates during degradation of TNT.The potato starch served as a readily degradable carbon source, which allowed the rapid establishment of anaerobiosis. The maintenance of a low to neutral pH was very important to minimise the polymerization of intermediates. In the case of the herbicide dinosed, no aromatic compounds remained in the bioreactors, whereas mineralization of TNT was poor. Although mass balances and biochemical mechanisms of conversion of TNT are largely lacking, to date, this is the only

commercially available system specifically developed for biologically remediating nitroaromatic contaminated soils (developed, patented and licensed by the University of Idaho).

RDX- and HMX-contaminated soil slurries were performed in an anaerobic pilot-scale reactor (Guiot et al. 1999). Although the initial concentrations used were extremely high (the RDX and HMX content of the soil were 24,060 and 7,860 mg/kg soil respectively), RDX was completely removed after about 45 days, while the HMX removal, which started once RDX was completely removed, took an additional 35 days.

2.16.2 Anaerobic/aerobic slurry reactors :

To eliminate the hydroxytoluenes or aminotoluenes remaining in the anaerobic bioreactors following the disappearance of TNT, second-stage aerobic reactors have been proposed to hasten the removal of those intermediates that are more rapidly degraded under aerobic conditions. Further research is required to demonstrate permanent immobilization and to investigate whether any residual toxicity remains. Anaerobic treatment of originally contaminated soil was carried out in a laboratory slurry reactor (Lenke et al. 1998). While fermenting glucose to ethanol, acetate, and propionate, the anaerobic bacteria completely reduced TNT to TAT, which led to complete and irreversible binding of the reduced products to the soil. A pilot-scale demonstration was exhibited on soil contaminated with TNT, RDX and HMX (Green 1999). The process was able to degrade TNT, RDX, and HMX to levels which would allow the reuse of the remediated soil as landfill cover. The combined concentration of TNT and amino intermediates was reduced by more than 99,5%. RDX was not appreciably degraded until the combined concentration of TNT and its reduction intermediates was less than the concentration of RDX.

2.16.3 Aerobic/anoxic slurry reactors :

An aerobic/anoxic soil slurry reactor study successfully removed TNT, RDX, HMX, and other contaminants present in the soil.[Boopathy et al.,1998] Molasses was selected as carbon source. Radio-labelled TNT incubated with reactor biomass showed that after 14 days 23% of I4C-TNT was mineralized, 27% converted to biomass, and 8 % adsorbed onto the soil. The rest of the 14C-TNT was accounted for as metabolites, including a ring cleavage product identified as 2,3- butanediol. Improvement to the reactor system by adding a food-grade surfactant (Tween 80) was tested (Boopathy and Manning 1999). Adding both Tween 80 and molasses greatly enhanced the degradation rate and might be cost effective in large-scale cleanup operations.

2.16.4 Fluidized bed reactors (FBR) :

A pilot-scale field demonstration was conducted to collect reliable cost and performance data for an aerobic, biological FBR system that treats groundwater contaminated with nitrotoluenes (Spain et al. 1999). The FBR was inoculated with a mixed culture of bacteria that had been acclimated to a mixture of mono- and dinitrotoluenes. It can be concluded that the FBR process can effectively remove mono and dinitrotoluenes from contaminated groundwater with an aerobic treatment process. However, the removal of TNT was poor due to the absence of TNT-degrading bacteria.

2.16.5 Activated sludge systems :

Feasibility studies for the bioremediation of TNT-contaminated water by activated sludge systems have been performed. TNT concentrations and those of its metabolites decreased, but no mineralization occurred in any of the systems studied. TNT metabolites were mainly associated with the lipid and protein components of the microflora present in the system under the form of amide polymers.[Zita et al.,2002]

2.17 MOLECULAR BIOLOGY ASPECTS OF DEGRADATION OF NITRO AROMATICS

Presently, biological approaches of bioremediation are constrained due to a number of factors, viz.:

(i) increasing load of pollutants,

(ii) higher concentration of other inhibitors along with the target compound,

(iii) presence of heavy metals in industrial effluents, and

(iv) slow rate of degradation requiring more time to reach a predetermined clean-up end point.

Cumulatively, these factors render use of biological systems rather inefficient. To circumvent these difficulties, molecular biology techniques have provided a useful handle to modify the genomic structure of the microorganisms to improve their qualitative and quantitative performance. For qualitatively superior performance, knowledge of catabolic pathways for degradation of nitro-aromatics and their generic deviations is essential. For quantitative improvement, optimization of process parameters is a must for designing an effective bioremediation .

Focus is thus placed on exploring novel metabolic routes, short-circuiting the process of natural evolution and designing microbes capable of detoxifying a wide range of pollutants. As a marker for ecological studies, a green fluorescent protein (gfp) gene has been introduced into E. coli, P. putida, Pseudomonas sp. UG14 and Moraxella sp. to facilitate soil bioremediation. This type of engineering allowed construction of

new strain(s) with higher capabilities under a broader range of environmental conditions. Engineered microbes can provide solutions to a variety of problems experienced during bioremediation. They are as follows:

(a) The existing biological waste treatment processes work at an exceedingly slow pace. The term slow pace refers to the rate of degradation of pollutants in the effluent within a given period, before new effluent arrives to place an additional load of pollutants. The degradation rates obviously will have to be such that mineralization takes place before the new volume of effluent arrives for degradation. This limitation can be alleviated by pinpointing a rate-limiting step and elevating the level of the rate-limiting enzyme or its regulatory protein by increasing either transcription/translation of encoding gene or its stability.

(b) Some pollutants are not completely mineralized by microbes. Instead, they are transformed to dead end products, which can serve as substrate for other microorganisms. By combining the genes from two parent strains, an effective organism can be engineered to achieve the ultimate goal of mineralization.

(c) Microbes do not metabolize many nitro-aromatics efficiently due to high substrate specificity of component enzymes. In vitro protein engineering can provide the desired modification to enhance the kinetics of degradation.

(d) Since industrial effluents are mixtures of toxic chemicals (instead of a single toxic compound), strains could be constructed which concomitantly destroy target nitro-aromatic along with other toxic pollutants.

(e) Combination of various pathways into a single metabolic pathway may create microorganisms able to act on a broader array of nitro-aromatics.

(f) The problem of toxic heavy metals in industrial effluents can be circumvented by construction of hybrid organisms which catabolize mixtures of pollutants in the presence of inhibitory levels of heavy metals.

(g) Microbes may be engineered for special in situ applications by

(i) manipulating genes for synthesis of constitutive enzymes,

(ii) improving their stability, and

(iii) enhancing resistance to environmental stress due to temperature, pH, ionic strength and nutrient concentration.

(h) Selection of natural bacteria or mutants with low adherence potential to the matrix in aquatic or soil systems is a basic prerequisite for bioremediation. Genetic modification has achieved this attribute in Ultramicrobacteria (UMB), a deep ocean isolate. Efforts are underway to exploit UMBs for clean up of waste dumps. The environmental application of genetically engineered microbes needs an appropriate cloning system, which can

(i) perform under particular environmental conditions,

(ii) be non-transmissible,

(iii) be cost-effective, and

(iv) environment friendly, while not fostering the spread of resistance to chemotherapeutic antibiotics.[Kulkarni , Chaudhari ,2007].

2.18 RESEARCH PAPERS

Boopathy R et al.(1994) studied **Metabolism of 2,4,6-trinitrotoluene by a Pseudomonas consortium under aerobic conditions**. An aerobic bacterial consortium was shown to degrade 2,4,6-trinitrotoluene (TNT). At an initial concentration of 100 ppm, 100% of the TNT was transformed to intermediates in 108 h. Radiolabeling studies indicated that 8% of [^{14}C]TNT was used as biomass and 3.1% of [^{14}C]TNT was mineralized. The first intermediates observed were 4-amino-2,6-dinitrotoluene and its isomer 2-amino-4,6-dinitrotoluene. Prolonged incubation revealed signs of ring cleavage. Succinate or another substrate—e.g., malic acid, acetate, citrate, molasses, sucrose, or glucose—must be added to the culture medium for the degradation of TNT. The bacterial consortium was composed of various Pseudomonas spp. The results suggest that the degradation of TNT is accomplished by co-metabolism and that succinate serves as the carbon and energy source for the growth of the consortium. The results also suggest that this soil bacterial consortium may be useful for the decontamination of environmental sites contaminated with TNT.

Crawford R.L(1995) in his study **The microbiology and treatment of nitroaromatic compounds** showed that Compounds those multiply nitrated aromatic compounds, such as 2,4,&trinitrotoluene (TNT), are extremely recalcitrant must now be re-examined.

Consideration was taken on :

a. Aerobic pure bacterial cultures that degrade nitroaromatic compounds in which he studied all the metabolic pathway of microbes degrading such compunds,

b. Fungi that transform nitroaromatic compounds

c. Other systems that transform nitroaromatic compounds d. Transformations of nitroaromatic compounds in natural environments and by consortia

e. Biotransformations of nitroaromatic compounds by anaerobic microorganisms

The conclusion drawn include Progress in understanding how microorganisms degrade nitroaromatic compounds has been impressive during the past years . It is clear that many mono- and dinitroaromatic molecules are readily degraded by aerobic bacteria through a variety of monooxygenase/dioxygenase-based

pathways. Anaerobic bacteria employ reductive pathways.Even TNT is completely degraded, especially under anaerobic conditions, but also under some aerobic conditions.

Spain JC(1995) worked on **Biodegradation of nitroaromatic compounds** and observed that Anaerobic bacteria can reduce the nitro group via nitroso and hydroxylamino intermediates to the corresponding amines. Isolates of Desulfovibrio spp. can use nitroaromatic compounds as their source of nitrogen. They can also reduce 2,4,6-trinitrotoluene to 2,4,6-triaminotoluene. Several strains of Clostridium can catalyze a similar reduction and also seem to be able to degrade the molecule to small aliphatic acids. Anaerobic systems have been demonstrated to destroy munitions and pesticides in soil. Fungi can extensively degrade or mineralize a variety of nitroaromatic compounds. Removal or productive metabolism of nitro groups can be accomplished by three different strategies : Formation of hydride-Meisenheimer complex, Monooxygenase enzymes can add a single oxygen atom and eliminate the nitro group from nitrophenols, Dioxygenase enzymes can insert two hydroxyl groups into the aromatic ring and precipitate the spontaneous elimination of the nitro group from a variety of nitroaromatic compounds and Potential applications of the above reactions include not only the biodegradation of environmental contaminants, but also biocatalysis and synthesis of valuable organic molecules.

Montpas S et al.(1997) worked on **Degradation of 2,4,6-trinitrotoluene by Serratia marcescens**. A strain of Serratia marcescens, isolated from the soil of a contaminated site, degraded 2,4,6-trinitrotoluene (TNT) as the sole source of carbon and energy. At an initial concentration of 50mg , TNT was totally degraded in 48h under aerobic conditions in a minimal salt medium. Reduction intermediates (4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene) were observed. The presence of a surfactant (Tween 80) is essential to facilitate rapid degradation.

Davis E.P et al.(1997) worked on **Use of Trinitrobenzene as a Nitrogen Source by Pseudomonas vesicularis Isolated from Soil**. An aerobic Gram-negative bacterium identified as Pseudomonas vesicularis was isolated from soil contaminated with 2,4,6-trinitrotoluene (TNT) and 1,3,5-trinitrobenzene (TNB). This bacterium used TNB as the sole source of nitrogen. The TNB was metabolized within 80 h of incubation. The major metabolites produced were dinitroaniline, dinitrobenzene (DNB), nitroaniline, nitrobenzene (NB), and ammonia. The concentrations of DNB and NB produced in the culture medium were nearly stoichiometric. The ammonia concentration in the culture medium increased during the course of incubation. The end product of TNB metabolism was NB, which did not undergo further

degradation even after long incubation time. This bacterium could be used in a syntrophic culture system with other NB-degrading bacteria to remove TNB completely from soil and water at contaminated sites.

Sherburne L.A et al.(1999) worked on **Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by Acetobacterium paludosum**. Substrates and nutrients are often added to contaminated soil or groundwater to enhance bioremediation. Nevertheless, this practice may be counterproductive in some cases where nutrient addition might relieve selective pressure for pollutant biodegradation. Batch experiments with a homoacetogenic pure culture of Acetobacterium paludosum showed that anaerobic RDX degradation is the fastest when auxiliary growth substrates (yeast extract plus fructose) and nitrogen sources (ammonium) are not added. This bacterium degraded RDX faster under autotrophic (H2-fed) than under heterotrophic conditions, even though heterotrophic growth was faster. The inhibitory effect of ammonium is postulated to be due to the repression of enzymes that initiate RDX degradation by reducing its nitro groups, based on the known fact that ammonia represses nitrate and nitrite reductases. This observation suggests that the absence of easily assimilated nitrogen sources, such as ammonium, enhances RDX degradation. Although specific end products of RDX degradation were not determined, the production of nitrous oxide (N2O) suggests that A. paludosum cleaved the triazine ring.

Diels L et al.(1999) worked on **Heavy Metals Bioremediation of Soil**. In this review a method is presented for the treatment of sandy soil contaminated with heavy metals. The system is based on the metal solubilization on biocyrstallization capacity of Alcaligenes eutrophus CH34. The bacterium can solubilize the metals (or increase their bioavailability) via the production of siderophores and adsorb the metals in their biomass on metal-induced outer membrane proteins and by bioprecipitation. After the addition of CH34 to a soil slurry, the metals move toward the biomass. As the bacterium tends to float quite easily, the biomass is separated from the water via a flocculation process. The Cd concentration in sandy soils could be reduced from 21 mg Cd/kg to 3.3 mg Cd/kg. At the same time, Zn was reduced from 1070 mg Zn/kg to 172 mg Zn/kg. The lead concentration went down from 459 mg Pb/kg to 74 mg Pb/kg. With the aid of biosensors, a complete decrease in bioavailability of the metal was measured.

Tope A.M and Jamil K(2000) researched on **Isolation of TNT Tolerant *Pseudomonas* Species (Strain KA) from TNT Contaminated Soil-Biotransformation of TNT. A** 2,4,6-trinitrotoluene (TNT) utilising bacterium, ***Pseudomonas*** species (strain ***KA)*** was isolated from soils of a munitions processing unit and studied for its ability to grow and metaholise TNT. The result indicated that the isolate could grow aerobically in a minimal salt medium containing 0.25 mM1l TNT at 30% It could completely transform 0.25

mMll TNT in 5 days giving 2-isomeric monoaminodinitrotoluenes, namely 4-aminodinitrotoluene and 2-aminodinitrotoluene. Products of TNT transformation were analysed and confirmed by thin layer chromatography and gas chromatography coupled with mass spectrometry. Both, growth of the isolate and biotransformation rates were better supported on sugar and ammonium salt when added to themedium separately. *Pseudomonas* species (strain KA***)*** showed maximum TNT transformation efficiency in the presence of mannose. For degradation of TNT, this strain can be employed to initiate the process in association with the other microbial members that can collectively degrade TNT.

Vidali M(2001) in **Bioremediation : An overview** discussed the development of bioremediation technologies. The major features and limitations are discussed, and an overview of the current state of the art in field applications is sketched. Studied principles and factors for Bioremediation. Moreover Bioremediation strategies were considered and then the advantages and disadvantages were overviewed.

Zhao J.S et al.(2002) worked on **Biodegradation of Hexahydro-1,3,5—Trinitro-1,3,5-Triazine and its Mononitroso Derivative Hexahydro-1-nitroso-3,5-Dinitro-1,3,5-triazine by Klebsiella pneumonia Strain SCZ-1 isolated from anaerobic sludge** .They isolated microbe from MIDI and the 16rRNA method from this sludge and employed it to degrade RDX. Strain SCZ-1 degraded RDX tp formaldehyde(HCHO), methanol(CH3OH)(12% of total carbon), carbon dioxide(CO2)(72% of total C), and nitrous oxide(N2O)(60% of total N) through intermediary formation of methylenedinitramine. Likewise hexahydro-1-nitroso-3,5-dinitro1,3,5-triazine(MNX) was degraded to HCHO,CH3OH and N2O with a removal rate similar to that of RDX. These findings suggested the possible involvement of a common initial reaction, possibly denitration followed by ring cleavage and decomposition in water. The trace amount of MNX detected during RDX degradation and the trace amount of hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine detected during MNX degradation suggested that another minor degradation pathway was also present that reduced – NO2 groups to the corresponding –NO groups.

Kim H.Y et al.(2002) worked on **Degradation of 2,4,6-trinitrotoluene by Klebsiella sp. isolated from activated sludge**. Klebsiella sp. strain C1 isolated from activated sludge metabolized 2,4,6-trinitrotoluene (TNT) by two different pathways. The typical metabolites in the nitro group reduction pathway of TNT, such as hydroxylamino-dinitrotoluenes and amino-dinitrotoluenes, were detected. Dinitrotoluenes and nitrite were also detected, possibly produced by a denitration pathway. After incubation of [U-^{14}C]TNT for 28 and 77 d, 2.4 and 6.24%, respectively, were released as $^{14}CO_2$. This mineralization rate was higher than those

reported by any other TNT degrading bacteria and might be due to the dual pathways of degradation in this bacterium.

Zita S et al.(2002) in their study on **Biological Remediation of Explosives and Related Nitroaromatic Compounds** studied The best-known nitro aromatic compound is the explosive TNT (2,4,6-trinitrotoluene). This article reviews the bioremediation strategies for TNT-contaminated soil and water. They comes to the following conclusion: The optimal remediation strategy for nitro aromatic compounds depends on many site-specific factors. Composting and the use of reactor systems lend themselves to treating soils contaminated with high levels of explosives. Compared to composting systems, bioreactors have the major advantage of a short treatment time, but the disadvantage of being more labour intensive and more expensive. Phytoremediation, although not widely used now, has the potential to become an important strategy for the remediation of soil and water contaminated with explosives.

Oh B.T (2003) et al. researched on **TNT biotransformation and detoxification by a Pseudomonas aeruginosa strain** and founded Successful microbial-mediated remediation requires transformation pathways that maximize metabolism and minimize the accumulation of toxic products. Pseudomonas aeruginosa strain MX, isolated from munitions contaminated soil, degraded 100 mg TNT L−1 in culture medium within 10 h under aerobic conditions. The major TNT products were 2-amino-4,6-dinitrotoluene (2ADNT, primarily in the supernatant) and 2,2_- azoxytoluene (2,2_AZT, primarily in the cell fraction), which accumulated as major products via the intermediate 2-hydroxylamino-4,6-dinitrotoluene(2HADNT). The 2HADNT and 2,2_AZT were relatively less toxic to the strain than TNT and 2ADNT. Aminodinitrotoluene (ADNT) production increased when yeast extract was added to the medium.

Zaripov S.A et al.(2004) studied **Initial Stages of 2,4,6-Trinitrotoluene Transformation by Microorganisms** under which they emphasized on Screening of a wide range of microorganisms (32 strains) isolated from various anthropogenic and natural environments. A number of collection strains showed that the early stages of 2,4,6-trinitrotoluene (TNT) transformation by the majority of the strains studied resulted in the formation of hydroxylamino dinitrotoluenes (HADNTs). The levels of HADNTs were in a number of cases comparable to the initial TNT level. The alternative reductive attack on TNT through the reduction of the aromatic ring was not characteristic of most of the prokaryotes studied. The susceptibility to the toxic effect of TNT was different for gram-positive and gram-negative bacteria.

Kroger M et al(2004) in their work **Biological reduction of TNT as part of combined biological-chemical procedure for mineralization** studied that The explosive 2,4,6-trinitrotoluene (TNT), one of the

most abundant and persistent contaminants at former armament factories and military sites, was cometabolically reduced by sludge (mixed culture) from a sewage plant in order to facilitate mineralization in a subsequent photochemical treatment. Under aerobic conditions, the main reduction products were aminodinitrotoluenes (ADNTs). A greater amount of the nitroaromatics (ca. 30%) was adsorbed by the sludge as was shown by a complete balance of the process using 14C-TNT. Under anaerobic conditions, TNT was further converted into ADNTs and diaminonitrotoluenes (DANTs) while only negligible adsorption to the sludge occured. Conclusion was the anaerobic transformation process results in a faster reduction of TNT than treatment under comparable aerobic conditions. Complete reduction was achieved within the first 12 h, while 20 h were necessary under aerobic conditions. This is a consequence of the redox potential since the reducing power of a solution is, by definition, proportional to its redox potential.

Popesku J.T et al.(2004) in their study **Metabolite production during transformation of 2,4,6-trinitrotoluene (TNT) by a mixed culture acclimated and maintained on crude oil-containing media** observed Metabolites formed during 2,4,6-trinitrotoluene (TNT) removal by a mixed bacterial culture acclimated and maintained on crude oil-containing medium and capable of high rates of TNT removal. In resting cell experiments in the absence of glucose, 46.2 mg/l TNT were removed in 171 h (87.5% removal), with a combined total formation of 7.7 mg/l amino-4,6- dinitrotoluene (ADNT) and 0.3 mg/l 4,4′ azoxytetranitrotoluene and 2′,4-azoxytetranitrotoluene, leaving 70% of the initial TNT unaccounted for. In the presence of glucose, resting cells removed 45.4 mg/l TNT in 49 h (95.5% removal), with 9.1 mg/l ADNT and 2.4 mg/l azoxy compounds being produced, leaving 70.3% of the TNT unaccounted for. Growing cells (glucose present) were capable of removing 44.2 mg/l TNT within 21 h (97.9% removal), with the concomitant formation of 1.8 mg/l ADNTs and 2.2 mg/l azoxy compounds. Denitrated TNT in the form of 2,6 dinitrotoluene was also produced in growing cells with a maximum amount of 1.31 mg/l after 28 h, followed by a slight decrease with time, leaving 88.5% of the initial TNT unaccounted for after 171 h. Radiolabeled 14C-TNT studies revealed 4.14% mineralization after an incubation period of 163 days with growing cells. Both resting cells of the mixed culture, in the presence and absence of glucose, and growing cells, were able to transform TNT rapidly with fractional conversion into nitroaromatic metabolites, fused aromatic azoxy compounds and nitrite.

Ye J. et al.(2004) studied **Biodegradation of nitroaromatics and other nitrogen-containing xenobiotics** and their research was focused on development of strategies to allow more recalcitrant compounds to serve as growth substrates for microorganisms. In this paper, an overview of recent developments on the biodegradation of nitrogen-containing xenobiotics is presented. Since many of the contaminated environments have combinations of nitroaromatic compounds present, this further complicates the

bioremediation efforts. They concluded research in exploration of new metabolic pathways has considerably improved understanding of the genes and enzymes responsible for key nitroaromatic biotransformation reactions. Although the field of metabolic engineering is still young, it has helped further understanding the nature of nitroaromatic biodegradation systems, which in turn is beginning to have an impact on the development of bioremediation solutions. A potential area to explore is the possibility of producing value-added products from nitroaromatic wastes. Development of biocatalytic processes that utilize relatively inexpensive nitroaromatic waste feedstocks for commercially useful products would potentially reduce waste at source and reduce complications in bioremediation efforts.

Thomas H, Gerth A et al.(2005) researched on **Enhanced Humification Of Tnt Bioremediation Of Contaminated Soil And Water In Practice** and founded The explosive 2,4,6-trinitrotoluene is a substance of environmental concern because of its toxicity. In reducing environments TNT is reduced to amino-metabolites that are immobilized into soil organic matter. The humification of TNT was tested with good results for the cleanup of contaminated soil and water. Molasses proved to be a suitable source of organic carbon for the biological reduction. The removal of TNT was 40-60% without a carbon source; after addition of molasses the removal was > 95% and the outflow concentration was less than 10 µg/L. The removal of 2,6- dinitrotoluene (2,6-DNT) was also measured (data not shown). It was initially 60-80% and after addition of molasses also reached > 95%. Adaptation phases for 2,6-DNT were longer than for TNT. They concluded from pilot-scale and field experiments, that it is possible to use a biological strategy for the remediation of soil and water.

Yin H et al.(2005) researched on **Reductive transformation of TNT by Escherichia coli: pathway description using aerobically grown Escherichia coli cultures**. In the absence of an external carbon or energy source, E. coli resting cells transformed TNT to hydroxylaminodinitrotoluenes (2HADNT, 4HADNT, with 4HADNT as the dominant isomer), aminodinitrotoluenes (4ADNT, with sporadic detection of 2ADNT), 2,4-di (hydroxylamino)-6-nitrotoluene (24D(HA)6NT), 2,4-diamino- 6-nitrotoluene (24DA6NT), and an additional compound which was tentatively identified as a (hydroxylamino) aminonitrotoluene isomer via gas chromatography/mass spectroscopy and spectral analysis. The resting cell assay, performed in an oxygen-free atmosphere, avoided formation of azoxy dimers and provided good mass balances. Significant preference for reduction in the para versus ortho position was detected. The formation of 24D(HA) 6NT, but not ADNT, appeared inhibited by the presence of TNT. The rate and extent of TNT reduction were significantly enhanced at higher cell densities, or by supplying an exogenous reducing power source, revealing the importance of enzyme concentration and reducing power. Whether the

oxygen-insensitive E. coli nitroreductases, encoded by nfsA and nfsB, directly catalyze the TNTreduction or account for the complete TNT transformation pathway, remains to be determined.

Popesku J.T et al.(2006) worked on **Trinitrotoluene removal in a soil slurry and soil box systems by an oil-degrading mixed bacterial culture**. They investigated the ability of the mixed bacterial culture acclimated and maintained on crude oil-containing medium to bioremediate TNT-spiked soil and artificially weathered soil slurry systems, as well as a soil box system. The culture was able to remove up to 302 ppm (mg/l) of TNT within 24 h in a spiked-soil slurry system, which is among the highest rates of TNT removal reported to date. The toxicity of artificially weathered TNT-spiked soil to Vibrio fischeridecreased over a period of 39 h from a 15-min EC_{50} of 15.7 to 32.5 ppm. Preliminary results of a soil box system, in which no agitation was used, showed similar TNT removal to the soil slurry system, with 100 ppm TNT being removed within 24 h.

Crocker F.H et al.(2006) researched **on Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20**. This paper reviews recent developments on the biodegradation of cyclic nitramines and the potential of genomics to identify novel functional genes of explosive metabolism. Biodegradation mechanisms for cyclic nitramines include (a) formation of a nitramine free radical and loss of nitro functional groups, (b) reduction of nitro functional groups, (c) direct enzymatic cleavage, (d) α-hydroxylation, or (e) hydride ion transfer. Pathway intermediates spontaneously decompose in water producing nitrite, nitrous oxide, formaldehyde, or formic acid as common endproducts. In vitro enzyme and functional gene expression studies have implicated a limited number of enzymes/genes involved in cyclic nitramine catabolism. Advances in molecular biology methods such as high-throughput DNA sequencing, microarray analysis, and nucleic acid sample preparation are providing access to biochemical and genetic information on cultivable and uncultivable microorganisms. This information can provide the knowledge base for rational engineering of bioremediation strategies, biosensor development, environmental monitoring, and green biosynthesis of explosives.

Claus H et al.(2007) worked on **Transformation of 2,4,6-trinitrotoluene (TNT) by Raoultella terrigena** and observed bacteria living in environments highly contaminated with 2,4,6- trinitrotoluene (TNT) and other nitroorganic compounds for their capacity for TNT degradation. One isolate, Raoultella terrigena strain HB, removed TNT at concentrations between 10 and 100 mg l)1 completely from culture supernatants under optimum aerobic conditions within several hours. Only low concentrations of nutrient supplements were needed for the cometabolic transformation process. Radioactivity measurements with ring-labelled 14C–TNT detected about 10–20% of the initial radioactivity in the culture supernatant and the residual 80–

90% as water-insoluble organic compounds in the cellular pellet. HPLC analysis identified aminodinitrotoluenes (2-ADNT, 4-ADNT) and diaminonitrotoluenes (2,4- DANT) as the metabolites which remained soluble in the culture medium and azoxy-dimers as the main products in the cell extracts. They concluded the accumulation of TNT metabolites within the bacterial cells offers the opportunity to clean up contaminated waters after separation of the biomass. R. terrigena strain HB removed TNT also under in situ-like conditions, i.e., in media with surface water and soil extracts contaminated with various nitroorganic compounds. As our new isolate is well adapted to different environmental conditions, e.g. low and high temperature, nutrient and redox states, R. terrigena strain HB may be a superior candidate for field applications, at least as long as biotransformation and not biomineralization is the state of art.

Kulkarni M, Chaudhari A.(2007) presented their work on **Microbial remediation of nitro-aromatic compounds: an overview** and observed that Several microbes catalyze mineralization and/or non-specific transformation of nitro-aromatics either by aerobic or anaerobic processes. Aerobic degradation of nitro-aromatics applies mainly to mono-, dinitro-derivatives and to some extent to poly-nitro-aromatics through oxygenation by: Dioxygenase catalyzed reactions, Meisenheimer complex formation, and Partial reduction of aromatic ring. Under anaerobic conditions, nitro-aromatics are reduced to amino-aromatics to facilitate complete mineralization. Presently, the qualitative and quantitative performance of biological approaches of remediation is undergoing improvement . Among them, degradative plasmids have provided a major handle in construction of recombinant strains. Although recombinants designed for high performance seem to provide a ray of hope, their true assessment under field conditions is required to address ecological considerations for sustainable bioremediation.

Newcombe D.A et al.(2007) in their study **Transformation and fate of 2,4,6-trinitrotoluene (TNT) in anaerobic bioslurry reactors under various aeration schemes**: Implications for the decontamination of soils founded trinitrotoluene (TNT)-contaminated soil subjected to treatment in strictly anaerobic bioreactors results in tight binding of TNT transformation products to soil organic matter. The research presented here examined the fate of TNT and its metabolites in bioreactors under three different aeration regimes. In all treatment regimes, the typical metabolites of aminodinitrotoluenes and diaminonitrotoluene were observed prior to irreversible binding into the soil fraction of the slurry. Significant transformation of TNT into organic acids or simple diols, as others report in prior work, was not observed in any of the treatments and is an unlikely fate of TNT in anaerobic soil slurries. These results indicate that aeration does not dramatically affect transformation or fate of TNT in reactor systems that receive a rich carbon source but does affect the rate at which metabolites become tightly bound to the soil. The most rapid

transformations and lowest redox potentials were observed in reactors in which an aerobic headspace was maintained suggesting that aerobes play a role in establishing conditions that are most conducive to TNT reduction.

George I. et al.(2008) studied **Effect of 2,4,6-trinitrotoluene on soil bacterial communities**. To gain insight into the impact of 2,4,6-trinitrotoluene (TNT) on soil microbial communities, they characterized the bacterial community of several TNT-contaminated soils from two sites with different histories of contamination and concentrations of TNT. The amount of extracted DNA, the total cell counts and the number of CFU were lower in the TNT-contaminated soils. Analysis of soil bacterial diversity by DGGE showed a predominance of Pseudomonadaceae and Xanthomonadaceae in the TNT-contaminated soils, as well as the presence of Caulobacteraceae. CFU from TNT-contaminated soils were identified asPseudomonadaceae, and, to a lesser extent, Caulobacteraceae. Finally, a pristine soil was spiked with different concentrations of TNT and the soil microcosms were incubated for 4 months. The amount of extracted DNA decreased in the microcosms with a high TNT concentration [1.4 and 28.5 g TNT/kg (dry wt) of soil] over the incubation period. After 7 days of incubation of these soil microcosms, there was already a clear shift of their original flora towards a community dominated by Pseudomonadaceae, Xanthomonadaceae, Comamonadaceae and Caulobacteraceae. These results indicate that TNT affects soil bacterial diversity by selecting a narrow range of bacterial species that belong mostly toPseudomonadaceae and Xanthomonadaceae.

Singh R. et al.(2009) researched on **Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria.** The effluent collected from HMX production plant containing acetic acid, ammonium nitrate and explosive residue with water and other organic nitro bodies was used. The diluted and neutralized effluent was subjected to biodegradation using Pseudomonas (HPB1) and two Bacillus (HPB2, HPB3) denitrifying bacterial isolates. Samples were analysed by HPLC for qualitative and quantitative analysis of remaining RDX and HMX. The results indicate that the HMX and RDX was biodegraded under denitrifying conditions. The isolate Pseudomonas (HPB1) was found to be an efficient biodegrading strain for HMX. However, the isolate Pseudomonas (HPB1) was found to have lower biodegradation activity for RDX as compared to the denitrifying strain Bacillus (HPB2). Denitrifying bacteria Bacillus (HPB2) was found to be the most efficient strain for the biodegradation of RDX and HMX containing effluent neutralized with sodium bicarbonate. Denitrifying bacteria Pseudomonas (HPB1) showed the maximum nitrate reduction in the presence of both the neutralizing agents- sodium bicarbonate and ammonia.

Cho Y.S et al.(2009) worked on **Comparative analysis of 2,4,6-trinitrotoluene (TNT)-induced cellular responses and proteomes in Pseudomonas sp. HK-6 in two types of media**. TNT-induced cellular responses and proteomes in Pseudomonas sp. HK-6 were comparatively analyzed in two different media: basal salts (BS) and Luria broth (LB). HK-6 cells could not degrade more than 0.5 mM TNT with BS medium, while in LB medium, they exhibited the enhanced capability to degrade as much as 3.0 mM TNT. Analysis of total cellular fatty acids in HK-6 cells suggested that the relative abundance of several saturated or unsaturated fatty acids is altered under TNT-mediated stress conditions. Scanning electron microscopy showed the presence of perforations, irregular rod formations, and wrinkled extracellular surfaces in cells under TNT stress. Proteomic analysis of soluble protein fractions from HK-6 cultures grown with TNT as a substrate revealed 11 protein spots induced by TNT. Among these, seven proteins (including Alg8, AlgB, NirB, and the AhpC/Tsa family) were detected only in LB medium containing TNT. These results provide a better understanding of the cytotoxicity and survival mechanism used by Pseudomonas sp. HK-6 when placed under TNT stress conditions.

Kanekar S.P et al.(2009) in their study **Bioremediation of nitroexplosives waste water by an yeast isolate Pichia sydowiorum MCM Y-3 in fixed film bioreactor** studied potential of a soil isolate of yeast Pichia sydowiorum MCM Y-3, for remediation of HMX wastewater in Wxed Wlm bioreactor (FFBR). The flask culture studies showed appreciable growth of the organism in HMX wastewater under shake culture condition within 5–6 days of incubation at ambient temperature (28 § 2°C). The FFBR process operated in both batch and continuous mode, with Hydraulic Retention Time (HRT) of 1 week resulted in 50– 55% removal in nitrate, 70–88% in acetate, 50–66% in COD, and 28–50% in HMX content. Continuous operation of the reactor showed better removal of nitrate as compared to that in the batch operation, while removal of acetate and COD was comparable in both the modes of operation of the reactor. Insertion of baffles in the reactor increased efficiency of the reactor. Thus, FFBR developed with baffles and operated in continuous mode is beneficial for bioremediation of high nitrate and acetate containing wastewater using the culture of P. sydowiorum.

Palleroni N.J , Pieper D.H and Moore E.R.B(2010) studied **Microbiology of Hydrocarbon-Degrading Pseudomonas.** In their study the genus *Pseudomonas* is summarily described with respect to the phenotypic and genomic properties of its member species, which have been considered in the development of the current taxonomic treatment. The chapter emphasizes the biochemical versatility of the species of the genus, which is manifested in the capacity of many strains to degrade a number of aliphatic, aromatic, poly-aromatic hydrocarbons and various derivatives, among a vast variety of miscellaneous organic compounds.

The observed degradation capabilities of *Pseudomonas* are considered with respect to the metabolic potential encoded in the genomes of strains for which the genome sequences have been determined. Strains of *Pseudomonas* species are able to grow in simple media and the nutritional versatility of a number of them includes the ability to utilize many compounds as substrates that may be toxic to most other prokaryotes and to higher organisms, as observed in environmental analyses of *Pseudomonas*. These properties point to Pseudomonas as particularly important agents in the design of bioremediation applications.

Jiménez J.L et al.(2010) studied **A Genomic View of the Catabolism of Aromatic Compounds in Pseudomonas**. Genomic analyses point to the existence of parologous genes likely involved in the degradation of aromatic compounds, suggesting that our current knowledge about the degradative potential of Pseudomonas is still far from complete. On the other hand, many aromatic compounds, e.g., hydrocarbons and phenolic compounds, simultaneously serve as potential nutrients to be metabolized by bacteria but also as cellular stressors. The transcriptomic and proteomic approaches carried out with some *Pseudomonas* strains provide some light on the biodegradation versus stress dilemma. All the basic knowledge generated so far about the metabolism of aromatic compounds in *Pseudomonas* paves the way for a wealth of biotechnological applications, e.g., bioremediation, biotransformations, biosensors, etc., and it is of great potential in Synthetic Biology. Therefore, *Pseudomonas* becomes a paradigmatic bacterial genus both for increasing basic knowledge and for applied research within the field of aromatic compounds degradation.

Douglas T.A et al.(2011) worked on **Desorption and Transformation of Nitroaromatic (TNT) and Nitramine (RDX and HMX) Explosive Residues on Detonated Pure Mineral Phases**. They detonated pure mineral phases with Composition B, a mixture of TNT and RDX, and investigated the fate of detonation residues in aqueous slurries constructed from the detonated minerals. The pure minerals included Ottawa sand (quartz and calcite), microcline feldspar, phlogopite mica, muscovite mica, vermiculite clay, beidellite (a representative of the smectite clay group), and nontronite clay. Energy dispersive X-ray spectrometry, X-ray diffraction, and gas adsorption surface area measurements were made of the pristine and detonated minerals. Batch slurries of detonated minerals and deionized water were sampled for 141 days and TNT, RDX, and TNT transformation products were measured from the aqueous samples and from the mineral substrates at day 141. TNT and RDX exhibited analyte loss in almost all batch solutions over time but loss was greater in vermiculite, beidellite, and phlogopite than in muscovite and quartz. Three important results emerge from this study illustrating how explosive detonation loads explosive residues to minerals in a manner markedly different than what is represented by aqueous addition. First, there are mineralogical changes to low temperature minerals (calcite and vermiculite) following detonation that show

soil mineralogies at impact areas may be altered by detonation events. Second, the mineralogical surface is altered by detonation. Third, some mineralogies are associated with an enhanced loss of explosive compounds from solution and this is potentially caused by transformation and/or sorption. Phlogopite, beidellite, and vermiculite samples were associated with the greatest loss of TNT and RDX from aqueous solutions over time. These minerals are all phyllosilicates and some (such as beidellite and vermiculite) have swelling capabilities.

Das M et al.(2012) studied on **Role of Microorganisms in Remediation of Contaminated Soil**. The review focuses on an overview of various physico-chemical methods used earlier for soil remediation purposes, what are the bioremediation techniques used nowadays and how it works; finally future perspectives of bioremediation techniques and conclusion. Traditional methods to cleanup or decontaminate the soil are expensive, labour intensive, do not always ensure that pollutants are completely removed or destroyed and often result in abrupt changes to the physical, chemical, and biological characteristics of the treated soil. Use of microorganism have shown promises in remediation of soil contaminated with heavy metals and radionuclide, organic compounds including chlorinated solvents like TCE; explosives such as TNT, RDX; petroleum hydrocarbons including PAHs; PCBs and pesticides such as atrazine and organophosphates. Aesthetically pleasing cleaning methodology, minimal disruption and preservation of top soil, usefulness in treating broad range of environmental contaminants and low cost (60–80% or even less costly than conventional methods) are the advantages associated with microorganisms mediated soil remediation technology and so it has gained increasing attention over the past 15 years.

Perreault N.N et al.(2012) worked on **Aerobic biotransformation of 2,4-dinitroanisole in soil and soil Bacillus sp.** 4-Dinitroanisole (DNAN) is a low sensitive melt-cast chemical being tested by the Military Industry as a replacement for 2,4,6-trinitrotoluene (TNT) in explosive formulations. Studied aerobic biotransformation of DNAN in artificially contaminated soil microcosms. DNAN was completely transformed in 8 days in soil slurries supplemented with carbon and nitrogen sources. DNAN was completely transformed in 34 days in slurries supplemented with carbons alone and persisted in unamended microcosms. A strain of Bacillus (named 13G) that transformed DNAN by co-metabolism was isolated from the soil. HPLC and LC–MS analyses of cell-free and resting cell assays of Bacillus 13G with DNAN showed the formation of 2-amino-4-nitroanisole as the major end-product via the intermediary formation of the arylnitroso (ArNO) and arylhydroxylamino (ArNHOH) derivatives, indicating regioselective reduction of theortho-nitro group. this paper provides the evidence of fast DNAN transformation by the indigenous microbial populations of an amended soil with no history of contamination with explosives and a first insight into the aerobic metabolism of DNAN by the soil isolate Bacillus 13G.

Maeda T and Ogawa H.I (2012) studied **Microbial Degradation of 2,4,6-Trinitrotoluene: Application to Explosives Sensor.** They overviewed that Sediments and soils beneath some industrial sites contain large amounts of nitro aromatics with up to 10 g of TNT per kg of soil being reported for some. The biodegradation studies have indicated that an explosive is highly recalcitrant for microbial biodegradation. Among them, in particular, TNT is more recalcitrant than other nitroaromatic compounds (e.g. mono- and dinitrotoluenes), because three nitro groups are located symmetrically on the aromatic ring which restrict the attack by classic dioxygenase enzymes involved in the microbial metabolism of aromatic compounds. Hence, TNT has strong cytotoxicity and mutagenicity in various living and is listed as class C potential human carcinogen by the US Environmental Protection Agency. In addition, in TNT-exposed humans, notable toxic manifestations have included aplastic anaemia, toxic hepatitis, cataracts, hepatomegaly, and liver cancer; therefore, it is significant to develop the bioremediation technology for TNT.

Arora P.K et al.(2012) researched on **Degradation of chlorinated nitroaromatic compounds** and founded Chlorinated nitroaromatic compounds (CNAs) are persistent environmental pollutants that have been introduced into the environment due to the anthropogenic activities. Bacteria that utilize CNAs as the sole sources of carbon and energy have been isolated from different contaminated and non-contaminated sites. Microbial metabolism of CNAs has been studied in their study , and several metabolic pathways for degradation of CNAs have been proposed. Detoxification and biotransformation of CNAs have also been studied in various fungi, actinomycetes and bacteria. Several physicochemical methods have been used for treatment of wastewater containing CNAs; however, these methods are not suitable for in situ bioremediation. They concluded Although metabolic pathways of CNAs have been characterized in bacteria, there is very little information about molecular characterization of genes involved in the microbial degradation of CNAs. Therefore, more efforts are needed to understand the mechanism of degradation of the CNAs at the molecular level.

Solyanikova I.P et al.(2012) researched on **Detoxification of High Concentrations of Trinitrotoluene by Bacteria**. The ability of the strains destructors of various aromatic compounds to utilize trinitrotoluene (TNT) up to concentration of 70 mg/l was shown. An increase in the TNT concentration from 100 to 150 mg/l did not inhibit its conversion rate by the Kocuria palustris RS32 strain. The Acinetobacter sp. VT11 strain utilized TNT as a sole substrate for growth; 3,5_dinitro_4_methyl anilide acetate and 2,6_dinitro_4_amino_ toluene were identified as intermediates of TNT degradation by active strains of *Pseudomonas* sp. VT_7W and Kocuria rosea RS51. At the same time, 4_methyl_3,5_dinitroformamide was discovered for the first time upon the TNT destruction by the bacteria strains of Rhdococcus opacus 1G and

Rhdococcus sp. VT_7. The active bacterial strains achieved an 82–90% destruction of TNT when they were introduced into the soil. The cultures chosen in the work effectively transformed TNT in the presence of an additional growth source. The isolated K. palustris RS32, R. opacus 1G, and Acinetobacter sp. VT11 cultures were capable to transform TNT at concentrations that entirely suppressed the growth of the strains. The experiment conducted in the soil showed the possibility to use selected bacteria for the decontamination of soil from TNT.

CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

This chapter discusses the materials used and methodology adopted during the study. All the chemicals used and reagents employed were of analytical grade with sufficient purity. The calibration curves were prepared prior estimation of unknown concentration and used for the study.

3.1 COLLECTION OF SAMPLE(from industry facility)

Water Sample Collected :

W1 : Outlet water from nitrate unit
W2 : Water from nitrate unit
W3 : Yellow water from Catch pit
W4 : Red Water
W5 : Outlet from ETP
W6 : Inlet Water to ETP
W7 : Pond Water

Soil Samples Collected :

SL1R1 : Sludge Sample
S1R1 : Soil Sample near titration plant at distance of 200m
S2R2 : Subsurface Soil
S3R1 : TNT Washing house at distance of 200-300 m surface
S4R1 : TNT washing house at distance of 500 m surface
S5R1 : TNT washing house at distance of 500m subsurface
S6R1 : Pond Site Surface
S7R1 : Pond Site Surface

3.2 PRETREATMENT OF SOIL AND WATER SAMPLES

3.2.1 For characterization of Nitroaromatics contaminated water samples :

Performed Gravity Filtration with the help of Whatmann Filter paper (15 cm)

3.2.2 For characterization of Nitroaromatics contaminated Soil samples :

i. Took out the soil sample and spread it on a filter paper sheet, firmly to be dried in air for 2-3 days.

ii. After the samples were dried , break the large chunks of soil into small or fine particles with the help of pestle and mortar.

iii. Transfer this soil threw a 10 mesh size screen to get fine layer of soil. This sieved soil is used for further analysis.[CSTP-NCR, 1980]

3.2.3 For HPLC Study Of Nitroaromatics Contaminated Water Samples :

Water sample is filtered through vacuum filter (0.45 micro metre) to be used further in HPLC machine , as larger particles present in the sample can block the column and will create problem in separation procedures in the column itself.[US EPA method 8330B, 2006]

3.2.4 Nitroaromatics contaminated soil sample for microbial study :

The soil samples used for microbial study were stored below room temperature , taken out 15 minutes before microbial analysis procedures on them.

3.3 EXTRACTION OF NITROAROMATICS CONTAMINATED SOIL AND WATER SAMPLES

For characterization of soil and water samples, after pre-treatment the samples should be extracted by various methods . Used HACH DR/890 COLORIMETER for all analysis and characterization of explosive contaminated water samples and HACH SIW-1 Soil and Irrigation water manual for all analysis and characterization of explosives contaminated soil samples, respectively. HACH has developed several procedures that are equivalent to USEPA approved methods. Even though minor modifications exist, the USEPA has reviewed and accepted certain procedures for reporting purposes. These methods are not published in the Federal Register, but are referenced to the equivalent USEPA method in the procedure.

3.3.1 Extraction Procedures for Soil samples :

Used the following methods for soil extraction :

3.3.1.1 Aqueous Extraction Method :

Used for Salinity and pH calculations, the procedure includes –

i) Take pre-treated, sieved soil samples.

ii) Using a 5gms scoop, measure 4 scoops = 20gms of the prepared soil sample into a 50ml plastic beaker.

iii) Using a 25ml graduated cylinder, accurately measure 20ml of deionized water and transfer it to 50ml beaker.

iv) Repeat steps 2-3 for each soil samples undergoing extraction.

v) Using spatula, stir the contents of the beaker for 1 minute at 10min. intervals over a 30minute period.

vi) After 30minutes, use the prepared sample for salinity and pH determination. [HACH,SIW-1]

3.3.1.2 Calcium Sulfate extraction Method :

Used for Nitrate – Nitrogen detection, the procedure includes –

i) Take pre-treated, sieved soil samples.

ii) Using a 5grms soil scoop, measure 2 scoops = 10grms of the prepared soil sample into a round sample bottle.

iii) Using the 0.1gm plastic spoon, add 1 level spoonful of Calcium Sulfate to the round sample bottle containing the sample.

iv) Using a 25 ml graduated cylinder, accurately measure 20ml of deionized water into the cylinder and transfer it into the round mixing bottle.

v) Repeat steps (i) to (iii) for each soil sample undergoing extraction.

vi) Cap and shake each bottle vigorously for 1 minute.

vii) Using a plastic funnel and filter paper, filter the contents of the bottle into another round sample bottle.

viii) Analyze this extract for nitrate nitrogen within 2hrs. If this is not possible, then extract may be refrigerated for 24hrs before analysis as test is temperature sensitive.

3.3.1.3 Mehlich 2 Extraction Method :

Used for Calcium + magnesium, phosphorus, potassium detection, the procedure includes

i) Take pre-treated , sieved soil samples.

ii) Using a 2-grams scoop, measure 1 scoop of the prepared soil sample into a round sample bottle.

iii) Using a 25-mL graduated cylinder, accurately measure 20 mL of the diluted Mehlich 2 Soil Extractant into the cylinder, then transfer it to the round sample bottle.

iv) Repeat steps (ii) and (iii) for each soil sample undergoing extraction.

v) Cap and shake each bottle for 5 minutes.

vi) Using a plastic funnel and filter paper, filter the contents of the bottle into another round sample bottle.

vii) Prepare /measure 20 ml of the Mehlich 2 soil extractant concentrate into a 25ml graduated cylinder.

viii) Transfer the extractant concentrate to one of the flip – top dispensing bottles provided.

ix) Add deionized water to the dispensing bottle until the volume reaches the bottom of the neck. Invert several times to mix.

vii) The mehlich 2 extractant is now ready to be used. It is stable for 24 hrs, If stored longer, refrigerate to prevent microbial growth. [HACH,SIW-1]

3.4 CHARACTERIZATION OF SOIL AND WATER SAMPLES CONTAMINATED WITH NITROAROMATICS

3.4.1 Soil Sample Analysis :

Used HACH SIW-1 Soil and Irrigation water manual for all analysis and characterization of nitroaromatics contaminated soil samples. Tested for chemical and Physical characteristics.

3.4.1.1 Chemical Analysis and Characterization:

Tested for pH, Salinity, Nitrate – Nitrogen, Phosphorous Reactive, Potassium(Exchangeable)

a. pH Test :

i) Prepare the Soil extractant from aqueous extraction method using pre-treated soil samples.

ii) Calibration of pH meter –

- Prepare a pH of 7.00 buffer solution by adding the contents of one pH 7.00 buffer powder pillow to a 50ml beaker containing 50ml of water.
- Swirl the beaker until the contents are dissolved, Slide the On/Off switch located on the top of the Pocket Pal pH tester to On.

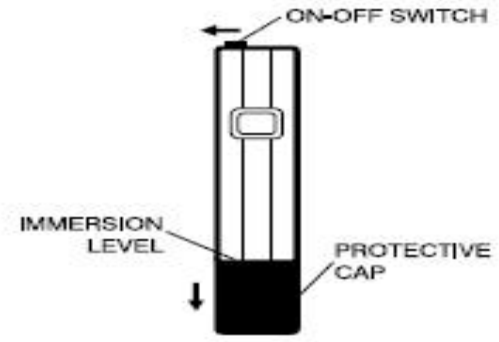


Fig 3.1 :Pocket Pal pH Tester

- Remove the protective cap from the bottom of the instrument and immerse the pH electrode tip one inch below the surface of the buffer solution. If necessary, calibrate the instrument by adjusting the calibration screw near the pocket clip with the trim tool(screw driver) provided to obtaina reading of 7.

iii) Now immerse the tip of the calibrated pocket pal pH tester one inch below the surface of the aqeous sample extract and stir gently until the soil is completely suspended.

iv) Record the stabilized pH reading to the nearest 0.1 pH unit.

viii) Rinse the pH electrode with deionized water. [HACH,SIW-1]

b. Salinity (Conductivity) Test :

i) Prepare the Soil extractant from aqueous extraction method and calcium sulfate extraction method using pre-treated soil samples.

ii) Calibration of conductivity tester –

- Add 50ml of the saturated calcium sulfate extraction solution to 50 ml plastic beaker.
- Slide ON/OFF switch on top of the pocket pal conductivity tester to on.
- Remove the protective cap from the bottom of the instrument. Immerse the electrode tip one inch below the surface of the calcium sulfate extraction solution. If necessary, calibrate the instrument by adjusting the calibration screw near the pocket clip with the trim tool(screw driver) provided. Adjust the value to 2200 µS or 2.2 mS.

iii) Immerse the tip of the calibrated pocket pal conductivity tester one inch below the surface of the sample extract prepared and stir gently until the soil is completely suspended. [HACH,SIW-1]

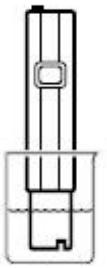


Fig 3.2 : Conductivity meter immersed in sample

iv) When the digital display stabilizes; record the value. The conductivity meter will show result in mS.

v) Rinse the electrode with deionized water, and remove the excess by wiping the tip with a tissue paper before continuing to the next sample.

vi) Once the conductivity of each sample has been determined, rinse electrode with deionized water. Slide the on/off switch towards off and cover the electrode with protective cap before storing. [HACH,SIW-1]

c. Nitrate – Nitrogen Test[Cadmium Reduction Method] 0 – 60 ppm :

i) Prepare/obtain the Soil extractant from calcium sulfate extraction method using pre-treated soil samples.

ii) Place the high range nitrate color disc into the color comparator. Make sure it is inserted so that mg/l values are visible through the window of the color comparator.

iii) Label one color viewing tube "S" for sample and another color viewing tube "B" for blank. Thoroughly rinse both color viewing tubes with deionized water. Shake the tubes to remove the remaining rinse water.

iv) Add a small amount of the sample extract (about $1/4^{th}$ deep) to the color viewing tube marked "S". Cap the tube with a rubber stopper and shake it for a few seconds. Discard this solution.

v) Add the extract to both tubes until the meniscus is even with 5ml mark(bottom of frosted area).

vi) Add the contents of one NitraVer 5 powder pillow to the tube marked "S". Cap and shake the tube exactly for 1 minute.

vii) Immediately place tubes "S" and "B" into the comparator with tube "B" in the outside hole and Tube "S" in the inside hole.

viii) Five minutes after completing step (vii), hold the color comparator up to a light source. Rotate the disc until the color in the window for tube "B" matches the color in the window for tube "S". Record the value in the scale window. Take two more readings of the sample, rotating the disc between each reading. Complete all three readings within 10min of completing step(vii).

ix) Take the average of the three readings and multiply by 2 for the available nitrate-nitrogen value in the soil. [HACH,SIW-1]

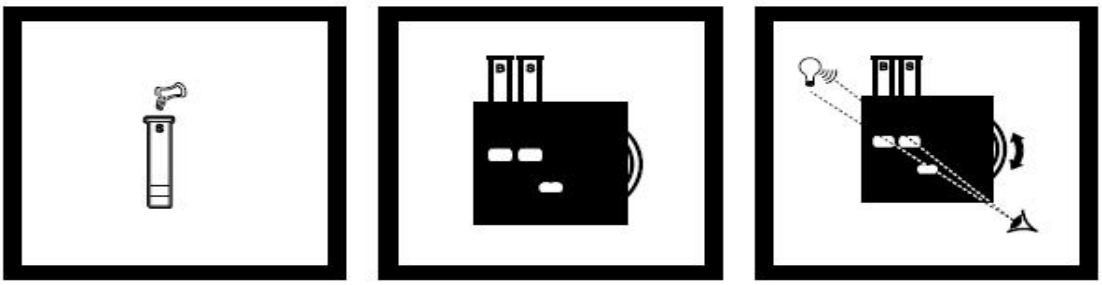


Fig 3.3: Nitrite – nitrogen test performed using color comparator

d. Phosphorus Test (0-130 mg/l or 0-130 ppm) – Ascorbic Acid Method :

i) Prepare/obtain the Soil extractant from mehlich 2 extraction method using pre-treated soil samples.

ii) Using the 2.5 ml dropper add 2.5 ml of the filtered sample extract prepared in step1 to a 25 ml graduated cylinder. Dilute to the 25ml mark with deionized water , stopper, and invert to mix.

iii) Label one color viewing tube “S” for sample and another color viewing tube “B” for blank. Thoroughly rinse both color viewing tubes with deionized water. Shake the tubes to remove the remaining rinse water.

iv) Add a small amount of the sample extract (about 1/4th deep) to the color viewing tube marked “S”. Cap the tube with a rubber stopper and shake it for a few seconds. Discard this solution.

v) Add the diluted mehlich 2 extract to both tubes until the meniscus is even with 5 ml mark(bottom of frosted area).

vi) Add the contents of one PhosVer 3 powder pillow to the tube marked “S”. Cap and shake the tube exactly for 1 minute.

vii) Immediately place tubes “S” and “B” into the comparator with tube “B” in the outside hole and Tube “S” in the inside hole.

viii) Three minutes after completing step (vii), hold the color comparator up to a light source. Rotate the disc until the color in the window for tube “B” matches the color in the window for tube “S”. Record the value in the scale window. Take two more readings of the sample, rotating the disc between each reading. Complete all three readings within 10min of completing step(vii).

ix) Take the average of the three readings and multiply by 3.3 for the available phosphate-phosphorus value in the soil. [HACH,SIW-1]

e. Potassium(Exchangeable) Test (0 – 250 mg/l or 0 – 250 ppm) – Turbidimetric Tetraphenyl Borate Method :

i) Prepare/obtain the Soil extractant from mehlich 2 extraction method using pre-treated soil samples.

ii) Using a 1 ml dropper, add 3.0 ml of the sample extract from step1 to a 25 ml cylinder.

iii) Add deionized water to the 21 ml mark on the cylinder. Firmly cap the cylinder with a rubber stopper and invert to mix.

iv) Add one potassium 2 reagent powder pillow and 3ml of alkaline EDTA solution to the cylinder and invert several times to mix. Allow the solution to stand for at least 3 minutes.

v) Add the contents of one Potassium 3 reagent powder pillow. Firmly cap and shake the cylinder vigorously for 10 seconds. Allow the solution to stand for at least 3 minutes but no longer than 10minutes. A white turbidity will develop.

vi) While looking straight down to the cylinder, slowly insert the potassium Dip stick vertically into the solution until the black dot is no longer visible from above the cylinder.

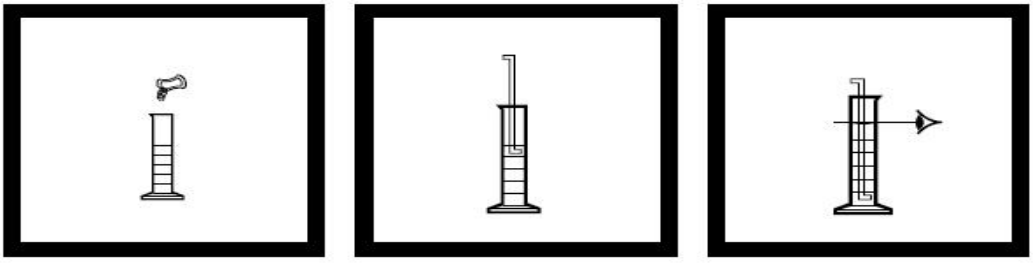


Fig 3.4: Potassium test using potassium Dip stick

vii) Hold the dipstick in that position and rotate the cylinder so you can see the scale on the dipstick. Record the number (in mm) on the dipstick scale where the surface of the sample meets the dipstick scale.

viii) Repeat step (vi) and (vii) two more times recording the values each time. Take the average of three readings. And then refer to potassium conversion table to determine level of potassium in the soil. [HACH,SIW-1]

Dipstick Reading (mm)	**mg/l (ppm) Potassium**	**lbs/A Potassium**	**Kg/Ha Potassium**	**meq/100 g Potassium**
80	87	174	194	0.22
75	94	188	210	0.24
70	101	202	225	0.26
65	109	218	243	0.28
60	118	236	263	0.30
55	129	258	281	0.33
50	143	286	319	0.37
45	159	318	355	0.41
40	180	360	401	0.46
35	207	414	462	0.53
30	243	486	542	0.62
25	294	588	656	0.75

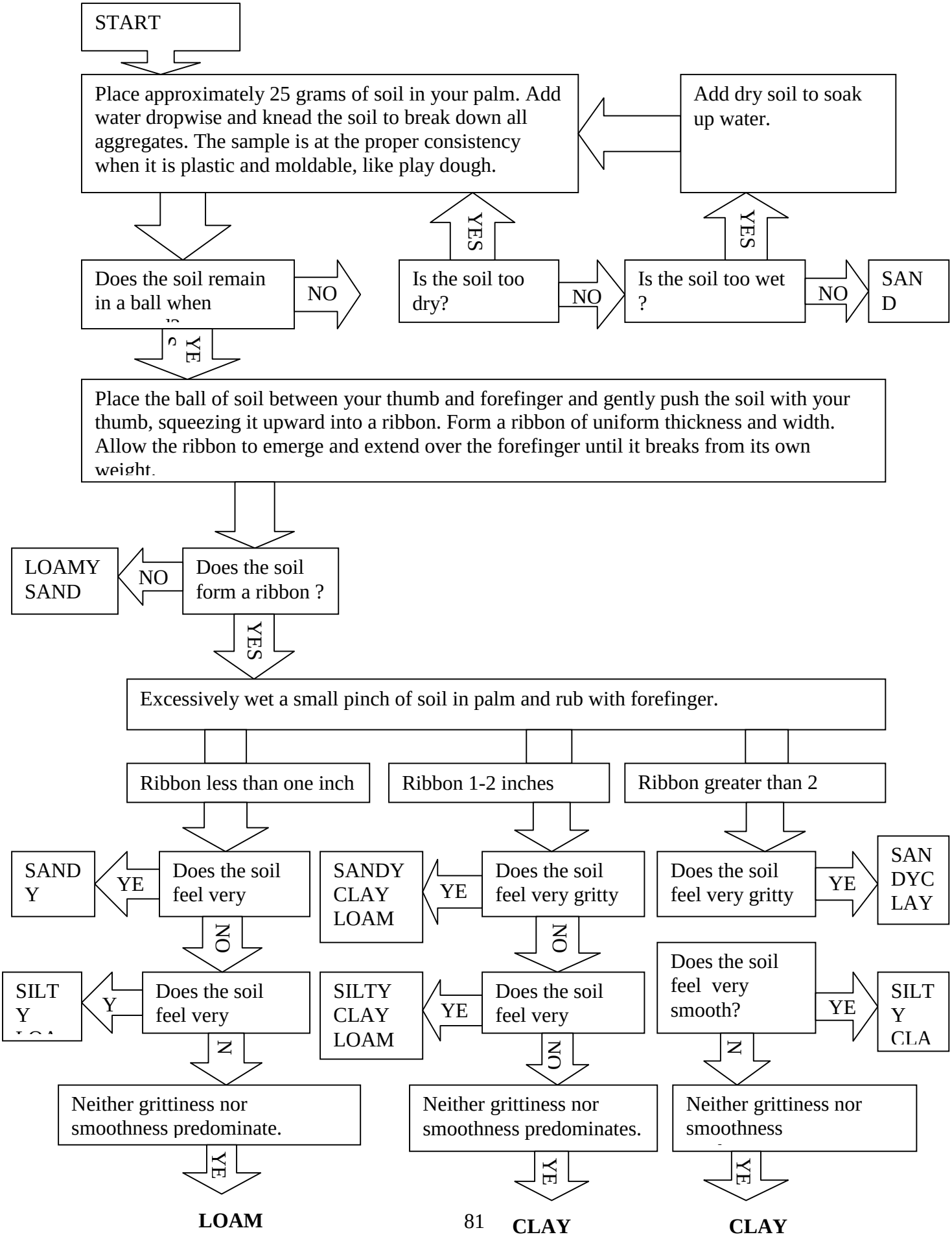
Table 3.1: Potassium conversion Table

3.4.1.2 Physical Analysis and Characterization:

Tested for Soil texture and Moisture Content.

a. Soil Texture Test :

Procedure (**Fig. 3.5**) was developed by Dr. David Whitney at Kansas State University. [HACH, SIW-1]


START
Place approximately 25 grams of soil in your palm. Add water dropwise and knead the soil to break down all aggregates. The sample is at the proper consistency when it is plastic and moldable, like play dough.
Add dry soil to soak up water.
YES
YES
Does the soil remain in a ball when
NO
Is the soil too dry?
NO
Is the soil too wet ?
NO
SAN D
YE
Place the ball of soil between your thumb and forefinger and gently push the soil with your thumb, squeezing it upward into a ribbon. Form a ribbon of uniform thickness and width. Allow the ribbon to emerge and extend over the forefinger until it breaks from its own weight.
LOAMY SAND
NO
Does the soil form a ribbon ?
YES
Excessively wet a small pinch of soil in palm and rub with forefinger.
Ribbon less than one inch
Ribbon 1-2 inches
Ribbon greater than 2
SAND Y
YE
Does the soil feel very
SANDY CLAY LOAM
YE
Does the soil feel very gritty
Does the soil feel very gritty
YE
SAN DYC LAY
NO
NO
SILT Y
Y
Does the soil feel very
SILTY CLAY LOAM
YE
Does the soil feel very
Does the soil feel very smooth?
YE
SILT Y CLA
N
NO
N
Neither grittiness nor smoothness predominate.
Neither grittiness nor smoothness predominates.
Neither grittiness nor smoothness
YE
YE
YE
LOAM
CLAY
CLAY

Soil Moisture Content Test :

i) Clean and dry the weighing bottle (petriplates) and weight to the nearest 0.01g.

ii) Take a measured sample of at least 30gm of soil and place it in the measured petriplates(weighed).

iii) Place the container with its content in the Oven and dry at 105 – 110 ° C for 16 – 24hrs.

iv) After drying, remove the container from the oven and place in the dessicator to cool.

v) Weight the container (petriplates) with contents(Soil samples) after drying and cooling to the nearest 0.01grms.[SSSTM]

Formula :

Moisture Content (%) = [(W2 – W3)/(W3-W1)] *100

Where : W1 = Weight of petriplate (in grms)

W2 = Weight of moist soil + weight of petriplate (in grms)

W3 = Weight of dried soil + weight of petriplate (in grms)[SSSTM]

3.4.2 Water Sample Analysis :

Used HACH DR/890 COLORIMETER for all analysis and characterization of nitroaromatics contaminated water samples. Tested for chemical characteristics.

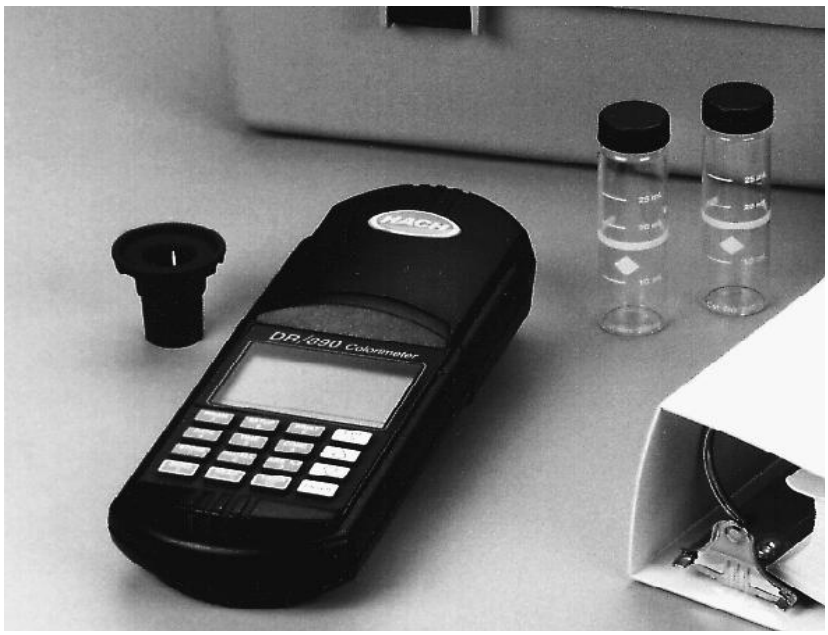


Fig. 3.6: DR/890 Colorimeter

3.4.2.1 Chemical Analysis and Characterization:

Tested for pH, Nitrate ,Nitrite, Phosphorous Reactive, Iron(Ferrous)

a. pH Test :

pH was measured by the same procedure as soil samples using Pocket Pal pH meter. [HACH,SIW-1]

b. Nitrate, Mid range (0 to 5.0 mg/l NO3-N) – Cadmium Reduction Method

i) Prepare water sample by pre-treating it through Whatmann filter paper.

ii) Enter the stored number for medium range nitrate nitrogen using powder pillows. Press : PRGM, The display will show PRGM?. Perform a reagent blank correction using deionized water.

iii) Press : 54 ENTER. The display will show mg/l, NO3-N and the ZERO icon.

iv) Fill two sample cells with 10ml of sample each. One cell will be the prepared sample, the other is the blank. Set the blank aside.

v) Add the contents of one Nitra Ver 5 Nitrate Reagent powder pillow to one cell (the prepared sample). Cap the cell.

vi) Press : TIMER ENTER. A one minute reaction period begins.Shake the sample vigorously until the timer beeps.

vii) After the timer beeps, the display will show : 5:00 TIMER 2, Press : ENTER, A five – minute reaction period will begin.(An amber color will develop if nitrate nitrogen is present).

viii) After the timer beeps, wipe off any liquid or fingerprints.

ix) Place the blank into the cell holder. Tightly cover the sample cell with the instrument cap.

x) Press : ZERO, The cursor will move to the right, then the display will show: 0.0 mg/l NO3-N

Fig 3.7: HACH DR 890 colorimeter with sample cell

xi) Place the prepared sample into the cell holder. Tightly cover the sample cell with the instrument cap. (Read the sample within two minutes after timer beeps).

xii) Press : READ, The cursor will move to the right, then the result in mg/l NO3-N (or NO3) will be displayed.[DR 890 colorimeter ;Method 8171]

c. Nitrite, High Range (0 to 150 mg/) – Ferrous Sulfate method

i) Prepare water sample by pre-treating it through Whatmann filter paper.

ii) Enter the stored program number for high range nitrite (NO2) , Press : PRGM , The display will show PRGM ?

iii) Press : 59 ENTER. The display will show mg/l, NO2 and ZERO icon.

iv) Fill a sample cell with 10ml of sample.

v) Add contents of one NitraVer 2 Nitrite Reagent powder pillow. Cap the cell and invert 5-7 times to mix(the prepared sample). A greenish –brown color will develop if nitrite is present.

vi) Press : TIMER ENTER. A ten-minute reaction period will begin. Do not move or disturb the sample cell during this reaction period.

vii) Fill another sample cell with 10ml of sample (the blank). Clean the outside of cells with a towel.

viii) Place the blank into the cell holder. Tightly cover the sample cell with the instrument cap.

ix) Press : ZERO. The cursor will move to the right, then the display will show: 0mg/l NO2.

x) After the timer beeps, gently invert the prepared sample twice. Place the prepared sample into the cell holder. Tightly cover the sample cell with the instrument cap.

xi) Press : READ. The cursor will move to the right, then the result in mg/l nitrite will be displayed.[DR 890 colorimeter ;Method 8153]

d. Phosphorus, Reactive(0 to 2.50 mg/l PO43-) – Ascorbic Acid Method :

i) Prepare water sample by pre-treating it through Whatmann filter paper.

ii) Enter the stored program number for reactive phosphorus, ascorbic acid method. Press : PRGM, The display will show: PRGM ?

iii) Press: 79 ENTER. The display will show mg/l, PO4 and the ZERO icon.

iv) Fill a sample cell with 10ml of sample.

v) Add the contents of Phos Ver 3 Phosphate Powder Pillows for 10-ml sample to the cell(the prepared sample). Shake for 15 seconds. A blue color will form if phosphate is present.

vi) Press: TIMER ENTER. A two-minute reaction period will begin. Perform steps (vii) to (viii) during this period.

vii) Fill another sample cell with 10ml of sample(the blank).

viii) Place the blank into the cell holder. Tightly cover the sample cell with the instrument cap.

ix) Press: ZERO. The cursor will move to the right, then the display will show: 0.00mg/l PO4

x) After the timer beeps, place the prepared sample into the cell holder. Tightly cover the sample cell with the instrument cap.

xi) Press: READ .The cursor will move to the right, then the result in mg/l phosphate will be displayed. [DR 890 colorimeter ;Method 8048]

e. Iron, Ferrous (0-3.00 mg/l) – Phenanthroline Method :

i) Prepare water sample by pre-treating it through Whatmann filter paper.

ii) Enter the stored program number for Ferrous iron (Fe2+) – powder pillows. Press: PRGM. The display will show mg/l, Fe and the ZERO icon.

iii) Fill a sample cell with 25 ml of sample(the blank).

iv) Place the blank into the cell holder. Tightly cover the sample cell with the instrument cap.

v) Press: ZERO. The cursor will move to the right, then the display will show: 0.00mg/l Fe

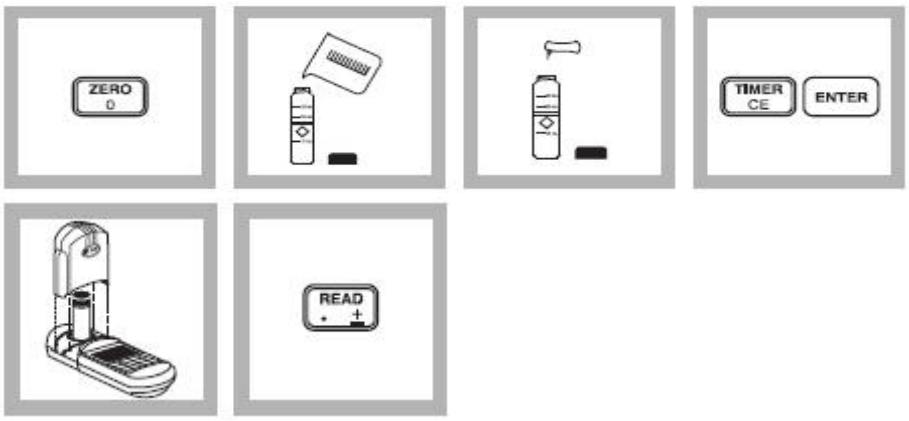


Fig 3.8: Process of calculating Iron(Ferrous) using HACH colorimeter

vi) Fill another sample cell with 25ml of sample.

vii) Add contents of one Ferrous iron reagent powder pillow to the sample cell(the prepared sample). Cap and invert to mix.

viii) Press: TIMER ENTER. A three minute reaction period will begin. An orange color will form if ferrous iron is present.

ix) Place the prepared sample into the cell with the instrument cap.

x) Press: READ .The cursor will move to the right, then the result in mg/l ferrous iron will be displayed. [DR 890 colorimeter ;Method 8146]

3.4.2.2 HPLC analysis of water samples for estimation of TNT and DNT concentrations :

High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures. HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing beads, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process: Hydrophobic (non-specific) interactions are the main ones in reversed-phase (RP) separations. Dipole-dipole (polar) interactions are dominant in normal phase (NP) (mode. Ionic interactions are responsible for the retention in ion-exchange chromatography. All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface.

Types of HPLC

1. Adsorption chromatography
2. Ion-exchange chromatography
3. Size exclusion chromatography

3.4.2.2.1 Instrumentation HPLC system

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometre size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder. Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computer, integrator, and other data processing equipment are frequently used.[StandardBase Techniques]

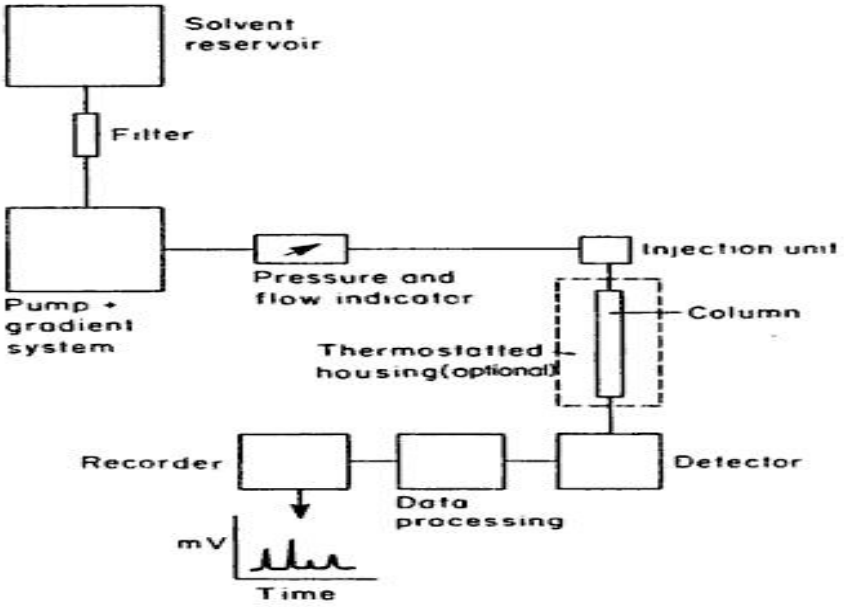


Fig 3.9: Block diagram of a HPLC instrument. [Lindsay S et al., 1992]

Functional description of the instrument :

(a) Solvent delivery system
(b) Sample injection system
(c) Columns
(d) Detectors
(e) Data handling system

1. ***Solvent Delivery Systems:*** The first component of the modern LC is a reservoir of pure degassed solvent to be delivered to the pump. The ideal pumping system for LC should also provide accurate, precise, pulse-free solvent delivery over a wide range of flow rates, be easy to change to a new solvent, be generally convenient to use and maintain, be able to draw from a large external reservoir, and be easily adapted to gradient elution Pumps are of two types (Constant pressure (pneumatic) or constant volume (mechanical). Dual piston reciprocating pump found to be today the most suitable pumping system having advantages of compatibility with micro-processor.
2. ***Sample Injection System:***The purpose of the injection system is to introduce the sample on to the pressurized column as a sharp column plug, with little loss in efficiency. The three common means of sample introduction are (i) syringe/on-stream injection (ii) Syringe septum less injection and (iii) valve injection. The injection systems utilizing the valves has proved over the years the ideal choice of the LC manufacturers. Manufacturers are now designing injection system, combining the flexibility of the valve, with the advantages of the syringe system. .[StandardBase Techniques]
3. ***Columns :*** The stationary phase may be a porous solid such as those used in adsorption, ion exchange and size exclusion chromatography. Today the most widely used packing in LC have the stationary phase chemically bonded to support particles. These are the so called bonded-phases which are very durable and require no conditioning or pre-saturation of the mobile phase. Bonded phases are prepared by chemical reaction between the surface hydroxyl groups of silica particles and a linear organic molecule or an organo- silane. Parameters which play an appreciable role in the LC separations include (i) adsorption (ii) Partition (iii) Ion exchange and (vi) size exclusion.
4. ***Detectors:*** The function of the detector is to provide an electrical signal to the recorder and data system, from which the qualitative and quantitative analysis are made .The most common LC detectors are RI and UV, followed by the infrared, conductivity and the electrochemical detector and fluorescence detector.
5. ***Data Processors:*** Data Processor technology facilitates computation in whatever format that may be desired by the use of specific programmes designed to meet the chromatographic requirement, e.g., area percentage, computation using internal standard method, external standard method, area normalization method etc. .[StandardBase Techniques]

3.4.2.2.2 Theoritical aspects of HPLC :

1. Retention time :

The retention time of the solute can be divided in to two parts tM and tR. These can be explained in the following way. During their passage through the column, the solute molecules spend part of their time in the mobile phase and the other part in the stationary phase. While in the mobile phase, they travel with its velocity, but while in the stationary phase they are static as they are retarded by the stationary phase. Thus,

$$t_{R'} = t_R - t_M$$

When a multi-component sample is analysed, each sample component will spend the same time in the mobile phase, but assuming that they are separated, each will spend a different amount of time in the stationary phase. This is the reason for their separation.

2. Capacity factor (K) :

This is an indication of how long each component can be retained on the column. Since tR' expresses the time the solute is retarded by the stationary phase and tM represents the time it spends in the mobile phase, their ratio would give this value

$$K = t'_R/t_m = t_R - t_M/t_M$$

Knowing the capacity ratio, we can calculate the retention time of a compound

$$t_R = t_M (1 + K)$$

3. Characteristics of the chromatographic peak :

A chromatographic peak corresponds to a normal distribution curve. Such a curve is usually characterized by its standard deviation σ. The peak width at any point can be expressed as a multiple of the standard deviation. Two characteristic values of the chromatographic peak are its area and height.

4. Peak area :

It is the integral under the peak and is proportional to the amount or concentration of the solute and is thus used for the quantitative evaluation of the chromatographic analysis.This is measured to the maximum of the peak and hence corresponds to the highest concentration in the zone. Since, however, a chromatographic peak corresponds only in ideal cases exactly to a gaussian distribution curve, peak area values are preferred.[Christie W.W, 1989]

5. Column Efficiency :

Sharpness of the chromatographic peak is an indication of how good a column is. In practice, the plate number is calculated by using one of the peak widths which can be measured directly from the chromatogram.. The plate number depends on column length. The longer the column length,

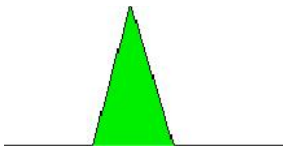
the larger the plate number. Therefore another term has also been introduced relating the plate number to column length called ***Height Equivalent to Theoritical Plate (HETP)***

$$H = L/n$$

The lower the plate height and the higher the plate number, the more efficient the chromatographic column.

c. Interpretation of HPLC result (Chromatograms)

The area under the peak is proportional to the amount of X which has passed the detector, and this area can be calculated automatically by the computer linked to the display. The area it would measure is shown in grey in the (very simplified) diagram.



If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:

This means that it is possible to calibrate the machine so that it can be used to find how much of a substance is present - even in very small quantities. . [Clark J, 2007]

Used Perkin Elmer HPLC for all study with Auto sampler and below given parameters:

S.no.	**Parameter description**	*DNT*	*TNT*
1	Detector wave length (nm)	254	254
2	Stationary phase used	C-8	C-8
3	Column length (mm)	150 x 4.6	150 x 4.6
4	Column particle size (µm)	5	3
5	Column temperature (°C)	30	30
6	Mobile phase	MeOH:H_2O 50: 50	MeOH : H2O 50: 50
7	Flow rate (ml/min)	1.0	1.0
8	Sample injection volume (µL)	10	10
9	Sample retention time (min)	5.8	4.3

Table 3.2: HPLC Operating Parameters for different explosive components

For preparation of standards to be used in water samples HPLC analysis and bioremediation of TNT in water samples further :

- Formula for standard preparation :

$$\frac{\text{(ppm required)}}{\text{(ppm available)}} \times \text{(volume to be made)} \quad \text{---------- eq. (1)}$$

3.4.2.2.3 Procedure for HPLC analysis of water samples contaminated with military waste(TNT, DNT) :

Preparation of Calibration curve :

- For 1000ppm of STOCK solution of TNT+DNT we took 0.1gm of TNT and 0.1 gm of DNT and dissolved it in 100 ml of ACN(Acetonitrile). As 1ppm = 1gm/litre
- Prepared INTERMEDIATE of 100ppm from this 1000ppm stock with the formula of eq.(1) where

  ppm required = 100

  Vol to be made = 100ml

  ppm available = 1000ppm

  Thus 10 ml of stock in 100ml of Distilled water will make 100ppm intermediate
- Then prepared 20, 40, 60 and 80 ppm of working standards by using eq.(1)

  For 20ppm ppm required = 20, Vol to be made = 10ml, ppm available = 100ppm

  So 2ml from 100ppm is added to 10 ml volumetric flask containing 8ml of distilled water.
- Similarly working standards of 40, 60, 80 ppm were constructed.

3.5 MICROBIAL ANALYSIS OF SOIL SAMPLES :

It includes :

3.5.1 Enumeration of microbial colony from TNT contaminated Soil samples :

i) Transferred 1grm of soil sample to 10 ml distilled water. After settling took 1ml of supernatant and transferred to another tube containing 9ml of distilled water to make it dilution of (-1) concentrations.

ii) Similarly transfer 1ml of supernatant after mixing from this -1 dilution to test tube containing 9 ml distilled water to make a dilution of -2.

iii) Make dilutions till 10^-5 both for sample A and B.

iv) From these dilutions transfer or culture less than 1ml of solution to Nutrient Agar plates from Spread plate method.

v) Keep at 30-35°C for 24hrs to view the colonies developed and to count them. [Reynolds J,2012]

THE STANDARD FORMULA

$$\text{CFU/ml} = \frac{\text{Colony count (CFUs) on an agar plate}}{\text{Total dilution of tube (used to make plate for colony count)} \ \mathbf{X} \ \text{volume plated}}$$

3.5.2 Isolation of microbial colony from TNT contaminated Soil samples :

3.5.2.1 Preparation of Primary culture plates using pour plate method:

i) Obtain a test tube containing 5ml of Trypticase Soy broth(TSB).

ii) Aseptically transfer a small amount of TNT contaminated soil sample – (Taken SL1R1 and S4R1 marked as A and B respectively) into the tubes of TSB.

iii) Mix tube to suspend any organisms from the soil into the broth. Let the test tube settle at 25 °C(RT) for about 30min. until the most of the soil particles have settled.

iv) Use upper liquid broth to pour on primary plates of Trypticase Soy Agar (TSA).

v) Put the inoculated plates in incubator at 30°C.

vi) Pull plates after 48hrs of incubation and store at 4°C for the next use.[Steubing P.M,1993]

3.5.2.2 Preparation of Secondary Culture Plates from primary culture plates using streak plate method :

i) Examined the pouring on primary plates done from soil extracts. Noticed the different types of colonies. Pick out three different colonies which might be of interest(look for bacterium to serve as the unknown) due to their color, texture, shape or frequency . {Do not pick a colony which is obviously filamentous suggesting a fungus}.

ii) Obtained three TSA plates label them and transfer the three different colonies onto it by streak plate method.

iii) Put the inoculated plates in incubator at 30° C.

iv) Pull plates after 48hrs of incubation and store at 4°C for next use.[Steubing P.M, 1993]

3.5.3 Identification of an unknown bacterium from secondary culture plates (By staining techniques):

3.5.3.1 Record colony characteristics of the pure cultures from their secondary plates by picking their unknown bacterium from one of the plates by looking at crystal violet stained smears.

i) Observed all secondary pure cultures. Recorded colony characteristics of each pure culture.

ii) Made one smear preparation of a colony from each of the secondary pure cultures.

iii) For smear preparation – Took a glass slide, with the help of a inoculating loop, took some unknown colony, transferred to the glass slides.

iv) After drying, heat fixed the slides by moving 2-3 times on the flame.

v) To stain cells, put a drop of crystal violet dye onto the glass slide.

vi) View in Microscope.[Steubing P.M, 1993]

3.5.3.2 To determine if the unknown bacterium is Gram positive(Gm+) or Gram negative(Gm-).

i) Took dried, contamination free glass slides, mark a circle/oval on back side of the glass slide and label it at one corner.

ii) With a sterile cooled loop, placed a drop of sterile water or saline solution on the slide. Sterilized and cooled the loop again and picked up a very small sample of a bacterial colony and gently stirred into the drop of water/saline on the slide to create an emulsion.

iii) Allowed the smear to dry , after the smear has dried, hold the slide at one end and pass the entire slide through a flame of a Bunsen burner two-three times with the smear side up.

iv) Placed the slide with heat fixed smear on staining tray and gently flood smear with crystal violet & let stand it for 1 minute.

v) Tilted the slide slightly and gently rinse with tap water or distilled water using a wash bottle.

vi) Gently flooded the smear with Gram's Iodine & set strand for 1 minute.

vii) Tilted the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear appeared as a purple cincel on the slide.

viii) Decolorized using 95% ethyl alcohol or acetone. Tilted the slide slightly and apply drop by drop for 5-10 sec. until the alcohol runs almost clear. Do not over decolorize.

ix) Immediately rinsed with water.

x) Gently flooded with safranin to counter stain and let stand for 45sec.

xi) Tilted the slide slightly & gently rinse with tap water or distilled water using a wash bottle.

xii) Blotted dry the slide with bi bulous paper.

xiii) Viewed smear using microscope.[Sridhar Rao PN]

3.5.3.3 To determine if the unknown bacterium is an endospore former.

i) Prepared smears of organisms to be tested for endospores and heat fixed it.

ii) Covered the smears with a piece of absorbent paper cut to fit the slide and placed the slide on a wire gauze on a ring stand.

iii) Saturated the paper with malachite green dye and holding the Bunsen burner in the hand. Heated the slide until steam can be seen rising from the surface.

iv) Removed the heat and reheated the slide as needed to keep the slide steaming for about three minutes. As the paper begins to dry added a drop or two of malachite green to keep it moist, taken care of not adding so much at one time that the temperature is appreciably reduced. Overheating is avoided strictly. The process was steaming and not baking.

v) Removed the paper with tweezers and rinsed the slide thoroughly with tap water.

vi) Drained the slide and counterstained 45 seconds with 0.5% safranin dye.

vii) Washed, blotted, and examined.

viii) The vegetative cells appeared red and the spores appeared green.[Microbiology Lab Procedures]

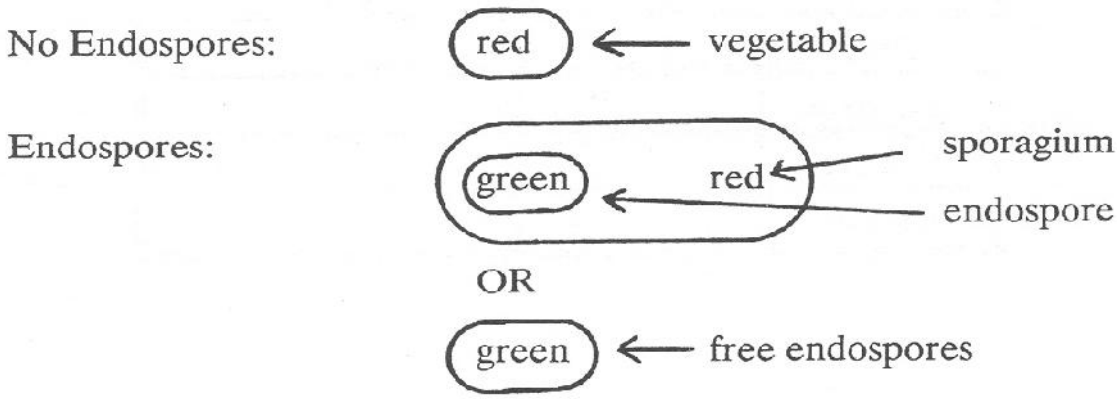


Fig. 3.10: Endospore staining

3.5.3.4 To determine if the unknown bacterium is acid-fast.

i) Prepared smears of organisms to be stained and heat fixed it.

ii) Cut or teared absorbent paper (bibulous paper) to fit the slide leaving one end for handling. Did not allow the paper to protrude beyond the slide, but the smears must be covered.

iii) Placed the slide on wire gauze on a ring stand.

iv) Saturated the paper with carbolfuschin.

v) Heated the slides with a hand-held Bunsen burner until steam can be seen rising from the surface. Alternately remove the burner and reheat the slide to maintain steaming for 3-5 minutes. As the paper begins to dry during the staining process added a drop or two of

carbolfuschin to keep the slide moist. {Adding too much stain will cool the slide (and drip on the bench). Overheating the slide or letting it dry will distort the cells. Under heating the slide will fail to stain acid-fast cells}.

vi) At the end of staining removed the paper with tweezers and washed the slide thoroughly.

vii) Drained the slide.

viii) Decolorized with acid-alcohol for 30 seconds.

ix) Rinsed, drained, and counterstained with methylene blue dye for 45 seconds.

x) Rinsed, blotted, and examined. Firstly observed each organism on its separate smear. Then examined the mixed smear.

vii) Acid-fast organisms will appear red and non-acid-fast organisms will be blue[Steubing P.M, 1993]

3.5.3.5 To determine if the unknown bacterium has a capsule.

i) Placed a single drop of India ink on a clean microscope slide, adjacent to the frosted edge.

ii) Using a flamed loop and sterile technique, removed some of the organism we wanted to stain and mixed it into the drop of India ink. There were no large clumps of organism, but we tried to avoid spreading the drop.

iii) Placed the end of another clean microscope slide at an angle to the end of the slide containing the organism. Spread out the drop out into a film. This is done by contacting the drop of India ink with the clean microscope slide and using the capillary action of the dye/ slide to spread the India ink across the smear.

iv) Allow the film to air dry. Did not heat it or blotted it dry as heating will melt the capsule.

v) Saturated the slide with crystal violet for 1 minute.

vi) Rinsed the slide gently with water.

vii) Allowed the slide to air dry. Did not heat it or blotted it dry as heating will melt the capsule.

viii) Observed the slide under the microscope, using proper microscope technique. [Smith,2010]

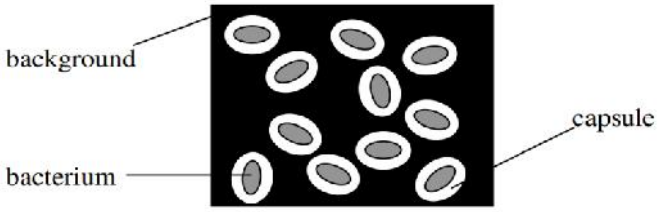


Fig 3.11: Capsule staining

3.5.4 Characterization of microbial cells taken from secondary culture plates on various optimized conditions :

3.5.4.1 To determine if the unknown bacterium will grow on nutrient agar, high-salt agar, and mannitol-salt agar (Growth on Selective and Differential Media)

i) Prepared three plates: nutrient agar, high-salt agar, and mannitol-salt agar.

ii) From secondary culture plates, took colonies to be used for characterization and inoculation.

iii) Used streak plate method to streak them on the prepared three culture plates of different media: nutrient agar, high-salt agar, and mannitol-salt agar

viii) Inoculated plates are then incubated at 30°C for 48 hours, and then stored at 4°C after analyzing the results. [Steubing P.M,1993]

3.5.4.2 To determine the unknown bacterium's tolerance for increasing concentrations of NaCl(Osmotic effects)

i) Prepared three plates: 0.5% NaCl in Typticase soy agar(TSA), 5% NaCl in TSA, 20% NaCl in TSA.

ii) From secondary culture plates, took colonies to be used for characterization and inoculation.

iii) Used streak plate method to streak them on the prepared three culture plates of different salt concentrations.

iv) Inoculated plates are then incubated at 30°C for 48 hours, and then stored at 4°C after analyzing the results.[Steubing P.M,1993]

3.5.4.3 To determine the temperature optimum of the unknown bacterium

i) Prepared three plates of Typticase soy agar(TSA) to be kept at three different temperatures (25°C, 37°C, 55°C).

ii) From secondary culture plates, took colonies to be used for characterization and inoculation.

iii) Used streak plate method to streak them on the prepared three culture plates to be kept at three different temperatures (25°C, 37°C, 55°C).

ix) Inoculated plates are then incubated at their respective temperatures for 48 hours, and then stored at 4°C after analyzing the results. .[Steubing P.M,1993]

3.6 BIOREMEDIATION OF TNT CONTAMINATED (PREPARED)WATER SAMPLES - With Microbe Isolated From TNT Contaminated Soil Sample And Tolerance Assessment To TNT By Growing In Growth Media Containing Different Concentrations Of TNT :

3.6.1 Preparation of synthetic TNT contaminated water samples :

Prepared three concentrations of TNT contaminated water samples these water samples are further used to make up their respective concentrations culture media onto which inoculation of isolated and cultured microbial strain is done and bioremediation of TNT is seen.

Three concentrations 120ppm, 80ppm and 40ppm are taken for Bioremediation study by the isolated microbial strain and to monitor its tolerance with increasing concentration of TNT (40ppm, 80ppm, 120ppm). [U.S EPA method 8330 B]

- Formula for sample preparation :

$$\frac{\text{(ppm required)}}{\text{(ppm available)}} \times \text{(volume to be made)} \quad \text{---------- eq. (1)}$$

- For 120ppm we took 0.12gm of TNT and dissolved it in 1 litre of distilled water. As 1ppm = 1gm/litre

- For 80ppm , according to eq. (1)

  (80/120) * 250 = 166.67 ml of 120ppm solution in Distilled water

  Where 80 = ppm to be made

  120= ppm available

  250= volume to be made

- For 40ppm, according to eq. (1)

(40/120) * 250 = 83.34 ml of 120ppm solution in distilled water

Where 40 = ppm to be made

120 = ppm available

250 = volume to be made

Thus TNT contaminated water solutions are made of 120ppm, 80ppm and 40ppm

3.6.2 Preparation of TNT contaminated culture media for growth of inoculated microbial colony onto it and therefore Bioremediating it :

Trypticase Soy Broth was taken as the culture media for growth of the inoculated colony taken from secondary culture plates. Colony was identified and wisely chosen for bioremediation activity.

Volume made was 250 ml each of 120ppm TNT contaminated media preparation, 80ppm TNT contaminated media preparation and 40ppm TNT contaminated media preparation.

Required amount of Trypticase Soy broth was added to each 120ppm, 80ppm, 40ppm marked flasks and make them up with respective 120ppm, 80pm and 40ppm TNT contaminated water solutions previously made.

3.6.3 Bioremediation Procedure :

i) All the three prepared concentrations (120ppm, 80ppm, 40ppm) of media with TNT contamination and the microbial colony are kept for a 9-day study.

ii) 10-day study was carried out by rotating the culture flask at 170 rpm on a shaker incubator at 30^{o}C continuously.

iii) Daily samples were taken for 8-days for evaluation of TNT degradation and microbial growth.

3.6.4 Evaluation of TNT degradation by HPLC(High-Performance Liquid Chromatography) :

i) Perkin Elmer's HPLC was used for all the study. A method was created and samples and standards were poured in HPLC vials. The sampler in this HPLC was Automatic so that the HPLC machine itself take required amount of sample to be poured on the column. The detector shows the amount of TNT in the solution which comes in the form of a peak in a chromatogram.

ii) Preparation of standards of TNT to be kept in HPLC :

a. Stock solution : 1000ppm stock solution was prepared by adding 0.1gm of TNT in 100ml flask containing acetonitrile(ACN).
b. Intermediate Solution : 500ppm of intermediate was prepared by same eq. (1) as
(500/1000) * 100 = 50ml of 1000ppm solution in 100 ml distilled water(HPLC grade).
Where 500 : ppm to be prepared
1000: ppm available
100 : volume to be made
c. Standard Solutions : Standards of 20ppm, 40ppm, 60ppm, 80ppm, 100ppm and 120ppm was made
   - 20 ppm : From eq. (1)
   (20/500) * 10 = 0.4ml of 500ppm solution on 10 ml distilled water(HPLC grade)
   Where 20 : ppm to be made
   500 : ppm available
   10 : volume to be made
   - 40 ppm : From eq. (1)
   (40/500) * 10 = 0.8ml of 500ppm solution on 10 ml distilled water(HPLC grade)
   Where 40 : ppm to be made
   500 : ppm available
   10 : volume to be made
   - 60 ppm : From eq. (1)
   (60/500) * 10 = 1.2ml of 500ppm solution on 10 ml distilled water(HPLC grade)
   Where 60 : ppm to be made
   500 : ppm available
   10 : volume to be made
   - Similarly for 80ppm, 100ppm and 120ppm standards were prepared and transferred in HPLC vials to be further used in study on HPLC. [U.S EPA method 8330 B]

iii) Preparation of TNT contaminated samples kept for microbial remediation to be kept in HPLC :

a. Daily for 8-days took 1ml of TNT contaminated samples of 120ppm, 80ppm and 40ppm each inoculated with desired microbe for microbial remediation.
b. These 1ml sample were taken in centrifuge tubes and centrifuged at 10,000 rpm for 15minutes.

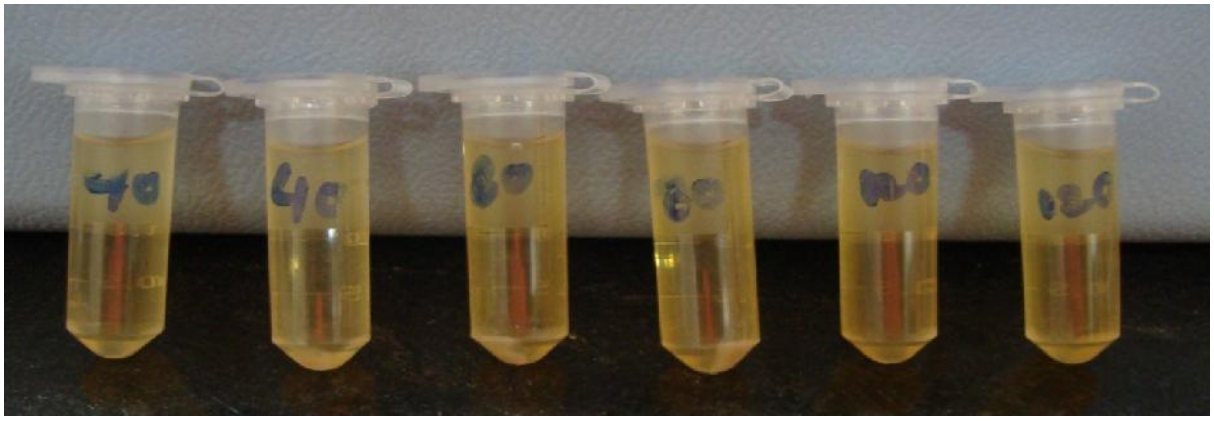


Fig. 3.12: Centrifuge vials after centrifuge with separate supernatant and microbial pellet(40,80,120 ppm) with replicates

c. Added 1ml of Acetonitrile (ACN) to this centrifuged sample for extraction purpose in the sample centrifuge tubes.

d. Three times vortex the tubes for 1minute for each sample .

e. Again centrifuge the vortexed sample plus ACN solution at 10,000 rpm for 15minutes.

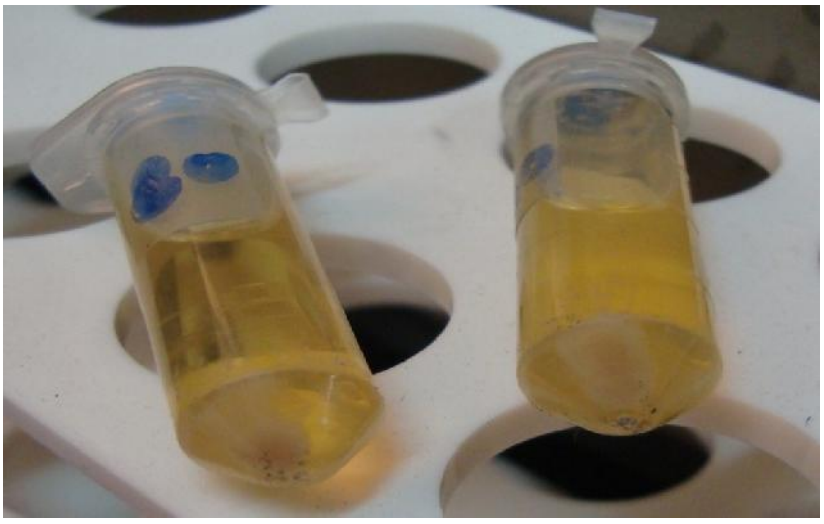


Fig. 3.13: Centrifuge vials after centrifuge with separate supernatant and microbial pellet for 80ppm concentration sample.

f. After the centrifugation microbial pellet and supernatant can be seen separately. Both can be further used for advanced study. Taken the supernatant and vacuum filtered it from 0.45micron filter.

g. Transferred the filtered sample in HPLC vials to be further used in study on HPLC.[Oh B.T et al.,2003]

Fig. 3.14: Centrifuge vials showing microbial cell pellet after removal of supernatant for HPLC study

3.6.5 Microbial Growth over 10-days of TNT degradation test :

a. Took 5 ml of TNT contaminated sample with cultured microbe in it for degradation of TNT, daily for 10 days during its incubation period.

b. Used HACH spectrophotometer to check the optical density of the solution prepared at 600nm.

c. As optical density increases, which shows more light is being scattered, which is due to increase in no. of microbes.[Claus H et al., 2005]

CHAPTER 4
RESULTS AND DISCUSSION

RESULTS AND DISCUSSIONS :

4.1 SOIL SAMPLES ANALYSIS :

4.1.1 Chemical analysis : For pH, salinity, Nitrate-nitrogen, Potassium, Phosphorus

S.No.	Soil Samples	pH	Salinity (mS)	Nitrate-Nitrogen(ppm) : Avrg of three readings*2	Potassium(ppm)		Phosphorus(ppm) : Avrg of three readings*3.3
					Avrg Reading	Potassium conc. from convertion table	
1	S1R2	7.4	0.59	6.667	85	Below 87	20.9
2	S2R2	7.5	0.02	11.867	85	Below 87	45.10
3	S3R1	7.3	0.15	4.134	83.33	Below 87	11.55
4	S4R1	8.3	1.91	88.667	31.667	243	63.799
5	S5R1	8.4	1.24	27.867	21.667	Above 294	46.2
6	S6R1	7.9	0.00	8.467	46.67	159	17.6
7	S7R1	8.1	0.33	5.667	80	87	22
8	SL1R1	8.1	0.03	10.334	85	Below 87	7.699

Table 4.1 : Results for soil sample analysis

Standard Parameters:

For Heavy soils(Bulk Density approximately 1.0 g/cm3) phosphorus level ranges from 25-35mg/l, potassium level ranges from 200 to 300mg/l and pH is 5.8 to 6.2. For light soil (Bulk Density approximately 0.5g/cm3) phosphorus, potassium and pH level ranges from 50-85mg/l, 200-400mg/l and 5.8 to 6.2 respectively.[Tamini Y.N et al.].

Nitrate-nitrogen if less than 10mg/l shows low fertility of soils, 10-20mg/l is medium fertility, 20-30mg/l is high fertility and above 30mg/l shows excessive fertility levels. Same is the case with phosphorus, as we have purely alkaline soil samples , so according to Olsen method less than 10mg/l of phosphorus conc. shows low fertility while 10-20mg/l, 20-40mg/l, above 40mg/l shows medium, high and excessive fertility levels. Likewise less than 75mg/l potassium is for very low fertile soil .75-150mg/l, 150-250mg/l, 250-800mg/l are for low, medium and high levels of fertility respectively. Above 800mg/l is for very high fertility rates.[Fulton A et al.]

Comparing these above parameters with our study results, S4R1 and SL1R1 are further used to isolate microbial colonies and according to their readings and standard readings its concluded that S4R1 is appreciably fertile soil, and SL1R1 is low in fertility.

Moreover all the soil sample ranges from less fertile to high fertility levels.

4.1.2 Physical analysis : Tested for Soil Texture and Moisture Content

i) Soil texture

The below diagram showing the diagram for estimating soil texture is one of the confirmation test for knowing exact soil type. While clays form good ribbons, Clay loams form medium one and Loams are poor on formation of ribbons.[HACH,SIW-1]

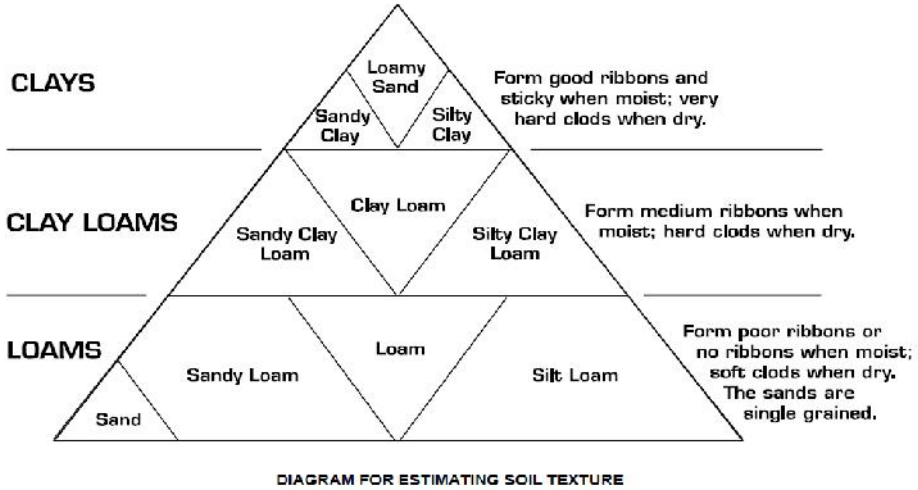


Fig. 4.1: Diagram for estimation and discussion of soil texture

S.No.	Soil Samples	Soil Texture
1	S1R2	Sandy Loam
2	S2R2	Loamy Sand
3	S3R1	Loamy Sand
4	S4R1	Loamy Sand
5	S5R1	Loamy Sand
6	S6R1	Loamy Sand
7	S7R1	Loamy Sand
8	SL1R1	Sandy Loam

Table 4.2 : Results for texture of soil samples

Most of the soil constituted Loamy soils. But the one used for culturing S4R1 and SL1R1 is Loamy Sand and Sandy Loam respectively.

ii) Moisture Content

S.No.	Soil Samples	Weight of Petriplates(W1) in grms	Weight of moist soil taken(in grms)	Weight of dried soil(in grms)	Moisture Content (%)
1	S1R2	28.5657	30	28.8232	4.0824
2	S2R2	50.6287	18.5465	17.6177	5.2719
3	S3R1	48.0122	30	28.9877	3.4921
4	S4R1	53.0108	30	28.7173	4.4660
5	S5R1	37.99106	21.4568	20.3878	5.2431
6	S6R1	50.2748	30	28.1989	6.3871
7	S7R1	41.8688	12.285	11.5589	6.2810
8	SL1R1	48.1149	30	27.1420	10.529

Table 4.3: Results for moisture content of soil samples

The highest moisture content(%) is in SL1R1 and lowest in S3R1. While S4R1 constituted one among the lowest moisture containing soil.

4.2 WATER SAMPLE ANALYSIS :

4.2.1 Tested for pH, Nitrate, Nitrite, Phosphorus Reactive, Iron(Ferrous)

S.No	Water Samples	pH	Nitrate (mg/l)	Nitrite (mg/l)	Phosphorus Reactive (mg/l)	Iron(Ferrous) (mg/l)
1	W1R1	2.6	5.5	1	0.41	1.60
2	W2R1	7.2	3.9	3	0.37	0.21
3	W3R2	1.0	5.5	4	0.25	0.03
4	W4R1	1.3	3.3	3	0.21	0.05
5	W4R3	1.4	4.7	3	0.23	0.06
6	W4R4	1.5	4.2	3	0.23	0.08
7	W5R2	7.3	2.1	0	0.26	0.00
8	W5R3	7.3	2.1	1	0.61	0.01
9	W6R1	2.4	5.5	1	2.75	0.09
10	W6R2	2.3	5.5	1	2.14	0.08
11	W6R5	2.3	5.5	2	0.88	0.10
12	W7R1	5.9	2.8	1	0.53	0.21

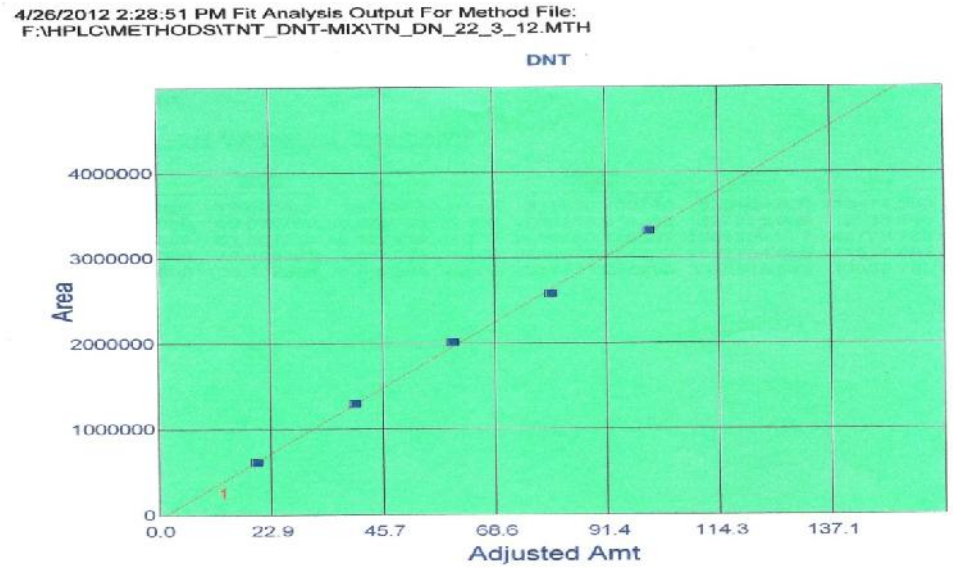
Table 4.4: Results for water samples analysis

According to USEPA, Nitrate and nitrite can act as severe contaminants if not in permissible limits in water. Permissible levels of Nitrate(mg/l) in drinking water is 10mg/l above which it is detrimental for infants if they drink such water excessively .Blue-Baby syndrome is one of the major symptoms of high nitrate in water. Permissible level of Nitrite is 1mg/l excess of which is also deadly for human consumption.[U.S EPA, Drinking water contaminants]

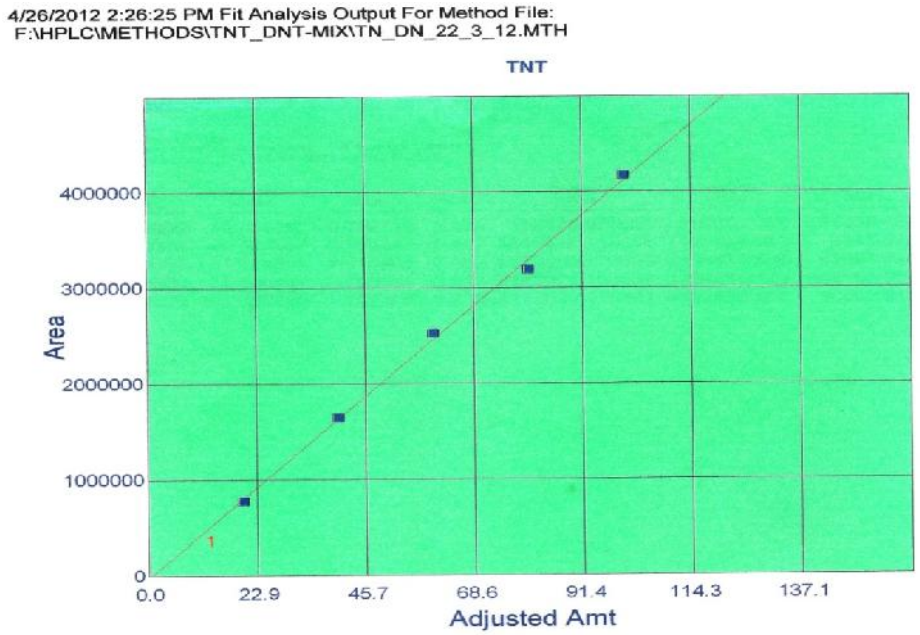
While Nitrate is under permissible limits in all water samples, nitrite is high in many (W2R2, W3R2, W4R1, W4R3, W4R4). pH of water should be nearly neutral for consumption and drinking purposes.

4.2.2 HPLC Analysis for water samples :

i) Calibration Curve for DNT : (Fig. 4.2)



ii) Calibration Curve for TNT : (Fig. 4.3)



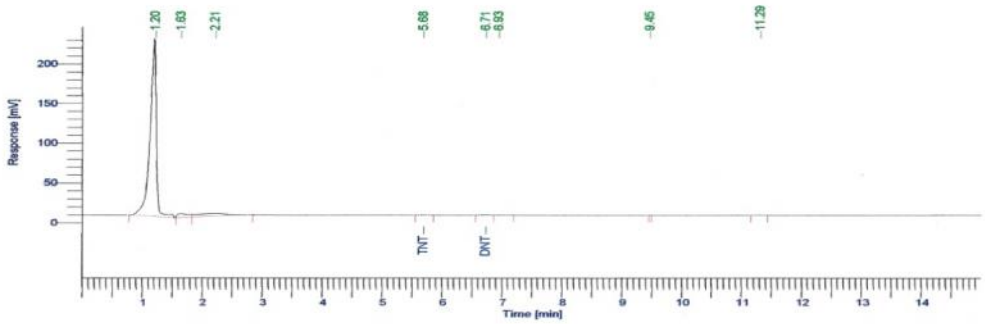
Above are the calibration curve for DNT and TNT respectively. The calibration curve was plotted for 20, 40, 60, 80, 100 ppm concentration of working standards from DNT plus TNT Stock solution of 1000ppm and intermediate solution of 100ppm.

Below are the HPLC report for all water samples contaminated with TNT and DNT . The respective peaks of TNT and DNT and area under it gave the concentration of TNT and DNT in the water samples.

iii) Result of W1R1 water sample (Fig. 4.4)

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:32:41 PM
Sample Name	: W1R1	Data Acquisition Time	: 4/25/2012 1:25:27 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/11	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

Result File :
Sequence File : F:\HPLC\Sequences\TN_DN_MIX\TN_DN_10.04.12.seq



DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.201	1653230.33	224833.93	88.10		BB	1.6532
2		1.631	64919.49	5385.87	3.46		BV	0.0649
3		2.207	151710.53	3964.08	8.08		VB	0.1517
4	TNT	5.677	1544.38	185.01	0.08		BB	0.4451
5	DNT	6.713	3092.58	331.16	0.16	-	BV	1.3118
6		6.932	1707.07	169.68	0.09		VB	0.0017
7		9.454	53.68	36.80	0.00		BB	0.0001
8		11.292	347.16	35.40	0.02		BB	0.0003
			1876605.22	234941.94	100.00			3.6289

iv) Result of W2R1 water sample(Fig. 4.5)

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:34:36 PM
Sample Name	: W2R1	Data Acquisition Time	: 4/25/2012 1:57:02 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/13	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

Result File :
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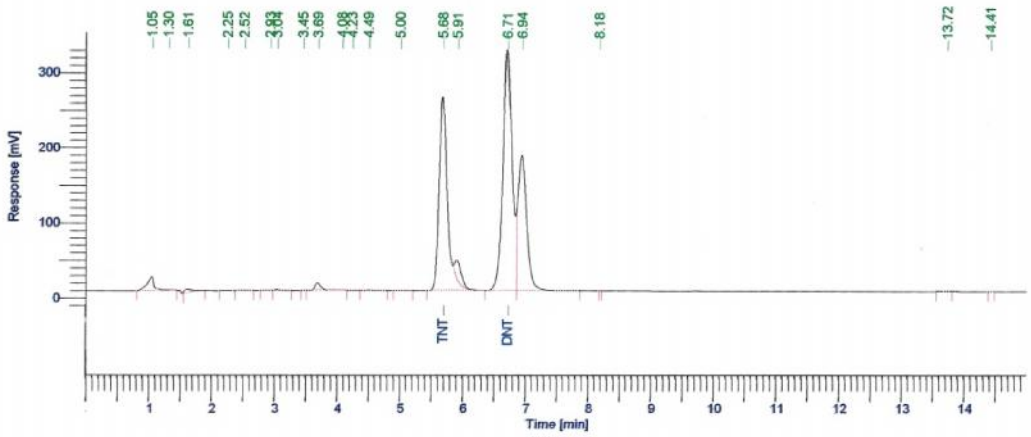


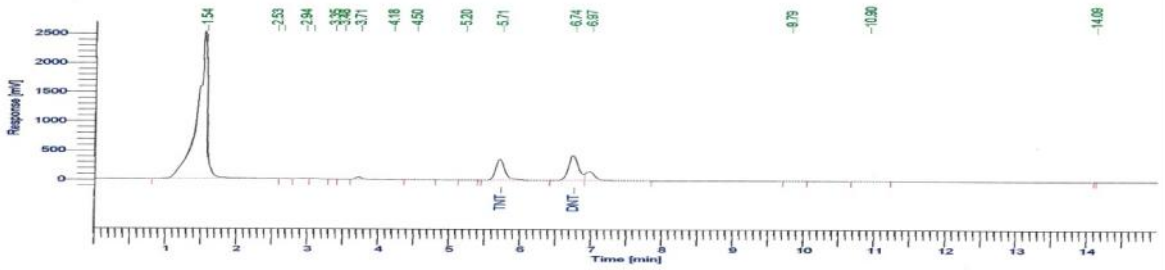
DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.047	139695.70	18701.29	1.74		BE	0.1397
2		1.303	3969.46	348.96	0.05		EB	0.0040
3		1.614	47405.72	4727.94	0.59		BB	0.0474
4		2.247	1201.93	177.32	0.02		BV	0.0012
5		2.516	2073.98	337.15	0.03		VB	0.0021
6		2.928	1713.85	362.20	0.02		BV	0.0017
7		3.037	8028.19	1220.48	0.10		VB	0.0080
8		3.450	123.42	46.48	0.00		BV	0.0001
9		3.688	71885.27	10592.72	0.90		VE	0.0719
10		4.076	2069.76	271.71	0.03		EV	0.0021
11		4.231	3112.24	387.83	0.04		VV	0.0031
12		4.492	5835.28	643.21	0.07		VB	0.0058
13		5.001	1366.16	167.27	0.02		BB	0.0014
14	TNT	5.684	2410005.29	258635.36	30.10		BE	58.6357

v) Result of W3R1 water sample : (Fig. 4.6)

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:35:39 PM
Sample Name	: W3R1	Data Acquisition Time	: 4/25/2012 2:28:39 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/15	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

Result File :
Sequence File : F:\HPLC\Sequences\TN_DN_MIX\TN_DN_10.04.12.seq



DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.545	26873249.17	2.55e+06	72.27		BE	26.8732
2		2.534	258404.24	8892.27	0.69		EV	0.2584
3		2.627	97218.74	9023.66	0.26		VV	0.0972
4		2.945	149931.26	14342.11	0.40		VV	0.1499
5		3.042	148125.79	13325.63	0.40		VV	0.1481
6		3.347	43048.26	5908.05	0.12		VV	0.0430
7		3.477	65532.12	6791.13	0.18		VV	0.0655
8		3.713	364179.01	46836.90	0.98		VE	0.3642
9		4.176	44331.59	2723.51	0.12		EV	0.0443
10		4.503	29647.28	2127.95	0.08		VB	0.0296
11		5.199	1160.19	146.89	0.00		BB	0.0012
12	TNT	5.710	3370616.97	358804.75	9.06		BB	81.8450
13	DNT	6.743	4190990.01	427876.76	11.27	+	BV	126.4610

vi) Result of W4R1 water sample : (Fig. 4.7)

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:36:53 PM
Sample Name	: W4R1	Data Acquisition Time	: 4/25/2012 3:00:15 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/17	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

Result File :
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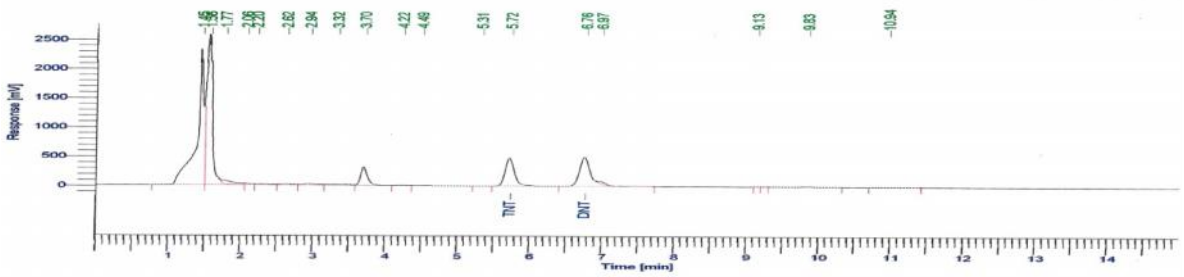

DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.448	14798410.11	2.33e+06	31.72		BV	14.7984
2		1.559	16152580.68	2.59e+06	34.62		VE	16.1526
3		1.768	1078859.67	74008.14	2.31		EV	1.0789
4		2.056	233242.48	33830.26	0.50		VV	0.2332
5		2.203	372730.94	24778.96	0.80		VV	0.3727
6		2.617	328379.84	26136.31	0.70		VV	0.3284
7		2.936	442662.45	35086.98	0.95		VV	0.4427
8		3.318	426575.52	20982.46	0.91		VV	0.4266
9		3.701	2219535.83	317132.70	4.76		VV	2.2195
10		4.219	126833.13	11107.80	0.27		VV	0.1268
11		4.489	151730.68	7089.15	0.33		VV	0.1517
12		5.311	13271.57	1134.52	0.03		VV	0.0133
13	TNT	5.720	4413067.15	476045.43	9.46	+	VB	107.0315
14	DNT	6.755	5394085.83	500953.02	11.56	+	BE	162.4138

vii) Result of W5R1 water sample : (Fig. 4.8)

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:38:54 PM
Sample Name	: W5R1	Data Acquisition Time	: 4/25/2012 3:31:50 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/19	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

Result File :
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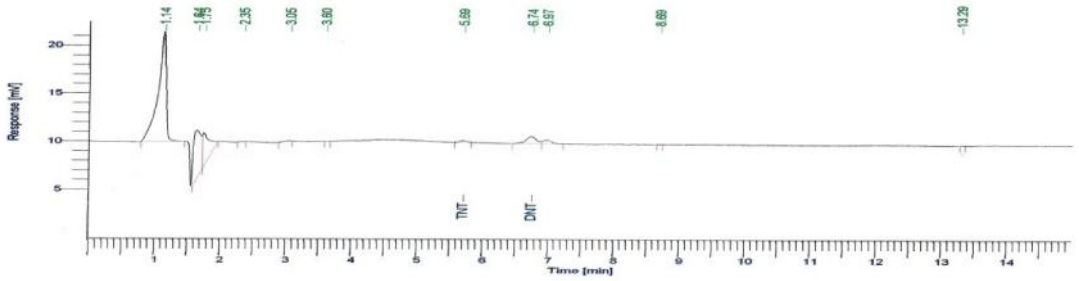

DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.137	100857.74	11535.00	58.13		BB	0.1009
2		1.642	34875.15	4903.73	20.10		BV	0.0349
3		1.747	23586.20	3327.77	13.59		VB	0.0236
4		2.345	147.97	30.90	0.09		BB	0.0001
5		3.048	493.37	40.56	0.28		BB	0.0005
6		3.598	75.44	26.01	0.04		BB	0.0001
7	TNT	5.693	1111.48	157.22	0.64		BB	0.4346
8	DNT	6.742	8365.21	762.41	4.82	-	BV	1.4693
9		6.971	3841.14	381.25	2.21		VB	0.0038
10		8.692	76.45	25.62	0.04		BB	0.0001
11		13.288	62.44	22.58	0.04		BB	0.0001
			173492.59	21213.05	100.00			2.0680

viii) Result of W6R1 water sample : (Fig. 4.9)

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:40:15 PM
Sample Name	: W6R1	Data Acquisition Time	: 4/25/2012 4:03:27 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/21	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

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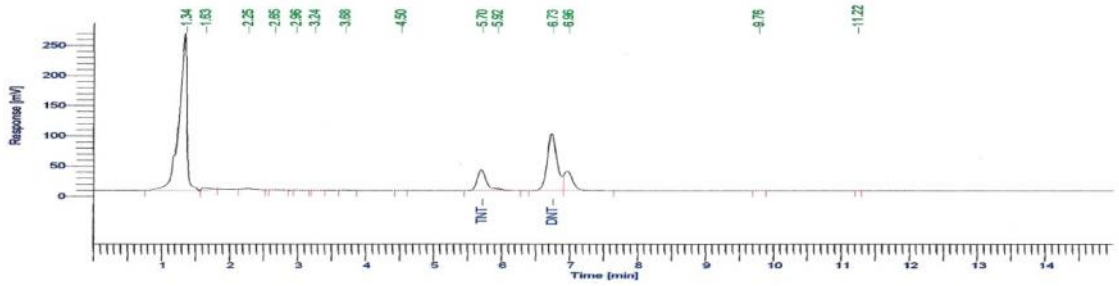

DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.345	2180320.89	266303.78	56.75		BB	2.1803
2		1.630	36263.92	4553.54	0.94		BB	0.0363
3		2.252	17120.97	1685.92	0.45		BB	0.0171
4		2.646	2047.36	195.16	0.05		BB	0.0020
5		2.959	1395.20	191.14	0.04		BB	0.0014
6		3.236	498.67	92.00	0.01		BB	0.0005
7		3.685	3707.31	547.90	0.10		BB	0.0037
8		4.500	480.92	74.93	0.01		BB	0.0005
9	TNT	5.698	318724.82	34480.66	8.30		BE	8.1085
10		5.924	19938.87	2292.45	0.52		EB	0.0199

ix) Result of W7R1 water sample : (Fig.

Software Version	: 6.3.2.0646	Date	: 4/26/2012 2:41:22 PM
Sample Name	: W7R1	Data Acquisition Time	: 4/25/2012 4:35:02 PM
Instrument Name	: PE_HPLC-2	Channel	: A
Rack/Vial	: 0/23	Operator	: cfees
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

Result File :
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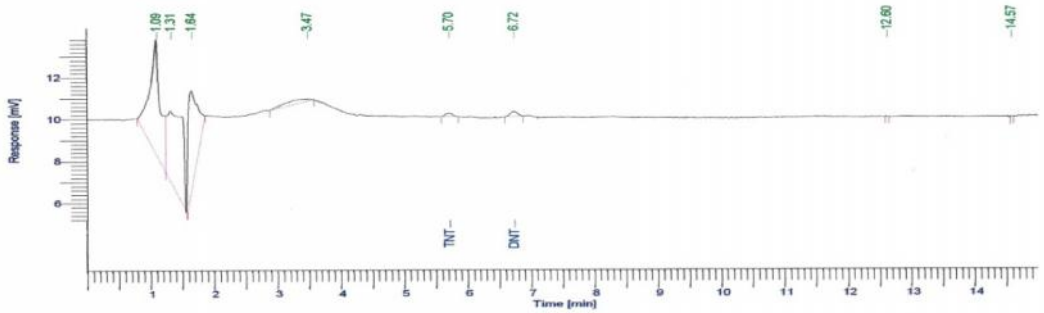


DEFAULT REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Cal. Range	BL	Adjusted Amount
1		1.090	63529.74	5646.25	34.61		BV	0.0635
2		1.309	69314.81	3450.45	37.76		VB	0.0693
3		1.639	41714.14	4540.76	22.73		BB	0.0417
4		3.469	5197.28	113.10	2.83		BB	0.0052
5	TNT	5.701	1494.77	181.22	0.81		BB	0.4439
6	DNT	6.723	2139.81	254.39	1.17	-	BB	1.2833
7		12.603	66.04	41.08	0.04		BB	0.0001
8		14.572	96.89	53.39	0.05		BB	0.0001
			183553.48	14280.63	100.00			1.9071

4.10)

S.No.	Water Samples	TNT Concentration in ppm	DNT Concentration in ppm
1	W1	0.4454	1.3118
2	W2	58.6357	96.8869
3	W3	81.8450	126.4610
4	W4	107.0315	162.4138
5	W5	0.4346	1.4693
6	W6	8.1085	29.2515
7	W7	0.4439	1.2833

Table 4.5: Concentration of TNT and DNT in water samples

- A Minimal Risk Level (MRL) of 0.0005 mg/kg/day has been derived for intermediate oral exposure to TNT .
- The oral slope factor for carcinogenic risk is 3 x 10-2 milligrams per kilogram per day(mg/kg/day).
- The EPA has established a Lifetime Health Advisory guidance level of 2 parts per billion (ppb) for TNT in drinking water. The health advisory for a cancer risk of 10-4 is 0.1 mg/L.
- EPA has calculated a resident soil screening level of 19 milligrams per kilogram (mg/kg) and an industrial soil screening level of 79 mg/kg .[U.S EPA,2012]

- According to USEPA study if administered dose of TNT in animal body(RATS) is 2ppm cases of tumor are 0 out of 55 while for 10ppm and 50ppm tumor cases are 1 out of 55 and 17 out of 55 respectively.[IRIS,DNT,2012]
- Similarly, if administered dose of DNT in animal body(RATS) is 15ppm cases of tumor are 12 out of 35 while for 100ppm and 700ppm tumor cases are 17 out of 27 and 34 out of 35 respectively.
- One of the USEPA regulation states —Maximum Concentration of Contaminants(DNT) for the Toxicity Characteristic is 0.13mg/l. [IRIS,TNT,2012]

Thus in the above water samples contaminated with explosive wastes minimum TNT level is shown in W5(0.4346ppm) and maximum in W4(107.0315ppm). Whereas maximum DNT level is shown in W4(162.4138ppm) and minimum in W7(1.2833ppm) samples. All the DNT concentrations in water sample are above the prescribed level of DNT(0.13mg/l), thus samples are highly toxic. Similarly concentration of TNT is also very much above the permissible limits making the samples highly toxic with TNT too.

4.3 MICROBIAL ANALYSIS OF SOIL SAMPLES :

For soil microbial analysis two soil samples were taken

A = SL1R1(Sludge Sample) and

B = S4R1(TNT washing house at distance of 500 m surface).

4.3.1 Enumeration(Colony Counting) of microbes in Soil sample:

Here A1 is for first dilution that is 1/10 and thus A2, A3, A4, A5 for second, third, fourth, fifth dilutions respectively. Same is the case with sample B. All the samples were inoculated in replications as A1 and A11, thus colony counted are average of the colonies founded on two plates. Same concept applies for all the samples.

S.No	Sample	Colony Counted	Volume plated(in ml)	CFU/ml
1	A1	575	0.1	57500
2	A2	174	0.1	174000
3	A3	42	0.1	420000
4	A4	10	0.1	1000000
5	A5	3	0.1	3000000
6	B1	167	0.1	16700
7	B2	27	0.1	27000
8	B3	17	0.1	170000
9	B4	5	0.1	500000
10	B5	1	0.1	1000000

Table 4.6: Results for colony counting of microbes in soil samples

As CFU/ml is indirectly proportional to colony counted in the culture plates[Reynolds J, 2012].Thus maximum number of colonies can be founded in sample A1 and B1 with least CFU/ml values. The same concept applies for all the samples. Some of the plates showing the grown colony :

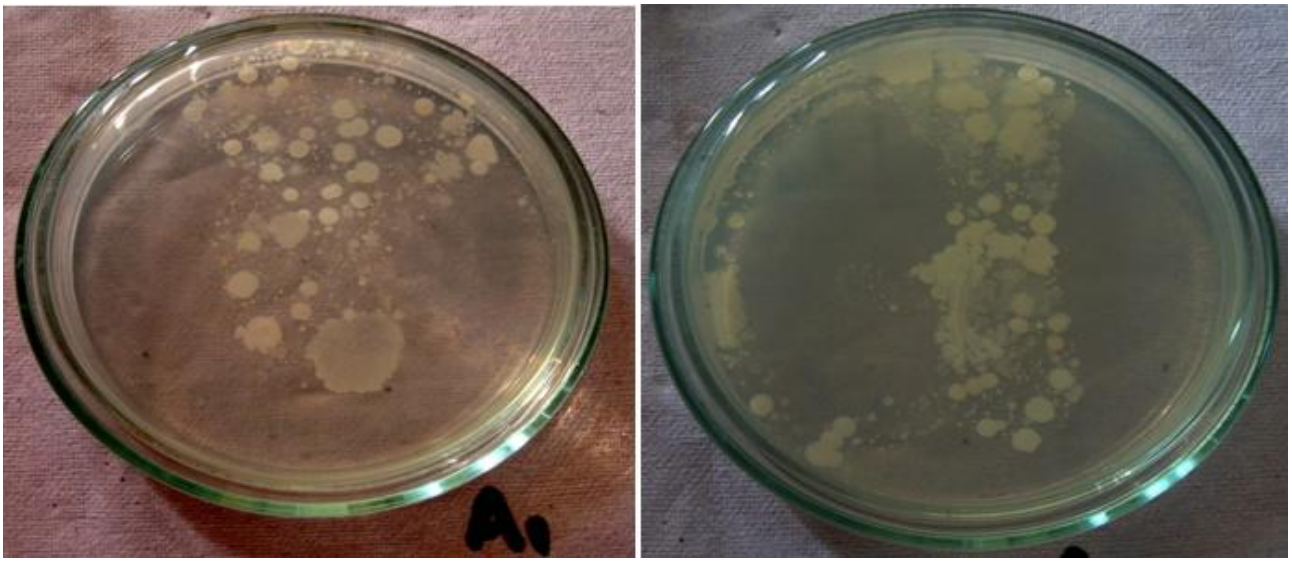
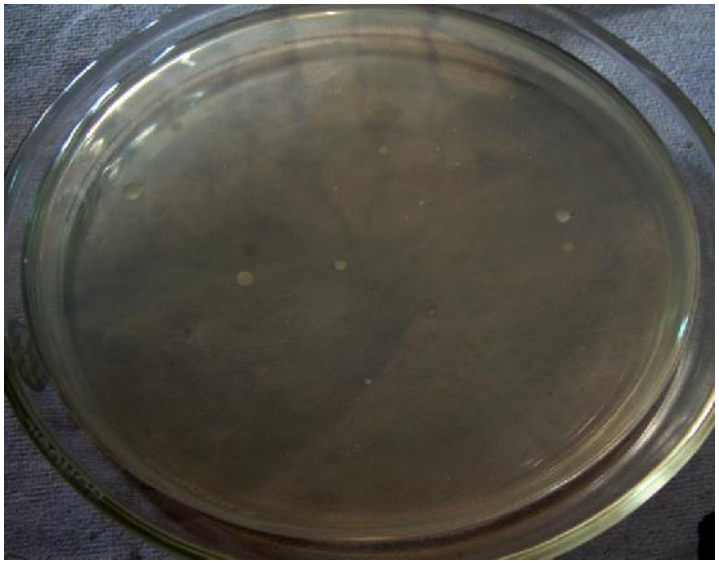


Fig. 4.11: Colony counting result for Sample A1 and Sample B1



Sample A4

4.3.2 Culturing of Soil samples contaminated with TNT :

i) Primary Culture Plates :

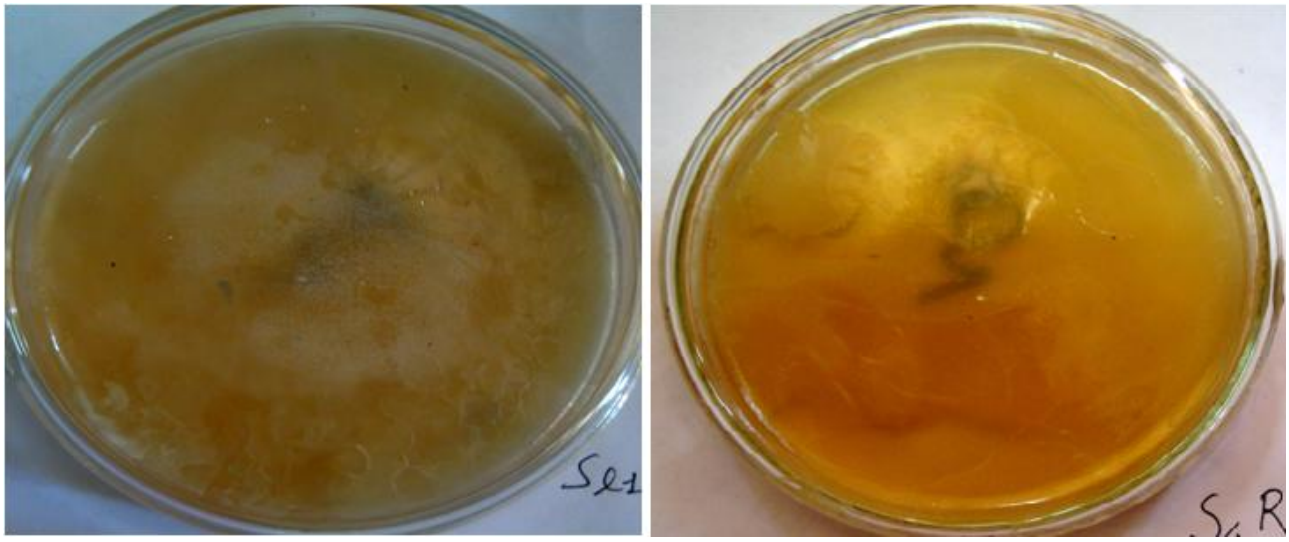


Fig. 4.12: Primary culture plates for Sample A and Sample B

The white patches observed over the Trypticase Soy Agar primary plates are colonies from soil samples which are further taken to be inoculated on secondary plates.

ii) Secondary Culture Plates :

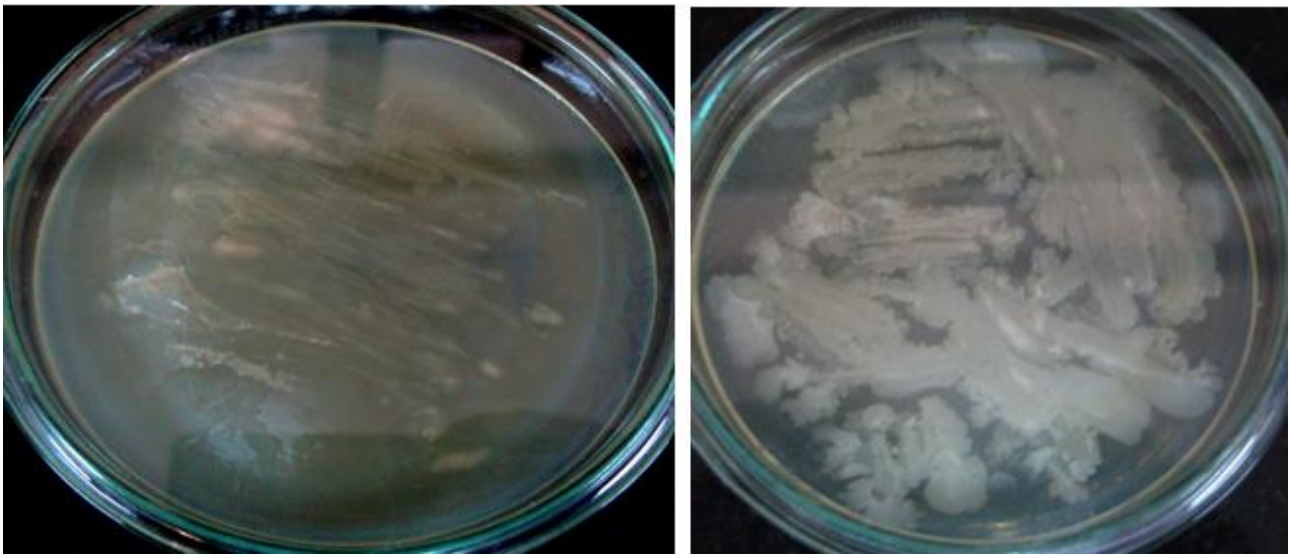


Fig. 4.13: Secondary culture plates: Sample A – Replicate 1 and Replicate 2

Above is the colonies formed by primary plates of trypticase Soy agar inoculated with TNT contaminated soil samples A (SL1R1). Culturing is done in replication as shown here above. Colonies are formed as mustard white colored in first replicate with dense(fused) straight colonies and replicate 2 showed white colonies with fried egg like appearance.

Fig. 4.14: Secondary culture plates: Sample B – Replicate 1 and Replicate 2

Above is the colonies formed by primary plates of trypticase Soy agar inoculated with TNT contaminated soil samples B (S4R1). Culturing is done in replication as shown here above. Colonies are formed as clear white colonies with fried egg like appearance in both the replicates showing one major microbe in the soil sample. Colonies were distinct, smooth and large in appearance.

4.3.3 Staining Tests :

i) Crystal Violet Test : For Bacteria

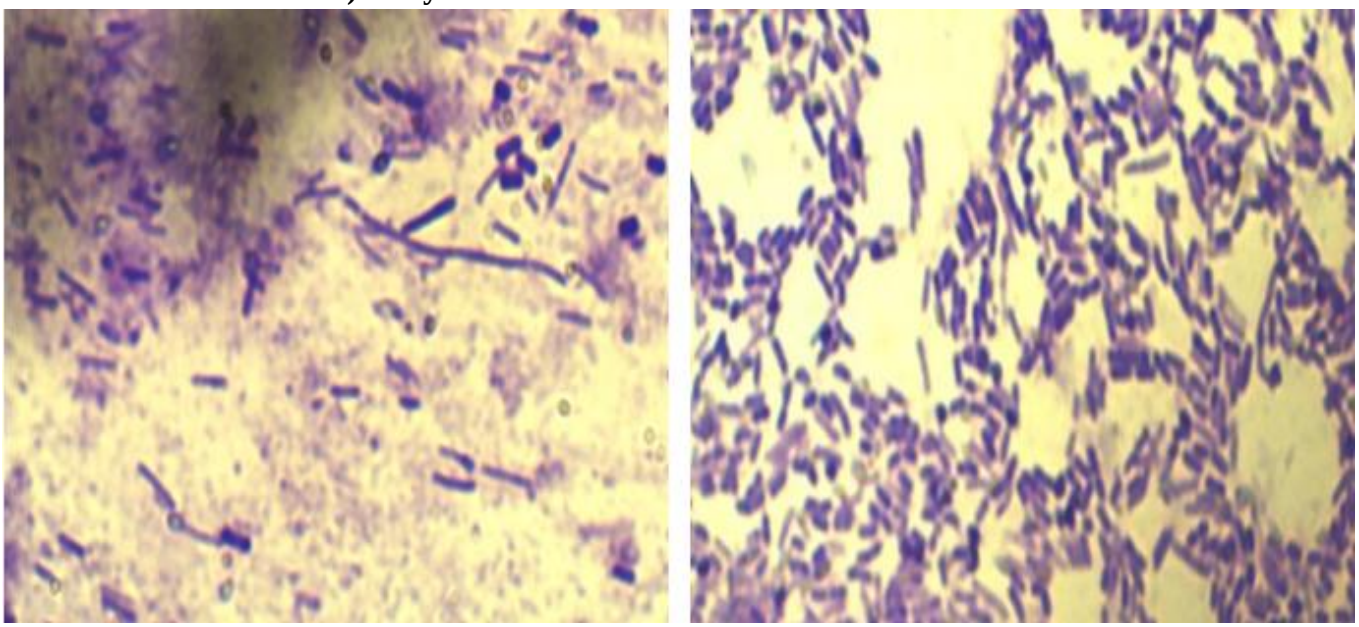


Fig. 4.15: Crystal Voilet staining results Sample A and Sample B

As per the colonies seen in Secondary culture plates and staining by Crystal Violet following conclusions were taken about the characteristics of the microbes (Bacteria) in the two samples(SL1R1, S4R1)

Sample	Colony	Colony			Surface		Shape
		Color	Texture	Edge	Elevation	Appearance	
A	1	Grey	Very Smooth	Straight/Slightly Curved	NO	Granular	Long Rod-shaped
	2	Yellowish Grey		Continuous Border	NO	Opaque Centre	Oval
B	3	White, Crème	Smooth	Flat -edge	YES	Fried-egg like	Rod shaped

Table 4.7: Characteristics of microbes encountered.

ii) Gram Staining :

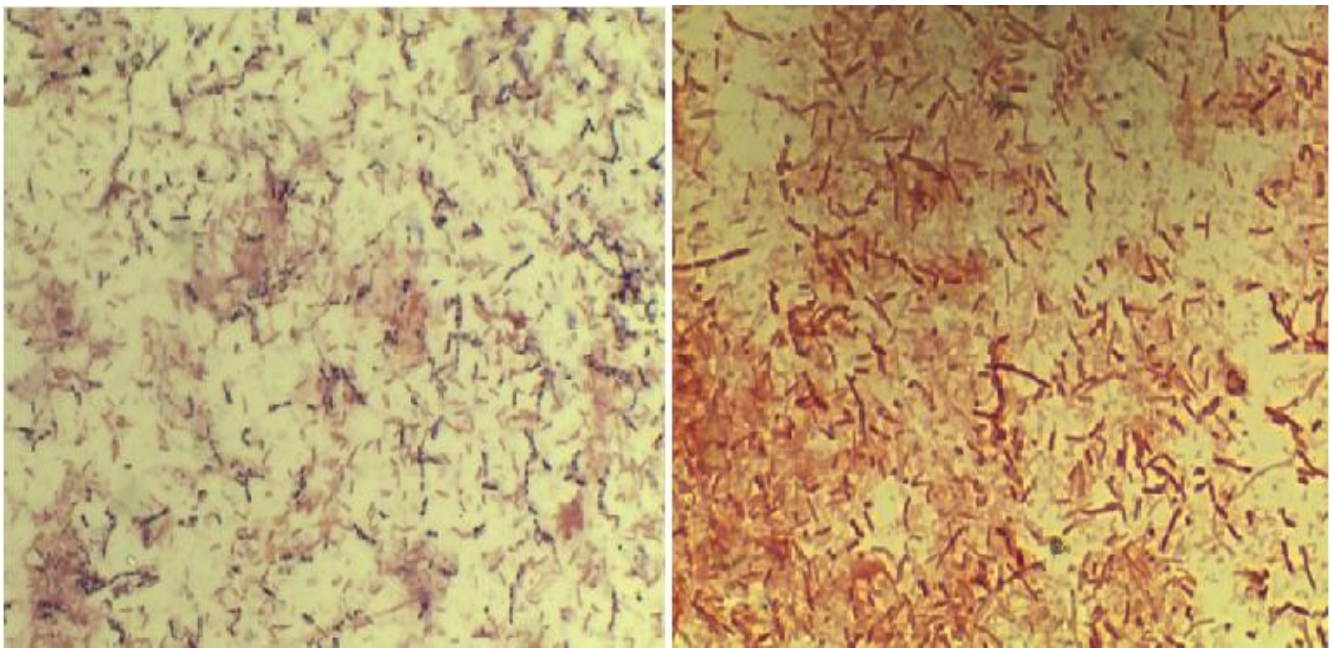


Fig.4.16: Gram Staining result for Sample A and Sample B

Above results for Gram staining of Sample A and B respectively shows presence of Gram(+) and Gram(-) both sort of bacteria in sample A while Sample B has only Gram(-) bacteria.

iii) Endospore Stain :

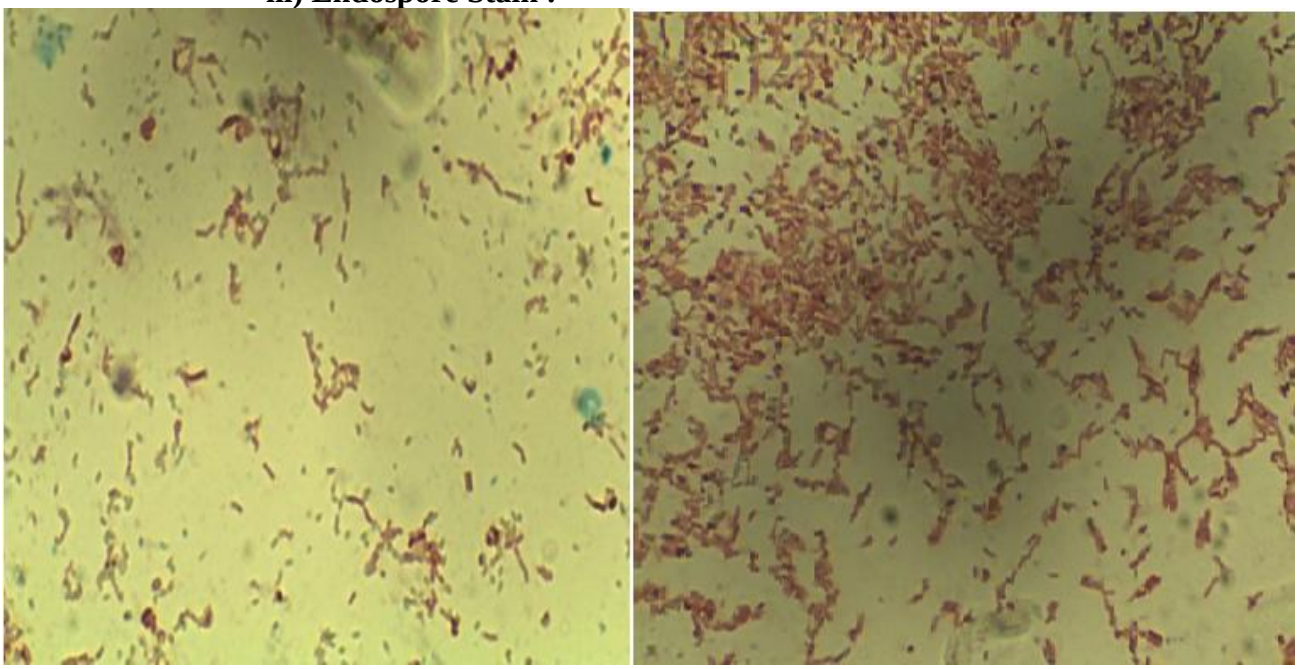


Fig. 4.17: Endospore staining results for Sample A and Sample B

As per the theoretical reviews, red colored stain shows no endospores. Thus both the samples(A and B) have no endospores.

iv) Acid – Fast Stain :

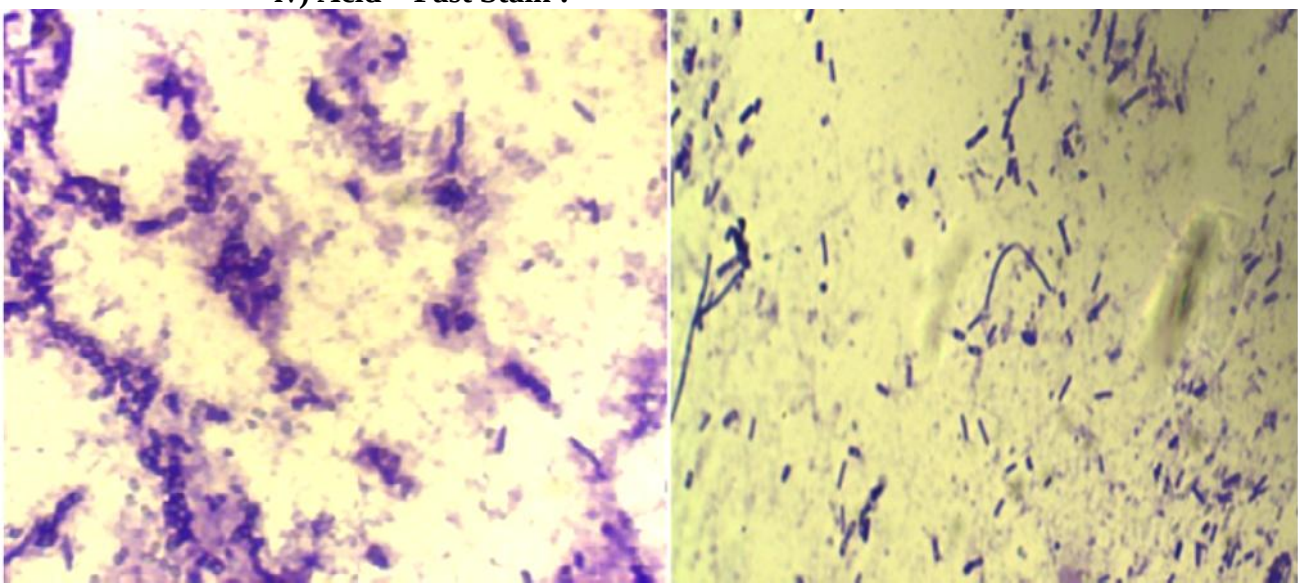


Fig. 4.18: Acid-Fast staining results for Sample A and Sample B

Blue colored colonies shows Non-Acid Fast bacteria. Both the samples(A and B) have negative results for Acid-fast staining.

v) Capsule Stain :

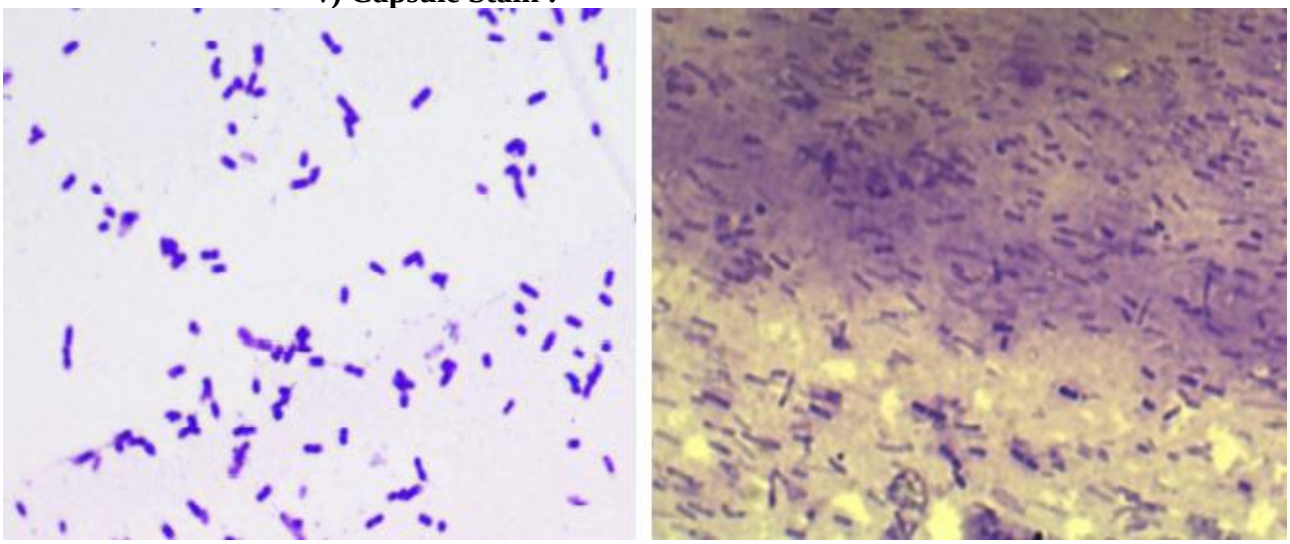


Fig. 4.19: Capsule Staining results for Sample A and Sample B

While Sample A showed some kind of capsules in the microscope, sample B was negative for capsule staining that is sample B do not have bacteria which has capsules.

S.No.	Staining Technique	Sample	Positive(+) OR Negative(-)	Shape
1	Gram	A	(+) and (-) both	Rod-shaped
		B	(-)	Rod-shaped
2	Endospore	A	(-)	Oval
		B	(-)	Rod-shaped
3	Acid-Fast	A	(-)	Oval, rod-shaped
		B	(-)	Elongated rods
4	Capsule	A	(+)	Very small rods
		B	(-)	Rod-shaped

Table 4.8: Results of all the staining techniques used on both the samples (A and B)

4.3.4 Characterization of microbial cells under different optimized conditions :

i) Growth on Selective and Differential Media

I. Nutrient Agar

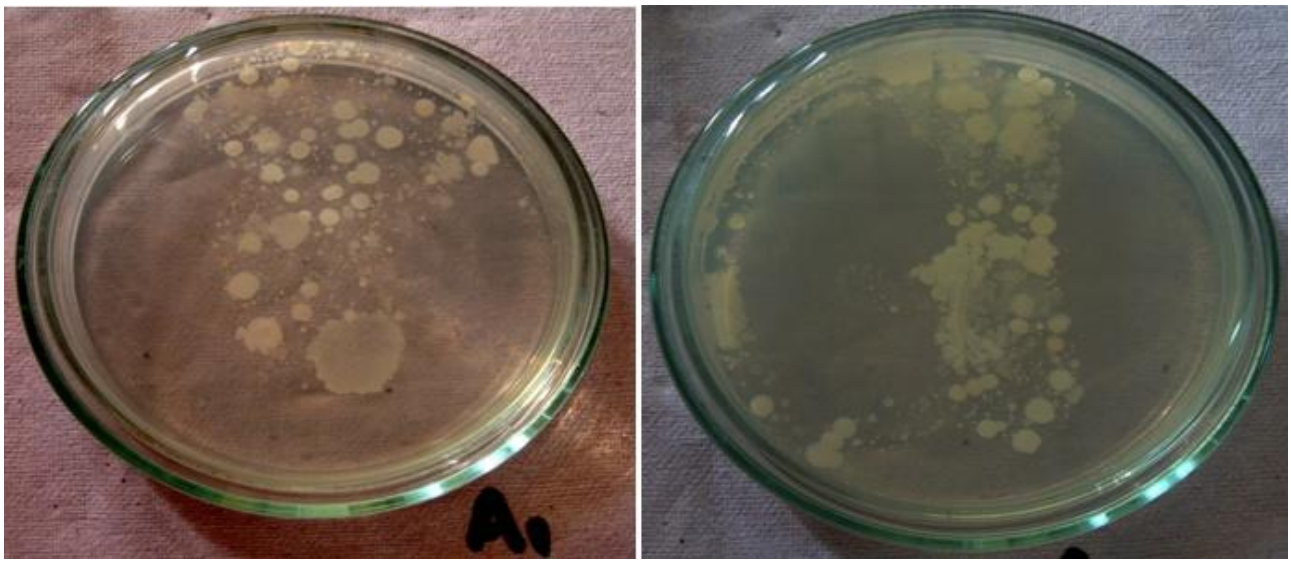


Fig. 4.20: Growth on Nutrient agar results for Sample A and Sample B

As shown in the above figure, both the samples A and B grows well on nutrient agar medium in the same interval of time and same conditions. The colonies were distinct and off-white in color.

II. High-salt agar

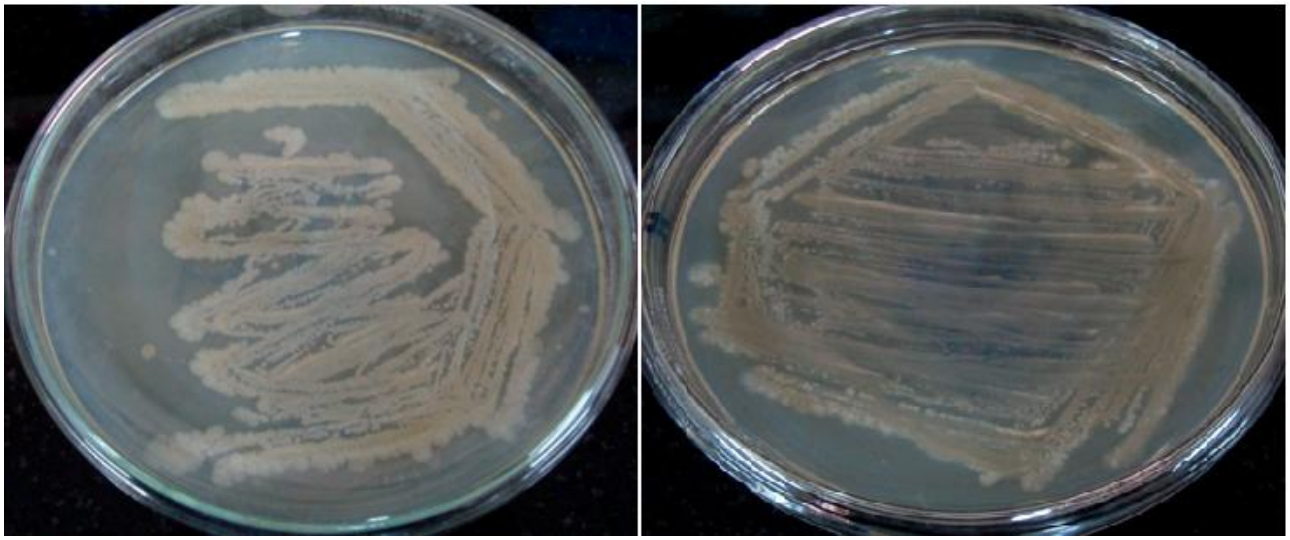


Fig. 4.21: Growth on High-Salt Agar results for Sample A and Sample B

As shown in the above figure, both the samples A and B grows well on High- salt agar in the same interval of time and same conditions but the colonies characteristics was different from Nutrient agar, off-white longitudinal, distinct colonies can be seen with fried-egg like appearance. The growth was less as compared to what results we get on normal trypticase soy agar culturing, and the color of colonies also differs somewhat from off-white on High-salt agar to white on Trypticase soy agar.

III. Mannitol-salt agar

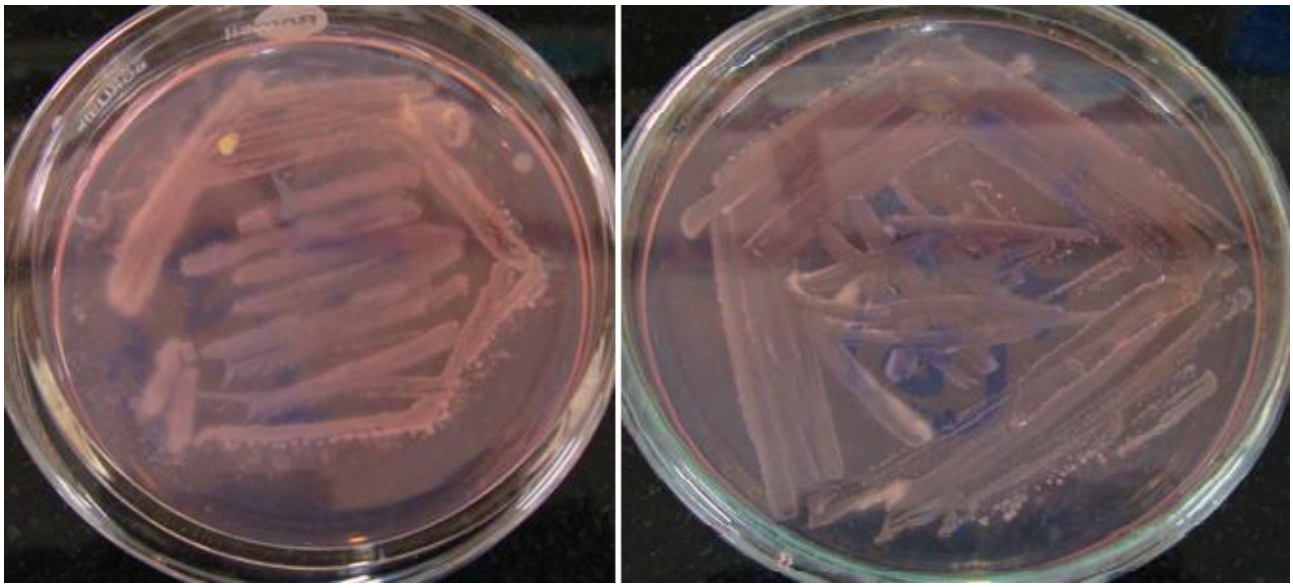


Fig. 4.22: Growth on Mannitol-Salt agar results for Sample A and Sample B

Mannitol-salt agar media is used to check whether the cultured colony acid- producing or not. If the grown colony turns the indicator present in media(Phenol Red) yellow, the colony is Acid producing. And if the colony turns the indicator present in media red, the colony is Non-acid producing. That is it do not produce any acid. The above results for both sample A and Sample B turned out to be red in color that shows the colonies isolated from both soil samples are not acid producing.

ii) Bacterium's tolerance for increasing concentrations of NaCl. (Osmotic effects)

I. 0.5% NaCl in Typticase soy agar(TSA)

Fig. 4.23: Growth on 0.5% NaCl in TSA results for Sample A and Sample B

With decreased concentration of salt in media onto which colonies isolated from soil samples are grown, Sample A shows increased growth with decreasing concentration of salt. While sample B has decreased growth with decreased salt concentration.

II. 5% NaCl in TSA

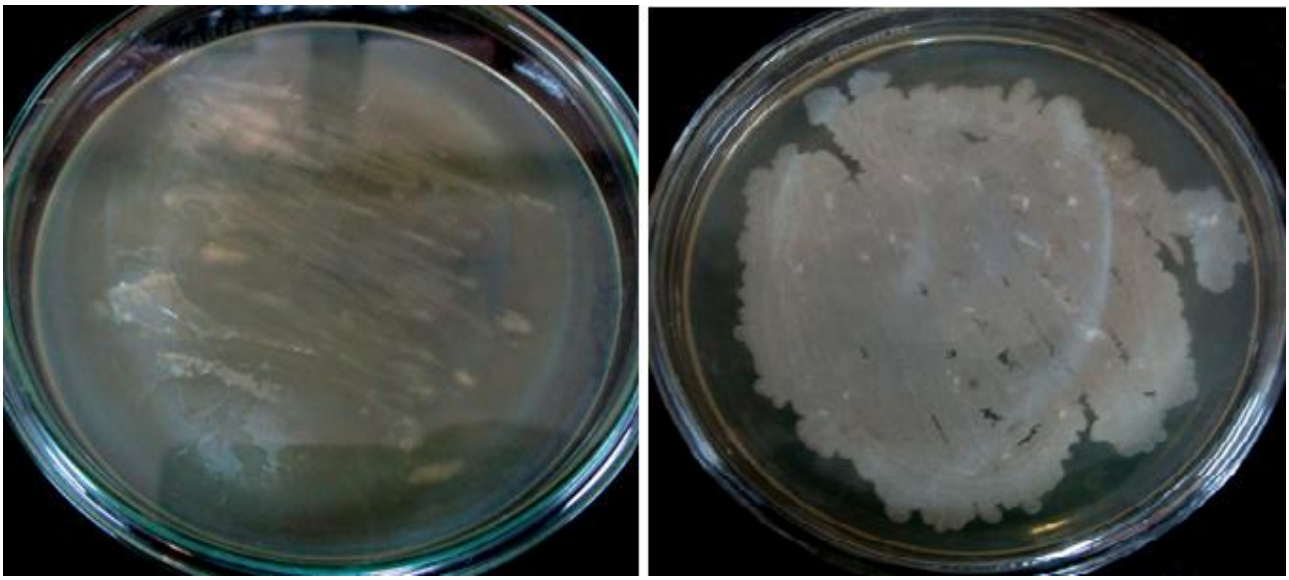


Fig. 4.24: Growth on 5% NaCl in TSA results for Sample A and Sample B

At 5% Salt concentration in TSA media , that is normal salt concentration , growth in sample A decreased and color of colonies changed from white to mustard –white. While for sample B colony encountered was of same type(White colored, distinct, fried-egg like appearance, smooth) but there was an increase in growth.

III. 20% NaCl in TSA

Fig. 4.25: Growth on 20% NaCl in TSA results for Sample A and Sample B

At increased concentration of salt, Sample A shows an abrupt growth in its colony and the characteristics appeared to be same as what seen at 0.5% salt concentration, White colored colony with fried-egg like appearance. While for Sample B the growth of colony increased with increased salt concentration but appearance changed a bit. As now the colonies encountered in Sample B are much smooth and are not distinct enough and do not have much of fried-egg like appearance.

iii) Temperature optimum of the unknown bacterium

I. At 25°C colony grown on Typticase soy agar(TSA)

No Growth was seen at 25 °C

II. At 37°C colony grown on Typticase soy agar(TSA)

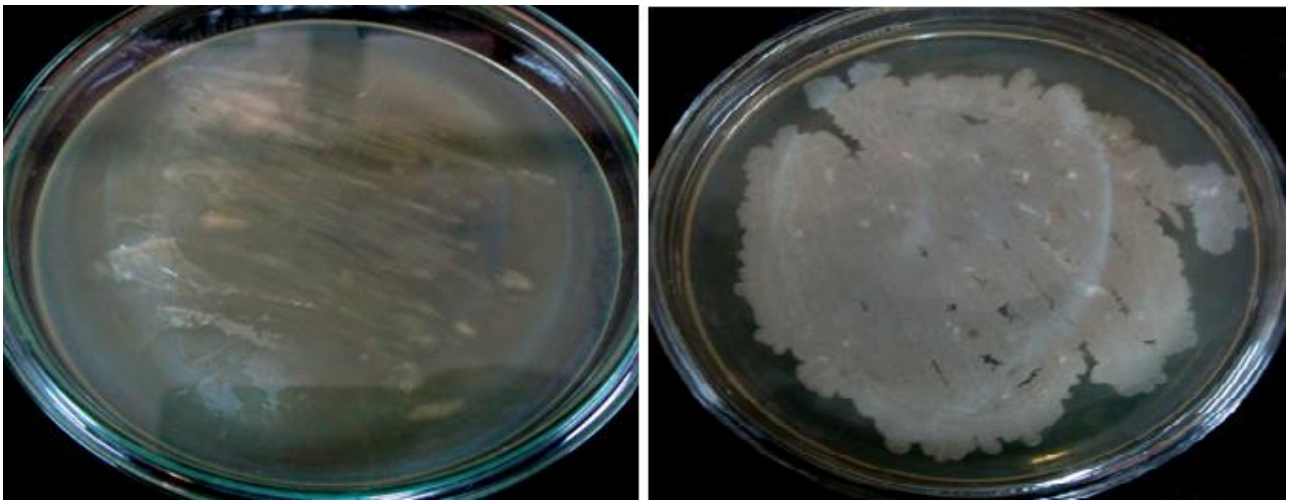


Fig. 4.26: Growth at 37°C temp. on TSA results for Sample A and Sample B

At 37°C in TSA media , that is normal temperature , growth in sample A is fused typed, mustard –white in color. While for sample B colony encountered was of same type(White colored, distinct, fried-egg like appearance, smooth) .

III. At 55 C colony grown on Typticase soy agar(TSA)

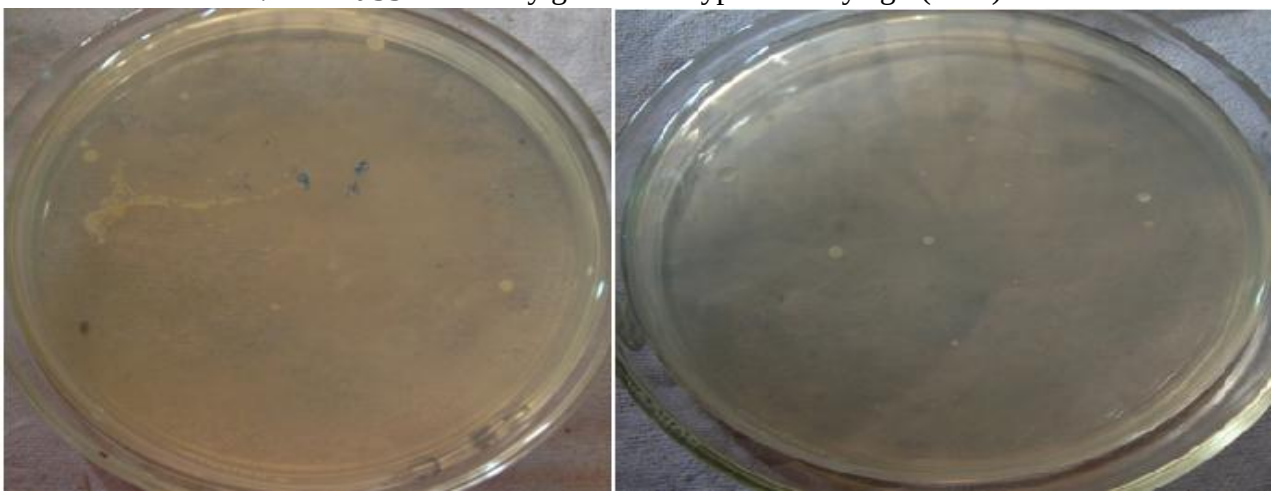


Fig. 4.27: Growth at 55°C temp. on TSA results for Sample A and Sample B

Almost negligible colonies were observed at 55°C. In sample A there was a cluster of dot-like colonies and in Sample B only 3-4 distinct colonies can be seen

From all the identification techniques(Staining and Characterization on optimized conditions) and literature study, following characteristics of the microbial strain isolated from sample B (S4R1) was observed :

S.No.	Properties/Test	Properties of Pseudomonas (Standard)	Properties of Pseudomonas(Observed)
1	Color	White	White, Crème
2	Texture	Smooth	Smooth
3	Edge	Flat-edge	Flat -edge
4	Surface Elevation	YES	YES
5	Appearance	Fried-egg like	Fried-egg like
6	Shape	Rod-shaped	Rod shaped
7	Gram stain	(-)	(-)
8	Endospore Stain	(-)	(-)
9	Acid-Fast Stain	(-)	(-)
10	Capsule Stain	(-)	(-)
11	Acid -producing	No	No
12	Mesophilic(grows well at 37°C)	Yes	Yes
13	Salt- Sensitive	Yes	Yes

Table 4.9: Comparative study of standard and observed characteristics of Pseudomonas

The microbe obtained during study was Gram (–ve), Rod Shaped, approximately 1.5 micrometer in length and 0.5-1.0 micrometer wide. Grows well aerobically and do not produce acid.

All these characteristics were similar to bacteria with genus ***Pseudomonas*** and thus this bacteria from sample B was taken for further Bioremediation study on water samples containing explosives(TNT).[Todar K],[Palleroni N.J, 2010] The growth of this microbe(Pseudomonas) was also monitored for 10-days under toxic conditions. Further Bioremediation study was performed by pseudomonas on different TNT concentrations to observe effect of different TNT concentration on microbes(tolerance assessment) and level of bioremediation by the microbe with increasing concentration of TNT in water samples (40ppm, 80ppm, 120ppm).

4.4 BIOREMEDIATION OF TNT CONTAMINATED(PREPARED)WATER SAMPLES : with microbe isolated from TNT contaminated Soil sample(SAMPLE B – S4R1) and tolerance assessment of microbe by growing in growth media containing different concentrations of TNT (HPLC Results)

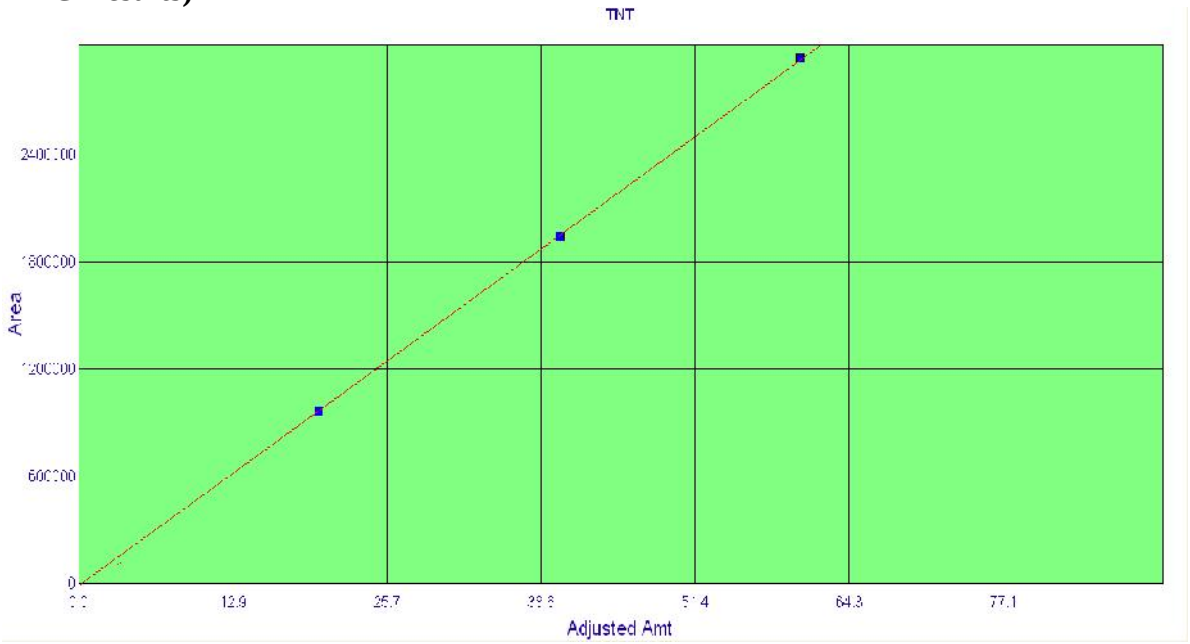


Fig. 4.28: Calibration curve for TNT(For Bioremediation)

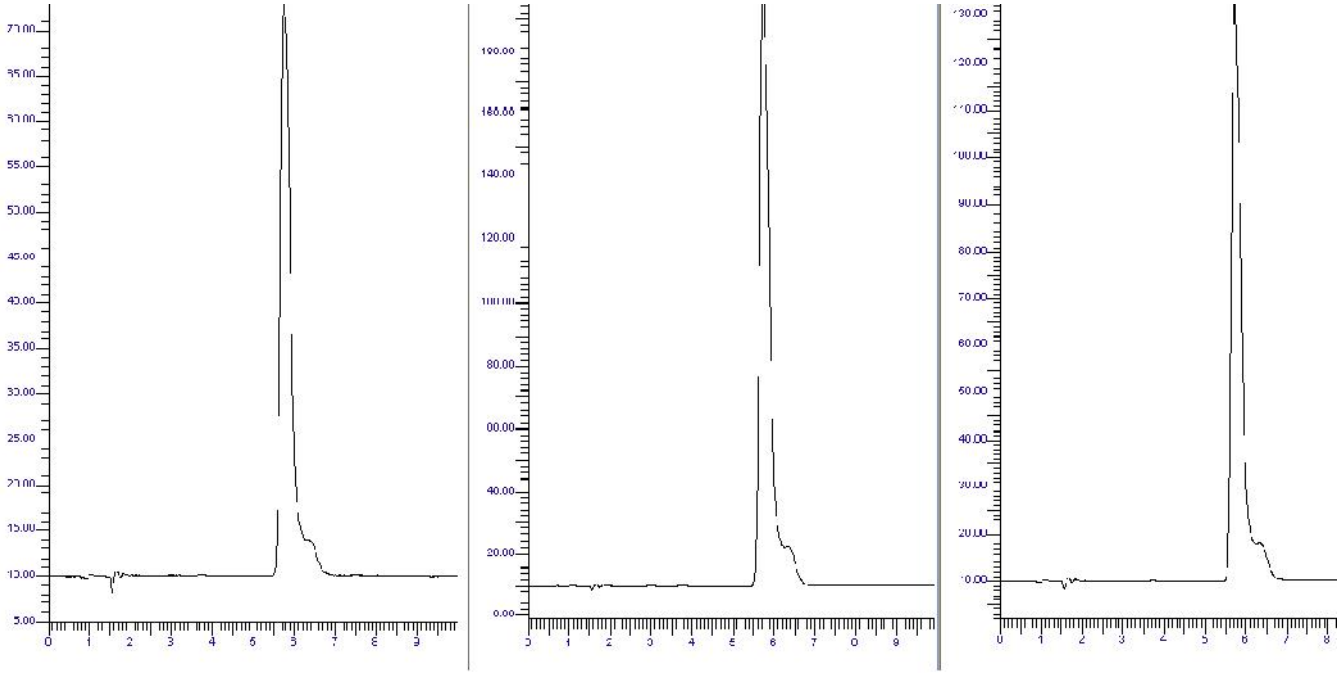


Fig. 4.29: Showing Standard Curve for 40ppm, 80ppm, 120ppm respectively. The peak observed is of TNT, obtained at 5 to 6 minute range.

Day 1 : 19hrs

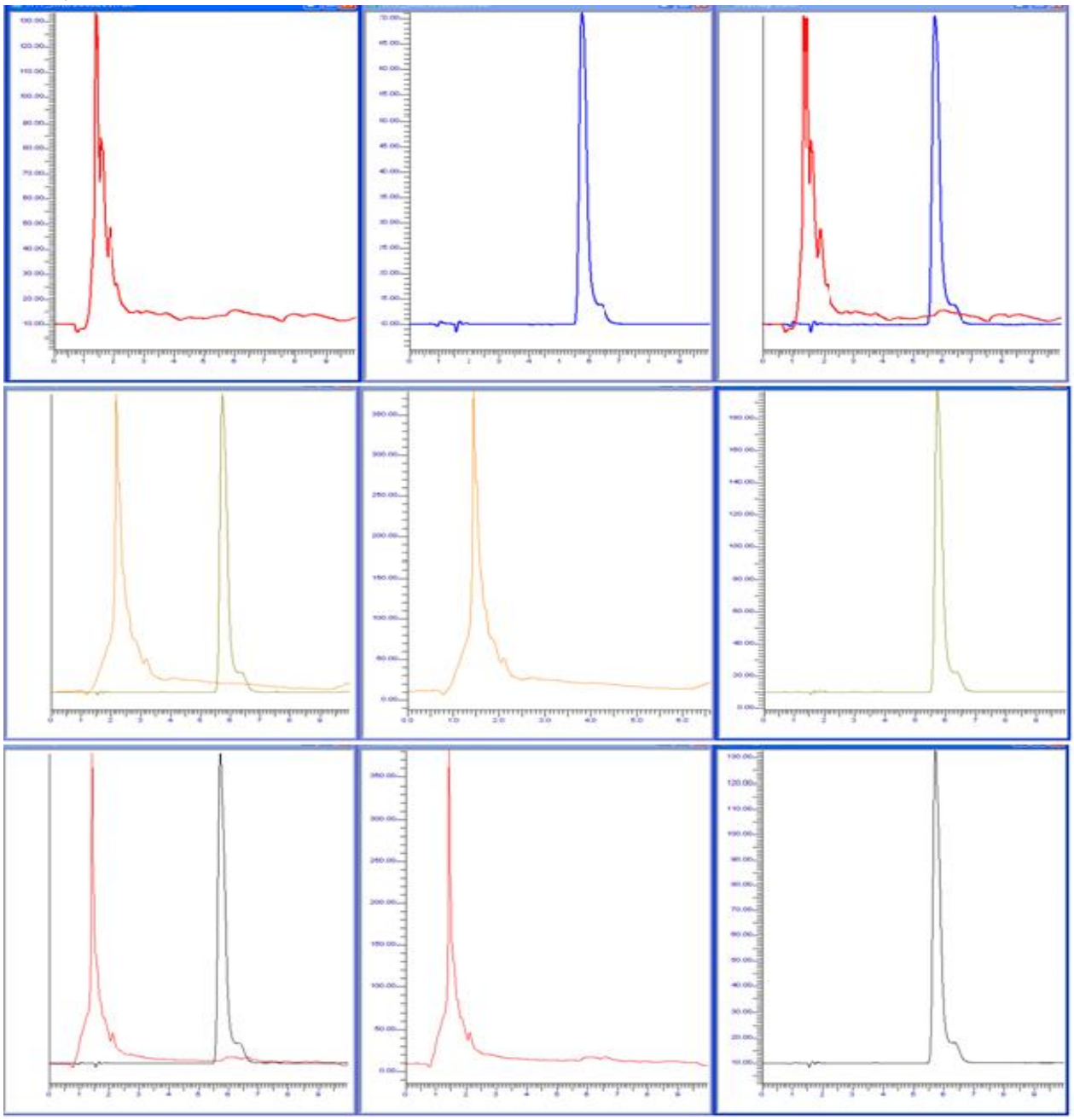


Fig. 4.30: Overview graphs of TNT removal at 40, 80 and 120 ppm for DAY1

In the above graphs, upper three are for 40ppm in which second graph shown 40ppm TNT standard solution curve, first graph shows sample after 19hrs. The peak observed on 40 ppm standard graph is of TNT which can't be observed at 40ppm sample after 19hrs, thus the peak in 40ppm sample solution is of compound formed due to action(bioremediation) by microbes, TNT is degraded and converted to another compound less toxic. Third graph shows comparison of both curves(Red depicting sample and blue depicting standard).

Similarly, middle three graphs are for 80ppm, in which third one is for standard 80ppm TNT, and the curve in it shows 80ppm standard TNT solution curve. While second graph is for 80ppm sample solution showing no peak at time interval where 80ppm standard peak was obtained, the peak obtained on 80ppm sample solution is of degraded compound. Lastly, the lower three graphs are for 120ppm, third one for 120ppm standard containing TNT, second for water sample containing 120ppm TNT which is degraded now in 19hrs .

Day3 : 67 hrs

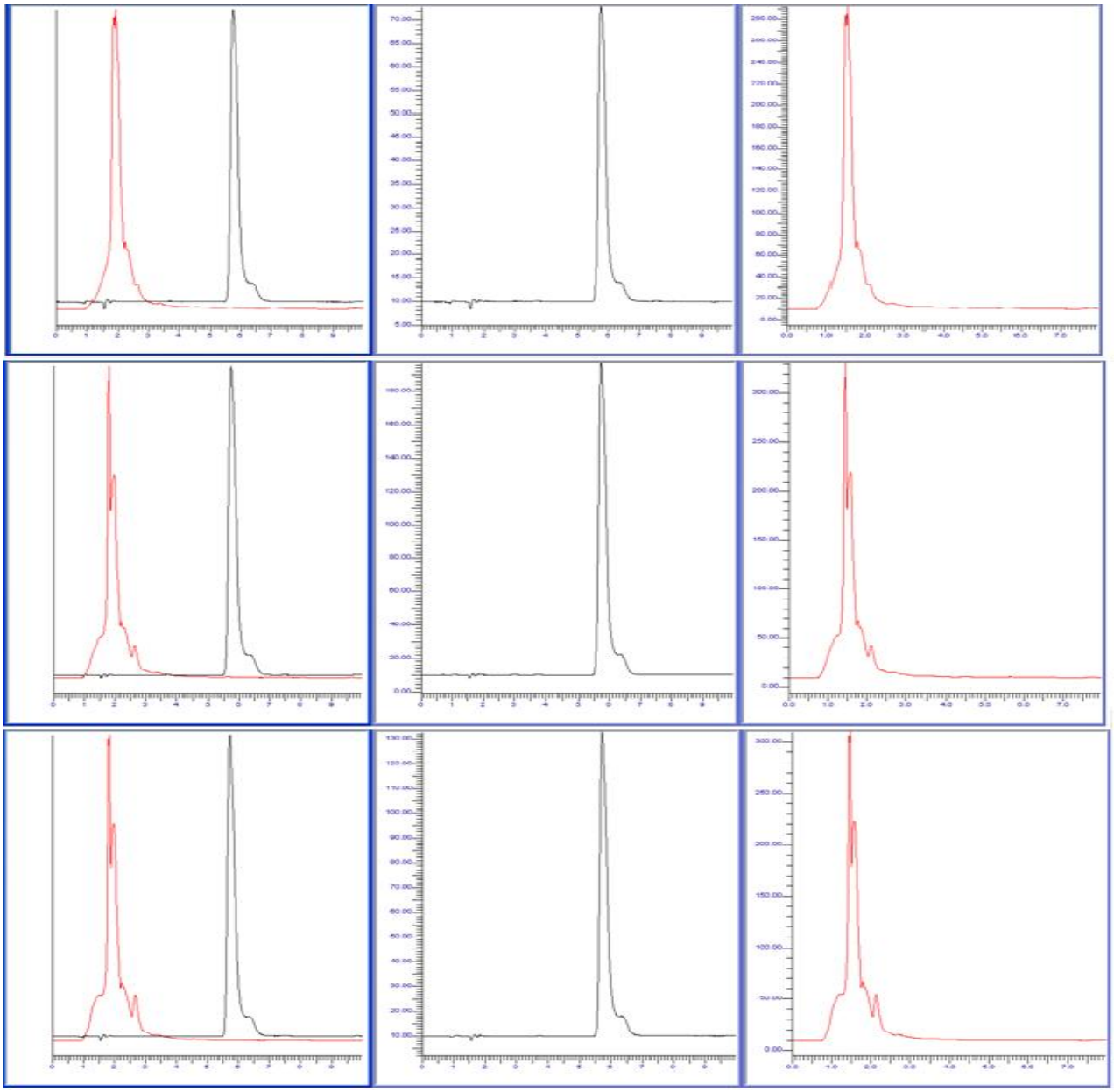


Fig. 4.31: Overview graphs of TNT removal at 40, 80 and 120 ppm for DAY3

For Fig. 4.31, Red graph for sample after 67hrs, Blue for standards. Upper three graphs are for 40ppm(overview graph, standard , sample after 67hrs), middle three are for 80ppm (overview graph,

standard , sample after 67hrs),lower three graphs are for 120ppm(overview graph, standard , sample after 67hrs).All the sample graphs do not show TNT peak as shown in their respective standards, hence HPLC study shows degradation of TNT.

Day7:163hrs

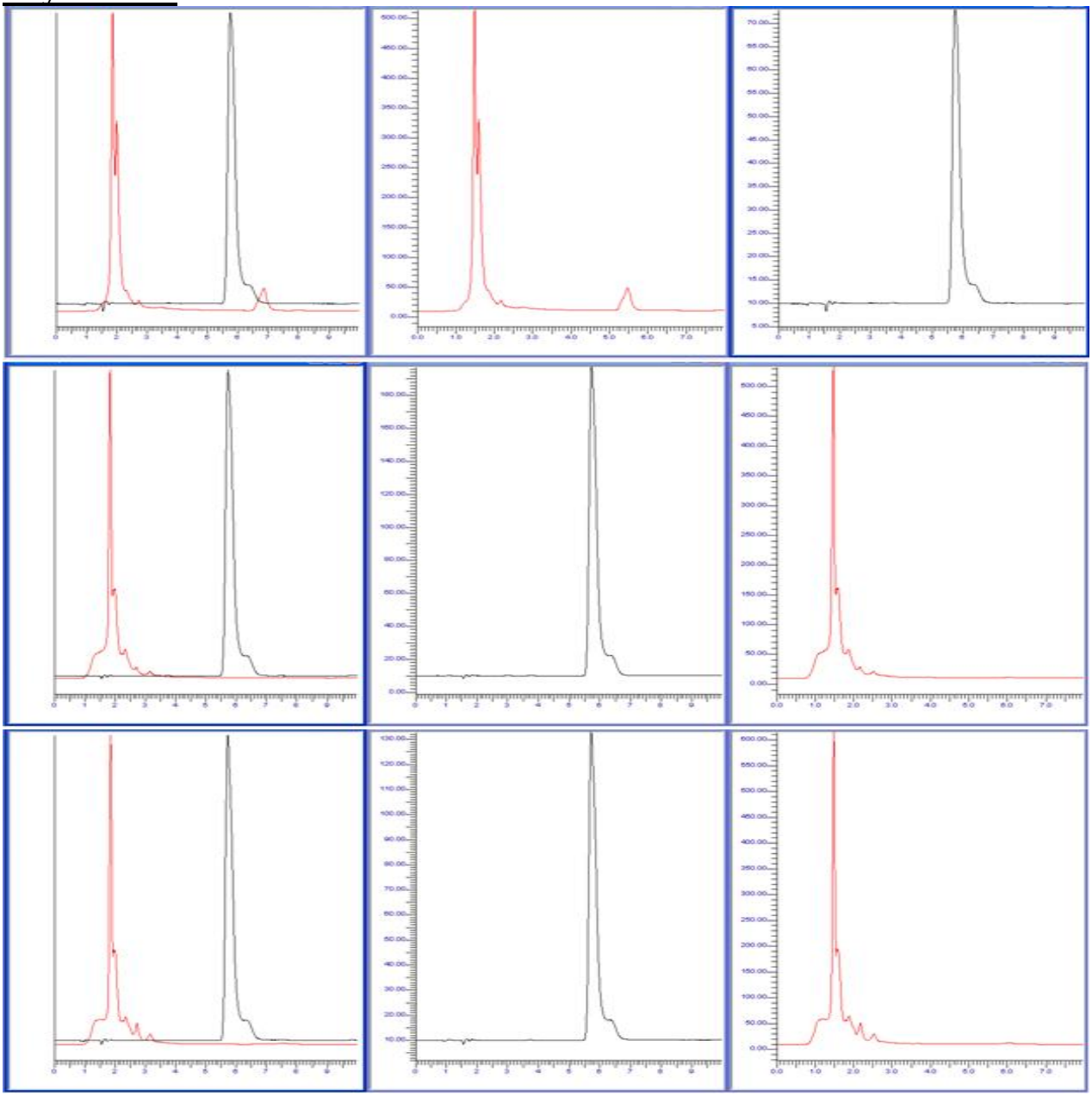


Fig. 4.32: Overview graphs of TNT removal at 40, 80 and 120 ppm for DAY7

For fig. 4.32, Red graph for sample after 163hrs, Blue for standards. Upper three graphs are for 40ppm(overview graph , sample after 163hrs,standard), middle three are for 80ppm (overview graph, standard , sample after 67hrs),lower three graphs are for 120ppm(overview graph, standard , sample after 163hrs).All the sample graphs do not show TNT peak as shown in their respective standards, hence

HPLC study shows degradation of TNT but the sample graph at 40ppm shows two peaks which depicts TNT is degraded in two compounds in 163 hrs.

Day8:187hrs

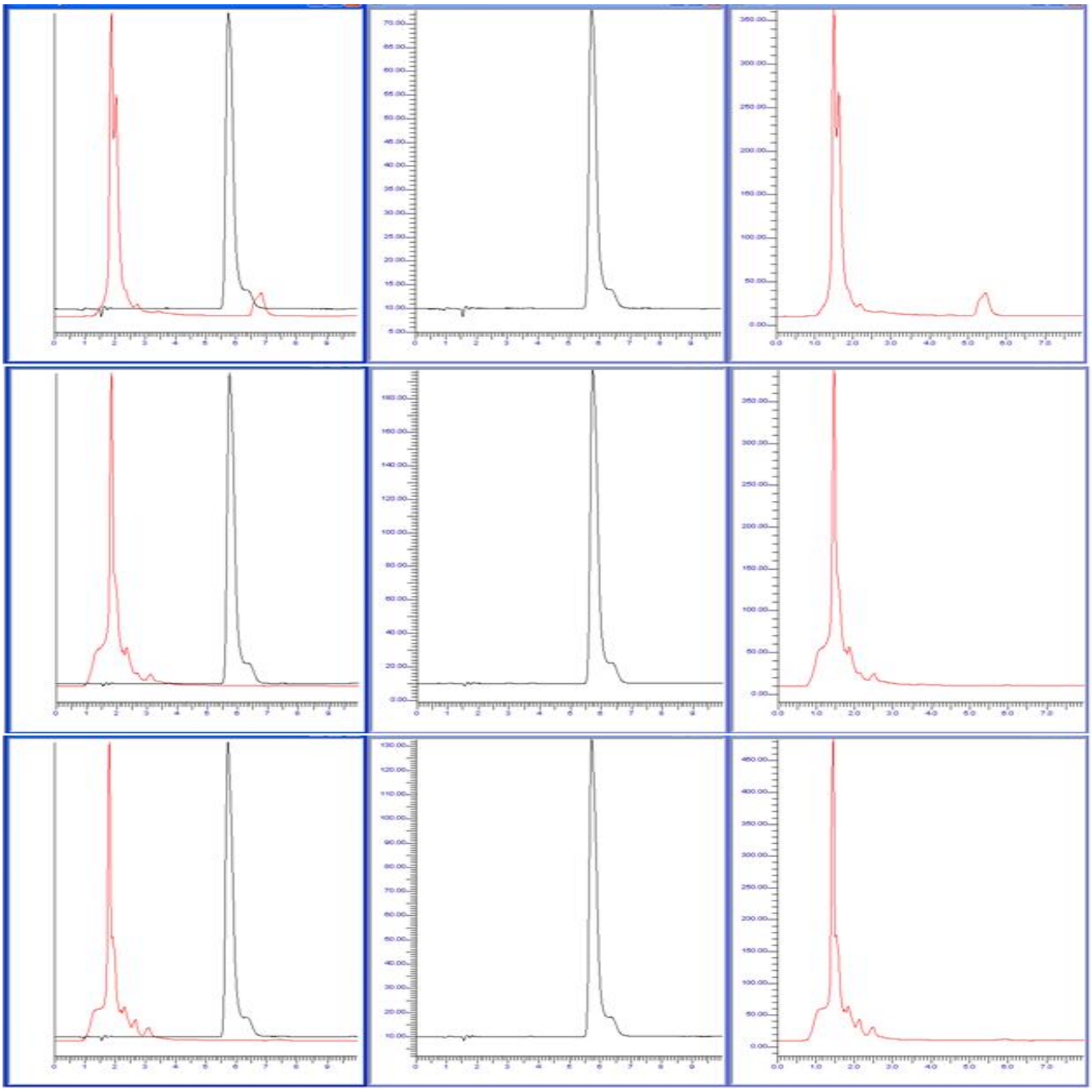


Fig. 4.33: Overview graphs of TNT removal at 40, 80 and 120 ppm for DAY8

For fig. 4.33, Red graph for sample after 187hrs(last day), Blue for standards. Upper three graphs are for 40ppm(overview graph , standard, sample after 187hrs), middle three are for 80ppm (overview graph, standard , sample after 187hrs),lower three graphs are for 120ppm(overview graph, standard , sample after 187hrs).All the sample graphs do not show TNT peak as shown in their respective standards, hence HPLC study shows degradation of TNT but the sample graph at 40ppm shows two peaks which depicts TNT is degraded in two compounds in 163 hrs.

4.5 MICROBIAL GROWTH OVER 10-DAYS OF TNT DEGRADATION TEST (USING HACH SPECTROPHOTOMETER)

At 600nm

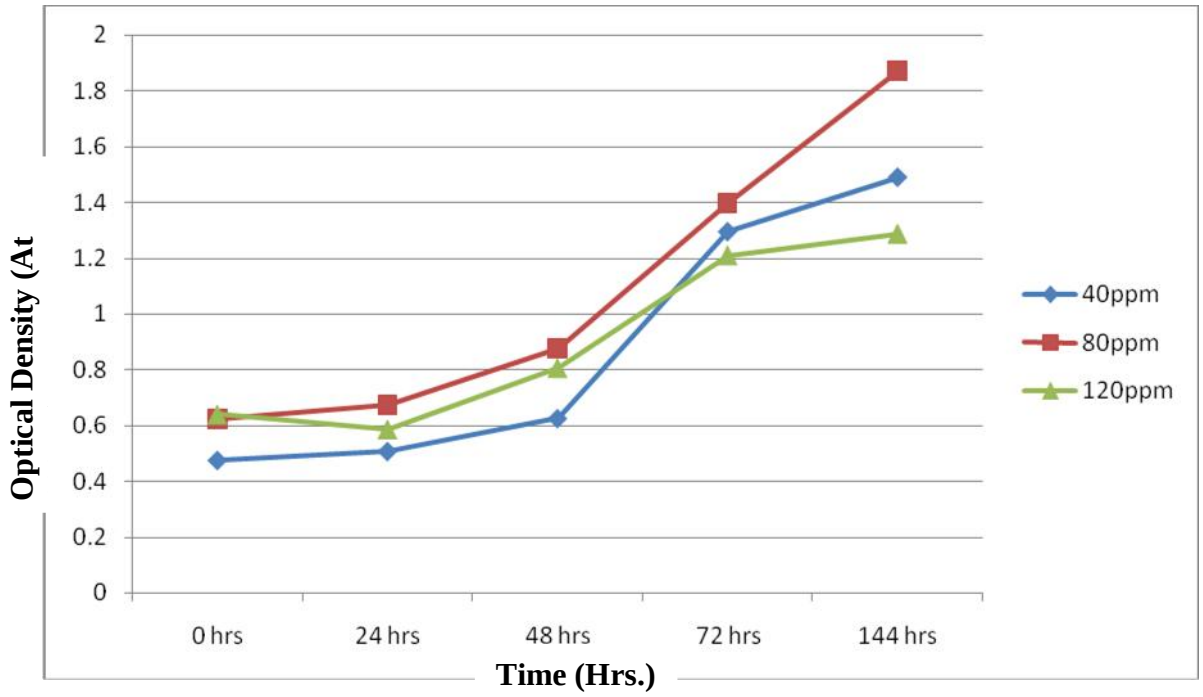


Fig. 4.34: Microbial Growth over 144hrs(7-days) of TNT degradation test at 600nm

* X-axis = Optical Density and Y-axis = No. of days

As microbial cells population is directly proportional to Optical Density because optical density value represents the amount of light that is scattered by the sample, and amount of light is scattered by the microbial population present in the sample. So an increase in OD means an increase in microbial population.[Kaiser G.E,1999]

At all three concentrations (40, 80, 120ppm),O.D increased from Day1 to Day 9,which shows growth of microbes increased from Day1 to Day9, the growth was eventually much more in 80ppm sample. O.D decreased from day9 to day 10 showing an eventual decrease in no. of microbes which is due to completion of life cycle of microbe under TNT contamination. There is a phase of approximately similar O.D from Day 4 to Day 8 in 40ppm and 80ppm showing stationed phase of microbial growth. TNT degradation study was done with the help of HPLC from Day 2 to Day 8, when O.D is increasing continuously and the result for TNT degradation was thus positive.

Time(in hrs.)	Concentration of sample contaminated with TNT(in ppm)		
	40ppm	80ppm	120ppm
0	0.474	0.622	0.64
24	0.506	0.674	0.587
48	0.624	0.875	0.807
72	1.294	1.396	1.21
144	1.489	1.872	1.287
168	1.572	2.134	1.478
192	1.764	2.467	1.588
216	1.456	2.123	1.343

Table 4.10: Optical density results for samples contaminated with TNT at 40, 80 and 120 ppm

CHAPTER 5

CONCLUSION

5. CONCLUSION

Extensive research has contributed to the recent development of treatment processes for the bioremediation of soils and waters contaminated with nitrosubstituted explosives. By elucidating the degradative pathways in both aerobic and anaerobic systems, we can determine the fate of the parent molecule and assess its effects on the environment. Further research into treating soil contaminated with various nitroaromatics is essential, since their incineration is not always a viable option, due to high cost and risk of pollution. Much has been learned about the bacterial metabolism of nitroaromatic compounds, but several fundamental aspects regarding their biosynthesis and biodegradation have yet to be explored.

Based on the study performed on Bioremediation of Nitroaromatic compounds, the following conclusion were made-

1. Microbes have the potential to degrade a highly toxic explosive (TNT) completely into other compounds as the results observed using HPLC were positive for the bioremediation.
2. These microbes can be isolated from TNT contaminated soils too. That means TNT contaminated soils have such microbes which can themselves degrade TNT in water samples contaminated with TNT.
3. Pseudomonas is quite a potential microbe(bacteria) to remediate TNT contaminated water.
4. Microbial strain(Like Pseudomonas) can clean TNT from 120ppm contaminated samples too(checked at different concentrations: 40ppm, 80ppm, 120ppm) and can sustain in it upto 10-days.

In a nut shell, In spite of using expensive, non-practical machines and services to clean toxic explosive like TNT ; microbes can be used inexpensively, isolated from TNT contaminated soil only, to remediate it. Need is to consider the technique of bioremediation with new prospects.

Future Scope :

1. Knowing which pseudomonas species is used for bioremediation of TNT in this study is a quite essential task.
2. Concentration of TNT in water samples can be increased further to observe degradation on increased concentration of TNT by microbe and to observe microbial growth pattern on increased TNT concentration.
3. Bioremediation is an economical method for treating toxic explosives and other hazardous wastes, the fate of other microbial colony for bioremediation can be tried to get even better results.

CHAPTER 6

REFERENCES

Arora P.K, Sasikala Ch. & Ramana Ch.V; Degradation of chlorinated nitroaromatic compounds; Appl Microbiol Biotechnol; 2012,March; Vol. 93; No. 6; 2265-2277

Arora P.K; Detoxification and Biotransformation of Chlorinated Nitro aromatic Compounds by Bacteria; World Congress on Biotechnology: 2011; doi:10.4172/2155-6199.1000001;

ATSDR- Agency for Toxic Substances and Disease Registry; U.S Department of Health and Human Services; Toxicological Profile for 2,4,6-Trinitrotoluene; 1995

Boopathy R; Bioremediation of tetryl-contaminated soil using sequencing batch soil slurry reactor; International Biodeterioration & Biodegradation : 2005,June; Vol55; No. 4

Boopathy R, Manning J, Montemagno C, Kulpa C; Metabolism of 2,4,6-trinitrotoluene by aPseudomonas consortium under aerobic conditions; Current Microbiology; 1994; Vol. 28; No. 3; 131-137

Cho Y.S, Lee B.U, Kahng H.Y and Oh K.H; Comparative analysis of 2,4,6-trinitrotoluene (TNT)-induced cellular responses and proteomes in Pseudomonas sp. HK-6 in two types of media; The journal of Microbiology; 2009; Vol. 47; No.2; 220-224

Christie W.W; Gas Chromatography and Lipids; P.J. Barnes & Associates (The Oily Press Ltd); 1989

Claus H, Bausinger T, Lehmler I, Perret N, Fels G, Dehner U, Preuss J & H. Konig H; Transformation of 2,4,6-trinitrotoluene (TNT) by Raoultella terrigena; Biodegradation; 2007,Feb; Vol. 18; No.1; 27-35

Clark J; High Performance Liquid Chromatography; Chemguide; 2007

Crawford Ronald L; The microbiology and treatment of nitroaromatic compounds; Current Opinion in Biotechnology: 1995; Vol 6 :329-336

Crawford Ronald L, Crawford Don L; Bioremediation: principles and applications; Cambridge University Press; 2005

CSTPR-NCR(Chemical Soil Test Procedure for the North Central Region); The North Dakota Agricultural Experiment Station- North Dakota State University; Bulletin No. 499; 1980

Crocker F.H, Indest K.J, Fredrickson H.L; Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20; Appl Microbiol Biotechnol; 2006,Nov.; Vol. 73; No.2; 274-90

Das M and Adholeya A; Role of microorganisms in Remediation of contaminated Soil; Microorganisma in environmental Management; 2012; 81-111

Davis E.P, Boopathy R and Manning J; Use of Trinitrobenzene as a Nitrogen Source by Pseudomonas vesicularis Isolated from Soil; Current Microbiology; 1997; Vol. 34; No. 3; 192-197

Diels L, Smet M.D, Hooyberghs L, and Corbisier P; Heavy Metals Bioremediation of Soil; Molecular Biotechnology; 1999; Vol. 12; No.2; 149-158

Douglas T.A, Walsh M.E, Weiss Jr C.A, McGrath C.J, Trainor T.P; Water Air Soil Pollut; Desorption and Transformation of Nitroaromatic (TNT) and Nitramine (RDX and HMX) Explosive Residues on Detonated Pure Mineral Phases; Water Air Soil Pollut; 2011,Nov.

DR 890 colorimeter ;HACH company(U.S.A);Procedures Manual; Method 8153,8146,8048,8171

Einschlag F.S.G, Carlos L and Nichela D; Degradation of Nitroaromatic Compounds by Homogeneous AOPs; Waste Water-Treatment and Reutilization; InTech ; 2011,April

Flathman P.E, Jager D.E, Exner J.H; Bioremediation Field Experience. Boca Raton; Lewis Publishers; 1994

Flores ER, Donlon B, Lettinga G, Field JA; Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge; FEMS Microbiology Reviews: 2006; Vol 20

FUNDAMENTAL PRINCIPLES OF BIOREMEDIATION(An Aid to the Development of Bioremediation Proposals); 1998, April

Fulton A, Advisor F, Tehama, Glenn, Colusa, and Counties S; Primary Plant Nutrient: Nitrogen, Phosphorus, and Potassium

Garbisu C, Alkorta I; Bioremediation: Principles and future; Journal Of Clean Technology Environmental Toxicology And Occupational Medicine; 1997; Vol. 6: Issue: 4, Pages: 351-366

Gatell X.M, Schraa G ; Engineers and microbiologists: a future together in bioremediation; Microbiologia

Madrid Spain; 1997, March; Vol. 13; No. 1; 85-7

George I, Eyers L, Stenuit B and Agathos S.N; Effect of 2,4,6-trinitrotoluene on soil bacterial communities; Journal of Industrial Microbiology and Biotechnology; 2008; Vol. 35; No. 4; 225-236

Gorontzy T, Küver J, Blotevogel KH; Microbial transformation of nitroaromatic compounds under anaerobic conditions; Journal of General Microbiology: 1993,June; Vol. 139; No. 6; 1331-1336

HACH,SIW-1; HACH Company(U.S.A), SIW-1 : Soil and Irrigation Water Manual, Model SIW-1, Kit 2496000

IRIS (Integrated Risk Information System); 2,4,6-Trinitrotoluene(TNT) (CASRN 118-96-7); U.S EPA; 2012

IRIS (Integrated Risk Information System); 2,4-/2,6-Dinitrotoluene mixture; no CASRN; U.S EPA; 2012

Jiménez J.I, Nogales J, García J.L and Díaz E; A Genomic View of the Catabolism of Aromatic Compounds in Pseudomonas; Handbook of Hydrocarbons and Lipid Microbiology; 2010; Vol. 14; No. 14; 1297-1325

Ju K.S and Parales R.E; Nitroaromatic Compounds, from Synthesis to Biodegradation; Microbiology Molecular Biology Rev. : 2010,June; Vol. 74; No.2; 250-272

Kaiser G.E; Lab4 : Enumeration of microorganisms; BIOL 230 Microbiology Lab Manual; 1999

Kim H.Y, Bennett G.N and Song H.G; Degradation of 2,4,6-trinitrotoluene by Klebsiella sp. isolated from activated sludge; Biotechnology Letters; 2002; Vol. 24; No. 23; 2023-2028

Kroger M, Schumacher M.E, Risse H & Fels G; Biological reduction of TNT as part of a combined biological-chemical procedure for mineralization; Biodegradation; 2004, Aug; Vol 15; No. 4; 241-248

Kulkarni M, Chaudhari A; Microbial remediation of nitro-aromatic compounds: an overview; Journal of Environmental Management : 2007,Oct; Vol 85; No. 2; 496-512

Lindsay S, Barnes J; High Performance Liquid Chromatography; John Wiley & Sons; 1992, Aug.; Second Edition; 0-337

Maeda T and Ogawa H.I; Microbial Degradation of 2,4,6-Trinitrotoluene: Application to Explosives Sensor; Microbial Degradation of Xenobiotic; 2012; 213-233

Martin A; Biodegradation and bioremediation; San Diego : Academic Press; 1994

Microbiology Lab Procedures; Endospore Stain; Biology 312; Western Michigan University
Montpas S, Samson J, Langlois E, Lei J, Piché Y and Chênevert R; Degradation of 2,4,6-trinitrotoluene by Serratia marcescens; Biotechnology Letters; 1997, March; Vol. 19; 291-294

NADCA(National Air Duct Cleaners Association; Understanding Microbial Contamination in HVAC systems; 2004

Nathan F; Bioremediation; 1999

National Research Council (U.S.). Committee on Intrinsic Remediation; Natural attenuation for groundwater remediation; National Academies Press; 2005

Newcombe D.A, Crawford R.L; Transformation and fate of 2,4,6-trinitrotoluene (TNT) in anaerobic bioslurry reactors under various aeration schemes: implications for the decontamination of soils; Biodegradation; 2007; Vol. 18; No.6; 741-754

Nepovíma A, Podlipnáa R, Soudeka P, Schröderb P, Vaněka T ; Effects of heavy metals and nitroaromatic compounds on horseradish glutathione S-transferase and peroxidase; Chemosphere : 2004, Nov; 57(8):1007-15

Oh B.T, Shea P.J, Drijber R.A, Vasilyeva G.K, Sarath G; TNT biotransformation and detoxification by Pseudomonas aeruginosa; Biodegradation; 2003, May; Vol 14; 309-319

Palleroni N.J, Pieper D.H and Moore E.R.B; Microbiology of Hydrocarbons- Degrading Pseudomonas; Handbook of Hydrocarbon and Lipid Microbiology; 2010; Vol. 19; 1787-1798

Perreault N.N, Manno D, Halasz A, Thiboutot S, Ampleman G and Hawari J; Aerobic biotransformation of 2,4-dinitroanisole in soil and soil Bacillus sp.; Biodegradation; 2012; Vol. 23; No. 2; 287-295

Peres CM, Agathos SN; Biodegradation of nitroaromatic pollutants: from pathways to remediation; Biotechnology Annual Review : 2000; Vol. 6: 197-220

Popesku J.T, Singh A, Alawi Y E, Ward O.P; Trinitrotoluene removal in a soil slurry and soil box systems by an oil-degrading mixed bacterial culture; World Journal of Microbiology and Biotechnology; 2006; Vol. 22; No. 10; 1075-1081

Popesku J.T, Singh A, Zhao J.S, Hawari J, Ward O.P; Metabolite production during transformation of 2,4,6-trinitrotoluene (TNT) by a mixed culture acclimated and maintained on crude oil-containing media; Appl Microbiol Biotechnol.; 2004; Vol. 65; No. 6; 739-746

Reynolds J; Serial Dilution Protocols; American Society for microbiology-Microbe Library; 2012

Rodgers J.D, Bunce N.J; Treatment methods for the remediation of nitroaromatic explosives; Water Research :2001, June; Vol. 35; No. 9; 2101-2111(11)

Singh R, Soni P, Kumar P, Purohit S and Singh A; Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria; World Journal of Microbiology and Biotechnology; 2009; Vol. 25; No.2; 269-275

Smith; Stain Protocols-BIOL 2420; 2010

Solyanikova I.P, Baskunov B.P, Baboshin M.A, Saralov A.I and Golovleva L.A; Detoxification of high concentrations of trinitrotoluene by bacteria; Applied biochemistry and microbiology; 2012, Jan; Vol. 48; No.1; 21-27

S. P. Kanekar, P. P. Kanekar, S. S. Sarnaik , N. P. Gujrathi, P. N. Shede, M. R. Kedargol , K. F. Reardon; Bioremediation of nitroexplosive wastewater by an yeast isolate Pichia sydowiorum MCM Y-3 in mixed film bioreactor; Journal of Industrial Microbiology and Biotechnology; 2009; Vol. 36; No. 2; 253-260

Sridhar Rao PN; Gram's Staining; Dept. of Microbiology, JJMMC, Davangere

StandardBase Techniques: Analytical Chemistry Laboratory Measurement; Drenthe College, The Netherlands; High Performance Liquid Chromatography

Steven D Aust; Bioremediation Monitoring; Environmental Health Perspectives: 2005; Vol. 113;

SSSTM(SOIL SURVEY STANDARD TEST METHOD); Soil Moisture content; Department of Sustainable Natural Resources.

Spain JC; Biodegradation of nitroaromatic compounds; Annu Rev Microbiology; 1995, Oct; Vol 49(1)

Sherburne L.A, Shrout J.D and Alvarez P.J.J; Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by Acetobacterium paludosum; Biodegradation; 2005; Vol. 16; No. 6; 539-547

Steubing P.M; Isolation of an Unknown Bacterium from Soil; in Tested studies for laboratory teaching; 1993; Vol. 14(C. A Goldman, Editor)

Talmage SS, Opresko DM, Maxwell CJ, Welsh CJ, Cretella FM, Reno PH, Daniel FB; Nitroaromatic munition compounds: environmental effects and screening values; Reviews of Environmental Contamination and Toxicology : 1999; 161:1-156

Tamini Y.N, Silva J.A, Yost R.S, Hue N.V; Adequate nutrient levels in soils and plants in Hawaii(general guide); CTAHR Publication AS-3; 1997,Nov.
Todar K; Todar's online textbook of bacteriology; Pseudomonas aeruginosa

Thomas H, Gerth A; Enhanced Humification of TNT Bioremediation of contaminated soil and water in practice; Use of humic Substances to remdiate Polluted Environments : From theory to Practice; 2005; Vol. 27; 353-364

Tope A.M and Jamil K; Isolation of TNT Tolerant Pseudomonas Species (Strain KA) from TNT; Indian Institute of chemical Technology, Hyderabad; Contaminated Soil-Biotransformation of TNT; 2001; Vol. 51; No.2

U.S. EXPLOSIVE ORDNANCE; A Bureau of Ordnance Publications: 1947; Vol.1

U.S EPA method 8330 B; NITROAROMATICS, NITRAMINES, AND NITRATE ESTERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC); 2006; Vol.2

U.S EPA; Technical Fact Sheet – 2,4,6-Trinitrotoluene(TNT); 2012

U.S EPA; Health and Ecological Criteria Division Office of Science and Technology – Office of Water; Drinking Water Health Advisory for 2,-Dinitrotoluene and 2,6-Dinitrotoluene; 2008

U.S EPA Drinking Water contaminants; EPA 816-F-09-0004; List of Contaminants & their Maximum Contaminant Level (MCLs); 2009

United States Environmental Protection Agency-US.EPA; EPA 542-F-01-001; Engineering Bulletin: In Situ Biodegradation Treatment; 1996,April

Vidali M; Bioremediation. An overview; Pure Appl. Chem. : 2001; Vol. 73; No. 7; 1163-1172

Ye J, Singh A and Ward O.P; Biodegradation of nitroaromatics and other nitrogen-containing xenobiotics; World Journal of Microbiology & Biotechnology: 2004; Vol. 20; No. 2; 117-135

Yin H, Wood T.K; Smets B.F; Reductive transformation of TNT by Escherichia coli: pathway description; Appl Microbiol Biotechnol; 2005; Vol. 67; No.3; 397-404

Zaripov S.A, Naumov A.V, Suvorova E.S, Garusov A.V and Naumova R.P; Initial stages of 2,4,6-Trinitrotoluene transformation by microorganisms; Microbiology: 2004; Vol. 73

Zhao J.S, Halasz A, Paquet L, Beaulieu C and Hawari J; Biodegradation of Hexahydro-1,3,5—Trinitro-1,3,5-Triazine and its Mononitroso Derivative Hexahydro-1-nitroso-3,5-Dinitro-1,3,5-triazine by Klebsiella pneumonia Strain SCZ-1 isolated from anaerobic sludge; Appl Environ Microbiol.; 2002,Nov; Vol. 69(11), 5336-41.

Zita S, Nepovim A, Taghavi S,Vangronsveld J,Vanek T, Lelie D.V; Biological Remediation of Explosives and Related Nitroaromatic Compounds; ESPR - Environ Sci & Pollut Res; 2002; Vol. 9; No. 1; 48-61

Websites :
http://www.globalsecurity.org/military/systems/munitions/explosives-nitroaromatics.htm