1.1 Introduction

Marine ecosystem is the largest ecosystem on the earth. Marine microorganisms have defined its chemistry over evolutionary time (Redfield, 1958). A mere teaspoon of seawater can contain on an average one million bacteria and up to ten million viruses. Microorganisms are simply the oldest forms of life, evolved on earth before 3.5 billion years and present as unicellular organisms for more than 2 billion years (Schopf, 2006). The field of environmental microbiology is a robust area encompassing research on the genetics, physiology, biochemistry and ecology of microorganisms in marine ecosystem.

1.1.1 WHAT WE KNOW? - Bacteria are Ubiquitous and Abundant

Bacteria inhabit an extraordinary array of habitats, from ideal conditions for living creatures to extreme environment to support most life forms. They inhabit the relatively benign and nutrient-rich environments of soils, lakes, oceans and other organisms, but they are also found in extreme environments such as hot springs nearly saturated salt brines (Anton et al., 2000), acid mine waters at pHs near zero (Baker and Banfield, 2003), deep in Antarctic ice (Christner et al., 2001; Price, 2000) and kilometres below the Earth’s surface (White et al., 1998). Bacteria are not only ubiquitous, but they are also incredibly abundant. The total number of bacteria on Earth may be as high as $4–6 \times 10^{30}$, with the largest proportion of bacterial cells possibly residing in the oceanic and terrestrial subsurface ($3.5 \times 10^{30}$ and $0.25–2.5 \times 10^{30}$, respectively). Perhaps most notably, bacterial cells are estimated to contain 60–100% of the total carbon found in plants. In addition, the prokaryotes contain $85–130 \times 10^{15}$ g of nitrogen and $9–14 \times 10^{15}$ g of phosphorus, i.e. approximately 10-fold more than found in plants (Whitman et al., 1998).

1.2 WHY THEY ARE SO DIVERSE? - Shaping the prokaryotic genome

1.2.1 Gene duplications

Gene duplication is considered an important mechanism of gene innovation, and consequently of genetic novelty, that has facilitated adaptation of microorganisms to changing environments and exploitation of new niches (Hooper et al., 2003).
1.2.2 Horizontal gene transfer
Besides gene duplication, microorganisms have an alternative mechanism for genetic adaptation to their environment. The introduction of novel genes or alleles by horizontal gene transfer (HGT) allows adaptation of microorganisms toward different ecosystem, which eventually might lead to bacterial diversification and speciation (Ochman et al., 2000; Cohan, 2001).

1.2.3 Gene loss
Bacterial genomes are not growing ever larger in size, rather than accumulating sequences, consequently gene acquisition (both by duplication and HGT) must be counter balanced by gene loss. Because bacterial genomes can only protect a finite amount of information against mutation and loss, chromosomal deletions will serve to eliminate genes that fail to provide a meaningful function, that is, the bulk of acquired DNA as well as superfluous ancestral sequences (Lawrence et al., 2001; Mira et al., 2001). Comparing Mycobacterium leprae to its relative Mycobacterium tuberculosis indicates that the Mycobacterium leprae lineage has discarded more than 2000 genes (Coenye et al., 2005).

1.2.4 Chromosomal rearrangements
Genome rearrangement is a driving force behind a constantly evolving genome organization (Moran, 2002). Gene order conservation could therefore be used as a phylogenetic measure to study relationships between species (Korbel et al., 2002).

1.3 History of microbial ecology: from Microscope to Pyrosequencing
Micro-organisms are ubiquitous and abundant on earth, it was not found until the mid seventeenth century. It was in 1665 when Robert Hook the English experimental philosopher, described microscopic observation of fungi and protozoa. Meanwhile, Antonie van Leeuwenhoek (1680) recovered bacteria from his own teeth and observed through his home made microscope. He has also given description of bacteria in rain water (natural habitat) and effect of pepper on microbes, thus providing not only description of earliest bacteria but also earliest description in microbial ecology (Handelsman, 2004). After 200 years Ferdinand Cohn, Botanist classified many bacteria
and described the life cycle of *Bacillus subtilis* based on his microscopic observations and it was the same time when Louis Pasteur had performed series of experiments to demolished the theory of spontaneous mutation. Step-by step evolution has mainly been promoted by methodological development. In 1880s, Robert Koch’s postulates and his own innovation in developing culture media. Further some evidence that drew attention of microbiologist to the presence of uncultured bacteria in ecosystem during the 1970s and 1980s. A study of oligotrophs indicated that incubation times longer than 25 days enhanced the recovery of certain organisms in culture (Whang and Hattori, 1988). One of the indicators that cultured microorganisms did not represent complete microbial diversity as often observed by “great plate count anomaly” (Staley and Konopka, 1987)—the discrepancy between the sizes of populations estimated by dilution plating and by microscopy. This discrepancy was particularly dramatic in some aquatic environments, in which plate counts and viable cells estimated by acridine orange staining differ by four to six orders of magnitude (Grimes et al., 1986). In 1985, an experimental advance radically changed the way we visualize the microbial world. Building upon the pioneering work of Carl Woese, a biophysicist turned evolutionary microbiologist, which showed that rRNA genes are the evolutionary chronometers (Woese, 1986), Pace and colleagues created a new branch of microbial ecology (Lane et al., 1985; Stahl et al., 1983) by direct analysis of 5S and 16S rRNA gene sequences in the environment to describe the diversity of microorganisms in an environmental sample without culturing microorganisms (Pace et al., 1986; Staley and Konopka, 1987). As 90 to 99% of microbes from natural environments are not cultivable in laboratory, the biggest boom in microbial ecology research undoubtedly came in 1990s from the application of culture-independent approach using molecular tools for studying diversity of microbial communities in fine detail (Desai et al., 2010). Over the years, such techniques have proved to be invaluable tools for the qualitative (e.g., fingerprinting techniques) and quantitative (e.g., dot blot and fluorescence in situ hybridization (FISH), or real-time PCR) analysis of environmental microbial communities. Moreover, this approach is also applicable in identification new catabolic operons of xenobiotics in environmental bacteria. However, their application is often time-consuming and therefore limited to a small number of samples, which does not allow the comprehensive
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characterization of any particular ecosystem. Next generation sequencing technologies such as Roche 454 Life Sciences GS FLX or GS XLR based pyrosequencing are simplifying and rapidly changing this current scenario. Initially, this approach was developed in the biomedical field, progressively applied in environmental microbiology and biotechnology. They offer the advantage of miniaturization, automation and massive parallelization of time consuming steps, allowing the simultaneous “real-time” analysis of numerous samples at a reasonable price. In parallel, expanding sequencing infrastructure and rapidly dropping genome-sequencing costs, has given dramatic increase in the number of sequenced bacterial genomes (including of bacteria involved in bioremediation) (Golyshin et al., 2003; Kube et al., 2005) and even of entire community genomes (Deutschbauer et al., 2006). In the near future, it is expected that the combined application of such genomic approaches with post-genomic techniques, like high-throughput functional analysis of metaproteomes will provide a comprehensive understanding of the composition and functioning of environmental microbial communities (Ram et al., 2005).

1.4 WHY MICROBIAL DIVERSITY? - Importance of studying microbial diversity

- Ancient microorganisms developed photosynthesis more than two billion years ago and helped to shape the chemical environment that allowed the evolution of multicellular organisms and complex biological communities, including human societies. Therefore, bacterial diversity can be used as models for understanding principles of ecology and evolution (Fuhrman, 2009).

- One of the most fascinating characteristics of microorganisms is their evolved ubiquity. Therefore study of microbial diversity is key factor for understanding the ubiquity of life (Malaterre, 2013).

- Marine microorganisms are central to all biogeochemical cycles and are crucial for the functioning of marine ecosystems. Such as marine phototrophic microorganisms (Cyanobacteria, diatoms and pico- and nanophytoplankton) are responsible for more than 50% of the oxygen production on Earth. Thus, studying the ecology of marine microbial communities is essential for an understanding of ecosystem function. Microbes are also responsible for the degradation of organic...
matter in the ocean and are thus key factor to maintain the balance between produced and fixed carbon dioxide (Bowler et al., 2009).

- Marine microorganisms represent a largely untapped source of novel bioactive compounds and metabolic pathways which could be exploited for new biotechnological applications and products (Fig. 1.1). Approximately 3000 natural products were identified from marine microorganisms by the end of 2008 (Laatsch, 2008; Prakash et al., 2013).

![Fig. 1.1 Application of culturable diversity in different areas of research and development including agriculture, bioenergy, industries, ecosystem services, and development of novel therapeutics](image)

- Marine microorganisms play an indispensable role in ensuring a sustainable supply of seafood products by occupying the critical bottom trophic level in marine food webs (Pomeroy, 1974).

- Microorganisms are sensitive to environmental stress since they are close in contact with surroundings due to high surface volume ratio. Giller et al., (1998) suggests that microorganisms are far more sensitive to the influence of contaminants than coexisting eukaryotic macrofauna. Bacterial community structure respond to concentrations of heavy metals at >60 µg of Cu g/1 in wet
sediment suggesting sensitivity of micro-organisms to low concentrations (Magalhães et al., 2011). In addition to their sensitivity, bacteria have a rapid generation time which is advantageous in facilitating early-detection of ecosystem stress (Ford et al., 1998). This is an important attribute in environmental indicators as it means that impacts may be assessed earlier in the ecosystem before loss of diversity and function takes place (Ogilvie and Grant, 2008). Community response to organic contaminants has also been shown to be dose-dependent (Ager et al., 2010). In addition to these, analysis of bacterial community structure helpful to identify hotspots area which is affected by contamination. Bacterial communities in sediments are taxa-rich (Lozupone and Knight, 2007), potentially providing a greater resolution for detecting a large range of anthropogenic contaminants across different environmental conditions. In terms of sensitivity, efficiency and ecological relevance, bacterial communities therefore present an attractive alternative to macrofaunal communities for use as indicators of ecosystem health. Microbial indicator are currently in use by European monitoring programmers which include bacterial diversity, soil respiration, microbial biomass, soil enzymes, C and N mineralization and bacterial growth rate (Winding et al., 2005).

In addition for its own sake, microbial diversity is also most rewarding when studied in relationship to general biodiversity and ecology, because microbes and macrobes are often involved in intimate associations, it is likely that they will display interrelated ecological phenomena (Bowler et al., 2009).

1.5 Approaches to study bacterial diversity
The detailed analysis of microbial diversity within an environment can be divided into two broad categories: culture dependent studies and culture independent studies (Juck et al., 2000).

1.5.1 Culture dependent approach
Culture dependent approach is “the technique that depends on culturing the microbes for studying their diversity”.

Chapter 1: General Introduction……
1.5.1.1 Dilution plating and plate count

Traditionally microbial diversity was assessed using culture dependent approach by isolation of different bacterial strains. The sample is diluted up to certain dilutions and spreaded on different selective media. The nucleic acid is extracted from isolated bacterial strains and identified by 16S rRNA gene sequencing. Advantages of this method are that it is fast and inexpensive. The biggest drawback in exploring bacterial biodiversity using culture dependent approach is that approximately 1% of the soil bacterial population can be cultured by these standard laboratory practices. However, it is likely that remaining 99% of microorganisms in ecosystem are phenotypically and genetically different from the 1%. Limitations for cultivation include the difficulty in dislodging bacteria from soil particles, biofilms, growth medium selection or growth conditions (temperature, pH, light) (Tabacchioni et al., 2000; Trevors, 1998). This is also because of environmental stress, bacteria can enter a state called “viable but non culturable” and thus microbes cannot be accessed (Rozak and Colwell, 1987). In addition, plate growth favors those micro-organisms with fast growth rates (Dix and Webster, 1995). These cultured microorganisms are considered as the “weeds” of the microbial world and constitute <1% of all microbial species (Hugenholtz, 2002). Most of the isolates cultured from soil samples belong to one of four phyla (the “big four”), Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, primarily due to their ease of cultivation under laboratory conditions. Current estimates of bacterial diversity indicate that there are about 61 distinct bacterial phyla, of which 31 cannot be cultured (Vartoukian, 2012). Many attempts have recently been made to cultivate previously uncultured microorganisms by the application of novel approaches, which have significantly contributed to our understanding of living microbes. These advances are: (i) use of modified media (ii) changes of growth conditions (iii) community culture and co-culture (iv) use of trans well plates (v) optical tweezers and laser micro dissection (vi) high-throughput microbioreactor (vii) simulated natural environments using diffusion chambers and (viii) assistance of culture-independent methods (Pham and Kim, 2012).
1.5.1.2 Sole carbon source utilization patterns/ community levels physiological profiling for measuring microbial diversity

Garland and Mills (1991) developed a technique using a commercially available 96-well microtitre plate to assess the potential functional diversity of the bacterial population through sole source carbon utilization (SSCU) patterns. There are 95 wells each containing different carbon source and one control well is without a substrate. Inoculated population is monitored over time for their ability to utilize substrate. The tetrazolium salt changes color from blue to yellow as the substrates is metabolized. Alternatively, researchers can use plates containing the growth medium and a tetrazolium salt from biolog, and add site specific carbon sources to analyze their samples (Campbell et al., 1997; Becker and Stattmeister, 1998).

1.5.2 Culture independent methods

Because of the inherent limitations of culture-based methods, microbial ecologists are turning increasingly to culture-independent methods of community analysis. Culture independent technique is the technique that does not rely on culturing microbes; rather the genome (Metagenomics) or protein (Metaproteomics) or total lipid (FAME) or RNA (Metatranscriptomics) or Metabolite (Metabolomics) of all the micro-organisms existing in the environmental sample extracted for the analysis (Desai et al., 2010).

1.5.2.1 Metagenomics and its applications in microbial ecology

Metagenomics is the study of genomic material collected directly from natural environmental samples. It is also termed as environmental genomics, ecogenomics and community genomics. Over the past two decades, metagenomic studies have provided valuable information in a number of fields including microbiology, medicine, energy, biotechnology, agriculture, environmental remediation and gut microbial ecology. It offers a powerful lens for viewing the microbial world which in turn presents potential to revolutionize understanding of the entire living world. All metagenomic studies begin with metagenomic DNA extraction from ecosystem followed by amplification of 16S and 18S rRNA (ribosomal RNA) for prokaryotes and eukaryotes, respectively and sequencing 16S or 18S rRNA gene to analyze bacterial community structure. Apart from the
phylogenetic marker, 16S rRNA and 18S rRNA genes, other catabolite specific genes have also been used to detect functional capabilities of microbial communities in ecosystem.

1.5.2.1 Extraction of metagenomic DNA from environment

Extraction of high molecular weight metagenomic DNA from environmental samples is the first and foremost requirement of analysis of microbial diversity using metagenomic approach. Till date, there is no single suitable method for the isolation of metagenomic DNA from various soil and sediment samples. Only combinations of different extraction and purification methods have been shown to yield PCR compatible metagenomic DNA. The similar physico-chemical properties of humic acids, fulvic acid and other phenolic compounds with nucleic acids (Harry et al., 1999) make it difficult to remove them during metagenomic DNA extraction. These contaminants inhibit restriction endonucleases and Taq polymerase (Porteous et al., 1991; Tsai and Olson, 1991; Tsai and Olson, 1992; Jacobsen et al., 1992). It becomes almost impossible to obtain PCR amplification from the extracted DNA containing humic acid content as low as 0.08μg/ml (Tebbe and Vahjen, 1993). However, restriction endonucleases can tolerate up to 0.5-17μg/ml humic acids content in the DNA (Tebbe and Vahjen, 1993). The presence of humic acid contamination in the DNA can be detected by the brownish color of the DNA extract and their level can be determined spectrophotometrically by the absorbance ratios. A high 260/230 ratio (>2) is indicative of pure DNA, while a low ratio is indicative of humic acid contamination since humic acids exhibit absorbance at both 230nm and 260nm (Yeates et al., 1997; Sharma et al., 2008).

1.5.2.1.2 Genetic Fingerprinting techniques based on PCR

Genetic fingerprinting techniques provide a specific pattern or profile of a given microbial community. They are based on the separation of amplicons after PCR amplification of phylogenetic (e.g., 16S rRNA or 18S r-RNA) or functional genes using universal or specific primers. These methods provide a rapid means for screening microbial communities from different environments or for comparing community dynamics at different spatial/temporal scales. Among all of the available fingerprinting
methods, PCR-DGGE is one of the most commonly used approaches for screening environmental microbial communities.

(a) **Amplified ribosomal DNA restriction analysis (ARDRA):**
Amplified Ribosomal DNA Restriction analysis (ARDRA) is based on sequence variations present in 16S rRNA genes (Smit et al., 1997). The PCR product amplified from environmental DNA is generally digested with tetra-cutter restriction endonucleases (e.g., *Alu*I, *Hha*I and *Hae*III), and restricted fragments are resolved on agarose or polyacrylamide gels. Although ARDRA provides little information in variation of different microorganisms present in the sample, the method is still useful for rapid monitoring of microbial communities over time, or to compare microbial diversity in response to changing environmental conditions.

(b) **Denaturing gradient gel electrophoresis (DGGE)/ Temperature gradient gel electrophoresis (TGGE):**
In denaturing-gradient gel electrophoresis (DGGE), the PCR products are obtained from environmental DNA using primers of a universal gene (e.g., 16S rRNA gene) or some specific gene and electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer et al., 1993). Temperature-gradient gel electrophoresis (TGGE) is based on the same principle of DGGE except that a temperature gradient rather than chemical denaturant is applied. This techniques allows separation on the basis difference in sequences. Both DGGE and TGGE involve the use of a 5′-GC clamped (30–50 nucleotides) forward primer during the PCR amplification. This is essential to prevent the two DNA strands from complete dissociation into single strands during electrophoresis. For determining the phylogenetic identities from DGGE/ TGGE fingerprints, the bands can be excised from the gel, reamplified and sequenced or blotted onto nylon membranes and hybridized to molecular probes specific for different taxonomic groups. In a microbial community investigation, DGGE was applied to soils collected from different agricultural fields in Norway and the USA that were under different agronomic treatments (crop rotation and tillage) (Nakatsua et al., 2000). **Denaturing High Performance Liquid Chromatography (D-HPLC)** separates DNA fragments within minutes using fast and repeatable reverse-phase ion-pair
chromatography. The separation of PCR products in D-HPLC is based on the elution of partially melted DNA molecules according to the column temperature and their interactions with the positively charged ion pairing reagent and the cartridge matrix of the system (Barlaan et al., 2005). Then, the fragments can be collected at the end of the column for further automated sequencing.

(c) Terminal-restriction fragment length polymorphism analysis (T-RFLP):
T-RFLP based on enzymatic restriction of PCR amplicons according to their size. The use of labeled primers allows a rapid, automated and high-throughput detection (the output is digital) of polymorphic terminal fragments. The separated fragments are visualized by an automated DNA sequencer to provide a pattern of peaks on an electropherogram. In addition, the identification of T-RFLP peaks can be directly obtained by comparing them to databases (Marsh et al., 2000).

(d) Length heterogeneity PCR (LH-PCR):
LH-PCR can be adapted to high-throughput in the same way, but the discrimination of amplicons originating from different organisms is based on natural variation in the lengths of small subunit rRNA genes rather than on restriction endonuclease sites (Suzuki et al., 1998).

(e) Single strand conformation polymorphism (SSCP):
SSCP is another technique initially developed for detection of human genetic variation, that is nowadays widely used to discriminate among 16S rRNA genes (Lee et al., 1996). It involves the denaturing of PCR-amplified fragments with the subsequent formation of sequence-specific secondary and tertiary structures of the single strands during non-denaturing gel electrophoresis (Kristensen et al., 2001). In its high-throughput version, fluorescent single strand conformation polymorphism or Capillary electrophoresis single strand conformation polymorphism (CE-SSCP) in which fluorescently labeled fragments are analyzed using automated capillary electrophoresis (i.e. an automated DNA sequencer).
(f) **Automated ribosomal intergenic spacer analysis (ARISA):**

ARISA targets the intergenic transcribed spacer (ITS) regions that are located between the 16S and 23S ribosomal genes. ARISA allows higher resolution in community profiling than 16S- or 23S-based techniques, because ITS regions display higher heterogeneity in both length and nucleotide sequence than their flanking genes. Qua et al., (2009) studied the population dynamics from the membrane reactor treating bromoamine acid wastewater using RISA technique.

1.5.2.1.3 **Clone library method**

The classical approach to study bacterial diversity is amplification of 16S r-RNA gene, cloning and sequencing rRNA genes from environmental DNA samples. The obtained sequences are compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP) and Greengenes. Clone libraries of 16S rRNA genes permit an initial survey of diversity and identify novel taxa. Studies have shown that environmental samples like soil may require over 40,000 clones to document 50% of the richness (Dunbar et al., 2002). However, typical clone libraries of 16S rRNA genes contain fewer than 1,000 sequences and therefore reveal only a small portion of the microbial diversity present in a sample. Despite its limitations (e.g., labor-intensive, time-consuming, and cost factor), clone libraries are still considered the “gold standard” for preliminary microbial diversity surveys (Desantis et al., 2007). Several diversity indices were also determined to estimate bacterial diversity.

1.5.2.1.4 **Functional metagenomics**

Construction of metagenomics libraries offers the possibility to retrieve novel sequence or novel gene from the environment, whereas methods relying on PCR amplification are based on prior knowledge of gene sequences. Construction of metagenomics libraries follows (i) generation of DNA fragments of appropriate size, (ii) ligation of the fragments into an appropriate cloning vector (e.g., cosmid, fosmid or bacterial artificial chromosome (BAC) vectors), (iii) introduction of the recombinant vectors into a suitable bacterial cloning host and (iv) screening of clones harboring particular activities, or containing specific sequences (Daniel, 2005). Metagenomic libraries could be screened
either by sequence-driven metagenomic analysis that involves massive high-throughput sequencing or by functional screening of expressed phenotypes. In function-driven metagenomic analysis (functional metagenomics), libraries are screened based on the expression of a selected phenotype on a specific medium. A wide variety of biochemical activities have been discovered in environmental metagenomic libraries. For example, novel antibiotics (e.g., turbomycin, terragine), microbial enzymes (e.g., cellulases, lipases, amylases), and proteins (e.g., antiporters) have been identified in soil metagenomic libraries (Rondon et al., 2000).

1.5.2.1.5 Quantitative PCR (Q-PCR)
Q-PCR, or real-time PCR, has been used in microbial investigations to measure the abundance and expression of taxonomic and functional gene markers (Bustin et al., 2005; Smith and Osborn 2009). Unlike traditional PCR, which relies on end-point detection of amplified genes, Q-PCR uses either intercalating fluorescent dyes such as SYBR Green or fluorescent probes (TaqMan) to measure the accumulation of amplicons in real time during each cycle of the PCR. Recently, a quantitative fingerprinting method combining real-time PCR and T-RFLP was developed for simultaneous determination of microbial abundance and diversity within a complex wastewater community (Yu et al., 2005).

1.5.2.1.6 DNA-DNA hybridization
This standard technique allows for comparison and measurement of genomic similarities between the total genome of two species under standardized conditions. However, this method is time-consuming and applicable in only culturable isolates. DNA microarrays have been used primarily to provide a comprehensive view of microbial communities in environmental samples. The PCR products amplified from total environmental DNA is directly hybridized to known molecular probes, which are attached on the microarrays. The hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. The microarray technique allows samples to be rapidly evaluated, which is a significant advantage in microbial community analyses. 16S rRNA gene Microarrays (PhyloChip) and Functional Gene Arrays (FGA) are the two major categories of the probes available. PhyloChip technology has been used for rapid
profiling of environmental microbial communities during bioterrorism surveillance, bioremediation, climate change and source tracking of pathogen contamination (Rastogi et al., 2010). Another microarray chip, FGA not only reveals the community structure, but also sheds light on the in situ community metabolic potential. FGA contains probes from genes with known biological functions; therefore, they are also useful in linking microbial community composition to ecosystem functions. In addition, environmental samples can be incubated in the presence of a radioactively labeled substrate prior to hybridization, in order to identify microorganisms involved in the metabolism of a specific substrate (“isotope arrays”) (Adamczyk et al., 2003; Wagner et al., 2006).

1.6 Marine microbial ecology: current status and perception
Marine environment is dominated by microscopic protists and prokaryotes. However, it is widely accepted that current and traditional culture based techniques are inadequate to study complete microbial diversity from environmental samples. Our understanding of marine microbial communities has increased enormously over the past two decades as result of culture independent studies (Fox et al., 1977). Microbial community structure from marine ecosystem are among the first to be investigated using culture-independent genomics approaches by Giovannoni et al. (1990), they found that communities are complex and contain heterogeneous micro-organisms including viruses, bacteria, archaea, and eukaryotic microorganisms. Phylogenetic analyses have identified numerous novel DNA sequences and phylogenetic groups from marine ecosystem. Similarly many other researchers also describe bacterial diversity from other marine samples using culture independent approach (Ward et al., 1992; Hugenholtz, 1998; Hentschel et al., 2001). This approach has dramatically changed our perspective in the area of microbial diversity leading to the development of a database that describes microbial diversity at the genetic level. Similarly, molecular methods have also been applied in the identification of isolated fungi and fungal diversity has been studied using 18Sr-RNA gene sequencing in environmental samples (Anderson and Cairney, 2004). In addition to these, Metagenomic libraries (Sequenced based and functional based approach) construction have led to other important discoveries for biotechnological applications such as production of the so called “cell factories” and novel genes encoding enzymes as well as identification of new drugs. Beja et al. (2000) identified a new class of genes of the rhodopsin family, named
proteorhodopsin, from an uncultivated Alphaproteobacteria SAR86. At that time, this rhodopsin family was known to exist only in extremely halophilic (salt-loving) Archaea and had never before been observed in cultured bacteria. Unlike the rhodopsin gene from Archea that does not express properly in laboratory strains, the proteorhodopsin gene from SAR86 expressed in *E. coli* and it functioned as a light-driven proton pump (Beja et al., 2001). Functional metagenomics focuses retrieve some novel gene responsible for enzymes production such as esterases, lipases, chitinases, etc. and other biotechnology products (Kennedy et al., 2010).

Advent of next-generation sequencing tools has increased the potential of metagenomics and environmental microbiology from marine ecosystem. One of the most extensive microbial metagenomic studies in the ocean was the shotgun sequencing of microorganisms of size ranges from 0.1 to 3.0 μm in the Sargasso Sea in the Atlantic Ocean near Bermuda (Venter et al., 2004). Their study generated almost 2 million sequence reads, yielding over 1.6 billion base pairs of raw DNA sequence. Based on sequence relatedness and unique rRNA gene counts, the analysis suggested that these DNA fragments were derived from at least 1800 genomic species including 148 previously unknown bacterial phylogenetic types (Venter et al., 2004). Up to now, there are 76 marine metagenomic projects available online, 23 of which have been completed based on the Genomes Online Database (http://www.genomesonline.org/cgi-bin/ OLD). Additionally, more and more marine microorganisms are becoming subjected to whole genome sequencing. Recently, a metagenomic study of the marine planktonic microbiota yields an extensive dataset consisting of 7.7 million sequencing reads (6.3 billion bp) which predicts 6.12 million proteins. These predictions add tremendous diversity to known protein families and cover nearly all known prokaryotic protein families, which provide a powerful protein database for identifying proteins in the marine ecosystem (Wang et al., 2013).

In addition to these, coastal areas are also repositories for waste discharges that accumulate a range of contaminants including hydrocarbons, pesticides, excess organic matter, and heavy metals. Traditionally, Pollution at coastal area has revolved around the use of benthic macrofaunal communities as indicators of sediment health. However, the analysis methods are time-consuming, labour intensive and require substantial taxonomic
expertise (Lear et al., 2009). Macrofaunal communities may also display limited change between pristine and contaminated sites, even when high throughput pyrosequencing is used for species observation (Chariton et al., 2010). Hence, assessment of bacterial communities, which comprise the bulk of biomass and chemical activity in sediments (Nealson, 1997), may be more applicable when examining contaminant impacts across different environments with wide ranging environmental conditions. From last decade, bacterial community structure has been also characterized from polluted sea coastal ecosystem such as Adriatic Sea, South China Sea (SCS) and Baltic Sea and shift in microbial community composition was observed due to environmental stress (Paisse et al., 2008; Besaury et al., 2012).

1.7 Anthropogenic pollution in marine ecosystem

Coastal zone of sea ecosystem, which occupies the upper edge of the World’s coasts extending over 1,600,000 km, is probably the most important coastal habitat including biological productivity and economic value; it comprises rocky platforms, sandy beaches, mudflats, estuaries, salt marshes, mangrove forests, certain coral reefs and human-made infrastructures (Bertness et al., 2001). However, coastal zones of the world’s oceans are increasingly subjected to the discharge of human waste products, ranging from domestic to industrial effluents. The discharge of excessive nutrients from municipal and industrial waste waters, urban and agricultural run leads to the enrichment of inorganic and organic material in marine waters. These organic pollutants are finally subjected to marine ecosystem. These input and accumulation of pollutant compounds is seen as a major threat for the sea coast ecosystem, resulting in eutrophication, Eutrophication can be defined as the process by which increasing nutrients cause a change of the nutritional status of a given body of water (Richardson and Jürgensen, 1996) which presents one of the major stresses to the marine environment. In addition to this, global climate change due to pollution is increasing sea-surface temperature and northward migrations of species. Increasing carbon dioxide (CO₂) levels reduce the pH of sea water and increase the solubility of calcium carbonate with potentially dramatic consequences for calcifying organisms, such as corals, mollusks, coccolithophorids, pteropods and forams. Eutrophication also resulted is a loss of sea grasses and related estuarine and marine
vegetation and the build-up of pathogenic bacteria and viruses. Recreational areas along the coasts become both public health hazards and an aesthetic loss for communities. Moreover, marine systems are more highly interconnected than terrestrial systems, so an alteration in microbial equilibrium in one part of the ocean can affect a geographically remote area. They have detrimental effects on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains (biomagnification) and in some instances, serious health problems and/or genetic defects in humans (Meyereil and Oster, 2000). The biological treatment, Bioremediation should be a more efficient, financially affordable and adaptable choice for removal of xenobiotic compounds compared to physicochemical treatment because it presents potential advantages such as the complete degradation of the pollutants, lower treatment cost, greater safety and less soil disturbance (Habe and Omori, 2003).

1.8 Bioremediation: Toward credible technology

Bioremediation utilizes the microbial ability to degrade and/or detoxify chemical substances such as petroleum products, aliphatic and aromatic hydrocarbons (including polycyclic aromatic hydrocarbons and polychlorinated biphenyls), industrial solvents, pesticides and metals. The presence of a large number of diverse bacterial species in environment expands the capability to degrade wide range of anthropogenic pollutants. It is an economical, versatile, environment-friendly and efficient treatment strategy and a rapidly developing field for environmental restoration. A well-known example of bioremediation which highlighted the usefulness of this treatment strategy and accelerated its development is the biological clean up (in addition to physico-chemical methods) of a large accidental oil spill by the tanker *Exxon Valdez* which ran around on Bligh reef in the Gulf of Alaska in March 1989, spilling approximately 41,000 m$^3$ of crude oil and contaminating about 2000 km of coastline. The *Exxon Valdez* and other similar incidents demonstrated the usefulness of bioremediation as a fairly complete solution to oil contamination. However, in contrast to these, Bioremediation could not be successful in treatment of Seal Beach and other ecosystem. They observed that there are many setbacks in the implementation of bioremediation technologies which should be studied and optimized (Swannell et al., 1996).
1.8.1 Biological factors affecting the biodegradation of hydrocarbons

Hydrocarbons in the environment are biodegraded primarily by bacteria and fungi. Individual organisms can metabolise only a limited range of hydrocarbon substrates, but bacterial consortium harbors broad enzymatic capacities that are capable of degrading mixtures of hydrocarbons such as crude oil and high molecular weight (HMW) PAHs in soils, freshwater and marine environments (Habe and Omori, 2003). An appropriate inoculum for bioaugmentation (the addition of appropriate microbes) in soils should contain a mixture of hydrocarbon degrading organisms that attack the range of hydrocarbon contaminants on site. Bioaugmented organisms should be compatible with indigenous flora of polluted ecosystem. Therefore analysis of bacterial community structure is essential for completion of successful bioremediation.

1.8.2 Physico-chemical factors affecting the biodegradation of hydrocarbons

1.8.2.1 Temperature

Temperature influences hydrocarbon biodegradation by its effect chemical composition of the hydrocarbon, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community. At low temperatures, the viscosity of the oil or hydrocarbon increases, the volatilization of toxic short-chain alkanes is reduced and their water solubility is increased, delaying the onset of biodegradation. (Margesin et al, 2003).

1.8.2.2 Concentration of the oil or hydrocarbons

High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, decrease availability of nutrients and oxygen which resulted in decreased biodegradation (Fusey and Oudot, 1984). The rates of mineralization of the higher-molecular-weight aromatic hydrocarbons, such as naphthalene and phenanthrene, are related to aqueous solubilities rather than total substrate concentrations (Johnsen et al, 2005).

1.8.2.3 Effect of pH

An important factor for the biodegradation activity is the pH of the soil or sediment as it may affect the solubility and bioavailability of the pollutants and nutrients. Generally
PAH degradation by bacteria favors in alkaline condition, fungi prefer the acidic environment for growth and PAH degradation. Even smaller pH change also dramatically affects the degradation of PAHs; one-unit pH shift changed the degradation rates of phenanthrene 4-fold (Lu et al., 2011).

1.8.2.4 Effect of surfactant
Low water solubility and adsorption of PAHs to soil are two major factors that limit their availability of PAH to microorganisms. Surfactants mediate interaction between two immiscible phases because of their hydrophobic and hydrophilic moieties. A surfactant forms micelles above the critical micelle concentration and solubilize PAHs in water phase. Surfactant-enhanced remediation has been suggested as a promising technology for the remediation of contaminated soil. In situ, surfactant is pumped into a contaminated site by introduction at an injection point and removal from an extraction point. In laboratory studies, surfactant solution is directly mixed with PAHs in the reactors through stirring (Debarati et al., 2005).

1.8.2.5 Oxygen
Oxygen has been identified as the limiting factor of the biodegradation of petroleum in soil and gasoline in groundwater. Oxygen availability depends on the rate of microbial oxygen consumption, the type of soil. Degradation of hydrocarbons by micro-organisms involves the initial oxidation of the hydrocarbon by dioxygenase incorporating oxygen from molecular oxygen (Cerniglia, 1992). Therefore, aerobic conditions are required for the oxidation of hydrocarbons.

1.8.2.6 Nutrients
Hydrocarbons released into aquatic environments, which contain low concentrations of inorganic nutrients, often produces excessively high carbon/nitrogen and/or carbon/phosphorus ratios, which are unfavourable for microbial growth (Atlas, 1981). Adjustment of carbon/nitrogen/phosphorus ratios by the addition of nitrogen and phosphorus stimulates the biodegradation of crude oil and individual hydrocarbons in seawater and soil (Coulon et al., 2004).
1.8.2.7 Soil moisture

In soils, water contents between 50 and 80% capacity are generally optimal for microbial activity (Morgan and Watkinson 1989). At lower water contents osmotic and matric forces limit the availability of water and subsequently microbial growth was also retarded. Hydrocarbon contamination can further reduce the water-holding capacity of the soils, because oil coating on the surface of soil particles makes the soil more hydrophobic (Dibble and Bartha 1979).

1.9 Alang-Sosiya ship breaking yard

Alang-Sosiya ship-breaking yard, which was established near Bhavanagar, India in 1982 and is world’s largest ship breaking zone with annual turn over of US$1.3 billion (Fig 1.2). It has a moderate slope with a hard and firm rocky bottom, which facilitates the incoming ships up to the scrapping yard with minimum investment and risk factors. To put Alang’s history into global context, about 5,600 deep-sea ships were scrapped worldwide between 1994 and 2006, for an average of about 430 a year.

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Fig. 1.2 Geographical location of the study area of Alang– Sosiya and Mahuva on the Gulf of Cambay
At present, ship-breaking industry is producing around 2 million tones of re-rollable steel per annum. This has also led to the development of other ancillary business including re-rolling mills, oxygen plants, etc. Although, economical prosperity is gift of industrialization, however, there are two sides of a coin. The other side of the coin which was overlooked and has recently gained focus is the rise in pollution level of the state. The wastes generated by ship breaking activity and which is dumped at the site includes all kinds of miscible and immiscible wastes in the form of solids, liquids and gases which accumulates over the sediment first and then migrate incrementally to the tidal zone, sub-tidal zone, subsequently to the deep sea water and into the respective sediment. The main pollutant of the ship scrapping industry and its associated wastes constitutes metals total petroleum hydrocarbons (PHCs), total polycyclic hydrocarbons (PAHs) and other xenobiotic compounds (Tewari et al., 2001; Reddy et al., 2003; Reddy et al., 2005; Basha et al., 2007).

Among all these contaminants petroleum hydrocarbons and heavy metals are the most serious pollutants because of their toxicity and persistence (Samanta et al., 2002; Zanardi et al., 1999). This has led to the deterioration of physico-chemical properties of sea water and intertidal sediments. COD (Chemical Oxygen Demand) and BOD (Biological Oxygen demand), used as indicators of water quality (organic degradation and tension in the system), are present at high levels. The inter-tidal zone around Alang-Sosiya Ship Breaking Yard (ASSBY) has practically no vegetation. Mangroves disappeared many years ago, soon after the industry began. The sea of ASSBY (Alang Sosiya Ship Breaking Yard) has very poor biological production potential with very low phytoplankton pigment concentration, low zooplankton standing stock, very poor macro benthic standing stock. Exotic species might have been carried in with ballast water, which represent a serious biological risk. Species tolerant to petroleum hydrocarbons seem to have adapted better to the environmental stress. Thus microbial communities tolerant to contaminants are known to colonize at the sites which make the site a big attraction for researchers to study the microbial diversity existing there, as they can discover different types of microorganisms that can degrade the contaminants and hence can be used for the bioremediation of the contaminated site and. Therefore, ASSBY is a one of major sites for microbial diversity studies.
1.10 Polycyclic aromatic hydrocarbons (PAHs): an environmental concern
PAHs are aromatic hydrocarbons with two or more fused benzene rings. The common sources of PAHs in environment include natural as well as anthropogenic activities. Natural sources are forest, land fires, oil seeps, volcanic eruptions and exudates from trees. Anthropogenic sources of PAH include burning of fossil fuel, coal tar, wood, garbage, refuse, used lubricating oil and oil filters, municipal solid waste incineration and petroleum spills and discharge (Haritash and Kaushik, 2009). The simplest PAH is benzocyclobutene (C8H6) (Cerniglia, 1992). PAHs classify in two groups on the basis of their benzene ring, three benzene rings PAHs are known as low molecular weight PAHs and those containing more than three benzene rings are known as high-molecular-weight PAHs (Van Hamme et al., 2003). As the molecular weight or benzene ring of PAHs increased, their solubility in water and vapor pressure decreases, whereas melting and boiling point increase (Patnaik, 1999). High molecular weight (HMW) PAHs (four or more rings) sorbs strongly to soils and sediments and are more resistant to microbial degradation. Therefore, high-molecular-weight PAHs are more stable and are of higher toxicity than low-molecular-weight PAHs (Cerniglia, 1992). The chemical structures of some commonly studied PAHs are given in Fig. 1.3.

![Chemical structure of PAHs](image)

**Fig. 1.3 Chemical structure of PAHs**
PAHs were, perhaps, the first recognized environmental carcinogens. Recently, the U.S. Department of Health and Human Services listed 16 PAHs as being carcinogenic and mutagenic. Degradation of PAHs become major environmental concern due to their ubiquitous occurrence, recalcitrance, bioaccumulation potential, mutagenic and/or carcinogenic activity, the PAHs have gathered significant environmental concern. PAHs are found in various physical forms like solubilized state, patched or adsorbed on soils and sediment or deposited in pores of sediments and organic matter (Samanta et al, 2001).

1.11 Microbial degradation of PAHs

1.11.1 Bacteria

A number of bacterial species are known to degrade PAHs including Achromobacter, Acidovorax, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Burkholderia, Comamonas, Corynebacterium, Flavobacterium, Microbacterium, Micrococcus, Moraxella, Mycobacterium, Neptunomonas, Nocardia, Paenibacillus, Porphyrobacter, Pseudomonas, Ralstonia, Rhodococcus, Sphingomonas, Streptomyces, Vibrio and Xanthomonas (Walter et al., 1991; Schneider et al., 1996; Cerniglia, 1992; Peng et al., 2008; Keum et al., 2005)

The first step in the microbial degradation of PAHs is the action of dioxygenase, which incorporates atoms of oxygen at two carbon atoms of a benzene ring of a PAH resulting in the formation of cis-dihydrodiol, which undergoes rearomatization by dehydrogenases to form dihydroxylated intermediates. Dihydroxylated intermediates subsequently undergo ring cleavage and form TCA-cycle intermediates (Fig. 1.4). In the absence of molecular oxygen, alternative electron acceptors such as nitrate, ferrous and sulphate ions can be used to oxidize aromatic compounds and recent studies have shown the anaerobic degradation of PAHs under both denitrifying and sulphate reducing conditions (Chauhan et al., 2008). During the past decade, a variety of microorganisms has been isolated and characterized for the ability to degrade different PAHs, and new pathways for PAH degradation has been elucidated. However, a highly efficient biodegradation of synthetic dyes, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and other organic
pollutants can be achieved by mixed microbial cultures that combine degradative enzyme activities inherent to individual consortium members (Mikesková et al., 2012).

A major limiting factor in hydrocarbon degradation is the lack of bioavailability of the hydrocarbon due to poor solubility and leading to accumulation of toxic and carcinogenic pollutants. But in nature, a few organisms tackle the hydrocarbon uptake/degradation problem either by direct contact, e.g., attaching themselves to these compounds or production of surface active compounds (biosurfactants) (Prabhu and Phale, 2000).

Once the PAHs have entered into the cell, the next step is the transcription of the degradative genes to produce the required enzymes. Generally the degradative genes are found to be inducible (Naphthalene dioxygenase), being expressed under certain condition only. The inducer molecule is often the pathway substrate (phenanthrene) and/or a pathway intermediate (salicylate) but some are structural analogues of the natural effectors (gratuitous inducers) which can also induce the pathway even if they are not themselves substrates for the corresponding catabolic enzyme. Several genes encoding PAH catabolic enzymes have been characterized. These genes are organized into operons which may be localized on chromosomal DNA or large, self-transmissible, catabolic plasmids. Analysis of the PAH catabolic genes are of interest in both fundamental and applied contexts. Fundamental knowledge of the catabolic genes in different species of bacteria can give useful information about the evolution of the encoded enzymes’ sequence-structure function relationships and the evolution and diversity of the catabolic genes via horizontal gene transfer, transposition events, DNA fusion, point mutation etc. This would help us to understand the molecular mechanisms by which bacteria adapt to the xenobiotics. In applied terms, the genetic information would help us to monitor the bacterial populations that degrade PAHs in the contaminated sites, and to engineer bacteria for developing bioremediation strategies. However, most of the information about metabolic pathways, enzymes and genes has been restricted to LMW PAHs (Chauhan et al., 2008).

Many organisms also harbor chemotaxis mechanism which may be help in increasing bioavailability of pollutants toward microorganisms. Chemotaxis is defined as the migration of microorganisms under the influence of a chemical gradient. Some pollutant-degrading bacteria such as Pseudomonas putida G7, a naphthalene degrader, have been
demonstrated to show chemotactic behaviour. Another naphthalene- and salicylate-degrading strain, RKJ1, also shows the chemotactic property and the genes for both its catabolic and chemotactic properties are present on its plasmid, pRKJ1. It is presumed that the chemotactic properties make the toxic molecule more bioavailable to the degrading bacteria. Cells displaying chemotaxis can sense chemicals such as those adsorbed to soil particles in a particular niche and swim towards them; hence, the mass-transfer limitations that impede the bioremediation process can be overcome (Samanta et al., 2002).

Fig. 1.4 Proposed pathways for microbial catabolism of polycyclic aromatic hydrocarbons.

1.11.2 Fungi

A diverse group of fungi have the ability to nonspecifically degrade a wide range of PAHs some of them degrading lignin (ligninolytic) while others are non ligninolytic. White rot fungi are ligninolytic, and are known to degrade PAHs that include the potent
carcinogen benzo[a]pyrene and to detoxify PAH-polluted soils and sediments. A battery of extracellular enzymes responsible for the degradation of lignin, lignin peroxidases (Lips), manganese peroxidases (MnP) and laccases, is believed to be involved in the degradation of PAHs. *Phanerochaete chrysosporium, Bjerkandera adusta,* and *Pleurotus ostreatus* are the common PAH-degrading fungi. A non ligninolytic fungus, *Cunninghamamella elegans*, utilized cytochrome P450 monooxygenases for the initial attack on PAHs. The product, arene oxide, was further metabolized by epoxide hydrolase to form a dihydriodiol with the trans-configuration. Thus, cytochrome P450 monooxygenases seem to play an important role in PAH biodegradation by fungi. (Harayama S, 1997).

Prokaryotic and eukaryotic photoautotrophic marine algae (i.e. cyanobacteria, green algae, and diatoms) also have potential to metabolize naphthalene to a series of metabolites (Cerniglia et al., 1979) though there are indications that cis-hydroxylation of naphthalene by Cyanobacteria, *Oscillatoria* and *Agmenellum* spp. involve pathways similar to fungus. Warshawsky et al., (1998) found that *Selenastrum capricornutum*, a freshwater green alga metabolizes BaP to cis-dihydriodils using a dioxygenase enzyme system as found in heterotrophic prokaryotes. Certain algae have been reported to enhance the removal fluoranthene and pyrene when present with bacteria. Borde et al., (2000) first reported case of photosynthesis-enhanced biodegradation of toxic aromatic pollutants by algal–bacterial microcosms in a one-stage treatment. PAH is also removed by phytoremediation and composting (Haritash and Kaushik 2009).

1.12 Monitoring bioremediation

- Effluent toxicity test can be assessed by monitoring biological responses of aquatic protozoan. One such organism used is *Daphnia similis*. In a version of this test, effluent toxicity was evaluated by comparing the responses of *D. similis* in medium in presence of contaminated environment as well in pristine environment. *D. similis* is an indicator organism of choice basically because of its rapid behavioural and physiological response to possible hazardous substances (Burton, 1998).
Measurement of CO\textsubscript{2} is also indication of microbial respiration in contaminated environment using hydrocarbon as carbon source. Hydrocarbon degradation rates calculated from CO\textsubscript{2} production rates can provide an accurate estimate of biodegradation and provide data for continuous application (Zucchi et al., 2003).

Nucleic-acid-based techniques are preferred for such monitoring because they preclude isolation and cultivation of bacteria. Preferably, molecular techniques focus on catabolic genes that code for specific pollutant degrading enzymes. DNA Micro array, FISH, Real-time PCR, DNA-DNA hybridization use to monitor actively growing bacteria in polluted environment. An alternative molecular tool is to use biomarkers as specific tags for the identification of specific bacteria or gene activity. A biomarker is defined as a DNA sequence, introduced in an organism, which confers a distinct genotype or phenotype to enable monitoring activity of inoculated organisms in a given environment. Bioluminescent, fluorescent, and chromogenic marker genes, such as \textit{luxAB} (bacterial luciferase), \textit{gfp} (green fluorescent protein, GFP), and \textit{xylE} (catechol 2, 3 oxygenase) genes, as well as reporter genes are examples of promising biomarkers (Vincenza et al., 2007).

Several analytical methods are available to evaluate the concentration of a pollutant and its depletion in a contaminated site. They include gas chromatography (GC), mass spectrometry (MS), infrared (IR), fluorescence spectroscopy, luminescence techniques, HPLC and others. GC/FID is suitable for the identification and determination of contaminants and has the advantage that FID response is linear in a wide range of concentrations and not influenced by temperature change. GC/MS is suitable to identify intermediates during degradation. Fluorescence spectroscopy, although generally accepted as a powerful and sensitive analytical tool to determine aromatic pollutants such as PAHs, has, however, limited applicability because the spectra of complex mixtures cannot be often resolved adequately. Stable isotope probe (SIP) is another technique to monitor in situ bioremediation that relies on the changes in stable isotope composition of the molecule of interest (Vincenza et al., 2007).
PAHs degradation in microcosm reflects study in real environment. There are many definitions of ‘microcosm’. A typical one is that of an intact, minimally disturbed piece of an ecosystem brought into the laboratory for study in its natural state (Prichard and Bourquin, 1984). In such experiments, it is important to include appropriate controls, such as sterile soil to determine effect of indigenous flora on biodegradation. Similarly, the biodegradation potential of study in its natural state can be assessed by using slurry reactors (10–15% soil: water w/v), which offer several advantages over the soil microcosms. Due to more efficient mixing, aeration and improved substrate bioavailability, the duration of a treatability study can be significantly reduced. During the treatability study, microcosms tests are usually monitored regularly for petroleum hydrocarbon degradation, by either sacrificing whole microcosm systems or by subsampling techniques. The rate of hydrocarbon degradation was determined by GC, GC-MS or IR analysis.

1.13 Application of bioremediation techniques

Bioremediation of contaminated soil can be processed by two types (1) in situ, which is carried out on site without soil removal; and (2) ex situ, which involves removal and transportation of contaminated material to a different location where it is treated biologically. In situ technologies are advantageous because there is no need to remove the contaminated soil and thus costs are reduced; however, the challenge is to deliver the required amendments. The advantage of ex situ approaches, including biopiles and land farming, is that the process can be better controlled. On the other hand, ex situ methods can be costly and disruptive due to soil excavation. Most commonly currently available In situ and Ex situ processes are listed in Table 1.1.
<table>
<thead>
<tr>
<th>Method</th>
<th>Principles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioaugmentation</td>
<td>Addition of exogenous microorganisms with ability of degrading the contaminants that are recalcitrant to the indigenous microbiota</td>
<td>Stormo and Crawford, (1992).</td>
</tr>
<tr>
<td>Biostimulation</td>
<td>Addition of nutrients that stimulate the growth and development of indigenous microorganisms, increasing their metabolic activity, thus elevating the degradation</td>
<td>Anderson et al., (2003).</td>
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<tr>
<td>Bioleaching</td>
<td>Specific microorganisms like <em>Thiobacillus ferroxidans</em> and <em>T. thiooxidans</em> promote the metals solubilization</td>
<td>White et al., (1998); Mishra et al., 2008</td>
</tr>
<tr>
<td>Biofilters</td>
<td>Specific microorganisms like <em>Thiobacillus ferroxidans</em> and <em>T. thiooxidans</em> promote the metals solubilization</td>
<td>Loutseti et al., (2009)</td>
</tr>
<tr>
<td>Biopiling</td>
<td>A biopile or biocell is an aboveground mound of soil similar to a compost pile, often constructed on an impermeable liner and fitted with aeration pipes; it can be amended with nutrients and/or moisture to stimulate biodegradation, and may be mixed mechanically.</td>
<td>(Filler et al., 2001)</td>
</tr>
<tr>
<td>Bioventing</td>
<td>Combination of venting of soil to remove the volatile compounds with bioremediation that uses the oxygen to degrade the organic contaminants</td>
<td>Lee and Swindoll, (1993)</td>
</tr>
<tr>
<td>Composting</td>
<td>Nutrients are added to soil that is mixed to increase aeration and activation of indigenous microorganisms</td>
<td>Nagel et al., 1998</td>
</tr>
<tr>
<td>Phytoremediation</td>
<td>Use of plants to extract, sequestrate or decontaminate terrestrial or aquatic environments</td>
<td>Burken and Schnoor, (1996).</td>
</tr>
<tr>
<td>Landfarming</td>
<td>Land farming or land application is a larger-scale land treatment method where thin layers of excavated contaminated soil are spread on ground that is amended with nutrients and/or moisture and tilled to achieve aeration.</td>
<td>Al-awadhi et al., (1996); Mancini et al., (2005)</td>
</tr>
<tr>
<td>Rhizoremediation</td>
<td>The plant releases exudates that will increase the rhizospheric microorganisms that will help plant growth and the degradation of contaminants</td>
<td>Yee et al., (1998).</td>
</tr>
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</table>
1.14 Objectives of the present study

Considering the present problem of marine ecosystem at Alang-Sosiya ship breaking yard, the study was planned with following objectives.

- Analysis of bacterial community structure from sediment of Alang-Sosiya ship breaking yard as well as from coastal pristine sediment (collected from 10km away from Alang-Sosiya ship breaking yard) near Bhavnagar, Gujarat
- Analysis of bacterial community structure from sea water of Alang-Sosiya ship breaking yard as well as from pristine sea water (which is not affected by ship breaking industries, 10km away from Alang-Sosiya ship breaking yard) near Bhavnagar, Gujarat
- Isolation and enrichment of PAH degrading bacterial consortia and isolates from Alang-Sosiya ship breaking yard, Bhavnagar and other hydrocarbon contaminated sediment.
- Optimization of various parameters to achieve better hydrocarbon degradation by isolated bacterial strain and bacterial consortia and effect of other xenobiotic compounds on PAH degradation.
- Application of developed bacterial consortium (ASP) and bacterial strain in stimulated microcosm for PAH degradation.
1.15 References


Chapter 1: General Introduction


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