CHAPTER - I

1.0. INTRODUCTION

The pressures of an ever-increasing population and industrial development have led to the addition of an array of man-made chemicals into the environment, leading to tremendous deterioration in the environmental quality. Contamination of soil, air, water, and food with pollutants is one of the major problems being faced by the industrialized world today. Significant regulatory steps have been taken to eliminate or to reduce the production and/or release of these chemicals into the environment. One of the major classes of these chemicals is chlorinated compounds, most of which are toxic and hazardous. The development and application of microbial processes to decontaminate environmental media polluted with these compounds will require a better understanding of why and how microorganisms can degrade them and utilize them for their own survival as well as in cleaning of the environment.

Environmental pollution caused by the release of a wide range of compounds as a consequence of industrial progress has now assumed serious proportions. Thousands of hazardous waste sites have been generated worldwide resulting from the accumulation of xenobiotics in soil and water over the years.

1.1. Spectrum of recalcitrant xenobiotic compounds

The recalcitrant xenobiotic compounds can be grouped into the following six types (EEC, 2011):

1. Halocarbons
2. Polychlorinated biphenyls
3. Synthetic polymers
4. Alkyl benzyl sulphonate
5. Oil mixture
6. Others

The structural features of these compounds that render them resistant to microbial degradation include the following:

1. Presence of halogens in the place of hydrogen in the molecule; the carbon-halogen bond is highly stable and its cleavage requires considerable energy.
2. Substitution of -H by other groups like nitro-, sulphonate, methoxy-, amino- and carbamoyl groups
3. Cyclic structures, aromatic compounds, cycloalkanes and heterocyclic compounds are more recalcitrant than linear chain or aliphatic compounds
4. The occurrence of branched linear chains resists biodegradation.

In general, as the complexity of the structure of a xenobiotic compound is increased, the compound is rendered more resistant to biodegradation. Thus other xenobiotics are also resistant to biodegradation due to their large molecular size and insolubility in water.

1.2. Biological Hazards from xenobiotic exposure

1. Many xenobiotics like halogenated and aromatic hydrocarbons are toxic to bacteria, lower eukaryotes and even to humans. At low concentrations they may cause various skin problems and reduce reproductive potential. Ex: *Para*-dichlorobenzene, pentachlorophenol, toluene, anthracene, naphthalene etc.
2. Certain halogenated hydrocarbons have been shown to be carcinogenic. Ex: Chlorophenoxy-herbicides, 2,4,6-Trichlorophenol, Polybrominated biphenyls, Polychlorinated biphenyls, Polychlorinated dibenzo-*p*-dioxins, Polychlorinated dibenzofurans.

3. Many xenobiotics are recalcitrant and persist in the environment so that there is a buildup in their concentration with time. Ex: halocarbons, polychlorinated biphenyls, oil mixtures, synthetic polymers, alkyl benzyl sulphonates etc.
4. Other xenobiotics, including polychlorinated biphenyls, are recalcitrant and lipophilic; as a consequence, they show bioaccumulation or biomagnifications often by a factor of 10^4-10^6 (EEC, 2011).

Biomagnification occurs mainly because of the following two reasons:

1. The compounds are continuously taken up over long exposures from the environment and accumulate in the lipid deposits of body.

2. These organisms are further consumed by other organisms in a sequential manner constituting the food chain, e.g., plankton-->small fish--> large fish-->sea-eagles; the concentration of xenobiotics builds up as we move up in the food chain. In case of DDT biomagnification, a 10^5- fold increase is observed in sea-eagles as compared to the concentration present in the aqueous environment. DDT and PCB's have been found in human tissues in high but sublethal concentrations in some countries where they have been used, even without direct contact with these chemicals.

3. Their large scale production also favors their accumulation in nature.

Among various xenobiotic pollutants that have proven recalcitrant to microbial attack are halocarbons. The carbon-halogen bond is highly stable. Cleavage of this bond is an endothermic reaction requiring a substantial energy input. As a result, halocarbons are chemically and biologically very stable. Some organochlorine insecticides are also highly recalcitrant (Alexander, 1999) due to the same reason.

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use as herbicides, insecticides,

fungicides, solvents, hydraulic and heat transfer fluids, plasticizers, and intermediates for chemical synthesis (Song et al., 2000). Because of their toxicity, bioconcentration, and persistence, the ubiquitous distribution of halogenated compounds in the biosphere has caused public concern over the possible effects on the quality of life. The widespread use of these compounds in industry, agriculture, health care, and household is an important source of soil and water contamination. Other sources of contamination are accidental spills, hazardous waste disposal sites, storage tanks, or municipal landfills (Fetzner & Lingens, 1994).

1.3. Halogenated aromatic pollutants

Over the last decades, halogenated aromatic compounds have been used extensively as pesticides and herbicides. Although some of these chemicals are generated by naturally occurring biotic and abiotic processes in the oceans and atmosphere, the widespread use of halogen-based chemistry in industrial-scale chemical processing over the past 100 years has introduced many additional man-made halocarbons into the environment. The occurrence of incomplete pathways and the accumulation of toxic intermediates are important factors in the apparent recalcitrance of environmental pollutants such as chloroaromatics, haloalkanes and chloroethenes (Jain et al., 2005). The recalcitrant nature of the haloaromatic compounds is due to their low electron density at the aromatic ring, and also due to inability of the usual enzyme oxygenase to initiate the degradation of these halogenated compounds. This reaction does not proceed through an electrophilic attack but *via* a nucleophilic attack. A variety of microbial enzyme systems have been found to effect cleavage of carbon–halogen bonds, providing the means for these compounds to be utilized as carbon sources or as alternative electron acceptors (Slater et al., 1997). An understanding of the fate of natural and man-made halogenated

aromatic compounds in the environment has become more important, as many of the most toxic and environmentally persistent pollutants are in this chemical class.

Organic chemical mixtures are prevalent in waste waters from industrial and municipal sources as well as in contaminated groundwater. Common examples of chemical mixtures that often become pollutants include chlorophenols, pesticides, and wood-treating substances. Chlorinated aromatic compounds are widely used as pesticides, in industrial applications, and produced unintentionally as trace contaminants during the industrial production of chlorinated compounds and incineration of chlorine-containing waste. Brominated aromatic compounds have been used to produce flame-retardants, while fluorinated and iodinated aromatic compounds have pharmaceutical applications. The chemical inertness and hydrophobicity of these compounds has further resulted in their wide distribution in the environment (Commandeur & Parsons, 1990). Contamination by chlorophenols of surface water and groundwater is an emerging issue in environmental science and engineering. After their usage as pesticide, herbicide and disinfectants, these organic compounds subsequently enter the aquatic environment through a number of routes. Some of the chlorophenols are slightly biodegradable, while others are more persistent and mobile in the aquatic environment. Many chlorinated phenols are introduced to the environment as a contaminating compound from chemical manufacturing companies (Freiter, 1979).

1.4. Halogenated compounds persistent contaminants in marine sediments

Some of the halogenated persistent contaminants in marine sediments are halophenols, halobenzenes, PCBs, halonaphthalenes, dioxins/furans and halobisphenols etc. (Fig.1.1) They have broad applications as; pesticides, herbicides and flame retardants.

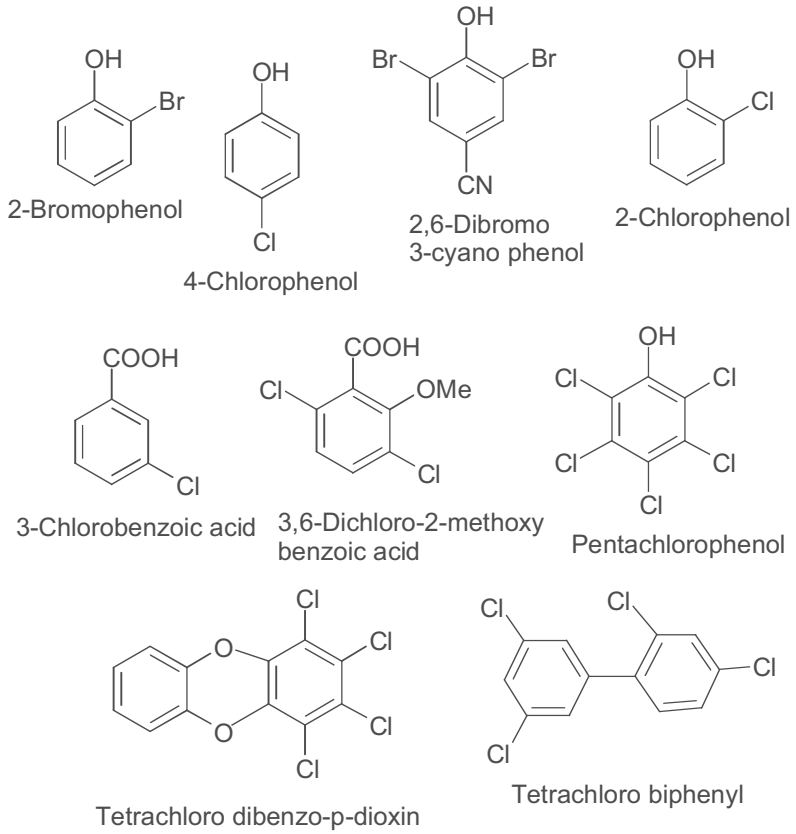


Fig. 1.1 Persistent halogenated compounds, in marine sediments (Haggblom, 1992)

1.4.1. Brominated aromatic pollutants

Brominated aromatic compounds have found use as flame retardants. Fluorinated and iodinated aromatic compounds are the components of pharmaceutical agents. The chemical inertness and hydrophobicity of many of these compounds has resulted in them becoming widely distributed in the environment; in particular accumulating in many terrestrial and aquatic organisms (Kushalatha et al., 2010). This coupled with their toxicity has given rise to concern about their fate in the environment. Some of the brominated persistent pollutants are 6-bromo-2, 4, 5-trichloro phenol, pentabromophenol, 3, 3′, 5, 5′ tetrabromobisphenol A, bromophenol and bromobenzoic acid. Brominated biphenyl ethers, are environmentally persistent class of organic pollutants, in “biosolids”. These compounds are widely used as flame retardants, and their presence suggests that the environmental consequences of land application of biosolids need further

investigation. Brominated biphenyl ethers have been frequently detected in wild-caught fish, indicating another pathway for human exposure. A diversity of natural aromatic brominated organic compounds can be found in a variety of biota, mostly aquatic species such as algae and sponges. *Aplysina aerophoba* (Fig.1.2) is an example of a marine sponge in which such compounds can be found (Ahn et al., 2003). Marine sponges are natural sources of brominated compounds. Brominated compounds may serve as a chemical defense against predators and for biofouling (Fig.1.3). Major secondary metabolites of *Aplysina aerophoba* are brominated compounds constituting 7-12% of the sponge on dry weight basis (Haggblom, 1992).

Fig.1.2. The marine sponge *Aplysina aerophoba,* a source of natural brominated compounds (Haggblom, 1992)

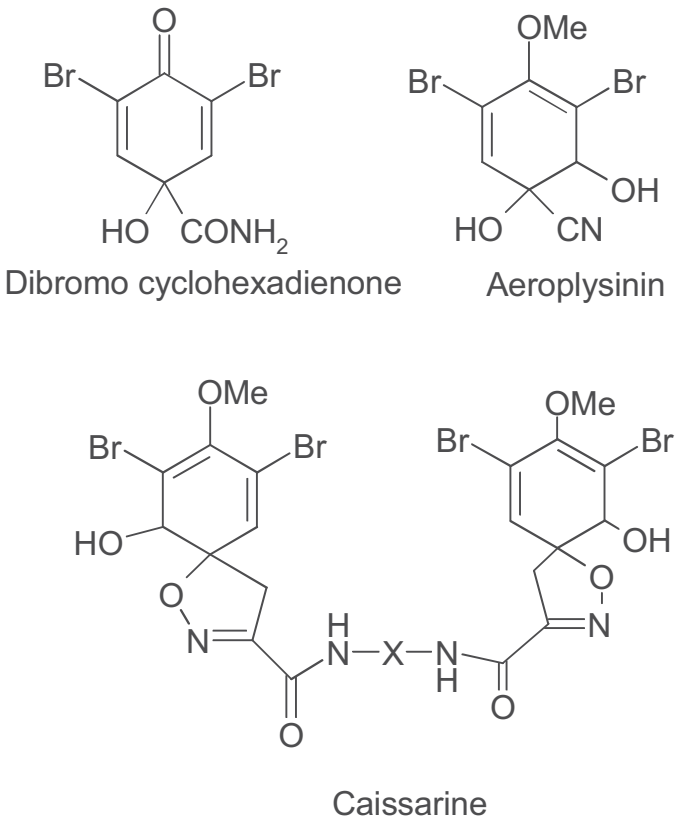


Fig.1.3. Natural aromatic brominated compounds from sponge (Puyana et al., 2003)

1.4.2. Fluorinated pollutants

Hazardous chemicals that have entered the environment due to leak out from products during use and waste treatment include chlorinated and brominated compounds from flame-retardants. The new groups of problematic micropollutants now being introduced are in the form of the fluorinated hazardous substances (Kushalatha et al., 2010). They are used in many types of products to achieve a smooth surface that is stain and water-repellent. The fluorinated chemicals can be found in certain cleaning agents, paint and varnish, wax, floor polishing agents, impregnation agents for textiles, carpets, paper, furniture and shoes, fire-extinguishing liquids and in photo paper.

1.4.3. Chlorinated aromatic pollutants

Chlorinated aromatic compounds are major environmental pollutants because they are often released in substantial quantities, are toxic and resistant to degradation, and accumulate in sediment and biota. Although some compounds are degraded only slowly by soil and aquatic microorganisms, others are metabolized relatively quickly. Some of the chlorinated aromatic compounds include chlorotoluene, chlorobenzenes chlorobenzoates, chlorophenols, 4-chlorophenylacetate and chlorophenoxyacetates. Chlorobenzenes are used extensively as solvents, fumigants, and intermediates in the production of pesticides, dyes, disinfectants, room deodorants and moth control agents (Kushalatha et al., 2010). Chlorinated phenols are used as wood preservatives, herbicides, fungicides, and general biocides are a large group of toxic xenobiotics that are serious environmental pollutants. Chlorinated derivatives of phenoxyacetates, such as Dichlorophenoxy acetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), have been released into the environment as herbicides over the past 40 years. Unlike many of the recalcitrant synthetic compounds, 2,4-D is rapidly degraded by soil microorganisms.

1.5. Applications of chlorinated aromatics

Among a large number of halogenated aromatic compounds some of the chlorinated aromatics and their applications are listed in (Table.1.1). These are widely used in pharmaceutical, photographic and chemical industries.

Chlorophenols have been found in several contaminated sites and the sites at which chlorophenols are found may increase in future. This information on the contaminated sites is important because exposure to this substance may harm us ensuing contaminant exposure from such sites.

Table.1.1 Major chloroaromatics and their applications (Bhatt et al., 2007)

Chloroaromatics	Major applications
Monochlorobenzene	Production of nitrophenol, nitroanisole, chloroaniline, phenylenediamine for the manufacture of dyes, crop protection products, pharmaceuticals and rubber chemicals.
1,2-Dichlorobenzene	Production of 1, 2-dichloro-4-nitrobenzene for the production of dyes and pesticides; production of disinfectants, room deodorants.
1,4-Dichlorobenzene	Production of disinfectants, room deodorants, moth control agent; production of insecticides; production of 2, 5-dichloronitrobenzene for the manufacture of dyes, production of polyphenylene sulfide-based plastics.
Chlorinated toluenes	Hydrolysis of cresol, solvent for dyes; precursors for dyes, pharmaceuticals, pesticides, preservatives and disinfectants.
Chlorophenols	Preparation of agricultural chemicals (herbicides etc).
Chlorophenoxy alkanoic acids	Herbicides.
Chloromethyl benzene (benzyl chloride)	Production of plasticizer, benzyl alcohol, phenyl acetic acid, quarternary ammonium salts, benzyl esters, triphenylmethane dyes, dibenzyl disulfide, benzyl phenol, benzylamines.
Dichloromethyl benzene (benzalchloride)	Production of benzaldehyde.
Trichloromethyl benzene (benzotrichloride)	Production of benzoylchloride; Production of pesticides; UV stabilizers and dyes.
Pesticides, herbicides and fungicides	For seed treatment, for treatment of diseases of plants, animals, and humans.

1.6. Chlorophenols

Chlorophenols are a group of chemicals in which chlorines (between one and five) have been added to phenol. Phenol is an aromatic compound derived from benzene, the simplest aromatic hydrocarbon, by adding a hydroxy group to a carbon to replace hydrogen. There are five basic types of chlorophenols: monochlorophenols, dichlorophenols, trichlorophenols, tetrachlorophenols, and pentachlorophenols. In all, there are 19 isomers of the chlorophenols, each containing between 1 and 5 chlorines (ATSDR, 1999). All members of the series are chlorine derivatives of phenol. They possess both acute and chronic toxicity which varies with the number of chlorines present. However, this profile is chosen on the basis of the following three criteria:

(1) Toxicity,

(2) Potential for human exposure, and

(3) Frequency of occurrence at hazardous waste sites.

Chlorophenols are a group of compounds that are used in a number of industries and products. Chlorophenols are widespread toxic compounds that are included in the U.S. Environmental Protection Agency (EPA) list of priority pollutants. EPA recommends that drinking water contain no more than 0.04 milligrams per liter (0.04 mg/L) of 2-chlorophenol (2-CP) for a lifetime exposure for an adult. For 2, 4-dichlorophenol (2,4-DCP), EPA recommends that drinking water contain no more than 0.03 mg/L for a 1-day, 10-day, or longer exposure for a child (ATSDR, 1999).

1.7. Physico-chemical properties of chlorophenols

Biological degradation of hydrocarbons in the environment is linked to a number of physical and chemical factors, including the concentration and chemical structure of contaminant, physicochemical properties of soil, the content of biogenic salts, moisture

content, oxygen and other terminal electron acceptor availability, organic compounds level, temperature and pH of soil (Hawrot, 2006). The physico-chemical properties of chlorophenols are listed in Table.1.2.

1.7.1. Molecular weight

The molecular weight of compound is expressed in g/mole. Generally, higher the molecular weight, the compound is less soluble in water. Molecular weight also affects the density of a compound.

1.7.2. Water solubility

Solubility is the measurement of the maximum concentration of a chemical that will dissolve in pure water at a specific temperature, measured in mg/L. water solubility plays a large role in a chemicals movement and distribution through soil and groundwater.

1.7.3. Polarity

Polarity is associated with charge on compounds. The polarity arises from the existence of a slightly negative charge on one part of a compound and a slightly positive charge on the other, which will cause the formation of a dipole. Water for instance, is considered a dipole because of its offset of positive and negative charges. Non polar compounds are hydrophobic, meaning they don't want to be attached to water molecules, and will be more likely to adsorb to the organic portion of a soil or they will volatilize. Polar compounds have an affinity for the liquid.

1.7.4. Specific density

The specific density is the ratio between the density of the actual component and the density of water. The density is measured as dry mass per volume [kg/m^3]. The

density of the contaminants influences the ability of the organic compounds to float on the water.

1.7.5. Octanol-water partition coefficient (K_{ow})

Octanol-water partitioning coefficient (K_{ow}) is the ratio of the solute concentration in an octanol phase to the solute concentration in the water phase of an octanol-water mixture. Octanol was chosen because it mimics the lipids found in organisms and provides a simple way to assess if a specific compound would accumulate in biological tissue or not. The *n*-octanol/water partition coefficients (K_{ow}) of chlorophenols increase with chlorination, indicating a propensity for the higher chlorophenols to bioaccumulate (US EPA, 1978).

1.7.6. Vapor pressure

The vapor pressure of a liquid is the pressure of the gas in equilibrium with respect to the liquid or solid at a given temperature. Vapor pressure represents a compounds tendency to evaporate and is essentially the solubility of an organic solvent in a gas. High vapor pressures mean that the compound is more likely to volatilize with ease out of solution.

1.7.8. Acid base properties

The behavior of weak acids and bases depends on the extent to which they exist in the neutral or charged state. This is determined by the pKa values of the chemical and the pH. The pH affects the toxicity of ionized chemicals. Generally chemicals are more toxic in their neutral unionized state. Pentachlorophenol (PCP) (pKa=4.69) and to a lesser extent, 4-chlorophenol (4-CP) (pKa=9.37) are more toxic at low pH values. The chlorophenols being weak acids at normal pH ranges they are dissociated, resulting in a higher LC_{50}.

1.7.9. Organoleptic properties

Chlorophenols generally have very low organoleptic thresholds. The taste thresholds in water for 2-CP, 2, 4-DCP, and 2, 4, 6-Trichlorophenol (2, 4, 6-TCP) are 0.1, 0.3, and 2 μg/L, respectively. Odor thresholds are 10, 40, and 300 μg/L respectively (Kozak et al., 1979). The taste and odor thresholds are quite low for chlorophenols.

1.7.10. Bioaccumulation

Bioaccumulation of chlorophenols appears to be moderate, and most bioconcentration factors (BCFs) fall roughly between 100 and 1000. The bioconcentration factor is usually a positive function of the chlorine number, and there are no obvious relationships between it and the type of organism (algae, plants, invertebrates, and fish). Once exposure is discontinued, chlorophenols clear rapidly from biota, indicating that the bioaccumulation observed in field studies is the result of long-term exposure rather than due to persistence (WHO, 1989).

1.8. Uses of chlorophenols

Chlorinated phenol compounds are solids at room temperature, except monochlorophenol which melts at 8^{o}C. They are toxic compounds but with many use (Table.1.3.). They are used as bactericides, fungicides and preservatives. Most chlorophenols are commercially applied in the form of a chlorophenol-organic solvent formulation. The salt forms of tri- and tetrachlorophenols find applications in aqueous based treatments. Generally, higher chlorinated phenols and their salt forms are used in wood preservation industry and in surface treatments for fresh-cut logs and lumber against sapstain fungi and mould (WHO, 1986).

Table.1.2. Physical and chemical properties of chlorophenols (Clayton & Clayton, 1981; Ding et al., 2008**)**

Property	**2-CP**	**2,4-DCP**	**2,4,6-TCP**	**3-CP**	**4-CP**	**2,3,4,6-TCP**	**PCP**
Molecular weight	128.56	162.0	196.5	128.56	128.56	231.0	266.34
Boiling point (°C)	175–176	210–211	246	214.0	220	164	309–310
Melting point (°C)	8.7	43–44	68	33.5	43-45	69.70	191
Density (g/cm^3)	1.24	1.38	1.49	1.245	1.2238	1.839	1.978
Vapour pressure (kPa)	0.133	0.133	0.133	0.119	0.087	_	0.16
Water solubility (mg/litre)	28000	4500	900	_	26 300	_	80
Log octanol–water partition coefficient	2.15	3.06	–	2.50	2.39	_	5.01
pka	8.49	8.09	6.21	8.85	9.18	5.62	4.35

Table.1.3. Principal uses of chlorophenols (US EPA, 1996)

Compound	Principal uses	Other uses
2-CP	Intermediate for further chlorination to 2,4-DCP, 2,4,6-TCP, and PCP	Polymer intermediate for fire-retardant varnishes; cotton fabric treatment to provide rot resistance; ingredient in coal processing
4-CP	Intermediate for higher chlorophenols; intermediate dyes, fungicides, and drugs	
2,4-DCP	Intermediate for production of 2,4-D and other herbicides; ingredient of antiseptics; starting material for higher chlorophenols	Intermediate for production of Sesone, Nitrofen, Nemacide, Genite-EM-923; raw material for polyester films; mothproofing; miticide.
2,4,5-TCP	Intermediate in manufacture of 2,4,5-T and related herbicides; fungicide, bactericide, algaecide	Germicides and ingredients of germicidal soaps
2,4,6-TCP	Precursor for higher chlorophenols; germicide, particularly for preservation of wood, leather, glue, and textiles; intermediate in preparation of insecticides and soap germicides	-
2,3,4,6-TCP, and its sodium salt	Fungicide and bactericide for wood preservation; sodium salt is sapstain inhibitor; pesticide	Preservative for latex and leather; preservative in glue for plywood
PCP	Herbicide, insecticide, fungicide, algaecide, disinfectant, and as an ingredient in antifouling paint	-

The lower chlorophenols serve as intermediates in the production of higher chlorophenols and various pesticides. 2-CP is used as a precursor in the production of higher chlorophenols (2, 4-DCP, 2, 4, 6- TCP, PCP) and dyestuffs, and as a preservative. 2, 4-DCP is used as a mothproofing agent, germicide and antiseptic, and in the production of the pesticide 2, 4-D.

Chlorophenols are used in leather tanning and finishing workshops. The monochlorophenols are used as synthetic intermediates for dyes and higher chlorinated phenols. 4-CP is a starting material for making germicides such as 2-benzyl-4-chlorophenol; it can also be converted to an analgesic like acetophenetidin. 4-CP is used as a disinfectant in homes, farms, hospitals, and as an antiseptic for root canal treatment. Their wide use has caused great damages to the environment and to living organisms. Among chlorophenolic compounds, 4-CP is a toxic and recalcitrant compound which is formed from wastewater chlorination in pulp mills from the breakdown of herbicides and the anaerobic degradation of more highly chlorinated phenols (Westerberg et al., 2000).

2, 4-DCP with formaldehyde forms methylene-bis compounds used as a mothproofing agent, an antiseptic, and a seed disinfectant. 2,4-DCP, with chloro acetic acid, forms 2,4-D, used as a selective weed-killer, systemic herbicide and defoliant, also used to increase the latex output of old rubber trees and in fruit drop control (WHO, 1989).

2,4,6-TCP is used as a bactericide and fungicide. The 2,4,5-isomer has similar applications and also can be converted into hexachlorophene or thio-bis (trichlorophenol) used as germicides in soap; into dimethyl trichlorophenyl phosphorothioate, a systemic agent effective against grubs in cattle; into 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) or 2,4,5-trichlorophenoxypropionic acid (2,4,5-TCPPA), both widely used as weed killers. 2,

4, 6-TCP is used in the production of 2, 3, 4, 6- tetrachlorophenol (2, 3, 4, 6-TCP) and PCP, and as a germicide, glue and wood preservative, and antimildew agent. They are used in industry primarily as biocides as well as preservatives for wood, glue, paint, vegetable fibers and leather (Muller & Caillard, 1986). 2,3,4,6-TCP is an insecticide and a bactericide and is used as a preservative for latex, wood, and leather.

PCP is a disinfectant, a fungicide, and the most heavily used preservative for wood. It is primarily used to protect timber from fungal rot and wood-boring insects, but the technical material may also be extensively used in cooling towers of electric plants, as additives to adhesives based on starch and vegetable and animal protein, in shingles, brick walls, concrete blocks, insulation, pipe sealant compounds, photographic solutions, textiles and in drilling mud in the petroleum industry.

In the past, PCP has been used as herbicide, insecticide, fungicide, algaecide, disinfectant, and as an ingredient in antifouling paint. Some applications were in the storage of agricultural seeds (for nonfood uses), leather, and masonry, wood preservation, cooling tower water, and rope and paper mill system. Its use has significantly declined in the recent years due to the high toxicity and its slow biodegradation (Fiege, 2000).

Lesser amounts of chlorophenols are used as wood preservatives in agricultural and domestic applications, and as additives to inhibit microbial growth in a wide array of products, such as adhesives, oils, textiles, and pharmaceutical products.

The use of 2,4,5-T has been discontinued in a number of countries. Chlorophenols with at least two chlorines either have been used directly as pesticides or converted into pesticides.

At one time, chlorophenol-treatment was widely used in agriculture, to prevent wood decay in buildings, food containers, and horticultural timbers. Recently, such

chlorophenol applications have been considerably restricted in some countries, and as a result, the quantities of non-PCP chlorophenols used in agriculture are minor (Jones, 1981).

1.9. Sources of haloaromatics

In addition to being produced commercially, small amounts of some chlorophenols, the mono- and di- chlorophenols may be produced when waste water or drinking water is disinfected with chlorine, if certain contaminants are present in the raw water. They are also produced during the bleaching of wood pulp with chlorine and during the manufacture of paper. The chlorination of phenol from dilute aqueous solutions (Barnhart & Campbell, 1972) and from sewage effluents has been demonstrated.

Direct chlorination of phenol leads to the formation of both 2-CP and 4-CP. These isomers can be separated by fractional distillation, since the difference in their boiling points is greater than 40°C. Most of the commercially used 2-CP is recovered as a byproduct of the manufacture of 4-CP by direct chlorination of phenol.

While some chlorophenols and related organohalogens occur naturally as metabolites of certain flora and fauna, these sources are thought to make a negligible contribution to overall environmental levels (Ahlborg & Thunberg, 1980). *O*-, *m*-, and *p*-Chlorophenols are synthetic organic compounds and have no known natural sources. PCP may be a product of fungal metabolism **(**Haggblom, 1992**)**.

More than 3000 organohalogen compounds have been found to occur naturally, and many thousands more surely exist. A detailed examination of one species of Okinawan acorn worm in a 1 km^2 study area showed that they released nearly 100 pounds per day of halogenated phenols, compounds previously thought to be unnatural pollutants.

Many organisms use organohalogen compounds for self-defense, either as feeding deterrents, as irritants to predators, or as natural pesticides. Marine sponges, coral, and sea hares, for example, release foul-tasting organohalogen compounds that deter fish, starfish, and other predators from eating them (Fig. 1.4). More remarkably, even humans appear to produce halogenated compounds as part of their defense against infection. The human immune system contains a peroxidase enzyme capable of carrying out halogenation reactions on fungi and bacteria, thereby killing the pathogen. Much remains to be learned, only a few hundred of the more than 500,000 known species of marine organisms have been examined — but it is already clear that organohalogen compounds are an integral part of the world around us.

Fig.1.4. Marine corals secrete organohalogen compounds that act as a feeding deterrent to starfish

1.9.1. Biogenic sources

A great variety of halogenated compounds are produced naturally, since they are part of the natural carbon cycle. Halogenated organic compounds should not be considered solely as anthropogenic contaminants. Brominated and chlorinated organohalides are produced most abundantly in nature. Iodinated compounds are found

less frequently, while fluorinated metabolites are very rare. The organisms that produce organohalides represent a diverse group and encompass both eukaryotic and prokaryotic kingdoms (Table.1.4) (Hagblom & Bossert, 2003).

Table.1.4. Natural sources of haloaromatics

Compound	Source
Chlorinated aromatics	
2,6-DCP	Tick species
3-Bromobenzoic acid	Coral
p-methoxy tetrachlorophenol	Bacidiomycetes
Brominated aromatics	
2,4-Dibromo phenol	Marine hemichordates(acorn worms)
2,6-Dibromophenol	Marine hemichordates(acorn worms)
2,4,6-Tribromophenol	Marine hemichordates(acorn worms)
Dibromo dibenzo-*p*-dioxins	Marine sponges
2,3,4,5-Tetrabromopyrrole	Marine bacterium
Iodinated aromatics	
Thyroxine	Mammals
Fluorinated aromatics	None

1.9.2. Geogenic sources

Geological origins of organohalides are generally associated with high temperature and pressure, such as found in volcanic eruption and forest fires. Chlorobenzenes are most abundant haloaromatics that have been detected in lava gas samples. Tetrachlorophenols and PCPs are generated during combustion of fresh wood. During combustion, the organohalides formed may volatalize or sorb on to fly ash resulting in their widespread airborne distribution. For example, chlorophenols most likely produced during forest fires are ubiquitously distributed in remote pristine areas and have been found in lake sediments located far from industrial sites.

1.9.3. Anthropogenic sources

The industrial production of organohalogen compounds has increased throughout last century. During this time period industrial haloorganics have been found increasingly as contaminants in the environment especially with improved methods for detection and quantification.

Chlorophenols are prepared by the alkaline hydrolysis of the appropriate chlorobenzenes or by the direct stepwise chlorination reaction of phenol or lower chlorinated phenols at a high temperature.

3-Chlorophenol (3-CP) is reported to be formed during chlorination of sewage and 4-CP is used as a denaturant for alcohol, as an antiseptic, and as a selective solvent for refining mineral. 4-CP is also formed inadvertently through chlorination of phenol-containing effluent and drinking water sources (Howard, 1990). The primary sources that have reported emissions of chlorophenols in California are hospital services, miscellaneous wood product manufacturing, and electrical services.

Chlorophenols may also be formed in the chlorination of surface waters and wastewaters. Additionally, chlorophenols have been found in waste water and sludges, sediments, ground water (due to leaching from contaminated soils), surface water (due to surface runoff or direct industrial waste discharges) and rainfall (Krumme & Boyd, 1988).

1.10. Discharge of chlorophenols into the environment

Releases of chlorophenols to the atmosphere may also occur through the incineration of chlorinated wastes. 2,4-DCP has been detected in atmospheric emissions from the combustion of municipal solid waste, hazardous waste, coal, wood, and 2,4-DCP-based herbicides (Oberg et al., 1989).

Patterns of losses to the environment appear similar in most industrialized countries. The majority of chlorophenol wastes are released in spills and leaching from treated lumber (PCP, NaPCP, NaT_4CP), and as contaminants or breakdown products of agricultural pesticides (2, 4-DCP, 2,4,5-Trichlorophenol (2,4,5-TCP). Substantial amounts of chlorophenol wastes (NaT_4CP, NaPCP) are released from sawmills, and the incineration of wood wastes.

Significant amounts of chlorophenols can be formed and subsequently released into the environment from the chlorine bleaching process in pulp and paper-mills, the chlorination of waste-water and drinking-water, and the incineration of municipal waste.

A significant amount of wastes is discharged from manufacturing sites. Losses during storage and transport are negligible. No estimates are available on the quantities of chlorophenols released as a result of the disinfection of waste-waters with chlorine, volatilization, or domestic uses of products containing these compounds.

A laboratory investigation reported that the addition of chloroperoxidase from the fungus *Culduriomyces fumugo*, hydrogen peroxide, and potassium chloride to swamp

water (pH adjusted to 3 with 100 mM phosphate) did result in the production of 2, 4, 6-TCP (Hodin et al., 1991). Chloroperoxidase could also chlorinate added phenol to form 2-CP and 4-CP. These results suggest that chloroperoxidase-mediated chlorination of natural organic matter does contribute to the levels of chlorophenols (especially 2, 4, 6-TCP) that are found in surface water. In laboratory studies, evaporation half-lives of 2-CP and 4-CP from water 0.38 cm deep were 1.35-1.6 hours and 12.8-17.4 hours, respectively (Chiou et al., 1980).

2, 4-DCP may be released to the environment in effluents from its manufacture and use as a chemical intermediate and also in small quantities from chlorination processes involving water treatment and wood pulp bleaching. It is also a degradation intermediate of the pesticide 2, 4-D and various other pesticides in soil.

1.11. Environmental transport, distribution, and transformation of chlorophenols

Chlorophenols adsorb strongly onto acidic soils, and those with a high organic content. Leaching is more significant in basic and mineral soils. Studies to date have not addressed the quantitative contribution of these processes to the transport of chlorophenols *in situ.*

Adsorption appears to play an important role in surface waters. Chlorophenols that are not degraded in the water body are incorporated into the sediments, most likely because they adsorb on sediment particulates where they persist for years. However, it is not known how important this process is for lower chlorophenols, since they should be adsorbed to a lesser extent than the tetrachlorophenols and PCP studied to date (WHO, 1989). While a large part of the chlorophenols entering natural waters are probably degraded, they are nonetheless fairly persistent and, thus, may be transported to considerable distances by water.

Although chlorophenols are principally water and soil contaminants, some atmospheric movement does occur, and low levels of PCP have been found in rain, snow, and in outdoor air. No corresponding measurements have been made for other chlorophenols, but it is highly probable that they too are transported in this manner.

1.12. Human Exposure to chlorophenols

Most of us are exposed to very low levels of chlorophenols in drinking water that has been disinfected with chlorine. Chlorophenols have been measured in chlorinated drinking water at parts per trillion concentrations. In lakes, rivers, and streams, chlorophenols were found in less than 1 percent of the water that was tested. Chlorophenols have been measured in city air at concentrations of less than a part per trillion (NOES, 1990). It has not been estimated how many people are exposed at work to the other chlorophenols. People who make chlorophenols or use them as pesticides are most likely to have high exposure to these chemicals. For example, mixtures of tetrachlorophenols are used at sawmills as wood preservatives. Skim contact while treating wood with the tetrachlorophenols is the most likely route of exposure. Another likely route of exposure is breathing air contaminated by mono- and dichlorophenols.

Chlorophenols have been detected in air samples collected from hazardous waste sites. In a study of 40 Canadian potable water treatment facilities, 4-CP, 2,4-DCP, and 2,4,6-TCP were the three halogenated phenols found most frequently in samples taken from chlorinated water supplies (Sithole & Williams, 1986). The frequency of detection ranged from 1 to 12 out of 40 samples. Mean values were <7 μg/L and the maximum values <130 μg/L. 2-CP has also been detected in treated drinking water in the Netherlands (1 μg/L) (Buikema et al., 1979).

Quantities of tetrachlorophenol range from trace to several µg/kg in carrots, potatoes (also 2, 4-DCP), turnips, cabbages, beets, and raw milk, though contamination from treated wood storage containers can elevate these levels considerably.

Recent restrictions on the agricultural use of chlorophenols have reduced this contamination. Tetrachlorophenols has been detected in poultry, but no reports of residues in other meat have been found (WHO, 1989).

1.13. Body diffusion of chlorophenols

When chlorophenols are ingested, almost all of it quickly distributes through the body. Chlorophenols can rapidly enter the body through the skin, and during breathing. The mono chlorophenols do not stay inside the body very long and are transformed to less harmful products, and they are excreted in the urine within 24 hours. The other chlorophenols (dichlorophenols, trichlorophenols, tetrachlorophenols), which also leave through the urine as less harmful chemicals, can stay in the body for several days (ATSDR, 1999)

1.14. Health effects and risk assessment

Chlorophenols are readily absorbed when administered by the oral, inhalation, ingested or dermal routes (percutaneous absorption). They accumulate mostly in the liver and kidney of experimental animals and to a lesser degree in the brain, muscle and fat. (WHO, 1986) The toxic effects of chlorophenols are directly proportional to the degree of chlorination (DNHW, 1986). Acute exposure to lesser chlorinated phenols in humans results in muscular twitching, spasms, tremors, weakness, ataxia, convulsions and collapse.

Probable routes of human exposure to chlorophenols are inhalation, ingestion, eye and dermal contact. Vapors of chlorophenols are eye and respiratory tract irritants. Direct

dermal contact is highly irritating. In general, exposure to the lower chlorinated phenols results in convulsions, whereas the higher chlorinated phenols are uncouplers of oxidative phosphorylation (Clayton & Clayton, 1994). Chlorophenols may be contaminated with polychloro-*p*-dibenzodioxins and polychlorodibenzofurans. Chlorophenols consist of many chemically distinct compounds and much of the toxicological information refers to the class as a whole. For example, the International Agency for Research on Cancer's (IARC) classification of possible human carcinogen is based on limited human evidence for chlorophenols as a class (IARC, 1987a).

There is evidence to suggest that people exposed to chlorophenols for a long time may have slightly higher incidences of cancer (ATSDR 1999). However, the people studied were exposed to other chemicals as well.

In animal studies, monochlorophenol, and 2, 4, 6-TCP, caused leukemia in rats and liver cancer in mice. The Department of Health and Human Services (DHHS) has determined that 2, 4, 6-TCP may reasonably be anticipated to be a carcinogen.

The toxic effect that chlorophenols can have on human health makes it a topic of concern. All the compounds are acutely toxic and have noticeable health effects at high concentrations. Exposure to these compounds from ground water systems is usually minimal but the exposures can be persistent over a long period of time.

The toxicity of chlorinated phenols tends to increase with their degree of chlorination and because few microorganisms can decompose them, the more highly chlorinated phenols tend to accumulate in the environment.

2-CP is poisonous; may be fatal if inhaled, swallowed or absorbed through skin. Irritating to skin and eyes; direct contact may cause burns. Rats receiving lethal doses *via* oral, subcutaneous or intra-peritoneal routes displayed similar symptoms: restlessness,

increased breathing rate and motor weakness followed by tremors, chronic convulsions, dyspnea, coma and death. Breathing 2-CP can irritate nose, throat and lungs causing coughing, wheezing and/or shortness of breath. Long term effects of 2-CP may cause damage to liver, immune system and kidney and also causes intestinal hemorrhage (Sittig, 2002).

2-CP is toxic to plants, fish and invertebrates. Chlorophenol spills have resulted in fish death **(**Fig. 1.5**)** (WHO, 1989).

Fig.1.5. Impact of 2-CP pollution on fauna

Exposure to large quantities of chlorophenol impairs algal reproduction and their primary production. Biodegradation of 2-CP in soils is likely to be reasonably rapid (days-weeks) and it binds moderately to soil/sediment particles, however for significant spills to land, leaching to groundwater may be possible (US EPA, 1980).

Excessive exposure to 2-CP may affect the eye, skin and still child birth. The Environment Agency aims to ensure that environmental exposures are too low to harm human health. Toxicity to humans, includes carcinogenicity, reproductive and developmental toxicity, neurotoxicity, and acute toxicity.

A chronic non-cancer Reference Exposure Level (REL) of 18 micrograms per cubic meter (μg/m3) is listed for 2-CP (US EPA, 1995a). The toxicological endpoints considered for chronic toxicity are the gastrointestinal system and liver. The United States Environmental Protection Agency (US EPA) estimated an oral Reference Dose as 5×10^{-3} milligrams per kilogram per day (mg/kg/d), based on reproductive effects. The US EPA estimates that consumption of this dose or less, over a lifetime, would not likely result in the occurrence of chronic, non-cancer effect.

4-CP is very hazardous in case of skin and eye contact (irritant). It is corrosive to eyes and skin. Eye contact can result in corneal damage or blindness. Skin contact with 4-CP can produce inflammation and itching, scaling, reddening, or, occasionally, blistering. Inhalation of dust containing 4-CP will cause irritation to gastro-intestinal or respiratory tract, characterized by burning, sneezing and coughing. Severe exposure can result in lung damage, choking, unconsciousness or death (ATSDR, 1999). 4-CP may also be toxic to liver, brain, gastrointestinal tract, upper respiratory tract, and central nervous system. 4-CP is toxic to aquatic organisms and may cause long term effects to the aquatic environment.

1.15. Fate of chlorophenols in the environment

Chlorophenols enter the environment while they are being manufactured or when applied as pesticides. Most of the chlorophenols released into the environment enter water and water bodies, while very little enters air. Among the chlorophenols, the mono- and di-chlorophenols that are most likely to be found in the air owing to their high volatility. Once in the air, sunlight helps to destroy these compounds and rain washes them out of the air. Chlorophenols stick to soil and to sediments at the bottom of lakes, rivers, or streams. However, low levels of chlorophenols in water, soil, or sediment are broken

down by microorganisms and are removed from the environment within a few days or weeks.

1.16. Degradation

Chlorophenol residues are removed from the environment both by biological and non-biological degradation. Laboratory studies have shown that ultraviolet radiation can break down chlorophenols in a matter of hours to days, and the shifts in the ratio of PCP to some of its breakdown products *in situ* suggest that this process is important in exposed habitats.

1.17. Natural processes for the removal of haloaromatics

Natural removal of haloaromatics from the environment can be achieved by photodecomposition and biodegradation. The biodegradation of chlorophenols has been studied in both, aerobic and anaerobic systems. Under anaerobic conditions chlorine substituents can be removed from the aromatic ring by reductive dechlorination. In this process chlorines are replaced by hydrogen, resulting in less toxic and less recalcitrant compounds. Anaerobic processes are reported to be suitable for the dechlorination of low to highly-chlorinated phenolic compounds while aerobic systems have a tendency to be more suitable for biodegrading the less halogenated phenolic compounds.

1.18. Biodegradability of chlorophenols

Halogenated compounds are not uncommon in nature and might well be important in the adaptation of microorganisms to utilize halogenated xenobiotics. A large number of bacteria and fungi from different habitats are able to degrade chlorophenols in the laboratory, sometimes eliminating tens of mg/L in a matter of hours or days. Degradation is generally slowest for the higher chlorinated phenols, and for those with chlorine in the "*meta*" position. Previous exposure to a given chlorophenol or a related compound

enables a microorganism to metabolize it immediately and/or at a faster rate, presumably by inducing the necessary enzymes. In general, anaerobic biodegradation of these compounds is much slower than aerobic metabolism. Considerable overlap appears to exist in the rates of biodegradation of the compounds in different habitats.

In treating chlorophenols, the biological method has attracted more attention than physical and chemical methods because of its relative inexpensiveness and reduced secondary pollution (Kargi & Konya, 2006). Moreover, many different types of microorganisms utilizing 4-CP as their sole carbon and energy source were isolated, such as *Arthrobacter ureafaciens* CPR706, *Arthrobacter chlorophenolicus* A6 (Backman & Jansson, 2004), and *Comamonas testosteroni* JH5 (Hollender et al., 1994).

1.19. Factors influencing biodegradabilities of the haloaromatics

The use of microbes for the degradation of the haloaromatics is limited by many factors (Utkin et al., 1995). Some of them are as follows: (1) Chemical structure of haloaromatics; (2) The microbes and (3) Environmental factors.

The less halogenated phenols usually are less toxic and undergo aerobic biodegradation more easily. It has been reported that mono-halogenated phenols and di-halogenated phenols could be degraded and mineralized to CO_2 and H_2O, after dehalogenation by pure cultures. However, the polychlorophenols are difficult to undergo biodegradation under aerobic conditions.

The degradabilities of chlorophenols by microorganisms are highly specific. The degradation of the three monochlorophenols under methanogenic cultures was investigated (Haggblom & Young, 1995). 4-CP was degraded the fastest; 3-CP removal was somewhat slower and 2-CP the slowest. However, the reverse biodegradation rates of isomers monochlorophenols were observed under sulfate-reducing conditions. Generally,

the concentration of bacterium cells can affect degradation rate of chlorophenols. Sometimes it can change degradation pathway of substrate and final products. The resting cells of *Azotobacter sp*. strain GP1, an isolate which uses 2, 4, 6-trichlorophenol (2,4,6-TCP) as carbon source for growth, degraded 2,4,6-TCP only and transform it to 2,6-Dichloroquinol at low cell density. However, the bacterium could degrade mono and dichlorophenols into CO_2 and H_2O at high cell densities completely or partially (Deng-Yu Li et al., 1991). Such an expansion of the spectrum for chlorophenols degradation *via* increasing cell concentration was earlier identified by Chu and Kirsch with a PCP degrader. Furthermore, environmental factors such as substrate concentration, medium pH, temperature and mineral salt components (Table.1.5) are important for biodegradation of chlorophenols (Utkin et al., 1995)

Table.1.5. Essential factors for microbial bioremediation

Factor	Desired conditions
Microbial population	Suitable kinds of organisms that can biodegrade all types of the contaminants
Oxygen	Enough to support aerobic biodegradation (about 2% oxygen in the gas phase or 0.4 mg/liter in the soil water)
Water	Soil moisture should be from 50–70% of the water holding capacity of the soil
Nutrients	Nitrogen, phosphorus, sulfur, and other nutrients to support good microbial growth
Temperature	Appropriate temperatures for microbial growth (0–40˚C)
pH	Optimum range is from 6.5 to 7.5

Based on the literature describing haloaromatic biodegradation pathways, several general traits might be concluded: (1) under anaerobic conditions degradation of haloaromatics includes reductive dehalogenation on the first step(s) that, nevertheless, not obligatory results in complete decomposition of initial substrate ; (2) under aerobic conditions mono- and di-, sometimes tri-halogenated phenols are converted to corresponding halocatechols. In that case dehalogenation takes place after splitting of the aromatic ring; (3) Under aerobic condition polyhalogenated phenols on the first step are subjected to oxygenative dehalogenation with the formation of (chloro)hydroxyquinol ((chloro)1,2,4-trihydroxybenzene) followed by *ortho*-cleavage of the aromatic ring with formation of maleylacetate (Haggblom, 1992).

1.20. Microbial diversity involved in degradation of haloaromatics

The focus on the biodegradation of the haloaromatics in recent years has resulted in the isolation of a number of microorganisms that can grow on these compounds as a sole carbon and energy source.

The representatives of both, Gram-positive and Gram-negative, bacteria are able to degrade haloaromatics. Among proteobacteria, chlorophenol degradation are studied most completely for strains of *Azotobacter sp.* GP1, *Burkholderia cepacia* AC1100 (Garrec et al., 2001), *Burkholderia pickettii*, *Pseudomonas cepacia*. *Arthrobacter urefaciens* CPR706 transforms *para*-substituted phenols. Some strains are able to degrade mono- and dichlorinated phenols, for instance *Pseudomonas pickettii* LD1, *Arthrobacter sp*., *Rhodococcus opacus* 1CP (Solyanikova & Golovleva, 2004), *Ralstonia eutropha* JMP134. *Rhodococcus chlorophenolicus* and *Streptomyces rochei* 303 carry out the degradation of the wide spectrum of chlorophenols, from mono- to penta-substituted ones.

Table.1.6. List of microorganisms degrading chloroaromatics (Wu Gaofeng et al., 2004)

Chloroaromatics	Microbes
2- CP	*Desulfovibrio dechloracetivorans （ATCC700921）, Alcaligenes sp., Ralstonia sp., Azotobacter sp., Pseudomonas putida Cystobacter sp., Ps.cepacia.*
3- CP	*Desulfomonile tiedjei*
4- CP	*Pseudomonas putida, Comamonas testosteroni JH5, Ps.cepacia, Rulstonie eutropha, Alcaligenes sp., Azotobacter sp, Ralstonia sp.*
2,3- DCP	*Desulfitobacterium dehalogenans JW/IU-DC1）, Desulfomomile tiedjei.*
2,4- DCP	*Desulfitobacterium dehalogenans （JW/IU-DC1）, Desulformonile tiediei, Ralstonia sp., Clostridium sp, Burkholderia cepacia, Pseudomonas pickettii (DTP0606).*
2,5- DCP	*Desulfomonile tiedjei, Desulfovibrio dechloracetivorans.*
2,6- DCP	*Desulfitobacterium dehalogenans （JW/IU-DC1）, Mycobacterium chlophenolicum. Ps.cepacia, Azotobacter sp. Ps.pickettii (DTP0606), Desulforibrio dechloracetivorans Ralstonia sp.*
3,4- DCP	*Pseudomonas pickettii (DTPO602)*
3,5-DCP	*Clostridium sp., Desulfomonile tiedjei.*
2,3,4- TCP	*Desulfovibrio dechloracetivorans （JW／IC－DC1）, Pseudomonas pickettii (DTPO602)*
2,4,6- TCP	*Ps.pickettii (DTPO602), Azotobacter sp, Desulfitobacterium dehalogenans （JW/IU-DC1）, Clostridium sp., Phanerochate chrysosporium.*
2,4,5- TCP	*Clostridium sp., Pseudomonas pickettii (DTPO602).*
T_4CP	*Pseudomonas pickettii (DTPO602), Ralstonia sp., Arthrobacter sp.*
PCP	*Flavobacterium sp. Desulfomonile tiedjei sp., Clostridium sp. Rhodococcus chlorophenolicus, Desulfitobacterium frappier (PCP-1). Desulfitobacterium dehalogenans (JW/IU-DC1), Pseudomonas cepacia (AC110).*

Some strains being lack the ability to grow on halophenols are known to be able to catalyze the incorporation of hydroxy group in *ortho*-position of mono-, di-, and trihalophenols with the formation of corresponding halocatechols (Boersma et al., 2001).

As indicated above, *Pseudomonas* sp. can dechlorinate various haloaromatics in suspension cultures. In fact, *Pseudomonas* sp. also can degrade many other aromatic compounds, such as other chlorinated aromatic compounds, nitrified aromatic compounds, aminophenols, and polycyclic aromatic hydrocarbon (Nishino et al., 2000). *Desulfomonile tiedjei,* a strictly anaerobic Gram-negative sulfate-reducing bacterium, is the best-described dechlorinating anaerobic bacterium (Table.1.6).

Among fungi, *Phanerochaete chrysosporium* that commonly involved in causing white rot is able to mineralize a variety of hazardous organic chemicals including chlorinated phenols.

1.21. Bioremediation

Use of biological organisms, such as, bacteria, fungi (usually), and plants (sometimes), to reduce or eliminate toxic pollutants from contaminated sites by degradation, assimilation or transformation in the atmosphere is called bioremediation. Degradation is the mode of elimination mostly in case of organic compounds, while heavy metals are assimilated.

It has been found for long time that microorganisms such as bacteria, fungi, actinomycete, protozoa, and others have performed the function of recycling organic matter. This natural process can be emulated, perhaps even accelerated, and used for detoxification. Microorganisms present in the environment can eventually transform most of the toxic organics, so the subject of biodegradation has been treated critically. Indigenous microbial populations, especially heterotrophic bacteria and fungi, are the

chief agents causing biodegradation. Microbes that transform specific compounds can be isolated, cultured, adapted, and enriched under laboratory conditions.

Typically, bioremediation provides an efficient and economical way to reduce environmental toxins, using indigenous or introduced microbes that naturally degrade contaminants. In the process of bioremediation, natural microbial populations are exploited to enhance the biodegradation process. This process may occur at the site of contamination (*in situ*) or in a designated area where the contaminant is removed from the original site (*ex situ*). Of particular concern is the carrying capacity of the microbial population, meaning the maximum toxic load that the population is able to withstand. Isolated microbes are capable of transforming or degrading a variety of organic and inorganic contaminants at levels beyond suspected health standards.

The major advantage of bioremediation is that it is a natural process and can be used at a much lower cost than many other treatment technologies. The first documented successful use of bioremediation on a large scale was the 1989 Exxon Valdez oil spill in Alaska (Margesin & Schinner, 1999). Other examples of microbial waste management can be found in the treatment of municipal water or waste that commonly involve natural populations of microbes to decompose suspended solids and reduce pathogenic organisms and other pollutants. Composting also involves use of microbial activity to reduce waste products to primary soil components and has been practiced since ancient times.

1.22. Sequential degradation of chlorinated compounds

Although degradation of chlorinated aliphatic and aromatic compounds has been reported both under aerobic and anaerobic conditions, sequential use of these processes always has an advantage over using them individually for complete mineralization of heavily chlorinated compounds. Aerobic bacteria that rapidly biodegrade

monochlorinated benzenes are usually unable to degrade heavily chlorinated benzene compounds (Zang & Wiegel, 1990). Therefore, it has been suggested that detoxification and complete mineralization of chlorinated wastes can be easily achieved by using a sequential treatment process, that is, anaerobic followed by aerobic treatment. For instance, the fungicide HCB (hexachlorobenzene) and polychlorinated biphenyl (PCB) undergo reductive dechlorination in anaerobic environments (Fathepure et al., 1988). The products are congeners bearing fewer chlorine substituents, which are more susceptible to biodegradation by aerobic bacteria. Thus a sequential treatment step will ensure total mineralization of these chlorinated toxic compounds.

1.23. Role cometabolism in the bioremidiation of chlorinated compounds

Cometabolism is defined as the degradation of a compound only in the presence of another organic material that serves as the primary energy source. A number of laboratory studies have demonstrated that several chlorinated hydrocarbons are transformed cometabolically by bacteria that degrade the chlorine unsubstituted aliphatic and/or aromatic hydrocarbons. Several studies on chlorinated solvents undergoing fortuitous dechlorination by microorganisms growing on other electron donors and acceptors have also been documented (Bhatt et al., 2007). A *Xanthobacter* has been reported to degrade 1, 3-DCP (dichlorophenol), and 2,3-DCP cometabolically. The phenomenon of cometabolism has been attributed to the production of broad-specificity enzymes. Both the primary substrate and the chlorinated compound compete for the same enzyme (McCarty, 1987). For a cometabolic mode, the degradation rate of the target chlorinated compound is dependent on the electron flow from the primary substrate.

1.24. Bioavailability as a limiting factor in bioremediation

Bioavailability or the amount of a substance that is physiochemically accessible to microorganisms is a key factor in the efficient biodegradation of pollutants. Chemotaxis or the directed movement of motile organisms towards or away from chemicals in the environment is an important physiological response that may contribute to effective catabolism of molecules in the environment. In addition mechanisms for the intracellular accumulation of aromatic molecules *via* various transport mechanisms are also important (Heider & Rabus, 2008).

1.25. Microbial bioremediation

Bioremediation of organic contaminants is primarily based on either microorganisms naturally present at the sites, or on microbial inoculants developed in the laboratory and introduced at the site. Certain bacterial, fungal and algal species are capable of accumulating some toxic inorganic contaminants as well.

Microorganisms can be isolated from almost any environmental conditions. Microbes are known to adapt and grow at subzero temperatures, as well as extreme high temperatures , desert conditions, in the presence of hazardous compounds or on any kind of waste stream. The main requirements are an energy source and a carbon source. Because of the adaptability of microbes and other biological systems to utilize pollutants, these can therefore be used to degrade or remediate environmental situations. Such environmental microorganisms can be subdivided in to the following groups; like aerobic, anaerobic, phototrophic, and methylotrophic bacteria, and ligninolytic fungi (Madigan, 2008).

1.25.1. Aerobic bacteria as bioremediation agents

These grow in the presence of oxygen. The aerobic bacteria that are recognized for their degradative capabilities belong to the microbial genera of *Pseudomonas, Alcaligenes*, *Sphingomonas, Rhodococcus,* and *Mycobacterium*. These microbes have often been reported to degrade pesticides, hydrocarbons, alkanes and polyaromatic compounds. Many of these bacteria use the environmental contaminants as the sole source of carbon and energy.

1.25.2. Anaerobic bacteria as bioremediation agents

This diverse group of bacteria grows in the absence of oxygen. Anaerobic bacteria are not as frequently encountered as compared to the aerobic bacteria. There is an increasing interest in anaerobic bacteria for the bioremediation of polychlorinated biphenyls (PCBs) in river sediments, and for dechlorination of the haloaromatics.

1.25.3. Methylotropic bacteria as bioremediation agents

These are subtypes among aerobic bacteria that grow by utilizing methane as source of carbon and energy. The initial enzyme in the pathway for aerobic degradation, in methylotrophs is methane monooxygenase, which has a broad substrate range and the same enzyme is active against a wide range of environmental pollutants, including the chlorinated aromatics.

1.25.4. Ligninolytic fungi as bioremediation agents

The fungi are the eukaryotic microorganisms characterized with saprophytic mode of nutrition. Among these are two groups the molds and the yeasts. The mold like the white rot fungus, *Phanaerochaete chrysosporium* has been shown to possess the ability to metabolize an extremely diverse range of persistant and toxic environmental pollutants.

The common substrates metabolized by fungi include straw, saw dust, or corn cobs in addition to a variety of synthetic organics.

1.25.5. Anoxygenic phototrophic bacteria as bioremediation agents

The phototrophic (photosynthetic) bacteria are capable of converting light energy into chemical energy and coupling this to reduction and assimilation of CO_2 into cellular materials. Most often ammonium salts are the nitrogen source in this process. Bacterial photosynthesis differs from that of the cyanobacteria and green plants in that it occurs under anaerobic conditions. Reduced sulfur compound, molecular hydrogen or organic compounds serve as electron donors for the reduction of CO_2. These physiological characteristics define the ecological niche of these organisms. Many phototrophic bacteria can fix nitrogen. They are typically found in aquatic environments, essentially anaerobic conditions where there is occasional light exposure. Therefore, phototrophic bacteria can be found in lakes, rivers, sulfur springs, oceans, and in moist or muddy soils. Purple bacteria have either bacteriochlorophyll a or b and the green sulfur bacteria have a bacteriochlorophyll c, d or e. The color of the bacteria is also influenced by the presence of the carotenoids. The purple non sulfur bacterium *Rhodospirillum* has only bacteriochlorophyll a.

1.26. Biodegradation of haloaromatic compounds

The concern about numerous aryl halide pollutants came up during the 1970s. Aryl halides include pesticides, solvents, heat transfer fluids, and waste products from many industrial processes. These compounds include halogenated anilines, benzenes, biphenyls, phenoxyacetates and phenols. Among them, chlorinated aromatic compounds are major environmental pollutants because they are often released in substantial quantities, are toxic and resistant to degradation, and may accumulate in sediments and

biota. The biological recalcitrance of halogenated compounds is related to the number, type, and position of the halogen substituents. The carbon-halogen bond is regarded as increasingly recalcitrant with increasing electronegativity of the substituent (Jain et al., 2005). Halogenated substances with one or few substituents are thought to be more readily degradable than the corresponding polyhalogenated compounds. The carbon-halogen bond can be cleaved either by enzymatic dehalogenation (catalyzed by specific enzymes) or by spontaneous chemical dehalogenation of unstable intermediates.

1.26.1. Halogen cycle in nature

There is now an increasing understanding of how natural organohalides are formed and degraded in the environment and how anthropogenic organohalides are incorporated in to a halogen cycle. A brief overview of the cycle (Fig.1.6) illustrates how biotic and abiotic processes contribute to the overall fate of halogens in the environment. These processes provide an effective means of reducing environmental load of harmfull organohalides in nature.

1.26.2. Halorespiration

Halorespiration is the use of halogenated compounds as sources of energy. The halogen serves as a terminal electron acceptor. It is also called dehalorespiration (Field, 2001) (Fig.1.7). Halorespiration is a major form of anaerobic respiration, and can play an important role in the microbial degradation. The most common substrates in the process are chlorinated aliphatics and chlorinated phenols.

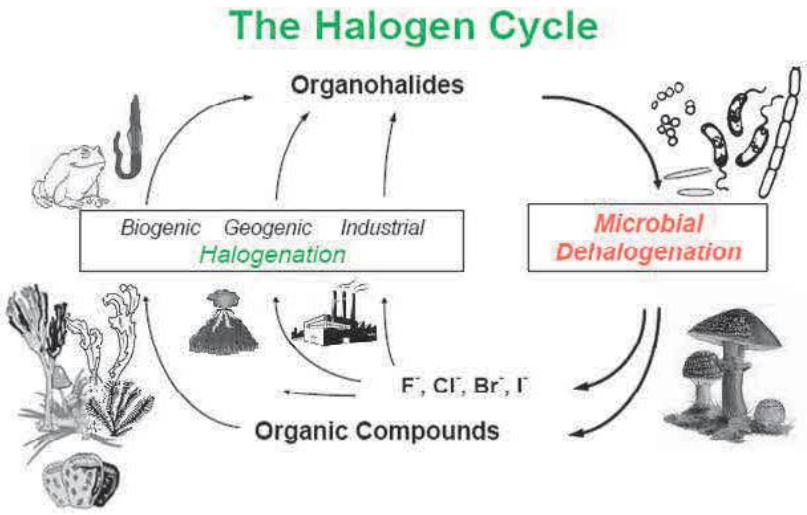


Fig.1.6. Microorganisms participate in the ultimate breakdown of halogenated organic compounds in the environment introduced from different sources through 'the halogen cycle'. (Häggblom & Bossert, 2003)

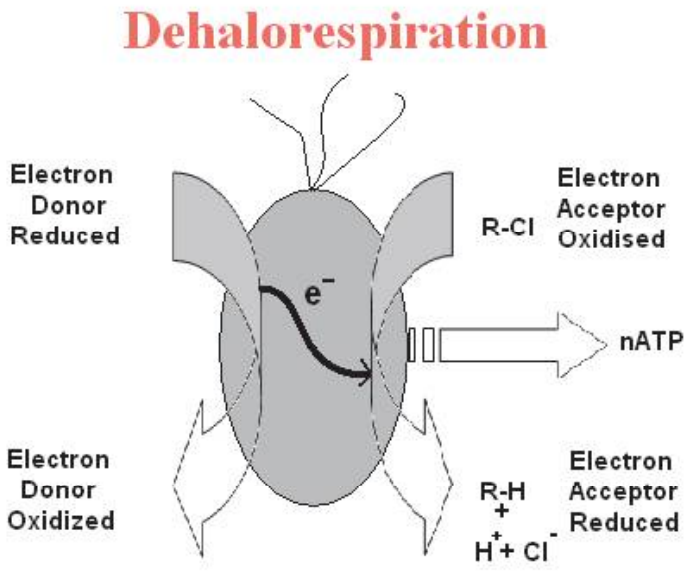


Fig.1.7. The dehalorespiration carried out by different organisms in nature (Löffler et al., 2003)

Halogenated compounds have long been doubted to be degradable in the absence of oxygen, but the isolation of hitherto unknown anaerobic hydrocarbon-degrading and reductively dehalogenating bacteria (Fig 1.8.) during the last decades provided ultimate proof for these processes in nature.

Fig.1.8. On the search of dehalogenating bacteria

1.27. Mechanisms of microbial degradation of haloaromatics

Dehalogenation is the first critical step in the bacterial degradation of many halogenated pollutants. The presence or absence of molecular oxygen plays a crucial role in determining the fate and biodegradation mechanisms of aromatic compounds (Krooneman et al., 1999). In general, under aerobic conditions, the chloroaromatics are transformed *via* oxidative dechlorination, while under anaerobic ambience they adopt reductive dechlorination pathways.

1.27.1. Oxygenolytic dehalogenation

These reactions are catalyzed by mono-oxygenases (or dioxygenases), which incorporate one (or two) atoms of molecular oxygen into the substrate (Fig.1.9). A number of proteobacterial pure culture isolates of the genera *Thauera*, *Pseudomonas*, and *Ochrobacterium* completely mineralized 3-chlorobenzoate and used this wide spread

chlorinated aromatic compound as a sole source of carbon and energy under denitrifying conditions (Song et al., 2000). No pure cultures, however, have so far been obtained that are able to oxidatively dehalogenate haloorganic compounds under methanogenic, sulfidogenic, or Fe(III)- and Mn(IV)-reducing conditions, although complete mineralization of a variety of organohalides under these conditions has been repeatedly observed for mixed cultures (Smidt & de Vos, 2004).

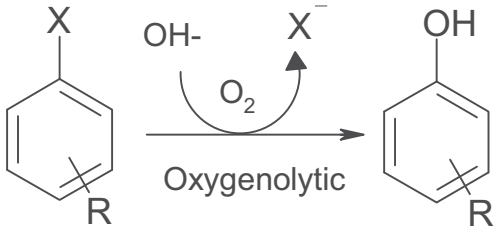


Fig.1.9. Representative biodegradation pathway for oxygenolytic dehalogenation

1.27.2. Hydrolytic dehalogenation

Hydrolytic dehalogenation represents a substitution reaction in which a hydroxyl group replaces a halogen on an organic molecule (Fig 1.10). In general, the anaerobic hydrolytic removal of halogen substituents from homocyclic aromatic compounds is rare (Kuhn & Suflita, 1989a), but has been observed under aerobic conditions.

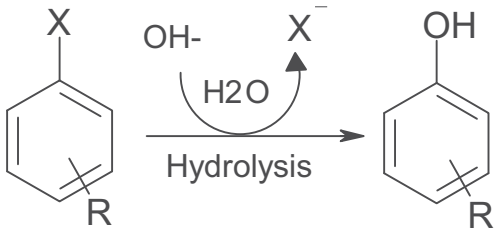


Fig.1.10. Representative pathway for reductive hydrolytic dehalogenation

Also, the enzymes involved have been shown to be active in reduced media, and some were inhibited by oxygen (Marks et al., 1984). Early work with 4-halobenzoic acids indicated a wide range of bacteria could hydrolyze the arene carbon-halogen bond

(Castro, 2003). These include *Micrococcus* sp., *Nocardia* sp., *Arthrobacter* sp. and *Pseudomonas* sp. particularly striking is the hydrolysis of 4-fluorobenzoic acid. Studies with ^{18}O water and oxygen demonstrated the hydroxyl oxygen emanated from water. The hydrolysis of halobenzoates also can occur anaerobically under denitrifying conditions, and suggests the capacity may be widespread in the environment due to the broad presence of denitrifying proteobacteria. Halophenols may also undergo an apparent hydrolysis of the C-Cl bond, but the process here is likely to be an oxygen (or hydrogen peroxide) reaction. The hydrogenolysis of aryl halides to arenes is conducted by a wide range of organisms under anaerobic conditions. The early conversions were observed with organisms from sludge and lake sediments and demonstrated the reduction of halobenzoic acids.

1.27.3. Reductive dehalogenation

Reductive dehalogenation reaction is a two-electron transfer reaction which involves the release of the halogen as a halogenide ion and its replacement by hydrogen (Fig.1.11). Reductive dehalogenation is the only known biodegradation mechanism for certain significant pollutants, including the highly chlorinated biphenyls, perchloroethene and chlorobenzenes (Sun et al., 2000).

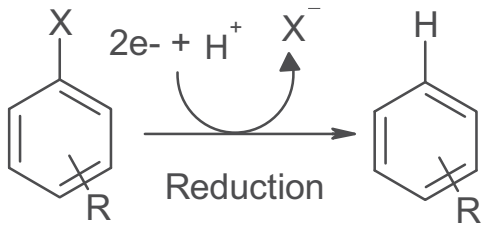


Fig.1.11. Representative pathway for reductive dehalogenation

Reductive dehalogenation usually makes xenobiotic compounds less toxic and more readily degradable by aerobes. Since ground waters and sediment

microenvironments are frequently oxygen-limited and accumulate pollutants, reductive dehalogenation can be a key initial step in achieving the biodegradation of chlorinated compounds in these environments.

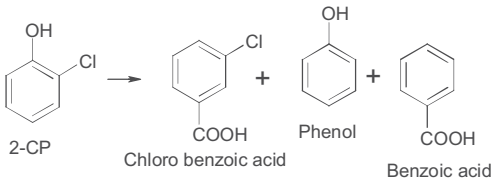
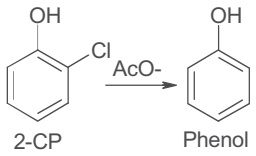
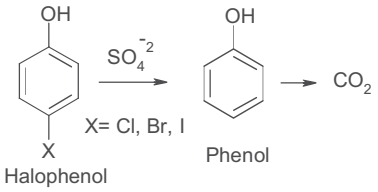
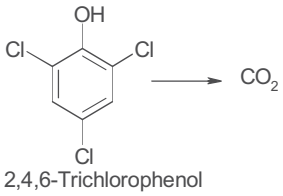
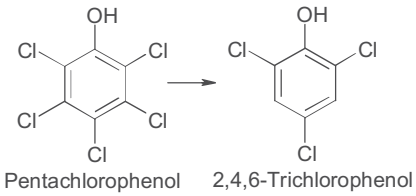
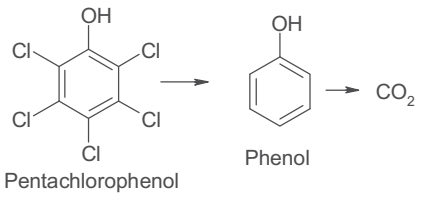
Many classes of halogenated aromatic compounds have been shown to be degraded by reductive dehalogenation processes. Evidence for the involvement of microorganisms in aryl or aromatic reductive dehalogenation reactions include: (1) the specificity of the reductive reaction; (2) characteristic lag periods required before significant dehalogenation ; (3) the absence of activity in autoclaved controls; and (4) the isolation of aryl dehalogenating bacteria. Reductive dehalogenation is rare in well-aerated environments. The dehalogenation of monochlorophenols and monochlorobenzoates under four anaerobic enrichment conditions: methanogenic, nitrate-reducing, sulfate-reducing, and bromoethane sulfonic acid (BESA)-amended has been investigated (Genthner et al., 1989a). BESA is a potent inhibitor of methanogenesis and was used to promote reductive dechlorination as a terminal electron process. The information about degradation of halophenols under different conditions is given (Table.1.7). Aquatic sediments were collected used as inocula from a salinity gradient that included both freshwater and estuarine environments and varying degrees of exposure to industrial effluents. Degradation was observed least often in enrichments with nitrate or sulfate, and most often when amended with 1 mM BESA. The sulfate-reducing bacteria did not participate in dehalogenation of 2,4-dibromophenol (DBP), a naturally occurring halogenated organic compound in some marine sediment, but did appear to degrade phenol, a metabolic product of DBP dehalogenation (King, 1988). The specificity of dehalogenation also is dependent on the position of halogens on the aromatic ring within a class of compounds. For example, chlorinated benzoates are generally more readily dehalogenated at the *meta* position, followed by the *ortho* and *para* positions (Suflita et

al., 1982; Genthner et al., 1989a). Hydroxy, alkoxy, and nitrogen-substituted aromatic compounds generally are dehalogenated faster at *ortho* and *para* halogens (Kuhn & Suflita, 1989a), however, the order of degradability of monochlorophenols was shown to be in the order of chlorination in *meta* > *ortho* > *para* substitutions (Genthner et al., 1989b). Three groups of acclimated microorganisms can act in concert to completely dehalogenate PCP to form phenol (Mikesell & Boyd, 1986). Each type of microorganism acclimated to one of three monochlorophenol isomers, transformed PCP by removal of halogens from the same relative ring positions at which they dehalogenated the monochlorophenol substrates. The 2-CP adapted cells dehalogenated PCP at the two *ortho* positions as well as from the *para* position. Similarly, 4-CP adapted cells cleaved the *para* chlorine of PCP in addition to the two *ortho* substituents. In contrast, the 3- CP adapted cells exclusively dehalogenated the *meta* position.

Other studies of PCP degradation have shown accumulation of tri- and tetrachlorophenol intermediates, which indicates that higher halogenated phenols tend to be more readily dehalogenated than their lesser halogenated congeners.

Reductive dehalogenation may require the induction of enzymes responsible for dehalogenation. The reductive dehalogenation of 3- chlorobenzoate using cell-free extracts of an anaerobic bacterium was established (DeWeerd & Suflita, 1990). The extracts exhibited the same substrate specificity as whole cells. Rapid dehalogenation activity was found only in extracts of cells cultured in the presence of the halogenated molecule, indicating that the enzymes responsible required induction. Dehalogenation was inhibited by sulfite, thiosulfate, and sulfide.

Table.1.7. Microbial reduction of halophenols (Castro, 2003)

Reaction path	Organism
	Anaerobic sediment
	2-CP-1 *Desulfovibrio* *Dechloracetivorans sp.*Nov
	Sulfidogenic anaerobic culture
	*Desulfomonile tiedjei* strain DCB-1
	*Alkaligenes eutrophus*
	Anaerobic mixed culture

Acclimation periods prior to detectable dehalogenation of halogenated benzoates in anaerobic lake sediments ranged from 3 weeks to 6 months. PCBs commonly thought to be resistant to biodegradative processes, have also been shown to be susceptible to

degradation by reductive dehalogenation (Brown et al., 1987). The dehalogenated products formed were less toxic than the original PCB congeners and may possible be more susceptible to oxidative biodegradation by aerobic bacteria.

Dechlorination of PCP in *Sphingomonas chlorophenollea* uses three different mechanisms (Fig.1.12).

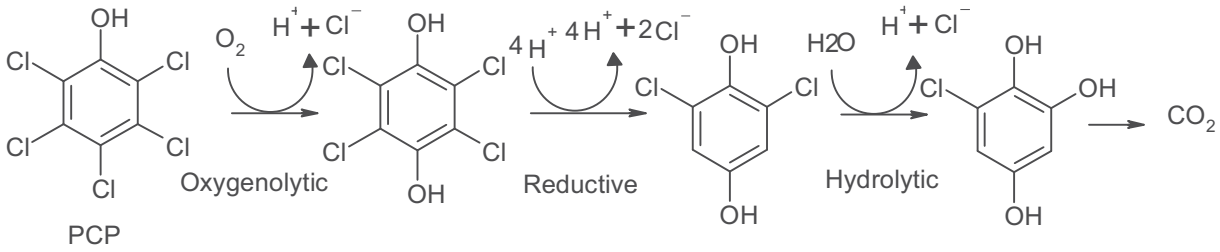


Fig.1.12. Different biodegradation mechanisms for pentachlorophenol

First dechlorination is catalyzed by a monooxygenase, next two dechlorinations are reductive and last dechlorination is hydrolytic (Steiert & Crawford, 1985).

1.28. Anaerobic biodegradation

Anaerobiosis usually occurs in any habitat where oxygen consumption exceeds its supply and is a common phenomenon in many natural environments. Examples include flooded soils, sediments, landfills, lagoons, anaerobic fresh and ocean waters, and some ground waters.

Within anoxic ecosystems, the availability of electron donors and acceptors play a crucial role in influencing the microbial activity and diversity (Berry et al., 1987). Presumably as a result of the ubiquitous nature of organic matter, organic carbon predominates as the electron donor in many anoxic environments and is required by many anaerobic microorganisms for their energy-yielding, oxidation-reduction reactions. In many anoxic ecosystems, food chains or syntrophic associations are necessary for anaerobic microorganisms to completely mineralize an organic substrate. For instance, it

is now believed that three major groups of microorganisms are essential for complete mineralization of organic carbon to CO_2 and CH_4 in anoxic dark sites and low in electron acceptors other than CO_2. These three groups of microorganisms are the fermenters, the proton reducers, and the methanogens.

Public concern about environmental contamination, particularly by halogenated industrial products, has led to extensive investigations of the fates of such compounds in anaerobic environments. Many studies have shown that reductive dehalogenation of aromatic as well as aliphatic compounds occurs fairly rapidly in anaerobic sediments (Wiegel & Wu, 2000), and a large number of pure cultures of anaerobes that carry out reductive dehalogenations have been isolated (Holliger et al., 2003).

A variety of anaerobic microbes have evolved that degrade organohalides by different mechanisms:

1. Organohalide as alternate electron acceptor; dehalogenate compound as a means of energy production, anaerobic “dehalorespiration”.

2. Organohalide as carbon substrate; dehalogenate compound to get at carbon-backbone and use it as a food source.

3. Fortuitous dehalogenation.

Anaerobic reductive dehalogenation is only one of the mechanisms available to remove halogens from organic compounds. Other anaerobic dehalogenation processes are identified (Kuhn & Suflita, 1989a). The reactions are classified according to the type of compound undergoing dehalogenation, i.e., aromatic or nonaromatic.

Two mechanisms of dehalogenation for aromatic compounds under anaerobic conditions have been observed: reduction and hydrolysis.

1.29. Aerobic biodegradation

Naturally occurring biological processes can significantly enhance the rate of organic mass removal from contaminated water aquifers. Biodegradation of chlorophenol is a reaction where the contaminant, e.g. 2-CP is transformed to CO_2 and water. Biologically mediated degradation reactions are oxidation/reduction (redox) reactions, involving the transfer of electrons from the organic contaminant compound to an electron acceptor. Oxygen is the electron acceptor for aerobic metabolism.

The aerobic degradation of halogenated aromatic compounds usually involves the following steps:

(i) Addition of -OH group by a di-oxygenase to yield chlorinated catechols.

(ii) Cleavage of the ring by *ortho* or *meta* cleavage

(iii) Elimination of the halogen from the aromatic ring, and finally,

(iv) Degradation of the aromatic hydrocarbon (non-halogenated) so produced. In case of phenols (which already have one -OH group), the step one reaction is catalysed by a hydroxylase, which adds another -OH group to yield the catechols (Solyanikova & Golovleva, 2004).

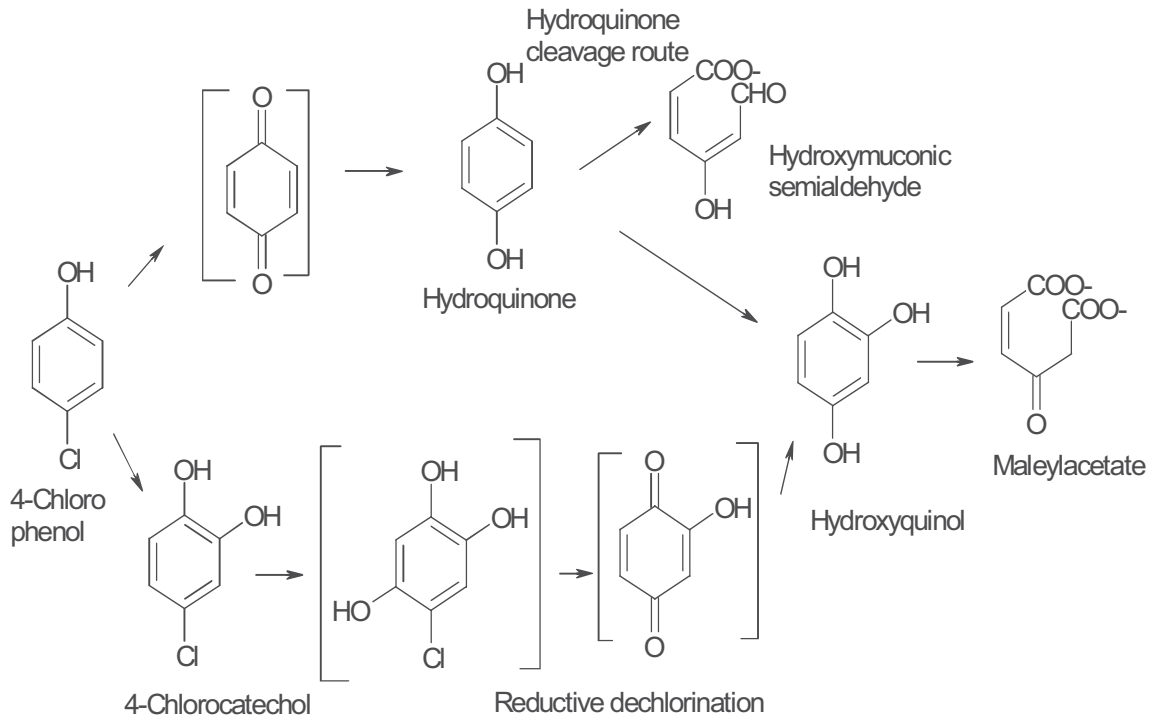


Fig.1.13. Proposed pathway for the 4-CP degradation in *Arthrobacter chlorophenolicus* A6 (Nordin et al., 2005)

It has been reported that 4-CP can be partially or completely degraded aerobically by several bacteria, including *Pseudomonas*, *Alcaligenes*, *Rhodococcus*, etc. The degradation of PCP by *Phanerochaete* spp. has been studied (Bhatt et al., 2007).

1.30. Photobiodegradation

Utilization of light energy by organisms for growth and survival is called phototrophy. The function of the anoxygenic photosynthetic apparatus is the transformation of light energy into an electrochemical gradient of protons across the photosynthetic membrane, which can be used for ATP production, active transport, motility, and other energy-consuming processes. Aromatic compounds, whether from natural or synthetic sources are ubiquitous in most ecosystems. Purple non sulphur photosynthetic bacteria are capable of degrading a wide variety of structurally diverse aromatic compounds anaerobically in the presence of light (Kushalatha et al., 2010).

Among them *Rhodopseudomonas palustris* is perhaps the most nutritionally versatile. Members of photosynthetic anoxygenic bacteria include purple sulfur bacteria and green and purple nonsulfur bacteria. Anaerobic soil environments would provide favorable conditions for the proliferation of these bacteria. In addition to cyanobacteria, these may contribute to the productivity through carbon fixation.

1.30.1. Phototrophic bacteria and halogenated aromatic compounds

Although the degradation of aromatic compounds by phototrophic bacteria has been known of for a long time, it is only recently that phototrophic mineralization of chloroaromatics has gained some attention. A phototrophic enrichment culture using acetate as carbon source partially dechlorinated 2,3,5,6-tetrachlorobiphenyl in the presence of light (Montgomery & Vogel, 1992). *Ortho* chlorines were removed preferentially. Two *R. palustris* strains, WS17 and DCP3, as well as the non-classified phototrophic bacterium H45-2, are able to photometabolize 3-chlorobenzoate when grown with benzoate and forming stoichiometric amounts of chloride (Kamal & Wyndham, 1990). In contrast to strains WS17 and H45-2, strain DCP3 is the only photoheterotroph capable of using 3-chlorobenzoate for growth independently of the presence of benzoate (Van der Woude et al., 1994). In addition, *R. palustris* DCP3, 3-chlorobenzoate-grown cells can also use 2- chloro-, 4-chloro-, and 3,5-dichlorobenzoate(Kushalatha et al., 2010).

Among all the halogenated aromatic pollutants the photobiodegradation of chlorobenzoates is well studied. However the reports on the photobiodegradation of other halogenated aromatic compounds are scanty. The degradation of chlorobenzoates by soil microorganisms have been reported by many researchers (Chaudhry & Chapalamadugu, 1991) and also the photobiodegradation of chlorobenzoates have been extensively studied

in the phototrophic bacteria like *R. palustris*. During the metabolic study of 3-chlorobenzoate by a mixed phototrophic culture in the presence of benzoate, *R. palustris* WS17 was the dominant phototroph (Kamal &Wyndham, 1990). In another degradation study, 3-chlorobenzoate was shown to be metabolized by *R. palustris* DCP3 under low-oxygen and phototropic conditions (Krooneman et al., 1999).

1.30.2. Dehalogenation by phototrophic bacteria

A few examples that phototrophic bacteria, including *Rhodospirillum* and *Rhodopseudomonas* sp., can grow phototrophically under anaerobic conditions using halocarboxylic acids has been reported (Thakur, 2007). Photosynthetic organism with dechlorination of halogenated aromatic compounds was first observed by Kamal and Wyndham, 1990. In all observed photosynthetic bacterial strains the catabolism of halogenated substrate required an initial dehalogenation step (Sikdar & Irvine, 1997). Dehalogenation follows a reductive pathway in *R.palustris*. Remarkably, most laboratory strains of *R. palustris* do not degrade chlorobenzoates, including *R. palustris* strain CGA009, for which the complete genome has recently been elucidated. However, these strains develop this activity after extended incubation in the presence of the organohalides, indicating that only a limited number of mutations are needed to acquire this function and providing a defined basis for studying this adaptation (Smidt & de Vos, 2004).

1.31. Enzymes of haloaromatic degradation

Microbial growth on halogenated substrates requires the production of catabolic enzymes that cleave carbon-halogen bonds. Such enzymes are commonly called dehalogenases. The substrate range of dehalogenases determines to a large extent the range of synthetic halogenated organic compounds that can be used as a growth substrate

by microbial cultures (Janssen et al., 1994). The lack of a suitable set of catabolic enzymes, rather than thermodynamics is responsible for the recalcitrance of many xenobiotic halogenated compounds. In addition, the kinetics of growth is influenced by the kinetics of initial catabolic enzymes, which are often dehalogenases. This critical role of dehalogenases has made them an important target for microbiological research on the bacterial degradation of xenobiotic compounds.

Microorganisms have evolved a diverse potential to transform and degrade halogenated organic compounds. They produce an array of enzymes that bring about dehalogenation and degradation of both aliphatic and chloroaromatic compounds. The reactions catalyzed by such enzymes can be broadly classified as follows (Table.1.8)

Table.1.8. Enzymes in haloaromatics degradation

Reaction	Enzymes
Oxidative dehalogenation	Mono- or dioxygenases
Substitutive dehalogenation	Halidohydrolases
Reductive dehalogenation	Dehydrohalogenases

Oxygenolytic dehalogenation of haloaromatic compounds is either catalyzed by specific oxygenases or occurs during a conversion, catalyzed by the enzyme for the corresponding unsubstituted substrate.

1.31.1. Hydrolytic dehalogenases

Hydrolytic dehalogenation of several heterocylic, aromatic, and aliphatic compounds has been reported. These reactions are catalyzed by halidohydrolases (Bhat et al., 2007). Hydrolytic dehalogenases were first detected in organisms growing on

chloroacetic acid, but they are also involved in growth on other 2-halocarboxylic acids. The hydrolytic dehalogenases catalyze a nucleophilic displacement reaction with water as the sole cosubstrate. No evidence indicates the involvement of cofactors or metal ions in their catalytic activity. The hydrolytic dehalogenases that belong to the hydrolase fold enzymes.

1.31.2. Phenol hydroxylases

Hydroxylation reactions being usual on the first step of aerobic microbial degradation of various aromatic compounds including chlorophenols are catalyzed by nonheme Fe containing oxygenases (Fetzner & Lingens, 1994). Phenol hydroxylases are monooxygenases and catalyze the incorporation of -OH group in the position *ortho* to hydroxy-group of phenol. Monooxygenases catalyze the incorporation of one oxygen atom in the aromatic ring; the second one is reducing to H_2O. The structure of phenol hydroxylases, their physico-chemical and catalytic properties are widely described in the literature. As an overview, all phenol hydroxylases can be divided into two groups: the one component and multi component enzymes. The former group includes monomers, homodimers and homotetramers with molecular mass of subunit 57–73 kDa. These enzymes contain FAD, need NADH or NADPH for the activity and possess wide substrate specificity. *P*-Chloro-mercuribenzoate inactivates these phenol hydroxylases by modification of cysteine residue of substrate-binding site. Multicomponent phenol hydroxylases consist of reductase, low molecular mass activator and heteromultimer ($\alpha\beta\gamma$) oxygenase component (Nordlund, 1990). Multicomponent monooxygenases differ from known multicomponents dioxygenases by the presence of protein-activator. At optimal concentration it prompts hydroxylation, while a high concentration inhibits it (Schirmer & Hillen, 1998). The reductase component is monomer with molecular mass 39 kDa, contains FAD and ferredoxin similar [2Fe-2S] cluster being of the same function

as reductase component of dioxygenases. The mechanism of phenol hydroxylase is analogous to one of other flavin containing aromatic hydroxylases and is well described in the literature (Massey, 1994). Hydroxylation of (halo) phenols by phenol hydroxylase results in formation of (halo) catechols–ones of central intermediates forming during conversion of various aromatic compounds. Few pathways of (chloro) catechol conversion are known which are widely distributed among prokaryotes (Solyanikova & Golovleva, 2004).

1.31.3. Hydroxyquinol 1, 2-dioxygenase

First step of aerobic degradation of chlorophenols bearing three–four substituents as a rule starts with the hydroxylation in *para*-position giving (chloro) quinol. The second hydroxylation leads to the formation of (chloro) - hydroxyquinol. Such pathway was found in *Rhodococci chlorophenolicus PCP-1, Rhodococcus sp. CP-2, Mycobacterium fortuitum CG-2, Flavobacterium sp.* degrading pentachlorophenols (Xun et al., 1992). *Azotobacter sp*. and *Pseudomonas pickettii* DTP0602, degrading 2,4,6-trichlorophenol, *Streptomyces rhochei 303* utilizing the whole spectrum of polychlorinated phenols and *Burkholderia cepacia* AC1100 degrading 2,4,5-T via formation of 2,4,5-TCP and further 5-chlorohydroxyquinol (Solyanikova & Golovleva, 2004). The key enzyme for opening of aromatic ring of (chloro) hydroxyquinol in this pathway is (chloro) hydroxyquinol 1,2-dioxygenase. It catalyzes splitting of aromatic ring of (chloro) hydroxyquinol with formation of maleylacetate, the common intermediate of modified *ortho*-pathways and hydroxyquinol pathway. (chloro) hydroxyquinol 1,2-dioxygenases were isolated and characterized from several microbes (Zaborina et al., 1995). These enzymes were homologous on the base of amino acid sequences, have the similarity in subunit composition and molecular mass. Nevertheless, enzymes from different microorganisms differ by their substrate specificity.

1.31.4. Chlorocatechol 1, 2-dioxygenase

Microbial degradation of chloro-substituted aromatics such as chlorobenzoates, chlorophenols, chlorobenzenes or chlorophenoxyacetates has been described *via* chlorocatechols as central intermediates, and a catechol- 1, 2-dioxygenase with relaxed substrate specificity and high activity against chlorocatechols was identified as a key activity in a variety of those organisms (Andrea et al., 1990). Mono- and dihalophenols are mainly converted *via* modified *ortho*-pathway, its 3-chloro- or 4-chlorocatechol branches depending on what catechol is formed as the central intermediate. At first, this pathway was described for *Pseudomonas* sp. B13 and *Pseudomonas putida* (Schlomann, 1994) degrading 3-chlorobenzoate. Chlorocatechol 1, 2-dioxygenase catalyzes the formation of 2- chloromuconate from 3-chlorocatechol.

Enzymes of this branch of the modified *ortho*-pathway of Gram-negative bacteria are under investigation for a long time and at present a lot of information about properties involved in it is available. Chlorocatechol 1, 2-dioxygenase are isolated from a number of Gram-negative bacteria and are shown to be homodimers with molecular mass 57.5–64.0 kDa (α_2Fe or α_2FeMn) with Fe^{3+} in the active center. They possess wider substrate specificity in comparison to catechol 1, 2-dioxygenase participating in an ordinary *ortho*-pathway and have the highest conversion rate with methyl substituted catechols. As the rule, the specificity constant of chlorocatechol 1, 2-dioxygenase towards chlorocatechol which is the intermediate of growth substrate conversion is higher than to other catechols (Van der meer et al., 1991).

1.31.5. Reductive dehalogenases

Reductive dehalogenases are the key catalysts in the respiratory chain of halorespiring microorganisms. Although different classes of dehalogenases from aerobic

bacteria such as haloalkane-, haloacid-, and 4-chlorobenzoyl-CoA dehalogenases have been studied extensively at the molecular level, including the elucidation of several crystal structures, detailed biochemical and molecular knowledge on dehalogenating enzymes from anaerobic microorganisms has only started to accumulate during the past five years (Smidt & de Vos, 2004). Reductive dehalogenases with a wide range of substrate specificities have now been purified and characterized from a number of halorespiring microorganisms (Holliger et al., 2003). All enzymes characterized thus far are more or less tightly associated with the cytoplasmic membrane, reinforcing their role in membrane-associated electron transport–coupled phosphorylation.

Analysis at the biochemical level has revealed that, with one exception, all reductive dehalogenases are monomeric, corrinoid dependent enzymes, and B_{12}-dependent reductive dehalogenases are now one of three currently recognized classes of B_{12} enzymes. In most cases, the involvement of a cobalamin (I) in the catalytic activity has initially been evidenced by the light-reversible inactivation of enzymatic activity by alkyl-iodides. In a number of cases, this has later been confirmed by the analysis of cobalt content in the enzyme and by extraction and electron paramagnetic resonance spectroscopic analysis of the corrinoid cofactor (Smidt & de Vos, 2004). The 3-chlorobenzoate- reductive dehalogenases from *Desulfomonile tiedjei* most probably does not contain a corrinoid. The enzyme has been found to be a heterodimeric complex, in which the small subunit presumably contains a heme, which, like corrinoids, is a transition metal ion-containing tetrapyrrole. Recently, the corrinoid cofactor of the *Streptomyces multivorans* perchloro ethene- reductive dehalogenases has been purified, and its crystal structure has been determined (Krautler, 2003). It differed from other known B_{12} cofactors in that it was lacking a methyl group of the cobamide moiety. In contrast, the corrinoid isolated from the perchloro ethene- reductive dehalogenases of

Dehalobacter restrictus had the same properties as those of commercially available cobalamin (Maillard et al., 2003). Interestingly, a nondechlorinating strain of *Streptomyces multivorans* proved inactive because it failed to produce the unusual reductive dehalogenases associated corrinoid, although conventional forms were still present. The dependence of the reductive dehalogenation activity on corrinoid cofactors has also been reinforced by the notion that for several halorespiring isolates growth required the addition of cyanocobalamin or B_{12}. With the exception of the *Desulfomonile tiedjei* enzyme, all reductive dehalogenases until recently isolated from anaerobic microorganisms contain two iron-sulfur (Fe-S) clusters as cofactors in addition to the corrinoid (Smidt et al., 2002).

1.32. Immobilization of microorganisms

Immobilization of microbial cells has received increasing interest in the field of wastewater treatment. Immobilized cells systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems since high densities of specialized microorganisms are used in immobilized cell systems.

The use of immobilized microorganism has many advantages over the conventional free cell system. Besides preventing wash out of biomass in continuous flow reactors, immobilization facilitates easy separation and imparts greater operational flexibility. Immobilized cells can be much more tolerant to high concentrations of toxic chemicals (Westmeier & Rehm, 1985). In addition, the cell density of immobilized cells is higher than that of the free cells, resulting in higher rates of biodegradation per unit volume of the reactor.

Immobilization of bacteria, yeast cells, and fungi has been done in a variety of ways. Matrices for entrapment include calcium alginate, carageenan, agar, cellulose,

polyacrylate, and polyamide (Chibata, 1983). These methods have their own problems associated with them, such as dispersion of cells, flow of nutrients into and wastes away from the cells (largely inhibited by the viscosity of the immobilization preparation), and purification of the desired cell product from the immobilization matrix.

One of the many critical parameters which affect the kinetics of immobilized microbe in the case of entrapment is the diffusion or mass transfer effect. Cell entrapment in alginate is rather a simple and non-toxic method for immobilization, but the gel may create a diffusion of substrates into and out of the alginate gel has been very well addressed by Tanaka et al., 1984. They found that the diffusion coefficients of substrates such as glucose (in general for substrates with molecular weight less than 20 kDa) into and from calcium alginate gel beads were the same as the diffusion coefficient of the substrates in the water systems. These results suggest that these substrates can diffuse into and out of calcium alginate gel beads. Similarly the agar and polyacrylamide matrices also exhibit a good substrate diffusion coefficient.

Immobilization matrices must prevent the bacteria from dislodging from the matrix and flowing downstream, yet still enable trafficking with the environment and signal transduction (Premkumar et al., 2002). An ideal immobilization matrix would be functional at ambient temperatures, survive harsh wastewater conditions including contaminated water and turbidity, and allow the flow of nutrients and oxygen and analytes through the matrix along with wastes and signal out. It would also prevent cell flow within the matrix. A simple schematic of a desirable immobilization matrix is shown in Figure 1.14.

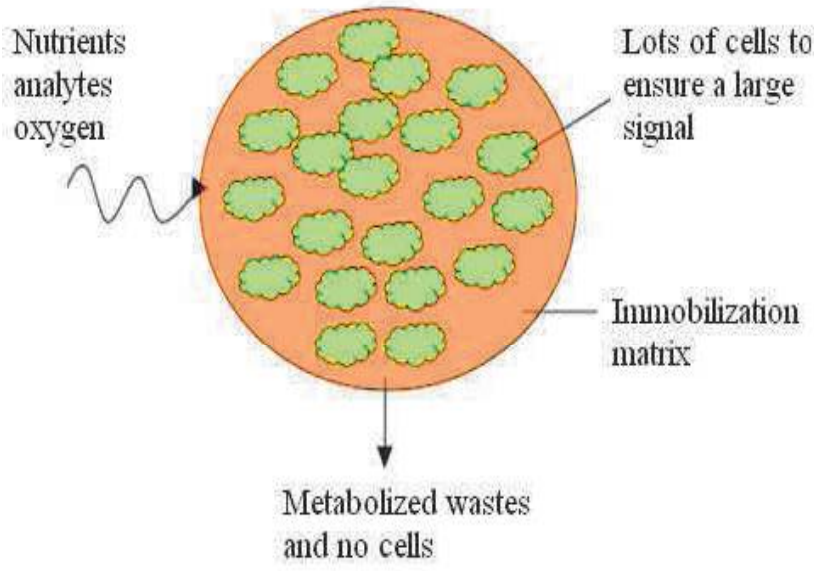


Fig.1.14. General schematic of a desirable immobilization matrix

Immobilization of living microorganisms has been described by several investigators (Chibata, 1983; Sunitha et al., 2008, Chandrashekhar & Karigar, 2009) as being useful in the production of specialty chemicals for industrial use. Fermentations which are currently performed in large vessels have problems with complete mixing of nutrients and biomass. Problems exist also with the purification of chemicals generated by microorganisms in fermentation vessels.

1.33. Nature of immobilization matrices

1.33.1. Alginates

Common immobilizing matrices include naturally occurring alginates. Alginates are produced by some strains of seaweed and are composed of a variety of polysaccharides. Alginates are formed by conversion of manuronic and guluronic acid into their salt forms of mannuronate (M) and guluronate (G). They are copolymers consisting of (1-4) linked β-D-mannuronic acid and α-L guluronic acid (Smidsrod et al., 1990). Alginates are linear polymers comprised of blocks of M and G or alternating GM blocks (Fig.1.15).

Alginates are often used in the food industry as gelling compounds such as in the production of the meat-like chunks found in pet food. Alginates are ionically cross linked between the carboxylic acid elements through divalent ones like Ca^{+2}. Because their cross links are ionic as opposed to covalent, they are easily broken apart by cationic scavengers such as sodium citrate and chelators such as EDTA (Smeds & Grinstaff, 2001). In addition to the weak bonded structure, these natural hydrogels biodegradation, making their use some what limited depending upon the cell type being immobilized.

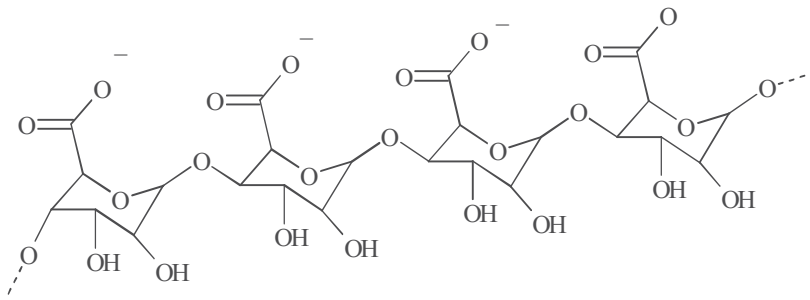


Fig.1.15. Partial structure of alginate

1.33.2. Agar

Agar is a complex polysaccharide and varies considerably depending on the source. Roughly these polymers consist mainly of alternating β-(1,3)-D and α-(1-4)-L linked galactose residues (Fig.1.16). Most of the α-(1-4) residues are modified by the presence of a 3-6, andydro bridge. The other modifications that can be found are mainly substance of sulphate, pyruvate, uronate or methoxy groups. Modern alkali treatment methods tend to increase the level of anhydro bridging in the molecule which subsequently improves the gel strength. The level of methoxy content appears to be one of the main structural moieties that determine the gel setting temperature with very low methoxy contents giving the lower setting temperatures. Agarose is typically high in

molecular weight and low in sulphate. Agaropectin is typically of a lower molecular weight and also higher in sulphate at about 5-8% xylose has been found in some agars.

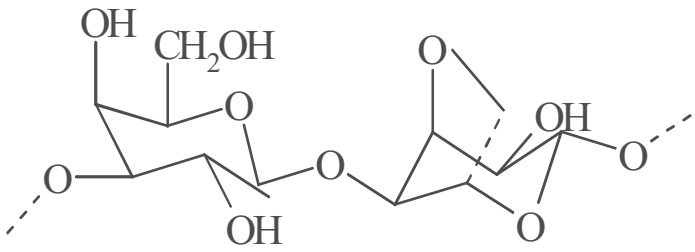


Fig.1.16. Partial structure of agar

1.33.3. Polyacrylamide

Polyacrylamide gels matrix is formed from the polymerization of acrylamide monomer in the presence of smaller amounts of N-N'-methyl bis-acrylamide (normally referred as 'bis'-acrylamide). During polymerization, acrylamide monomer links in a head to tail fashion into long chains and occasionally a bis-acryamide molecule is built into the growing chain, thus introducing a second site for chain extension (Fig.1.17). The polymerization of acrylamide is an example of free radical catalysis, and is intiated by the addition of persulphates and the base N,N,N^1,N^1-tetramethylenediamine (TEMED). TEMED catalyses decomposition of the persulphate ion to generate free radicals.

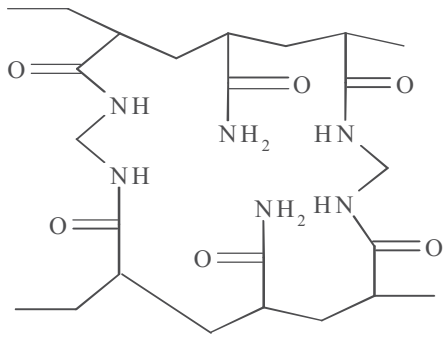


Fig.1.17. Partial structure of polyacrylamide gel

Different stable and non-degradable materials are presently being employed as matrices. Cells of *Enterobacter agglomerans* were immobilized in calcium alginate,

polyacylamide, cooper beech, and vermiculite, and were used for the decolorization of methyl red from synthetic water by using a fluidized bed bioreactor. Gel-entrapped cells of *Pseudomonas luteola* in calcium alginate and polyacrylamide were utilized for azo-dye decolorization in continuous mode. Immobilized titanium (IV) oxide nanoparticles were used for the photocatalytic degradation of acid red 14 **(**Battmann & Rehm, 1984*)* **v**arious approaches have been developed to treat the textile effluents. The constraints are the availability of the suitable microorganisms that can overcome their culturing limitations from their natural habitats to the effluent conditions. The method of cell immobilization seems to be promising in the development of the biotechnology for the removal of various xenobiotic bearing effluents (Murugesan*,* 2003). Since the entrapped cells remain viable for a considerable duration they would be a better alternative against free cells for the bioremediation applications of variety of toxic organics from effluents.