MOLECULAR METHODS FOR MONITORING ENVIRONMENTAL CONTAMINANTS AND BIOREMEDIATION: AN OVERVIEW

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ABSTRACTS

Biodegradation is the most important parameter influencing the toxicity, persistence and ultimate fate of pollutants in soils as a principle abatement process in the environment. Among various biological, physical and chemical methods developed for decontamination of polluted sites, bioremediation methods utilizing microorganisms provide a cost-effective and contaminant/substrate specific treatment technology. A successful bioremediation approach requires sufficient proof for the degradation of the contaminants and detoxification of the contaminated soil or water. Current environmental regulations require appropriate monitoring practices determining of the disappearance of the contaminants and their degradation products to a regulatory levels. Studies during the last decade have indicated that the microbial community response may be a better indicator of residual toxicity and can be used to complement the disappearance or sequestration of contaminants. Differential morphological, physiological and metabolic characters are the basis of traditional culture-dependent methods. This includes isolation and cultivation on solid media, most probably number (MPN) assays and BIOLOG substrate utilization patterns. Culture- independent methods for community analysis are based on direct examination of metabolically active microorganisms using differential strains, denaturing gradient gel electrophoresis (DGGE).

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INTRODUCTION

Bioremediation is one of the most rapidly growing areas of environmental biotechnology .the use of bioremediation for environmental clean-up is popular due to low costs and its public acceptability. Indeed bioremediation stand to benefit greatly and advanced even more rapidly with the adoption of molecular techniques developed originally from other areas of biotechnology .the 1990s was the decade of molecular microbial ecology(time of using molecular techniques in environmental biotechnology) . Adoption of these molecular techniques made scientist realize that microbial population in natural environment made diverse than previously thought using traditional culture techniques. (Grazyna *et.. al* 2001)

The population explosion in the world has resulted in an increase in the area of polluted soil and water. As the number of people continues increasing day by day it also brings with it a growing pressure on our natural resources i.e. air, water and land resources. In order to outfit to the demands of the people, the rapid expansion of industries, food, health care, vehicles, etc. is necessary. But it is very difficult to maintain the quality of life with all these new developments, which are unfavorable to the environment in which we live, if proper management is not applied. In nature there are various fungi, bacteria and microorganisms that are constantly at work to break down organic compounds but the question arises when pollution occurs, who will do this clean up job? Since the quality of life is inextricably linked to the overall quality of the environment, global attention has been focused on ways to sustain and preserve the environment. This endeavor is possible by involving biotechnology. The types of contaminants that Environmental Biotechnology investigators have expertise with include chlorinated solvents, petroleum hydrocarbons, polynuclear aromatic hydrocarbons, ketones, TNT, inorganic nitrogen (NO3, NH4), Tt, , Pu, Np, Cr, U and other heavy metals.

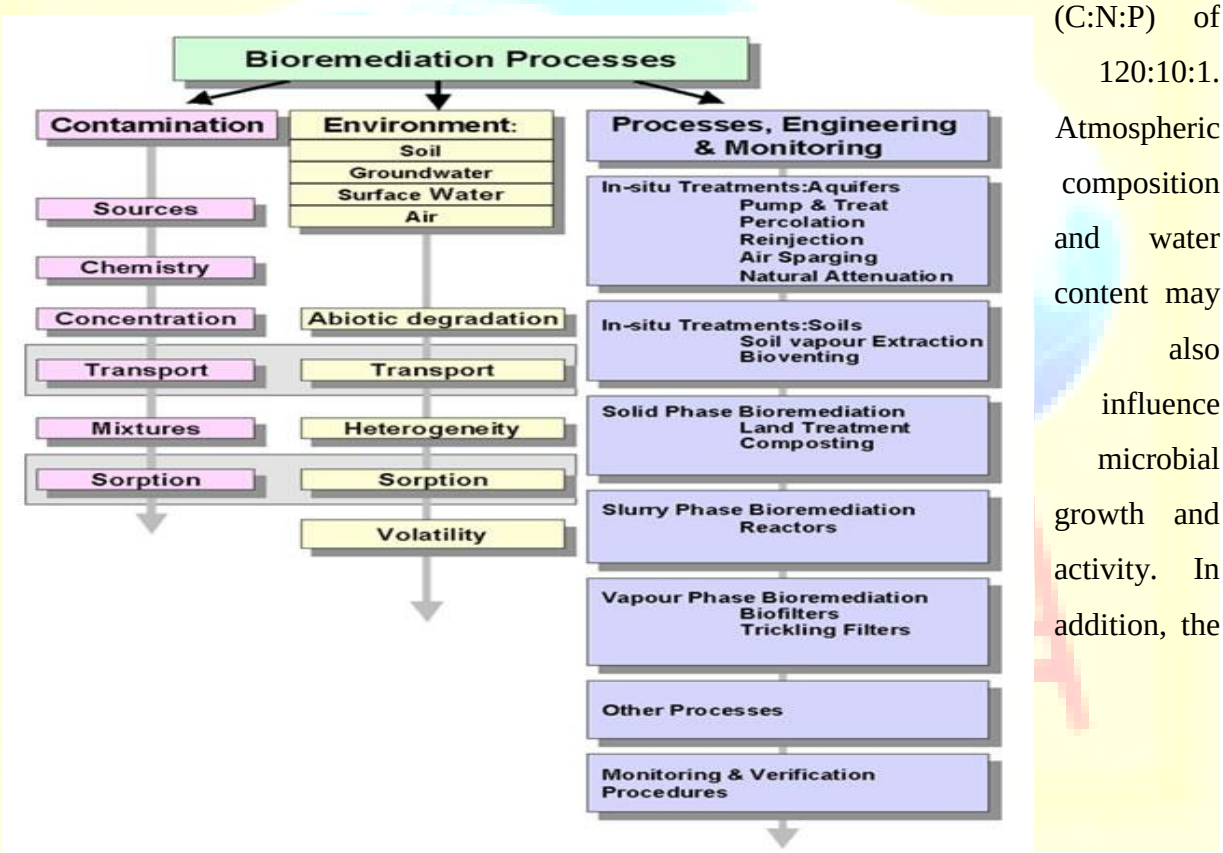
1.0 BIOREMEDIATION

Bioremediation refers to the use of biological system usually microorganisms to clean up the contaminated environments. This approach had a number of advantages over physical and chemical treatment , primarily because it is lower in cost and more environmentally friendly but in addition, organic pollutants can be completely mineralized or biodegraded to simple in organic compounds, example co_2 ,H_2O ,CL^- using bioremediation, whereas physical and chemical processes e.g. vaporization, adsorption and extraction ,destroy soil structure and disturb natural

processes in environment and quite often only transfer the contaminant from one environment to another e.g. soil to the atmosphere.(Semple*et al.*.,2001).

Recent studies in molecular biology and ecology offers numerous opportunities for more efficient biological processes. Notable accomplishments of these studies include the cleanup of polluted water and land areas. Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Mueller 1996). By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. Biodegradation of a compound is often a result of the actions of multiple organisms. When microorganisms are imported to a contaminated site to enhance degradation we have a process known as bioaugmentation. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products (Vidali 2001). As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. Like other technologies, bioremediation has its limitations. Some contaminants, such as chlorinated organic or high aromatic hydrocarbons, are resistant to microbial attack. They are degraded either slowly or not at all, hence it is not easy to predict the rates of cleanup for a bioremediation exercise; There are no rules to predict if a contaminant can be degraded. Bioremediation techniques are typically more economical than traditional methods such as incineration, and some pollutants can be treated on site, thus reducing exposure risks for cleanup personnel, or potentially wider exposure as a result of transportation accidents. Since bioremediation is based on natural attenuation the public considers it more acceptable than other technologies. Most bioremediation systems are run under aerobic conditions, but running a system under anaerobic conditions (Colberg and Young 1995) may permit microbial organisms to degrade otherwise recalcitrant molecules.

Advanced technique emerged from biotransformation this technique is known as Biotransformation and biodegradation. Biotransformation is any alteration of the molecular or atomic structure of a compound microorganism. Biodegradation is the breaking down of organic or inorganic components. These transforming and degrading components process occur as a result of microorganism using the contaminants as a source of nutrients or energy, challenging them through various metabolic reactions .bioremediation depends on the presence of the appropriate microorganisms in the correct amounts and combination and on the appropriate environmental conditions optimum environment's for growth of microbes typically consist of temperature ranging between 15^{o}C and 45^{o}C ,$_{p}$H values between 5.5 and 8.5,nutrient ratios (C:N:P) of 120:10:1. Atmospheric composition and water content may also influence microbial growth and activity. In addition, the



contaminants must be in close enough proximity to the microbes and in a form that the microbes can utilize (Grazyna *et al.,2001).*

FIG 1 SHOWING BIOREMEDIATION PROCESS

2.0 MICROBES INVOLVED IN BIOREMEDIATION

Microbes can adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirements are an energy source and a carbon source (Vidali 2001). Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. Natural organisms, either indigenous or extraneous (introduced), are the prime agents used for bioremediation (Prescott et al., 2002). The first patent for a biological remediation agent was registered in 1974, being a strain of *Pseudomonas putida*(Prescott et al., 2002) that was able to degrade petroleum. In 1991, about 70 microbial genera were reported to degrade petroleum compounds (U.S Congress, 1991) and almost an equal number has been added to the list in the successive two decades (Glazer and Nikaido, 2007).

2.1 Some groups of microbes

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

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

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

4.**Methylotrophs**: Aerobic bacteria that grow utilizing methane for carbon and energy.

The initial enzyme in the pathway for aerobic degradation, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds, including the chlorinated aliphatic trichloroethylene and 1, 2dichloroethane

TYPES OF BIOREMEDIATION

- ✓ In situ bioremediation
- ✓ Ex situ bioremediation

In situ biodegradation involves supplying oxygen and nutrients by circulating aqueous solutions through contaminated soils to stimulate naturally occurring bacteria to degrade organic contaminants. It can be used for soil and groundwater. Generally, this technique includes conditions such as the infiltration of water containing nutrients and oxygen or other electron acceptors for groundwater treatment (Vidali 2001). Most often, in situ bioremediation is applied to the degradation of contaminants in saturated soils and groundwater. It is a superior method to cleaning contaminated environments since it is cheaper and uses harmless microbial organisms to degrade the chemicals. In situ bioremediation have more advantages over ex situ methods because no pumping or excavation is required. Also in situ may be less hazardous, as there is no exposure to the contaminant during treatment.(Grazyna *et al..,2001)*

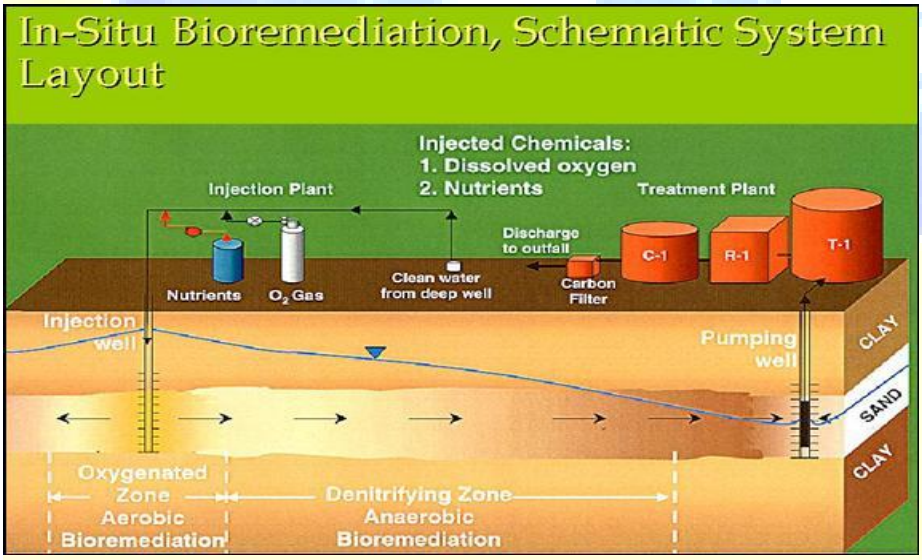


FIG 2 SHOWING *IN SITU BIOREMEDIATION*

Ex situ bioremediation

This process requires excavation of contaminated soil or pumping of groundwater to facilitate microbial degradation. This technique has more disadvantages than advantages. Ex situ bioremediation techniques involve the excavation or removal of contaminated soil from ground.

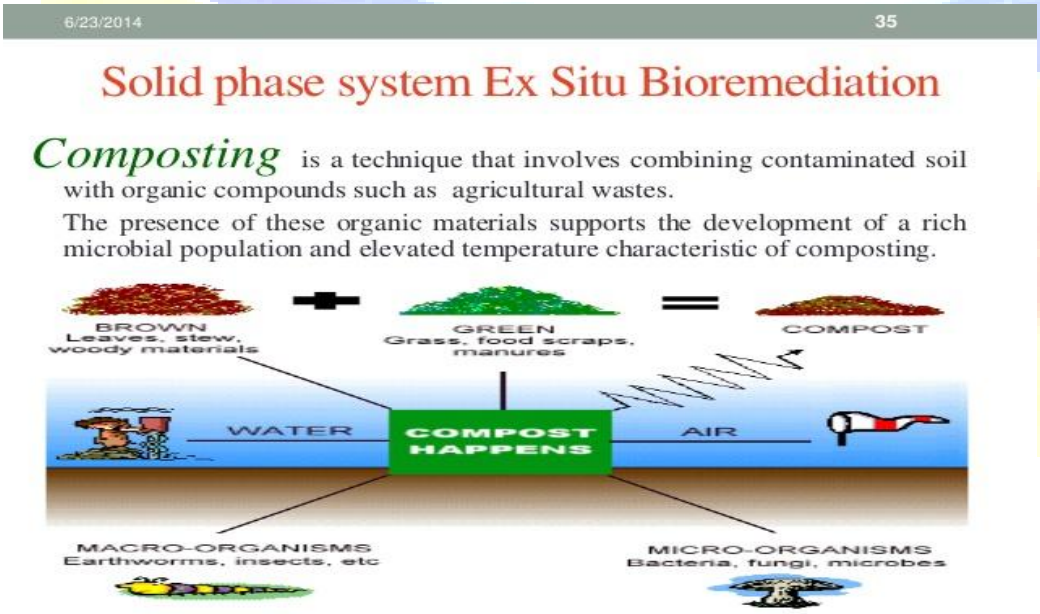
ex situ bioremediation is classified as:

1. Solid phase system (including land treatment and soil piles)

2. Slurry phase systems (including solid liquid suspensions in bioreactors)

Solid phase treatment: It includes organic wastes (leaves, animal manures and agricultural wastes) and problematic wastes e.g. domestic and industrial wastes, sewage sludge and municipal solid wastes. Solid phase soil treatment processes include Land farming, soil bio piles, and composting.(Kumar *et al..,2011*)

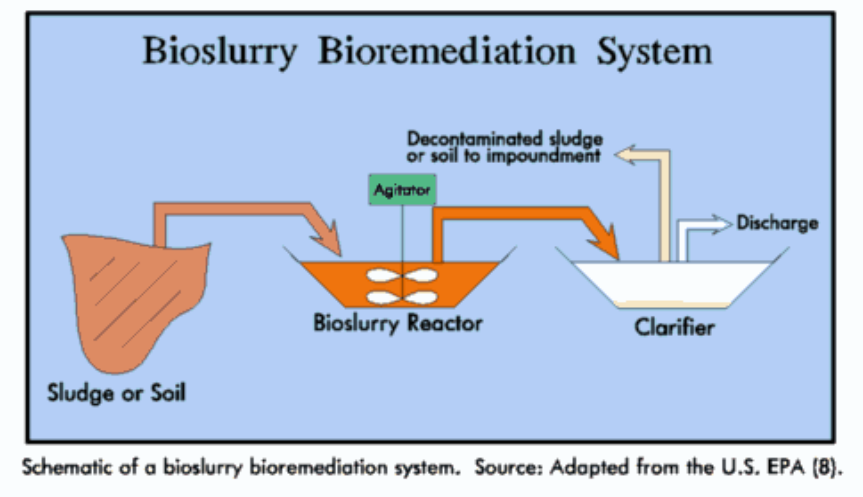
Slurry Phase Bioremediation: Slurryphase bioremediation is a relatively more rapidprocess compared to the other treatment processes. Contaminated soil is combined withwater and other additives in a large tank called a bioreactor and mixed to keep the microorganisms, which are already present in the soil, in contact with the contaminants in the soil. Nutrients and oxygen are added and conditions in the bioreactor are controlled to create the optimum environment for the microorganisms to degrade the contaminants. When the treatment is completed, the water is removed from the solids, which are disposed of or treated further if they still contain pollutants. **(**Kumar *et a*l.,2011**).**



(Source: https://www.google.co.in/search?q=bioremediation+images)

a)

b)



Schematic of a bioslurry bioremediation system. Source: Adapted from the U.S. EPA (8).

Fig 3: showing *ex situ bioremediation. (A) solid phase. (B) slurry phase*

MOLECULAR TECHNIQUES IN BIOREMEDIATION

Numerous techniques have been developed to monitor the presence and or activity of microorganisms or determining microbial diversity in environmental samples from the contaminated sites. These methods include the followings:

DNA EXTRACTION

DIRECT DNA extraction techniques are very important part microbial ecology investigations .these techniques are use in detection of the presence of native bacteria or bacteria introduced into environments, GMOs or GEMs (Torsvik *et a*l 1995). A large fraction, often 90-99% of microbial cells present in environmental sample are not cultivable on microbiological media. Viable microbial cells can only be partially recovered from complex environmental samples by traditional plating methods. Their detection through molecular techniques requires a true understanding of the environmental microbial ecology.

Two different techniques for DNA isolation from soil include:

-the cell extraction method and subsequent lysis, or

-direct lysis method.

cell extraction method and subsequent lysis involves extraction of microbial cells from *soils* ,the second techniques DNA is directly extracted from the soil, water contaminants (Zhau*et al.,).*both methods aimed at the same goal: the highest yield of DNAallows for advanced molecular

techniques. The quantity of DNA extracted can be assessed on agarose gel compared with DNA markers .DNA concentrations are expressed as nano gram of dry soil/sediment or nanogram per milliliters of water.

The pure DNA obtained can be use advanced molecular techniques such as DNA-DNA hybridization, the terminal restriction fragment length polymorphism(TRFLP) analysis or the amplification by PCR(Sambrook et al)

Environmental samples	Sample size	Method of DNA extraction	Cells numbers per gram	DNA yield
Water	>1 liter	Direct lyses/ethanol precipitation or centrifugation	10^6	1ng
soil	50g	Cell lysis after dispersion and PVPP treatment	10^6	ND
Sediment	100g	Direct lyses/ethanol precipitation or centrifugation	10^9	350µg
sediment	100g	Direct lyses incorporating glass beads /DNA precipitation	10^7	2,6µg

ND- not determined, PVPP-polyvinylopolypyrrolide * cells per ml.

PCR BASED TECHNIQUES

The use of PCR in environmental microbiology was reviewed by Bej and Mahbubani (1992). The development of this technique was a major methodological discovery in molecular biology (Grazyna et al., 2001).PCR is commonly used for specific detection of microorganisms in environmental samples. The high specifity, sensitivity and reproducible consistency of PCR detection detection in complex environmental samples has contributed significantly to the advancement of knowledge in environmental microbiology and many more areas of research(eg detection of microbial pathogens ,clinical diagnosis ,detection of mutation ,generation of DNA probes by PCR, and the cloning of PCR product)(Pillai *et al* .,1991;Toze:2000; Watson and

Blackwell 2000).Generally PCR involves repetitive cycling between a high temperature to thermally denature DNA, are relatively low temperature to allow primer to hybridize (anneal) with the complementary region of the target DNA ,and an intermediate temperature for primer extension or replication. (Grazyna et al., 2001)

In environmental studies PCR method is used for detection of microorganisms ,e.g. genetically engineered microorganisms (GMOs and GEMs), pathogens ,indicator organisms ,Steffan and Atlas 1998 used PCR to amplify specific regions of specific regions of a 1.0_kilobase(kb) length which was an integral portion of a larger 1.3kb repeated sequence present in the genome of the herbicide degrading bacteria *Pseudomonas cepacia*AC1100 to increase the sensitivity of dot blot detection of microorganisms. Bacterial DNA was isolated from sediment samples. After amplification, P.cepaciawas positively detected at a concentration of 1 cell per gram of sediment samples.

PCR techniques are also use in analysis techniques or ribosomal RNA sequences for identification and phylogenetic characterization of microorganisms. This is a major advancement in the study of microbial ecology. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation in the review of Amann et al (1995).

Manz *et al* (1994) showed how specific oligonucleotides probes could be applied for rapid in situ characterization of microbial communities in activated sludge of two wastewater treatment plants. In a another research bacteria cells and soil and sediments were detected by PCR (Tsai and Olson,1991).

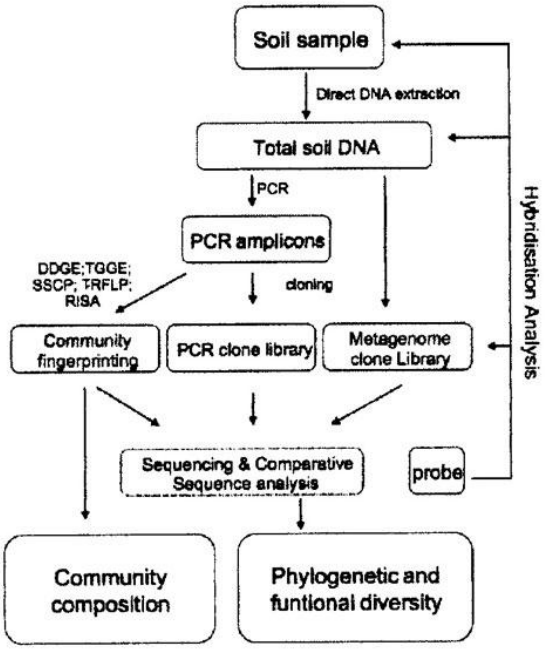


FIG 5:Culture-independent molecular methods for the analysis of microbial communities. PCR: polymerase chain reaction; DGGE: denaturant gradient gel

3. Hybridization –based Techniques

Hybridization methods allows for the total DNA from the community in a single step and be used to track different microorganisms in a quantitative manner. DNA microarray technique can be a powerful tool for analyzing microbes and their activities in environmental sample. For monitoring bioremediation process ,DNA microarray can be potentially useful when bioaugmenting with a culture containing multiple organisms . it is possible to detect several genes in a degradation pathways to assess the functional diversity and distribution of selected genes.

Fluorescent in situ hybridization analysis (FISH) is relatively new technique that can be used to visualize, quantify, and identify environmental microorganisms directly without culturing .cells are hybridized with a probe that is tagged with fluorescent molecules .this enable microscopic detection.

Several reporter genes have been developed to allow for more sensitive monitoring for activity and property of the presence and activity of introduced microorganism during bioaugmentation study bacterial *Luciferase* (lux) and jellyfish green fluorescent protein (grp) genes are currently the most widely used for environmental application since both lux and gpf can emit signals without the addition of an external substrate ,microbes containing lux and gpf have been added to contaminated soils for the assessment of contaminant bioavailability and remediation. In one of the first field studies in the United States of genetically engineered microorganisms for bioremediation, *pseudomonas fluorescence* HK44, containing *lux* genes fused together with naphthalene degradative pathway was added to the soil contaminated with anthracene and detected by luminescence based fiber optic /photon multiplier tube techniques.

Gene probe may be used for accurately evaluating bacteria degradative potential. Gene probes have been applied following the EXXON Valdes spill to detect the bacterial populations containing both *xylE and* alkB genes in the environmental samples.(Ashok 2009).

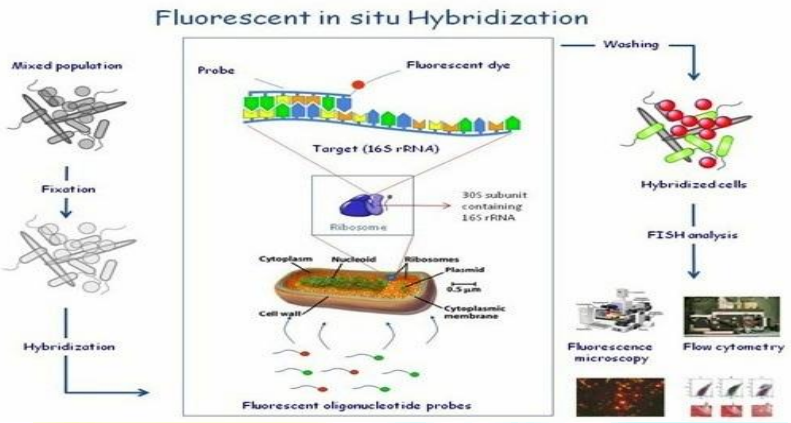


FIG 6:FISH

BACTERIAL BIOSENSORS

biosensors measures the interaction of specific compounds through high sensitive bio recognition processes that can be within 3% range of those measured by standard GC-MS techniques . Bacterial biosensors represent a breakthrough for the monitoring of pollutants in contaminated matrices because element they have the unique ability to measure the interaction of specific compounds with biological systems through highly sensitive recognition processes.

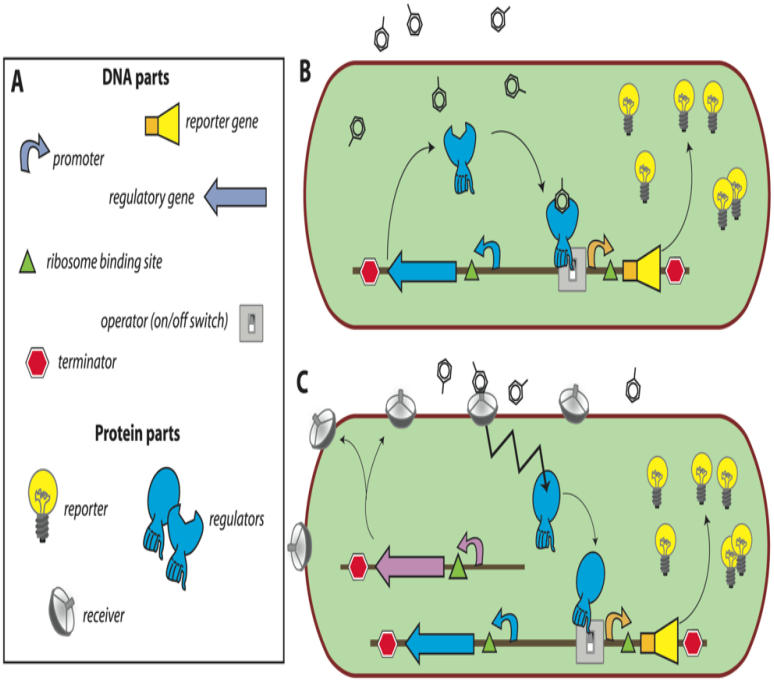
Whole cell biosensor constructed by fusing a reporter gene to a promoter element induced by the target compound, offer possibility to characterize,identify,quantify and determine the biodegradability of specific contaminants present in a complex mixture ,without pretreatment of the environmental samples. The genetic information, located on plasmid vector, is inserted into a bacterial strain so that the engineered fusion replicates along with the cell's normal DNA.

The biosensor systems include a wide range of integrated devices that employs enzymes, *antibodies*, tissues or living microbes as biological recognition element. Broad specificity biosensors are used for toxicity testing and a good example include the MICROTOX assay that is used for measuring the toxicity of environmental samples by monitoring the light production naturally bioluminescent marine bacteria photobacterium*phosphorium*. Since bacterial bioluminescence is tied directly to cellular respiration ,any inhibition of cellular metabolism due to toxicity results in a decrease in the light emission of the affected cells.

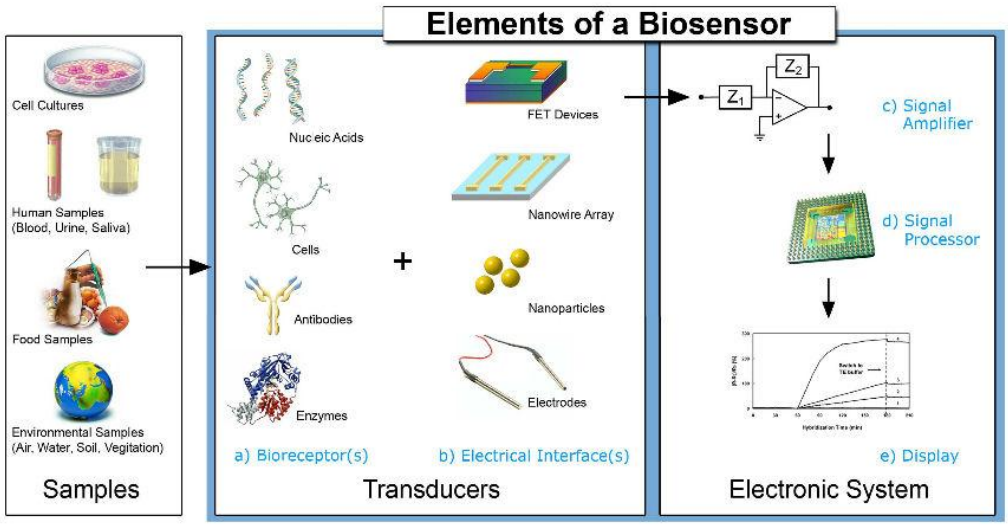
Bioluminescent biosensors have been developed for the detection of benzene, toluene, ethylbenzene and xylene isomers. Cells interfaced with integrated circuit called Bioluminescent

Bioreporter Integrated circuit (BBIC)represent an advanced system capable of detecting optical signals ,distinguishing it from noise, performing signal processing, communicating the results also carry out position sensing.(Ajit*et al.,)*

FIG 7



Schematic depiction of targets for bacterial biosensor development.



Biosensors: Novel immunosensors for direct detection of Geno toxicants

TOXICOLOGICAL TEST

Toxicity test using biomonitors can be used to evaluate the cumulative effects of pollutants on an organisms or system. Generally toxicity varies with the pollutants types and concentration, soil type and properties, microbial communities and plant species .biomonitors are useful tools in assessing the risks associated with contaminated soil and in monitoring bioremediation processes, bacteria, fungi, algae, and plants are useful biomonitors, whereas carnivore's fish are used to measure bioaacumulative pollutants in aquatic systems. Toxicity tests involve exposure of the organism or cultured cell-lines to toxic compounds under defined experimental conditions followed by monitoring biological endpoints such as mortality, reproduction, growth and behavioral changes. Toxicity testing using biomonitorshave been implemented in the remediation of pentachlorophenol (PCP), polycyclic aromatic hydrocarbons (PHA) petroleum_ and lead _contaminated sites. Three types of biomarkers are used for toxicity assessments that are indicators of cellular or biochemical responses to a pollutant. Some biomarkers are developed for in response to exposure to organic and inorganic pollutants such as heat shock proteins,metallothiones, and antioxidant enzyme . Second category of biomarkers can detect biological effects and disturbed structures and functions such as population size, DNA mutations, and enzyme inhibition. The third category measures activities of detoxifying enzymes, cytochrome P450 monooxygenese and DNA repair enzymes.(Zhou 2003**)**

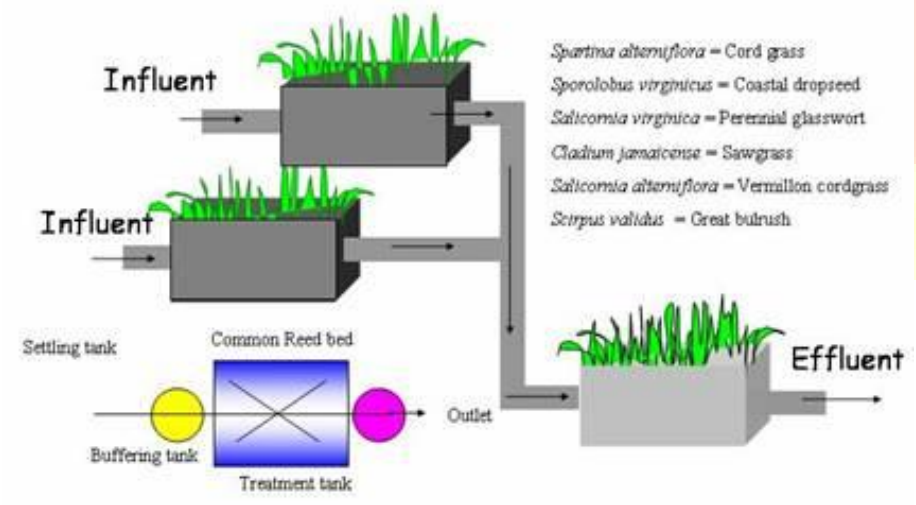


Figure 5: Cascade model of constructed Aquaplant with cmmonreed beds or grasses for the removal of xenobiotics and treatment of saline waste streamsS

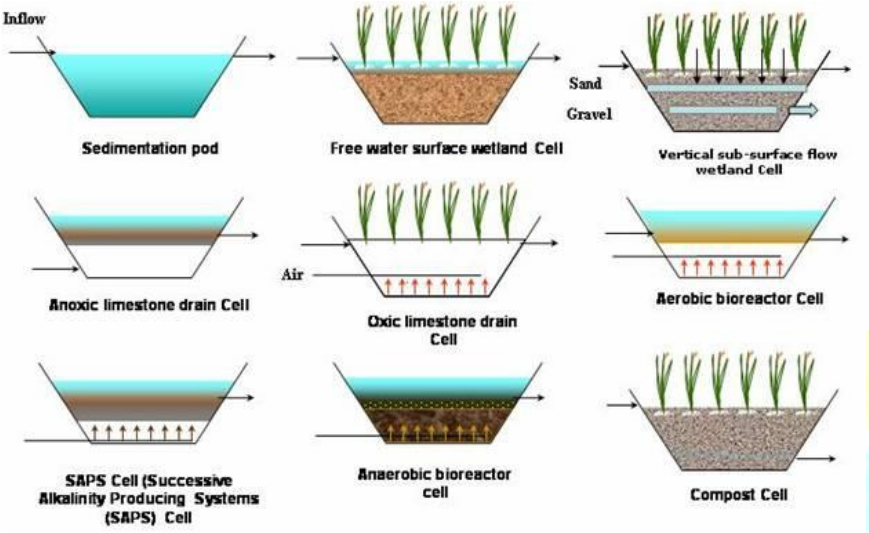


Figure 3: Removal of Arsenic from ground water using macrophytes by phytovolatilization(Aksorn and Visoottiviseth 2004

CONCLUSION

The clean-up of contaminated soil, is a priority task due to the risks contaminants pose to the groundwater, drinking water and soil fertility. Challenges in monitoring assessing, and directing microbial processes during bioremediation of contaminated sites have represented a major research pursuit for developing more rapid and accurate methods over the past two decades .molecular diagnostics in bioremediation have been advanced during the last decade. Qualitative detection methods are being replaced with quantitative detection measurements of specificmicrobial population present in the contaminated soils. The presence of toxic compounds and ecological risks can be determined using various advanced molecular techniques

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