Application of Molecular Microbiology Techniques in Bioremediation of Hydrocarbons and Other Pollutants

Chioma Blaise Chikere[1*]

[1]*Department of Microbiology, University of Port-Harcourt, P.M.B. 5323, Port Harcourt, Rivers State, Nigeria.*

Author's contribution

This review article was prepared by the author who drafted and critically revised the manuscript.

ABSTRACT

Molecular microbiology techniques have revolutionized microbial ecology by paving the way for rapid, high-throughput methods for culture-independent assessment and exploitation of microbial communities present in complex ecosystems like crude-oil/hydrocarbon polluted soil. The soil microbial community is relatively diverse with a high level of prokaryotic diversity. This soil species pool represents a gold mine for genes involved in the biodegradation of different classes of pollutants. Currently, less than 1% of this diversity is culturable by traditional cultivation techniques. The application of molecular microbiology techniques in studying microbial populations in polluted sites without the need for culturing has led to the discovery of novel and unrecognized microorganisms and as such complex microbial diversity and dynamics in contaminated soil offer a resounding opportunity for bioremediation strategies. The combination of PCR-amplification of metagenomic DNA, microbial community profiling techniques and identification of catabolic genes are ways to elucidate the composition, functions and interactions of microbial communities during bioremediation. In this review, an overview of the different applications of molecular methods in bioremediation of hydrocarbons and other pollutants in environmental matrices and an outline of the recent advances in this fast-developing field are given.

*Corresponding author: Email: chioma.chikere@uniport.edu.ng;

1. INTRODUCTION

Modern molecular techniques provide an exciting opportunity to overcome the requirement for culturing and have greatly increased our understanding of bacterial diversity and functionality during bioremediation of oil-polluted soils. Unfortunately, only a fraction of the microorganisms involved in the biodegradation of pollutants in different ecosystems can currently be cultured using standard laboratory agars and conditions [1-10]. Comparisons between classical culture-dependent and molecular/metagenomic methods have revealed that only about 1% of the total microorganisms are amenable to culture [11]. In a few cases, understanding the phylogenetic diversity has led to the development of improved culturing methods such as extended incubation periods, improvement of media and growth conditions such as pH, temperature and atmosphere [10,12]. However, estimating the total microbial diversity/dynamics in any environment is a persisting challenge especially during bioremediation to cleanup hydrocarbon contaminants [13-15]. It has been observed using cultivation-based techniques that fast-growing strains best adapted to particular culture conditions grow more than those which are not and therefore do not accurately represent the actual microbial community composition during aerobic biodegradation of pollutants like crude oil [16-27]. To elucidate how microbial communities change due to the effects of environmental disturbances like pollution, investigations need to rely on rapid methods that can characterize cellular constituents such as nucleic acids, proteins (enzymes) and other taxa-specific compounds [28-31]. These molecules can be extracted directly from the soil without the need for culturing and thus can be used to elucidate the microbial community composition in such polluted environments during bioremediation [32]. This review evaluates current molecular microbiology applications in the assessment of microbial diversity and dynamics in polluted ecosystems during bioremediation in order to identify predominant microbial communities or degradative genes.

2. POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) was first discovered and performed with non-heat-stable polymerase, thus subsequently new polymerase was added after each heat cycle. Then, the Taq polymerase was added into the mix, resulting in overwhelming success of PCR [5].

In 1988, a thermally stable DNA polymerase from a thermophilic bacterium was used to perform PCR. This extremely sensitive technique allows the amplification of millions of copies of a portion of a desired gene, entire gene or gene clusters with high fidelity within 3 to 4 h facilitating their cloning and study. It is the most widely used method for the amplification of 16S rRNA, or its gene, prior to fingerprinting studies. The 16S rRNA gene is essential as it encodes the small subunit of the prokaryotic ribosome and is therefore present in all prokaryotes. In bacteria, the rRNA genes are transcribed from the ribosomal operon as 30S rRNA precursor molecules and then cleaved by RNase III into 16S, 23S and 5S rRNA molecules [5,33]. The ribosomal operon size, nucleotide sequences, and secondary structures of the three rRNA genes are conserved within a bacterial species [12]. Since 16S rRNA is the most conserved of these three rRNAs, it has been proposed as an "evolutionary clock", which has led to the reconstruction of the tree of life [34]. For the past two decades, microbiologists have primarily relied on 16S rRNA gene sequences (hereafter,

16S sequences) for the identification and classification of bacteria. The 16S sequence analysis is used in two major applications: (i) identification and classification of isolated pure cultures and (ii) estimation of bacterial diversity in environmental samples without culturing through metagenomic approaches. Several criteria that make the 16S rRNAs and their genes the most widely studied phylogenetic molecular markers include:

- The function of ribosomes has not changed for about 3.8 billion years;
- The rRNA genes are present in all living organisms;
- The size of 1,540 nucleotides makes them easy to analyze;
- There are extensive databases available (e.g. the GenBank) for pairwise and/or multiple comparative analysis;
- The genes act as molecular chronometer;
- The genes allow for culture-independent analysis of unknown microbial communities;
- The primary structure is an alternating sequence of invariant DNA, more or less conserved to highly variable regions, and
- Lateral gene transfer is either totally absent or exceedingly rare [2].

PCR-based methods have also been used in the detection and quantification of microorganisms found in soil and water [35]. The technique can also be applied for the analysis of catabolic genes involved in the biodegradation of organic contaminants [36]. With PCR, phylogenetic relationships can be assessed by pairwise and/or multiple alignments/similarities (this can be done by submitting unknown sequences to the GenBank 16S rRNA sequences database in National Center for Biotechnology Information [NCBI] website http://www.ncbi.nlm.nih.gov/blast/). One hundred percent similarity found between a pair of 16S rRNA sequences obtained from comparative tools such as BLAST (basic local alignment search tool) indicates very close relatedness, if not identity of the investigated organisms. The lower the value, the more unrelated the compared organisms [2,12]. For the detection of organisms or genes from contaminated environments, simple and/or multiplex PCR techniques can be used. Simple PCR uses a pair of primers in a single amplification reaction, while multiplex PCR uses multiple primer pairs simultaneously to amplify several genes in a single reaction [37-40]. PCR amplification is dependent on the extraction and purification of nucleic acids of sufficient yield and quality from environmental samples. Insufficient lysis of cells could result in the preferential extraction of DNA from Gram-negative bacteria, while excessively harsh treatments may result in the shearing of DNA from readily lyzed cells [41]. In addition, PCR amplification efficiency can severely be hampered by the presence of inhibitory substances which are co-extracted with nucleic acids which include humic acids, organic matter and clay particles [42]. Methods employed for sample collection, transportation and storage prior to nucleic acid extraction are important in the way that bias may be introduced into subsequent microbial analysis of native communities [43] of particular concern is the temperature at which samples are stored and their exposure to oxygen [44]. With extended sample storage, both these factors may alter the microbial composition hence the need to extract DNA/RNA after sample collection [41].

Margesin et al. [45] studied the prevalence of alkane and PAH-degradative genes in hydrocarbon contaminated and pristine Alpine soils using PCR amplification and hybridization analysis of different alkane monooxygenases and aromatic dioxygenases. They found out that there was a statistically significant correlation between the level of contamination and the prevalence of these genotypes in the Alpine soils. Alquati et al. [46] used the 16S rRNA and naphthalene dioxygenase genes amplified by PCR to identify

naphthalene degrading bacteria from a petroleum contaminated soil. Of the thirteen isolates they obtained belonging to *Rhodococcus*, *Arthrobacter*, *Nocardia* and *Pseudomonas* genera, nine showed PCR fragment amplification with naphthalene dioxygenase specific primers of *Rhodococcus* spp. while four were detected with primers of naphthalene dioxygenase specific for *Pseudomonas* spp. Other independent studies have also used PCR amplification of different hydrocarbon degradative enzymes using specific primers to study the diversity and composition of bacterial populations in hydrocarbon contaminated environments [47-56, 71]. Knaebel and Crawford [57] applied multiplex PCR to detect the petroleum-degrading microbial population in a petroleum contaminated soil. Baldwin et al. [58] Also employed a multiplex PCR technique for the detection of naphthalene dioxygenase, biphenyl dioxygenase, toluene dioxygenase, xylene monooxygenase, phenol monooxygenase and ring-hydroxylating toluene monooxygenase genes in a single PCR reaction. Although multiplex PCR can save time and resources in the detection of microorganisms or genes involved in biodegradation, successful application depends on the combination of several primer pairs being able to perform reliably in a single reaction. Primer dimer formation between the various primers is more likely to occur and this may lead to poor sensitivity and preferential amplification of certain targets [59]. Another variant of the PCR technique which can simultaneously detect and quantify the amplified product while the reaction is occurring is a real-time PCR. This approach enables the detection and quantification of PCR amplicons during the early exponential phase of the reaction [60,61]. It involves the use of fluorescent markers, and the amount of fluorescence measured at the end of each cycle is directly related to the amount of product in the PCR reaction. Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or C_T. Since the C_T value is measured in the exponential phase when reagents are not limited, real-time PCR can be used to reliably and accurately calculate the initial amount of template present in the reaction. The C_T of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, C_T. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, C_T. This relationship forms the basis for the quantitative aspect of real-time PCR [62,63]. Real-time PCR has been used in several environmental studies such as the monitoring of carbazole 1,9a-dioxygenase gene (*carAa*) numbers in soil slurry microcosms [64], the measurement of the alpha-subunit of benzylsuccinate synthase gene (*bssA*) and the atrazine catabolic gene (*atz*) [65, 66] and the identification and quantification of the arsenate reductase gene (*arsC*) in soil and aromatic oxygenase genes [67,68]. Real-time PCR targeting the 16S rRNA genes and *Dehalococcoides* reductive dehalogenase (*RDase*) gene was used in the monitoring of *Dehalococcoides* strains [69]. It has also been used in quantifying the proportion of microorganisms containing alkane monooxygenase and the subsequent assessment of microbial community changes in hydrocarbon-contaminated Antarctic soil [70]. The advantages that real-time PCR offers include speed, sensitivity, accuracy and the possibility of robotic automation [70]. Although real-time PCR can measure gene quantity, the results obtained do not link gene expression with a specific measurable microbial activity or population. RNA extracted from soil and water samples are low in yield and often do not represent the soil microbial population [63]. Also, specific probes used in the amplification reactions may fail to capture the sequence diversity that is present within environmental samples [65]. PCR molecular techniques have completely revolutionized the detection of

DNA/RNA especially in microbial ecological studies. However, differential amplification of target genes such as 16S rRNA can bias PCR-based diversity studies [41]. For example, sequences with lower guanine plus cytosine content are thought to separate more efficiently in the denaturing step of PCR and hence could be preferentially amplified [41]. Also products seen on gels or in real-time may be as a result of artefacts or chimeric PCR product formation [72]. PCR is a very sensitive technique and in some cases may produce false-positive signals due to contamination [73]. Quantitative reverse transcription PCR (qRT-PCR) is used for monitoring transcription of a specific gene. This technique first uses the enzyme reverse transcriptase to produce a complementary DNA (cDNA). The primers used to prime the reverse transcriptase reaction are random hexamers. Then PCR amplifications of the cDNA are done, using specific primers to amplify the cDNA corresponding to the RNA being quantified. Where this differs from reverse transcriptase-PCR is that an attempt is made to estimate the amount of cDNA template and hence the amount of original RNA by measuring the rate of accumulation of PCR product rather than the amount of end product which is measured in standard PCR amplifications [5]. Alonso-Gutierrez et al. [74] Used a RT-PCR to analyze the alkane degrading properties of *Dietzia* sp., a key player in the Prestige oil spill biodegradation. According to them, three putative alkane hydroxylase genes (one *alkB* homologue and two *CYP153* gene homologues of cytochrome *P450* family were PCR-amplified from the bacterium of which *alkB* and *CYP153* gene expression were observed to be constitutive in this alkane degrader. In an earlier investigation, [75] examined thoroughly bacterial communities from shoreline environments (Costa da Morte, Northwestern Spain) affected by the Prestige oil spill by a triple-approach method based on different cultivation strategies and culture-independent techniques such as PCR, DGGE and screening of 16S rRNA gene clone libraries. Their results revealed that members of the *α-proteobacteria* and *Actinobacteria* were the prevailing groups of bacteria detected among the alkane and polyaromatic hydrocarbon degraders. For PCR analysis to be successful, critical analysis of primer sets to be used is necessary to ensure that the target gene is efficiently amplified [76, 77].

3. DENATURING   GRADIENT   GEL   ELECTROPHORESIS (DGGE)/TEMPERATURE GRADIENT GEL ELECTROPHORESIS (TGGE)

Denaturing gradient gel electrophoresis (DGGE) was introduced in 1993 by [78] as a fingerprinting technique very useful for analysing complex microbial communities. DGGE was not unknown in science as it was already developed in the 1980s for the detection of point mutations in defined genes [79].

DGGE [80,81] or TGGE [82,78] separate PCR-amplified 16S rRNA fragments of the same length but with different base pair compositions. The separation of bands in both DGGE and TGGE is dependent on the decreased electrophoretic mobility of partially melted double stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants or a linear temperature gradient [78,83,84]. A GC-rich sequence (known as GC clamp) is usually attached to the 5′-end of either the forward or reverse primer to prevent complete melting of the DNA fragments [85]. The PCR-amplified DNA fragments are generally limited in size to 500 bp and are separated on the basis of sequence differences, not variation in length. The number of bands produced during DGGE or TGGE is proportional to the number of dominant species in the sample. The final result of the electrophoresis of the mixed amplicons from a complex community through denaturing gradients will be a fingerprint consisting of bands at different migration distances in the gel. The DGGE/TGGE is a method of choice when the desired information does not have to be as phylogenetically exhaustive as that provided by 16S rRNA gene clone libraries, but is still

precise to determine the dominant members of microbial communities with average phylogenetic resolution [86]. For environmental or contaminated source samples where microbial diversity is largely unknown [87], DGGE/TGGE technique provides the opportunity for the identification of the microbial population through the excision of selected bands followed by their reamplification, cloning and sequencing that can lead to the phylogenetic affiliation of the ribotypes [88,89,79]. DGGE in particular has been widely used for the assessment of microbial community structure in contaminated soil and water in a number of studies [90-99]. Apart from microbial community profiling, the DGGE technique has also been used to examine gene clusters such as dissimilatory sulphite reductase beta-subunit (*dsrB*) genes in sulphate-reducing bacterial communities [100] and benzene, toluene, ethylbenzene and xylene (BTEX) monooxygenase genes from bacterial strains obtained from hydrocarbon-polluted aquifers [101]. Coulon et al. [104] used DGGE analysis of reverse-transcribed bacterial 16S rRNA from the upper 1.5cm of a hydrocarbon polluted sediment in coastal mudflats to study the central role of dynamic tidal biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms in the biodegradation of hydrocarbons. Their investigation revealed the presence of phylotypes associated with aerobic straight chain and polycyclic hydrocarbons degradation such as *Cycloclasticus*, *Alcanivorax*, *Oleibacter* and *Oceanospirillales* strain ME113.

The main advantages of DGGE/TGGE are that; it enables monitoring of spatial/temporal changes in microbial community structure and provides a simple view of dominant microbial species within a sample. The limitations of DGGE/TGGE in microbial community studies include; sequence information derived from microbial populations is limited to 500 bp fragments of 16S rRNA sequences which may lack the specificity required for the phylogenetic identification of some organisms [59]. Due to the existence of multiple copies of rRNA in an organism, multiple bands for a single species may occur [102,103]. In addition, different 16S rRNA sequences may have identical mobilities. Another shortcoming of PCR is that band intensity may not truly reflect the abundance of microbial population (strong band may just mean more copies of a particular gene from the same organism) and perceived community diversity may be underestimated.

4. AMPLIFIED RIBOSOMAL DNA RESTRICTION ANALYSIS (ARDRA)

In amplified ribosomal DNA restriction analysis, PCR-amplified 16S rRNA fragments are digested or cut at specific sites with restriction enzymes and the resulting digest separated by gel electrophoresis. Different DNA sequences will be cut in different locations and will result in a fingerprint unique to the community being analyzed [79,11]. Divergence of the community rRNA restriction pattern on a gel is highly influenced by the type of restriction enzyme used [105]. Banding patterns in ARDRA can be used to screen clones or be used to measure bacterial community structure [42]. ARDRA is simple, rapid and cost-effective, and as a result has been used in microbial identification [106-108] and microbial community studies [109,105,110,111]. Microbial community composition and succession in an aquifer exposed to phenol, toluene and chlorinated aliphatic hydrocarbons were assessed by ARDRA with the aim of identifying the dominant microbial community involved in the biodegradation of trichloroethene (TCE) following biostimulation [112]. In another study, [105] used ARDRA to examine the microbial differences in activated sludge from treatment plants fed on domestic or industrial wastewater. It was observed that the bacterial communities in activated sludge were different from industrial and domestic waste water treatment plants. Hohnstock-Ashe et al. [113], using ARDRA as a fingerprinting technique also observed that the microbial community composition in well waters contaminated with TCE had shifted toward a highly diverse community dominated by *Dehalococcoides*

ethenogenes-like microorganisms. Babalola et al. [111] used ARDRA to study the phylogenetic relationships of actinobacterial populations associated with Antarctic valley mineral soils. Further sequencing of the of the amplicons restricted singly with endonucleases *RsaI*, *BsuRI* or *AluI* revealed that the phylotypes were most closely related to uncultured *Pseudonocardia* and *Nocardioides* spp. whereas complementary culture-dependent studies isolated more of *Streptomyces* spp. which were detected at a low frequency in the metagenomic analysis. ARDRA is useful for detecting structural changes in microbial communities but is unable to measure microbial diversity or detection of specific phylogenetic groups within a community fingerprinting profile [114]. Optimization with restriction enzymes is required and is often difficult if sequences are unknown. As a result, further optimization may be required to produce fingerprinting patterns characteristic of the microbial community [106,73]. In addition, banding patterns in diverse communities become too complex to analyze using ARDRA [42]. In recent studies, ARDRA has been combined with other molecular techniques such as T-RFLP and DGGE to characterize microbial communities from contaminated sources [115,116,11]. A major challenge in using ARDRA lies in the interpretation of the fingerprints obtained from complex microbial communities.

5. RIBOSOMAL INTERGENIC SPACER ANALYSIS (RISA)

The RISA method makes use of the length and sequence heterogeneities that are present in the intergenic spacer (IGS) region between the small (SSU) and the large subunit (LSU) rRNA genes in the rRNA operon [79]. It is a PCR-based technique that amplifies the region between the 16S and 23S rRNA genes [5]. The IGS region, depending on the species, has both sequence and length (50–1500 bp) variability [117] and this unique feature facilitates taxonomic identification of organisms [5,73]. RISA has been used to distinguish between different strains and closely related species of *Staphylococcus* [118,119], *Bacillus* [120,121], *Vibrio* [122,123], and other medically important microorganisms. In environmental studies, RISA has been used to detect microbial populations involved in the degradation of PAH at low temperature under aerobic and nitrate-reducing enriched soil conditions [124]. RISA has also been used to define microbial diversity and community composition in freshwater environments [125]. RISA is a very rapid and simple fingerprinting method but its application in microbial community analysis from contaminated sources is limited partly due to the limited database for ribosomal intergenic spacer sequences is not as large or as comprehensive as the 16S sequence database [73]. As a result of the limited database, community analysis using RISA could have reduced utility for the identification of unknown or unculturable microbial species from contaminated sources. Furthermore, RISA sequence variability may be too great for environmental applications. Its level of taxonomic resolution is greater than 16S rRNA and hence may lead to very complex community profiles [73]. A modification of RISA is ARISA (automated ribosomal intergenic spacer analysis) which detects the abundance and size of PCR amplicons by measuring the fluorescence emission of labelled primers. Fragment length and abundance can be measured by comparison with labelled internal standards [79]. Kostka et al. [126] used ARISA to determine the diversity of hydrocarbon degrading bacteria and bacterial community response in beach sands impacted by the Deepwater Horizon oil spill in the Gulf of Mexico. Their findings indicated that oil contamination from the Deepwater Horizon had a profound impact on the abundance and community composition of autochthonous bacteria in the beach sands. Also members of the *γ-proteobacteria* (*Alcanivorax*, *Marinobacter*) and *α-proteobacteria* (*Rhodobacteraceae*) were identified as the key players in crude oil degradation.

6. TERMINAL-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)

Terminal-restriction fragment length polymorphism is a major modification and improvement of the ARDRA method. The main advancement of T-RFLP over ARDRA lies in the fact that per organism detected, only the terminal restriction fragments (T-RFs) will be detected. The PCR primers used in T-RFLP analysis are fluorescently labelled at the 5′-terminus and the resultant PCR products are visualised and quantified [114,2,127]. T-RFLP relies on variations in the positions of restriction sites among sequences and the determination of the length of fluorescently labelled terminal restriction fragments by high-resolution gel electrophoresis on an automated DNA sequencer. The electropherogram represents the profile of a microbial community as a series of peaks varying in migration distance [79]. The use of fluorescently tagged primers limits the analysis to only the terminal fragments of the digestion [128]. This simplifies the banding pattern, hence enabling the analysis of complex communities as well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype [129]. Dojka et al. [130] monitored microbial diversity in a hydrocarbon- and chlorinated solvent- contaminated aquifer undergoing intrinsic bioremediation with T-RFLP and found sequence types characteristic of *Syntrophus* spp. and *Methanosaeta* spp. They hypothesized from their findings that the terminal step of hydrocarbon degradation in the methanogenic zone of the aquifer was aceticlastic methanogenesis, with these organisms existing in a syntrophic relationship. Bordenave et al. [131] studied bacterial community changes in microbial mats following exposure to crude oil using T-RFLP. Their results demonstrated clear succession of different bacterial populations with operational taxonomic units that were related to *Chloroflexus*, *Burkholderia*, *Desulfovibrio* and *Cytophaga* genera. In another related study, Bordenave et al. [132] used T-RFLP to assess the impact of the Erika oil spill on microbial communities in the Northern French Atlantic coast. Cluster analysis of T-RFLP fingerprints of eubacterial communities indicated that contaminated and uncontaminated communities evolved differently, suggesting that Erika oil had an impact on the evolution of the bacterial communities structure. Kaplan and Kitts [21] investigated bacterial succession in a petroleum land treatment unit with T-RFLP analysis. The T-RFLP patterns they obtained separated into five PCA clusters that reflected total petroleum hydrocarbons (TPHs) degradation phases and trends in aerobic heterotrophic bacterial counts. Further analysis revealed that *Flavobacterium*, *Pseudomonas* and *Azoarcus* phylotypes were the most abundant bacteria during the fast degradation phase. Restriction fragment length polymorphism (RFLP) analysis of 16S rRNA gene libraries was used by Allen et al. [133] to investigate the interdependence between geoelectrical signatures at underground petroleum plumes and the structures of subsurface microbial communities. The 16S rRNA gene inserts were screened for their RFLP patterns by using the restriction endonuclease MspI. Clones with the same RFLP patterns as well as those with sequence similarities of greater than 97% were considered to represent the same phylotypes. Their results revealed that the zone contaminated with residual hydrocarbons above the free-phase petroleum contained aromatic hydrocarbon degraders such as *Sphingomonas aromaticivorans*, *Brachymonas petroleovorans* and large populations of methylotrophs and methanotrophs. The microbial community composition of lake sediments contaminated with copper as a consequence of mine milling disposal over a 100-year period was studied using T-RFLP [134]. T-RFLP has also been used to characterize microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer [135] and dechlorinating bacteria from a basalt aquifer [136,137] using T-RFLP observed that the chronic presence of benzene in groundwater reduced bacterial diversity and community composition compared with that of clean groundwater sources. In addition, the reliability of T-RFLP for monitoring microbial populations characterized by low diversity and high relative abundances of a few dominant

groups was assessed in a hydrocarbon-polluted marine environment [138]. In contaminated soils, T-RFLP has also been used successfully in describing bacterial communities of polychlorinated biphenyl (PCB) contaminated soils [139] and microbial communities that reductively dechlorinate TCE to ethene [140]. Coulon et al. [104] investigated the dominance and dynamics of aerobic hydrocarbonoclastic bacteria in the biodegradation of hydrocarbons in oil-polluted coastal mudflats using T-RFLP employing the methods of Fahy et al. [137] but rather used a Genescan 500 TAMRA (5-carboxytetramethylrhodamine) internal standard. The use of automated detection systems and capillary electrophoresis in T-RFLP analysis allows high throughput and more accurate quantitative analysis of microbial community samples than with any of the other genetic fingerprinting methods. Despite the high resolution and sensitivity, T-RFLP is highly dependent on PCR amplification of 16S rRNA which is affected by DNA extraction method, PCR biases and the choice of universal primers [42]. Different enzymes will produce different community fingerprints and incomplete digestion by the restriction enzymes may lead to an overestimation of diversity [141,142]. It is therefore important to use at least two to four restriction enzymes [129] as T-RFLP profiles generated by a single restriction enzyme in a complex microbial community may lead to erroneous conclusions about the abundance of a particular strain or species [143]. A major drawback of T-RFLP in comparison with DGGE/TGGE is the impossibility of retrieving suitable phylogenetic information from the T-RFs generated since fragments are difficult to isolate and are usually too short to be sequenced properly and would not yield enough sequence information for phylogenetic analysis [79].

7. FLUORESCENT *IN SITU* HYBRIDIZATION (FISH)

An important step towards determining the diversity of microbes in environmental samples is to harness the information obtained from the direct sequencing of rRNA genes extracted from such samples. The full-cycle rRNA approach basically uses the sequence information of cloned, rRNA-encoding genes from environmental habitats to develop phylogenetic oligonucleotide probes that allow specific hybridization to the target region of the ribosomal RNA in fixed permeabilized cells. This technique is called fluorescent *in situ* hybridization (FISH) [79].

FISH is used to quantify the presence and relative abundance of microbial populations in a community sample. Microbial cells are treated with fixatives, hybridized with specific probes (usually 15–25 bp oligonucleotide-fluorescently labelled probes) on a glass slide then visualised with either epiflourescence or confocal laser microscopy [79,11]. Hybridization with rRNA-targeted probes enhances the characterization of uncultured microorganisms and also facilitates the description of complex microbial communities [144]. FISH is a taxonomic method that is mostly used for the examination of whether members of a specific phylogenetic affiliation are present and provides direct visualisation of uncultured microorganisms and also facilitates the quantification of specific microbial groups [86]. FISH use alone does not provide any insight to metabolic function of microorganisms. However, it can be coupled with other techniques such as micro autoradiography to describe the functional properties of microorganisms in their natural environment [145]. Two types of FISH probes based on conserved or unique regions of 16S rRNA genes can be developed; domain or-group specific probe and strain-specific probes. Domain-or-group specific probes discriminate or identify members of larger phylogenetic groups, while strain-specific probes quantify or assess the abundance of a specific species or strain within a microbial community [146]. Wagner et al. [147] and Wagner et al. [148] used both group- and species-specific rRNA-targeted oligonucleotide probes to define *Acinetobacter* from activated sludge. Richardson et al. [140] combined group-specific FISH and T-RFLP in the characterization of

microbial communities engaged in TCE biodegradation. From the FISH analysis, the authors observed that the number of organisms such as *Cytophaga*, *Flavobacterium* and *Bacteroides* were more abundant than the TCE degrader *Dehalococcoides ethenogenes* in the microbial consortium. However, the lack of functional gene analysis in the study meant that the relative abundance of these organisms and their ecological importance for TCE biodegradation could not be established. FISH techniques are often used in conjunction with other genetic fingerprinting methods such as DGGE [149-152] and T-RFLP [140,153,154,152] for the enumeration and characterization of microbial population from contaminated sources. The drawback of FISH is that a limited number of probes can be used in a single hybridization experiment and background fluorescence can be problematic in some samples [146,86]. A prior knowledge of the sample and the microorganisms most likely to be detected is necessary (i.e. rRNA sequence) for the design of specific probes.

A major obstacle of the standard FISH technique is its limited sensitivity because bacterial cells with reduced ribosome contents which often occur in oligotrophic environments like most soil habitats are not satisfactorily stained for the microscopic analyses. Two modifications of FISH used to increase sensitivity and reliability are catalyzed reporter deposition-FISH (CARD-FISH) and microautoradiography-FISH (MAR-FISH). In CARD-FISH, signal intensities of hybridized cells are increased by enzymatic signal amplification using horseradish peroxidase (HRP)-labeled oligonucleotide probes in combination with the tyramide signal amplification (TSA). TSA is based upon the patented catalyzed reporter deposition (CARD) technique using derivatized tyramide. In the presence of small amounts of hydrogen peroxide, immobilized HRP converts the labeled substrate (tyramide) into a short-lived, extremely reactive intermediate. The activated substrate molecules then very rapidly react with and covalently bind to electron rich regions of adjacent proteins. This binding of the activated tyramide molecules occurs only immediately adjacent to the sites at which the activating HRP enzyme is bound. Multiple deposition of the labeled tyramide occurs in a very short time (generally within 3-10 minutes). Subsequent detection of the label yields an effectively large amplification of signal [79]. The combination of FISH with microautoradiography allows for the identification of bacteria and concomitantly gives an indication of their specific *in situ* activity using suitable isotope-labeled substrates (especially β-emitters such as ^{14}C and ^{3}H [79]. The substrate uptake patterns in FISH-labeled bacteria can be investigated *in situ* in mixed natural communities at a single-cell level even if the bacteria are yet unculturable.

8. DNA MICROARRAY TECHNOLOGIES

Microarrays ('chips') containing nucleic acids as probes represent a major advancement in molecular detection technology. They are ideal for the high-throughput study of the sequence diversity of 16S rRNA genes as well as of functional genes in environmental samples [79,11].

DNA microarray technology is a very powerful taxonomic and functional tool that is widely used to study biological processes, including mixed microbial communities involved in pollutant degradation. This technique is similar to FISH, but provides a means for simultaneous analysis of many genes [155]. DNA microarray is a miniaturized array of complementary DNA probes (~500–5000 nucleotides in length) or oligonucleotides (15–70 bp) attached directly to a solid support, which permits simultaneous hybridization of a large set of probes complementary to their corresponding DNA/RNA targets in a sample [156]. Microarray technology has been used successfully in the analysis of global gene expression in pure culture studies [157,158], but is complicated for environmental samples due to

numerous challenges such as specificity, sensitivity and quantification [159]. Despite these challenges, three major forms of environmental microarray formats namely, functional gene arrays (FGA) [160,161,162], community genome arrays (CGA) [163,164] and phylogenetic oligonucleotide arrays (POA) [165,166,167] have been developed for microbial community analyses of environmental samples. Functional gene arrays (FGA) identify or measure genes encoding key enzymes in a metabolic process [168,161]. Such an approach provides vital information about the presence of important genes as well as the expression of the genes in the environment by measuring the mRNA [169]. Many studies have used the FGA approach to investigate microbial involvement in environmental processes such as nitrogen fixation and nitrification [170,171,169]. For biodegradation of contaminants, FGA techniques have been developed for the detection of specific aromatic oxygenase genes in a soil community degrading PCB [172] and the presence and expression of naphthalene-degrading genes from soil contaminated with PAH (Rhee et al. [161], 2004). Community genome array (CGA) is similar in concept to reverse sample genome probing (RSGP) [173] except that CGA uses nonporous hybridization surfaces and fluorescence based detection systems for high throughput analysis but shows decreased sensitivity [163]. Wu et al. [163] initiated the development and testing of CGA as a tool to detect specific microorganisms within a natural microbial community. CGA has been shown to achieve species-to-strain level differentiation depending on hybridization temperature and has an added potential for the determination of genomic relatedness of isolated bacteria [169]. The major disadvantage of CGA is that culturable organisms are needed in the array preparation thus making the CGA application on the field apart from laboratory studies almost impossible. POA rely on the use of 16S rRNA for the identification of microorganisms in the environment. Due to a high throughput capacity of microarrays and the availability of extensive rRNA sequence databases, POA provides a very convenient means of simultaneously identifying many microorganisms from a sample. Several studies have employed POA in environmental investigations of microbial populations in water [174,175], soil [176,177] and activated sludge [178]. The application of microarrays in environmental microbiology, specifically in the examination of microbial populations engaged in biodegradation has the potential for organism identification as well as defining their ecological role [160,161]. However, more rigorous and systematic assessment and development are needed to realize the full potential of microarrays for microbial detection and community analysis [168]. Microarrays detect only the dominant populations in many environments [161]. In addition, probes designed to be specific to known sequences can cross-hybridize to similar or unknown sequences and may produce misleading signals [169]. Moreover, soil, water and sediments often contain humic acids and other organic materials which may inhibit DNA hybridization onto microarrays [179,79]. Finally, limitations in quality RNA extraction from many environmental samples imply that advances in RNA extraction and purification and amplification methods are needed to make microarray gene expression analysis possible for a broader range of samples [169,11].

9. APPLICATION OF METAGENOMIC DNA IN STUDYING MICROBIAL DIVERSITY AND DYNAMICS

The use of metagenomic DNA by direct isolation of total community DNA from the environment has been a good tool for evaluating microbial diversity and dynamics. Commercially available DNA extraction kits circumvent most of the challenges associated with DNA extraction using readily available and cheap reagents. Microbial population changes during bioremediation of an experimental oil spill was investigated by Macnaughton et al. [90] using metagenomic 16S rDNA PCR-denaturing gradient gel electrophoresis

(DGGE) to identify the bacterial community members responsible for the decontamination of the site. Prominent DGGE bands which were excised for sequence analysis indicated that oil treatment encouraged the growth of Gram-negative microorganisms within the α-*Proteobacteria* and *Flexibacter-Cytophaga-Bacteroides* phylum. Consequently, α-*Proteobacteria* were never detected in unpolluted controls. Hamamura et al. [180] examined soil bacterial population dynamics in several crude-oil-contaminated soils to identify those organisms associated with alkane degradation and to assess patterns in microbial response across disparate soils using metagenomic DNA. The DNA sequences of prominent DGGE bands corresponding to bacterial populations selected during crude-oil degradation revealed that phylogenetically diverse populations related to β- and γ-*Proteobacteria*, *Actinobacteria*, and candidate division TM7 were identified across the set of contaminated soils. Surridge [181] characterized microbial communities in PAH and PCB-contaminated soils using DGGE of metagenomic soil DNA. This investigation revealed the involvement of novel bacterial genera such as *Burkholderia*, *Sphingomonas*, *Pseudomonas*, *Staphylococcus, Bacillus, Providencia, Burkholderia, Methylobacterium, Klebsiella, Rhodococcus* and *Pseudomonas* and many uncultured bacterial clones in contaminant degradation. Bacterial communities from shoreline environments affected by the Prestige oil spill in Spain were thoroughly examined by Alonso-Gutierrez et al. [75] using PCR-DGGE and the screening of 16S rRNA gene clone libraries. Their findings revealed that members of the classes *α-Proteobacteria* and *Actinobacteria* were the prevailing bacterial groups associated with hydrocarbon degradation of which *Dietzia* sp. HOB [74] was later identified as an excellent alkane degrader expressing alkane hydroxylase genes such as *alkB* and *CYP153* constitutively. Chikere [182] used the soil metagenome from crude oil polluted soil to study the bacterial diversity and dynamics during bioremediation. The investigation recorded distinct and dominant ribotypes obtained using denaturing gradient gel electrophoresis (DGGE) and sequencing that corresponded with known hydrocarbon degrading, Gram positive bacteria after PCR-amplification of 16S gene. Coulon et al. [104] used a metagenomic approach comprising DGGE of PCR-amplified cDNA from reverse transcribed 16S rRNA, T-RFLP, cloning and 454 pyrosequencing to investigate aerobic hydrocarbon degraders in hydrocarbon-polluted mudflat mesocosms. Throughout the experiment they discovered that phylotypes associated with degradation of alkanes and PAHs such as *Cycoclasticus*, *Alcanivorax*, *Oleibacter* and *Oceanospirillales* ME113 significantly increased in the oil-polluted mesocosms. A recent review by Chikere et al. [13] extensively discussed both culture-dependent and -independent techniques employed in monitoring microbial hydrocarbon remediation in the soil. This review presented the chromosomal or plasmid locations of some degradative enzymes and genes such as dioxygenases and alkane hydroxylases in known hydrocarbon degrading bacterial genera. Such nucleic-acid based analyses can only be done using molecular microbiology techniques such as PCR, DGGE and DNA sequencing.

10. METAGENOMIC ANALYSIS

Metagenomic approaches have enabled us to understand the genomic potential of the entire microbial community in an ecosystem by cloning and analyzing microbial community DNA directly extracted from environmental samples [33]. The construction of metagenomic libraries and other DNA-based metagenomic projects are initiated by isolation of high quality DNA suitable for cloning and cover the microbial diversity present in the original sample [183,32]. The large amount of cloning and sequencing required for such a task was prohibitive during the early development of metagenomics. More recently, researchers have transitioned to new direct and high throughput sequencing technologies (e.g. 454 pyrosequencing), often bypassing cloning steps that were essential previously. Direct

sequencing increases the depth of microbial community DNA sequences analyzed, probing deeper into the metagenome of an ecosystem, and to date, has been applied to a variety of environments ranging from the termite gut (471 Mbp of sequence), to the human intestinal tract (478 Mbp), to marine environments [46 Gbp (billion base pairs)] and hydrocarbon-contaminated sites [180,181,33]. This direct, deep-sequencing, metagenomic approach has been accepted widely and has even been termed 'megagenomics' in acknowledgement of the amount of effort required to achieve a comprehensive coverage.

Functional screening of metagenomic libraries have led to the discovery of novel genes encoding polyphenol oxidase [184], ester and glycosyl hydrolase [185] and also given indications in the diversity of extradiol dioxygenases in coke plant waste water [186]. Brennerova et al. [187] revealed the diversity and abundance of meta cleavage pathways in microbial communities from soil highly contaminated with jet fuel (aromatic and aliphatic hydrocarbons) under air sparging bioremediation using metagenomics. Moreover, the extradiol dioxygynase diversity was assessed by functional screening of a fosmid library in *Escherichia coli* with catechol as substrate. The 235 positive clones from inserts of DNA extracted from contaminated soil were equivalent to one extradiol dioxygenase-encoding gene per 3.6 Mb of DNA screened, indicating a strong selection for genes encoding this function. Three subfamilies were identified as being predominant, with 72, 55 and 43 fosmid inserts carrying genes, related to those encoding *TbuE* of *Ralstonia pickettii* PK01 (*EXDO-D*), *IpbC* of *Pseudomonas* sp. JR1 (*EXDO-K2*) or *DbtC* of *Burkholderia* sp. DBT1 (*EXDO-Dbt*), respectively, whereas genes encoding enzymes related to *XylE* of *Pseudomonas putida* mt-2 were not observed. Genes encoding oxygenases related to isopropylbenzene dioxygenases were usually colocalized with genes encoding *EXDO-K2* dioxygenases. Later on, [188,189] used the *E. coli* library from the study conducted by Suenaga et al. [186] to screen for EDO activity using catechol as a substrate and 38 clones were subjected to sequence analysis. As a result, various gene subsets were identified that were not similar to those involved in previously reported degradation pathways. The distribution of these genes among the different genome segments was reported in some isolated sphingomonads [190]. Iwai et al. [191] applied gene-targeted-metagenomics and pyrosequencing to aromatic dioxygenase genes to obtain greater sequence depth than possible by other methods. A PCR primer set designed to target a 524 bp region that confers substrate specificity of biphenyl dioxygenases yielded 2000 and 604 sequences from 5′ and 3′ ends of the PCR products, respectively, that passed set validity criteria. Sequence alignment showed three known conserved residues as well as another seven conserved residues not previously reported. Ninety-five and 41% of the valid sequences were assigned to 22 and 3 novel clusters in that were not included in any previously reported sequences at 0.6 distance by complete linkage clustering for the sequenced regions. Although they designed their primers using only toluene/biphenyl dioxygenase, interestingly the deeper sequencing technique applied enabled them to obtain a much broader range of apparent dioxygenase genes. For example, clusters F24 and R5 contained all well-known toluene/biphenyl dioxygenase genes and clusters F35 and R7 contained all well-known naphthalene dioxygenase genes. This approach is likely to be most useful for genes directly responsible for important ecosystem functions or ecological processes such as biogeochemical cycles and biodegradation. Korenblum et al. [71] employed PCR-amplification of 16S rRNA gene sequences using the primers U968 and L1401 to amplify the V6-V8 variable regions in the *Escherichia coli* small subunit rRNA genes to determine the bacterial communities in crude oil samples from two Brazilian offshore petroleum platforms. The PCR products were then cloned and a total of 156 valid 16S rRNA gene sequences obtained was analyzed for taxonomic affiliation by the ribosomal database project (RDP) classifier. Among the clones, 122 were γ-*proteobacteria* and most were affiliated with the family *Pseudomonadaceae*. The remaining clones were

affiliated with *β-proteobacteria* (representative families were *Comamonadaceae* and *Burkholderiaceae*), *Moraxellaceae*, *Enterobacteriaceae*, *Alteromonodaceae* and *Xanthomonadaceae*. A recent review by Suenaga et al. [192] focused on 'targeted metagenomics' studies, which combine metagenomic library screening and subsequent sequencing analysis. This approach is a more effective means to understanding the content and composition of genes for key ecological processes in microbial communities especially those involved in pollutant degradation such as hydrocarbons. Metagenomics have some biases that make the technique not completely fool-proof. Despite vast sequencing efforts, the complete coverage of a metagenome based on multiple-fold redundancy or the complete assembly of individual genomes within a community remains largely unattained. Such difficulties are expected, given the typical microbial community complexity and unevenness (i.e. a few numerically predominant populations and vast numbers of low abundance ones).

11. CONCLUSION

Cultivation-independent molecular microbiology techniques have in recent times unravelled the complexities of microbial populations, functions and interactions in hydrocarbon-polluted sites. These techniques have been applied in the monitoring of the progress of bioremediation of hydrocarbons and allied pollutants and invariably have greatly increased the acceptance of biological treatment of these pollutants as a result of the application of DNA-based techniques [193]. In recent years, the studies of gene expression and protein biosynthesis have emerged to complement DNA-based microbial community analysis [194]. Therefore, metagenomics offer significant promise to advance the prediction of the *in situ* microbial responses, activities and dynamics during hydrocarbon/pollutant bioremediation [195-199].

ACKNOWLEDGEMENT

COMPETING INTERESTS

Author declares non-existence of competing interests.

REFERENCES

1. Chikere BO. The impact of Eleme petrochemical effluents on the microbial ecology of Eleme River. Ph.D. Thesis. Microbiology Department, University of Port Harcourt, Rivers State, Nigeria; 2000.
2. Philp JC, Whitely AS, Ciric L, Bailey MJ. Monitoring bioremediation. In: Atlas RM, Philp J, editors. Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup. Washington DC: American Society for Microbiology (ASM) Press. 2005;237-292.
3. Chikere BO, Chijioke–Osuji C. Microbial diversity and physicochemical properties of a crude oil polluted soil. Nig J Microbiol. 2002;20:1039-46.
4. Theron J, Cloete TE. Microbiological Detection Techniques In: Cloete TE, Atlas RM. editors. Basic and Applied Microbiology. 1st ed. Pretoria: Van Schaik Publishers. 2006;185-208.

5. Snyder L, Champness W. Molecular Genetics of Bacteria. 3rd ed. Washington DC: American Society for Microbiology (ASM) Press; 2007.
6. Chikere CB, Okpokwasili GC, Surridge AKJ, Cloete TE. Impact of oil contamination and biostimulation on bacterial diversity in soil microcosms. Proceedings of Department of Petroleum Resources 13th Health, Safety and Environment (HSE) Biennial International Conference on the Oil and Gas Industry in Nigeria, Abuja; 2008a. pp 1-7.
7. Chikere, CB, Okpokwasili GC, Surridge AKJ, Cloete TE. (2008b). Population dynamics of indigenous bacteria during bioremediation of crude oil-polluted soil. Proceedings of Nigerian Society for Microbiology (NSM) 32nd Annual National Conference and General meeting, ABSU; 2008.
8. Chikere CB, Okpokwasili GC, Chikere BO. Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. Afr J Biotech. 2009a;8:2535-40.
9. Chikere CB, Okpokwasili GC, Ichiakor O. Characterization of hydrocarbon utilizing bacteria in tropical marine sediments. Afr J Biotech. 2009b;8:2541-44.
10. Zengler K. Accessing uncultivated microorganisms: from the Environment to Organisms and Genomes and Back. Washington DC: American Society for Microbiology (ASM) Press. 2008;153-186.
11. Malik S, Beer M, Megharaj M, Naidu R. The use of molecular tools to characterize the microbial communities in contaminated soil and water. Environ Int. 2008;38:265-276.
12. Rajendhran J, Gunasekaran P. Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. Microbiol Res. 2011;166:99-110.
13. Chikere CB, Okpokwasili GC, Chikere BO. Monitoring of microbial hydrocarbon remediation in the soil. 3Biotech. 2011a;1(3):117-38.
14. Chikere CB, Surridge KJ, Cloete TE, Okpokwasili GC. Phylogenetic diversity of dominant bacterial communities during bioremediation of crude oil-polluted soil. Revis Ambi Agua. 2011b;6(2):61-76.
15. Chikere CB, Ughala E. Preliminary screening of hydrocarbon utilizing bacteria habouring plasmids. TWOWS Africa Int J Sci Technol. 2011c;202:26-36.
16. Okpokwasili GC, Somerville CC, Sullivan M, Grimes DJ, Colwell RR. Plasmid mediated degradation of hydrocarbons in estuarine bacteria. Oil Chem Pollut. 1986;3:117-29.
17. Okpokwasili GC, Amanchukwu SC. Petroleum hydrocarbon degradation by *Candida* species. Environ Int. 1988;14:243-47.
18. Okpokwasili GC, Odokuma LO. Tolerance of *Nitrobacter* to toxicity of some Nigerian crude oils. Bull Environ Contam Toxicol. 1994;52:388 – 95.
19. Okpokwasili GC, James WA. Microbial contamination of kerosene, gasoline, and crude oil and their spoilage potentials. Material und Organismen. 1995;29:147-56.
20. Okpokwasili GC, Ibe SN. Genetically engineered microbes and oil degradation In: Proceedings of International seminar on petroleum industry and the Nigerian environment. Nigerian National Petroleum Corporation (NNPC), Lagos. 1998;97–103.
21. Kaplan CW, Kitts CL. Bacterial succession in a petroleum land treatment unit. Appl Environ Microbiol. 2004;70:1777–86.
22. Ollivier B, Magot B. Petroleum Microbiology. Washington DC: American Society for Microbiology (ASM) Press; 2005.
23. Okpokwasili GC. Microbes and the Environmental Challenge. Inaugural lecture series No. 53. University of Port Harcourt Press. Port Harcourt. 2006;1-77.
24. Okpokwasili GC, Ibiene AA. Enhancement of recovery of residual oil using a biosurfactant slug. Afr J Biotech. 2006;5:453-56.
25. Okpokwasili GC, Oton NS. Comparative applications of bioreactor and shake-flask systems in the laboratory treatment of oily sludge. Int J Waste Mgt. 2006;1:49-60.

26. Chikere CB, Okpokwasili GC, Chikere BO. Bioreactor-based bioremediation of hydrocarbon-polluted Niger Delta marine sediment, Nigeria. 3Biotech. 2012a;2:53-66.

27. Chikere CB, Surridge K, Okpokwasili GC, Cloete TE. Dynamics of indigenous bacterial communities associated with crude oil degradation in soil microcosms during nutrient-enhanced bioremediation. Waste Manag Res. 2012b;30:225-36.

28. Maila MP. Microbial ecology and bio-monitoring of total petroleum contaminated soil environments. Ph.D. Thesis, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa; 2005.

29. Maila MP, Randima P, Surridge K, Drønen K, Cloete TE. Evaluation of microbial diversity of different soil layers at a contaminated diesel site. Int Biodeterio Biodeg. 2005;55:39–44.

30. Maila MP, Randima P, Drønen K, Cloete TE. Soil microbial communities: Influence of geographic location and hydrocarbon pollutants. J Soil Biol Biochem. 2006;38:303-10.

31. Montiel C, Quintero R, Aburto J. Petroleum Biotechnology: Technology trends for the future. Afr J Biotech. 2009;8:2653-66.

32. Simon C, Daniel R. Metagenomic analysis: Past and future trends. Appl Environ Microbiol. 2011;77:1153-61.

33. Morales SE, Holben WE. Linking bacterial identities and ecosystem processes: can'omic' analyses be more than the sum of their parts? FEMS Microbiol Ecol. 2011;75:2-16.

34. Woese CR. Bacterial evolution. Microbiol Rev. 1987;51:221–271.

35. Wilson VL, Tatford BC, Yin X, Rajki SC, Walsh MM, LaRock P. Species specific detection of hydrocarbon-utilizing bacteria. J Microbiol Methods. 1999b;39:59–78.

36. Wilson MS, Bakermans C, Madsen EL. In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. Appl Environ Microbiol. 1999a;65:80–87.

37. Markoulatos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: a practical approach. J Clin Lab Anal. 2002;16:47–51.

38. Heiss-Blanquet S, Benoit Y, Marechaux C, Monot F. Assessing the role of alkane hydroxylase genotypes in environmental samples by competitive PCR. J Appl Microbiol 2005;104:251-59.

39. Brons JK, van Elsas JD. Analysis of bacterial communities in soil by use of denaturing gradient gel electrophoresis and clone libraries, as influenced by different reverse primers. Appl Environ Microbiol. 2008;74:2717-27.

40. Kumar M, Khanna S. Diversity of 16S rRNA and dioxygenase genes detected in coal tar-contaminated site undergoing active bioremediation. J Appl Microbiol. 2010;108:1252-62.

41. Wintzingerode FV, Gobel UB, Stackebrandt E. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev. 1997;21:213–29.

42. Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H. Methods of studying soil microbial diversity. J Microbiol Methods. 2004;58:169–88.

43. Schneegurt MA, Dore SY, Kulpa CF Jr. Direct extraction of DNA from soils for studies in microbial ecology. Curr Issues Mol Biol. 2003;5:1–8.

44. Jackson CR, Roden EE, Churchill PF. Changes in bacterial species composition in enrichment cultures with various dilutions of inoculum as monitored by denaturing gradient gel electrophoresis. Appl Environ Microbiol. 1998;64:5046–5048.

45. Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG. Characterization of hydrocarbon degrading microbial populations in contaminated and pristine Alpine soils. Appl Environ Microbiol. 2003;69:3085-92.

46. Alquati C, Papacchini M, Riccardi C, Spicaglia S, Bestetti G. Diversity of naphthalene-degrading bacteria from a petroleum contaminated soil. Annals Microbiol. 2005;55:237-42.

47. Abed RM, Safi NM, Koster J, de Beer J, El-Nahhal Y, Rullkotter E, et al. Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. Appl Environ Microbiol. 2002;68:1674-83.

48. Liu C, Shao Z. *Alcanivorax dieselolei* sp. nov., a novel alkane degrading bacterium isolated from sea water and deep sea sediment. Int J Sys Evol Microbiol. 2005;55:1181-86.

49. Kloos K, Munch JC, Schloter M. A new method for the detection of alkane monooxygenase homologous genes (*alkB*) in soils based on PCR-hybridization. J Microbiol Methods. 2006;66:486-96.

50. Popp N, Schlomann M, Mau M. Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. Microbiol. 2006;152:3291-304.

51. Zeinali M, Vossoughi M, Ardestani SK, Babanezhad E, Masoumian M. Hydrocarbon degradation by thermophilic Nocardia otitidiscaviarum strain TSH1: Physiological aspects. J Basic Microbiol. 2007;47:534-39.

52. Quatrini P, Scaglione G, De Pasquale C, Reila S, Puglia AM. Isolation of Gram-positive n-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. J Appl Microbiol. 2008;104:251-59.

53. Higashioka Y, Kojima H, Sato S, Fukui M. Microbial community analysis at crude oil-contaminated soils targeting the 16S ribosomal RNA, xylM, C23O and bcr genes. J Appl Microbiol. 2009;107:126-35.

54. Liu FH, Wang SB, Zhang JS, Zhang J, Yan X, Zhou HK, et al. The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis. J Appl Microbiol. 2009;106:952-66.

55. Rodrigues DF, Sakata SK, Comasseto JV, Bicego MC, Pellizari VH. Diversity of hydrocarbon-degrading *Klebsiella* strains isolated from hydrocarbon-contaminated estuaries. J Appl Microbiol. 2009;106:1304-14.

56. Wu Y, Lai Q, Zhongewn Z, Qiao N, Liu C, Shao Z. *Alcanivorax hongdengensis* sp. nov., an alkane-degrading bacterium isolated from surface sea water of the straits of Malacca and Singapore, producing a lipopeptide as its biosurfactant. Int J Sys Evol Microbiol. 2009;59:1474-79.

57. Knaebel DB, Crawford RL. Extraction and purification of microbial DNA from petroleum-contaminated soils and detection of low numbers of toluene, octane and pesticide degraders by multiplex polymerase chain reaction and Southern analysis. Mol Ecol. 1995;4:579–91.

58. Baldwin BR, Nakatsu CH, Nies L. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl Environ Microbiol. 2003;69:3350–3358.

59. Gilbride KA, Lee DY, Beaudette LA. Molecular techniques in wastewater: understanding microbial communities, detecting pathogens, and real-time process control. J Microbiol Methods. 2006;66:1–20.

60. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnol. 1993;11:1026-30.

61. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res. 1996;6:986-94.

62. Lerat S, England LS, Vincen ML, Pauls KP, Swanton CJ, Klironomos JN. Real-time polymerase chain reaction quantification of the transgenes for roundup ready corn and roundup ready soybean in soil samples. J Agric Food Chem. 2005;53:1337–42.

63. Saleh-Lakha S, Miller M, Campbell RG, Schneider K, Elahimanesh P, Hart MM. Microbial gene expression in soil: methods, applications and challenges. J Microbiol Methods. 2005;63:1–19.

64. Widada J, Nojiri H, Yoshida T, Habe H, Omori T. Enhanced degradation of carbazole and 2, 3-dichlorodibenzo-p-dioxin in soils by *Pseudomonas resinovorans* strain CA10. Chemos. 2002a;49:485–91.

65. Beller HR, Kane SR, Legler TC, Alvarez PJ. A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. Environ Sci Technol. 2002;36:3977–84.

66. Devers M, Soulas G, Martin-Laurent F. Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. J Microbiol Methods. 2004;56:3-15.

67. Baldwin BR, Nakatsu CH, Nies L. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl Environ Microbiol. 2003;69:3350–58.

68. Sun Y, Polishchuk EA, Radoja U, Cullen WR. Identification and quantification of arsC genes in environmental samples by using real-time PCR. J Microbiol Methods. 2004;58:335-49.

69. Ritalahti KM, Amos BK, Sung Y, Wu Q, Koenigsberg SS, Loffler FE. Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple Dehalococcoides strains. Appl Environ Microbiol. 2006;72:2765-74.

70. Powell SM, Ferguson SH, Bowman JB, Snape I. Using real-time PCR to assess changes in the hydrocarbon-degrading microbial community in Antarctic soil during bioremediation. Microb Ecol. 2006;52:523–32.

71. Korenblum E, Souza DB, Penna M, Seldin L. Molecular analysis of the bacterial communities in crude oil samples from two Brazilian offshore petroleum platforms. Int J Microbiol. 2012;2012:1-8.

72. Liesack W, Weyland H, Stackebrandt E. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb Ecol. 1991;121:191–98.

73. Spiegelman D, Whissell G, Greer CW. A survey of the methods for the characterization of microbial consortia and communities. Can J Microbiol. 2005;51:355-86.

74. Alonso-Gutierrez J, Teramoto M, Yamazoe A, Harayama S, Figueras A, Novoa B. Alkane-degrading properties of *Dietzia* sp. HOB, a keyplayer in the Prestige oil spill biodegradation (NW Spain). J Appl Microbiol. 2011;111:800-10.

75. Alonso-Gutierrez J, Figueras A, Albaiges J, Jimenez N, Vinas M, Solanas AM, et al. Bacterial communities from shoreline environments (Costa da Morte, Northwestern Spain) affected by the Prestige oil spill. Appl Environ Microbiol. 2009;75:3407-18.

76. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Oslen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol. 2008;74:2461-70.

77. Iwai S, Johnson TA, Chai B, Hashsham SA, Tiedje JM. Comparison of the specificities and efficacies of primers for aromatic dioxygenase gene analysis of environmental samples. Appl Environ Microbiol. 2011;77:3551-57.

78. Muyzer G, de Waal DC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 1993;59:695-700.

79. van Elsas JD, Jansson JK, Trevors JK. Modern Soil Microbiology. 2nd ed. New York: CRC Press. 2007;387-429.

80. Fischer SG, Lerman LS. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. Cell. 1979;16:191-200.

81. Fischer SG, Lerman LS. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc Natl Acad Sci. 1983;80:1579–83.

82. Rosenbaum V, Riesner D. Temperature-gradient gel electrophoresis. Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts. Biophys Chemist. 1987;26:235-46.

83. Muyzer G, Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek. 1998;73:127–141.

84. Muyzer G. DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol. 1999;2:317-22.

85. Ferris MJ, Muyzer G, Ward DM. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol. 1996;62:340-46.

86. Sanz JL, Kochling T. Molecular biology techniques used in waste treatment: an overview. Process Biochem. 2007;42:119-33.

87. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995;59:143–69.

88. Evans FF, Rosado AS, Sebastian GV, Casella R, Machado PLOA, Holmstrom C, et al. Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms. FEMS Microbiol Ecol. 2004;49:295-305.

89. Forney LJ, Zhou X, Brown CJ. Molecular microbial ecology: Land of the one-eyed king. Curr Opin Microbiol. 2004;7:210–20.

90. Macnaughton SJ, Stephen JR, Venosa AO, Davis GA, Chang YJ, White DC. Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol. 1999;65:3566-74.

91. Chang YJ, Stephen JR, Richter AP, Venosa AD, Bruggemann J, Macnaughton SJ. Phylogenetic analysis of aerobic freshwater and marine enrichment cultures efficient in hydrocarbon degradation: effect of profiling method. J Microbiol Methods. 2000;40:19–31.

92. Ralebitso TK, Roling WF, Braster M, Senior E, van Verseveld HW. 16S rDNAbased characterization of BTX-catabolizing microbial associations isolated from a South African sandy soil. Biodegrad. 2000;11:351-57.

93. Watanabe, K, Kodama Y, Syutsubo K, Harayama S. Molecular characterization of bacterial populations in petroleum-contaminated groundwater discharged from underground crude oil storage cavities. Appl Environ Microbiol. 2000;66:4803–4809.

94. Kleikemper J, Schroth MH, Sigler WV, Schmucki M, Bernasconi SM, Zeyer J. Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon- contaminated aquifer. Appl Environ Microbiol. 2002;68:1516-23.

95. Cummings DE, Snoeyenbos-West OL, Newby DT, Niggemyer AM, Lovley DR, Achenbachl LA. Diversity of *Geobacteraceae* species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. Microb Ecol. 2003;46:257–69.

96. El-Latif Hesham A, Khan S, Liu X, Zhang Y, Wang Z, Yang M. Application of PCR-DGGE to analyse the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil. Yeast. 2006;23:879-87.

97. Gertler C, Gerdts G, Timmis KN, Yakimov MM, Golyshin PN. Populations of heavy fuel oil-degrading marine microbial community in presence of oil sorbent materials. J Appl Microbiol. 2009a;107:590-605.

98. Gertler C, Gerdts G, Timmis KN, Golyshin PN. Microbial consortia in mesocosm bioremediation trial using oil sorbents, slow release fertilizer and bioaugmentation. FEMS Microbiol Ecol. 2009b;69:288-300.

99. Mahmoud HM, Al-Hasan RH, Sorkhoh NA, Eliyas M, Radwan SS. Attenuation of oil pollutants in the Arabian Gulf water by bacteria naturally associated with live fish. Int Biodeterio Biodeg. 2009;63:615-20.

100. Geets J, Borremans B, Diels L, Springael D, Vangronsveld J, van der Lelie D. DsrB gene-based DGGE for community and diversity surveys of sulphate-reducing bacteria. J Microbiol Methods. 2006;66:194-205.

101. Hendrickx B, Dejonghe W, Faber F, Boenne W, Bastiaens L, Verstraete W. PCR DGGE method to assess the diversity of BTEX mono-oxygenase genes at contaminated sites. FEMS Microbiol Ecol. 2006;55:262-73.

102. Nubel U, Garcia-Pichel F, Muyzer G. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol. 1997;63:3327-32.

103. Murray AE, Preston CM, Massana R, Taylor LT, Blakis A, Wu K. Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. Appl Environ Microbiol. 1998;64:2585-95.

104. Coulon F, Chronopoulou PM, Fahy A, Paisse S, Goni-Urriza M, Peperzak L, et al. Central role of dynamic tidal biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms in the biodegradation of hydrocarbons in coastal mudflats. Appl Environ Microbiol. 2012;78:3638-48.

105. Gich FB, Amer E, Figueras JB, Abella CA, Balaguer MD, Poch M. Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). Int Microbiol. 2000;3:103–6.

106. Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos P, Claeys G, et al. Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. J Clin Microbiol. 1995;33:11–15.

107. Kita-Tsukamoto K, Wada M, Yao K, Kamiya A, Yoshizawa S, Uchiyama N. Rapid identification of marine bioluminescent bacteria by amplified 16S ribosomal RNA gene restriction analysis. FEMS Microbiol Lett. 2006;256:298-303.

108. Krizova J, Spanova A, Rittich B. Evaluation of amplified ribosomal DNA restriction analysis (ARDRA) and species-specific PCR for identification of *Bifidobacterium* species. Syst Appl Microbiol. 2006;29:36–44.

109. Weidner S, Arnold W, Puhler A. Diversity of uncultured microorganisms associated with the sea grass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl Environ Microbiol. 1996;62:766–71.

110. Bai Y, Yang D, Wang J, Xu S, Wang X, An L. Phylogenetic diversity of culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China. Res Microbiol. 2006;157:741-51.

111. Babalola OO, Kirby BM, Roes-Hill ML, Cook AE, Cary SC, Burton SG, et al. Phylogenetic analysis of actinobacterial populations associated with Antarctic dry valley mineral soils. Environ Microbiol. 2009;11:566-76.

112. Fries MR, Hopkins GD, McCarty PL, Forney LJ, Tiedje JM. Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene co-metabolism. Appl Environ Microbiol. 1997;63:1515-22.

113. Hohnstock-Ashe AM, Plummer SM, Yager RM, Baveye P, Madsen EL. Further biogeochemical characterization of a trichloroethene-contaminated fractured dolomite aquifer: electron source and microbial communities involved in reductive dechlorination. Environ Sci Technol. 2001;35:4449-56.

114. Liu WT, Marsh TL, Cheng H, Forney LJ. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol. 1997;3:4516-22.
115. Watts JE, Wu Q, Schreier SB, May HD, Sowers KR. Comparative analysis of polychlorinated biphenyl-dechlorinating communities in enrichment cultures using three different molecular screening techniques. Environ Microbiol. 2001;3:710-19.
116. Haack SK, Fogarty LR, West TJ, Alm EW, McGuire JT, Long DT. Spatial and temporal changes in microbial community structure associated with recharge-influenced chemical gradients in a contaminated aquifer. Environ Microbiol. 2004;6:438-48.
117. Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J, Nazaret S. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl Environ Microbiol. 2001;67:4479-87.
118. Mendoza M, Meugnier H, Bes M, Etienne J, Freney J. Identification of Staphylococcus species by 16S-23S rDNA intergenic spacer PCR analysis. Int J Syst Bacteriol. 1998;48:1049-55.
119. Bes M, Saidislim L, Becharnia F, Meugnier H, Vandenesch F, Etienne J. Population diversity of *Staphylococcus intermedius* isolates from various host species: typing by 16S–23S intergenic ribosomal DNA spacer polymorphism analysis. J Clin Microbiol. 2002;40:2275-77.
120. Bourque SN, Valero JR, Lavoie MC, Levesque RC. Comparative analysis of the 16S to 23S Ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. Appl Environ Microbiol. 1995;61:1623-26.
121. Daffonchio D, Cherif A, Brusetti L, Rizzi A, Mora D, Boudabous A. Nature of polymorphisms in 16S–23S rRNA gene intergenic transcribed spacer fingerprinting of Bacillus and related genera. Appl Environ Microbiol. 2003;69:5128-37.
122. Chun J, Rivera IN, Colwell RR. Analysis of 16S–23S rRNA intergenic spacer of *Vibrio cholerae* and *Vibrio mimicus* for detection of these species. Methods Mol Biol 2002;179:171–78.
123. Ghatak A, Majumdar A, Ghosh RK. Molecular phylogenetic analysis of Vibrio cholerae O1 El Tor strains isolated before, during and after the O 139 outbreak based on the inter-genomic heterogeneity of the 16S–23S rRNA intergenic spacer regions. J Biosci. 2005;30:619–25.
124. Eriksson M, Sodersten E, Yu Z, Dalhammar G, Mohn WW. Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. Appl Environ Microbiol. 2003;69:275–84.
125. Fisher MM, Triplett EW. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl Environ Microbiol. 1999;65:4630-36.
126. Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, et al. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the deep horizon oil spill. Appl Environ Microbiol. 2011;77:7962-74.
127. Smalla K, Oros-Sichler M, Milling A, Heuer H, Baumgarte S, Becker R, et al. Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: Do the different methods provide similar results? J Microbiol Methods. 2007;69:470-79.
128. Marsh TL. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr Opin Microbiol. 1999;2:323-27.

129. Tiedje JM, Asuming-Brempong S, Nusslein K, Marsh TL, Flyn SJ. Opening the black box of soil microbial diversity. Appl Soil Ecol. 1999;13:109-122.

130. Dojka MA, Hugenholtz P, Haack SK, Pace NR. Microbial diversity in a hydrocarbon- and chlorinated-solvent contaminated aquifer undergoing intrinsic bioremediation. Appl Environ Microbiol. 1999;64:3869-77.

131. Bordenave S, Fourcans A, Blanchard S, Goni MS, Caumette P, Duran R. Structure and functional analyses of bacterial communities changes in microbial mats following petroleum exposure. Ophelia. 2004a;58:195-203.

132. Bordenave S, Jezequel AR, Fourcans A, Budzinski H, Merlin FX, Fourel T, et al. Degradation of the "Erika" oil. Aqua Living Res. 2004b;17:261-67.

133. Allen JP, Atekwana EA, Duris JW, Werkema DD, Rossbach S. The microbial community structure in petroleum-contaminated sediments corresponds to geophysical signatures. Appl Environ Microbiol. 2007;73:2860-70.

134. Konstantinidis KT, Isaacs N, Fett J, Simpson S, Long DT, Marsh TL. Microbial diversity and resistance to copper in metal-contaminated lake sediment. Microb Ecol. 2003;45:191–202.

135. Reardon CL, Cummings DE, Petzke LM, Kinsall BL, Watson DB, Peyton BM. Composition and diversity of microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer. Appl Environ Microbiol. 2004;70:6037–46.

136. Macbeth TW, Cummings DE, Spring S, Petzke LM, Sorenson KS Jr. Molecular characterization of a dechlorinating community resulting from *in situ* biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture. Appl Environ Microbiol. 2004;70:7329-41.

137. Fahy A, Lethbridge G, Earle R, Ball AS, Timmis KN, McGenity TJ. Effects of long-term benzene pollution on bacterial diversity and community structure in groundwater. Environ Microbiol. 2005;7:1192-99.

138. Denaro R, D'Auria G, DiMarco G, Genovese M, Troussellier M, Yakimov MM. Assessing terminal restriction fragment length polymorphism suitability for the description of bacterial community structure and dynamics in hydrocarbon-polluted marine environments. Environ Microbiol. 2005;7:78-87.

139. Fedi S, Tremaroli V, Scala D, Perez-Jimenez JR, Fava F, Young L. T-RFLP analysis of bacterial communities in cyclodextrin-amended bioreactors developed for biodegradation of polychlorinated biphenyls. Res Microbiol. 2005;156:201-10.

140. Richardson RE, Bhupathiraju VK, Song DL, Goulet TA, Alvarez-Cohen L. Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. Environ Sci Technol. 2002;36:2652-62.

141. Dunbar J, Ticknor LO, Kuske CR. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol. 2000;66:2943-50.

142. Osborn AM, Moore ER, Timmis KN. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ Microbiol. 2000;2:39-50.

143. Osborne CA, Rees GN, Bernstein Y, Janssen PH. New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. Appl Environ Microbiol. 2006;72:1270-78.

144. Edgcomb VP, McDonald JH, Devereux R, Smith DW. Estimation of bacterial cell numbers in humic acid-rich salt marsh sediments with probes directed to 16S ribosomal DNA. Appl Environ Microbiol. 1999;65:1516-23.

145. Wagner M, Nielsen PH, Loy A, Nielsen JL, Daims H. Linking microbial community structure with function: fluorescence in situ hybridization-micro autoradiography and isotope arrays. Curr Opin Biotechnol. 2006;17:83–91.

146. Dubey SK, Tripathi AK, Upadhyay SN. Exploration of soil bacterial communities for their potential as bioresource. Bioresour Technol. 2006;97:2217-24.

147. Wagner M, Amann R, Lemmer H, Schleifer KH. Probing activated sludge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl Environ Microbiol. 1993;59:1520-25.

148. Wagner M, Erhart R, Manz W, Amann R, Lemmer H, Wedi D. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Appl Environ Microbiol. 1994;60:792-800.

149. Straub KL, Buchholz-Cleven BE. Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate-reducing bacteria from diverse European sediments. Appl Environ Microbiol. 1998;64:4846–56.

150. Onda S, Hiraishi A, Matsuo Y, Takii S. Polyphasic approaches to the identification of predominant polyphosphate-accumulating organisms in a laboratory-scale anaerobic/aerobic activated sludge system. J Gen Appl Microbiol. 2002;48:43–54.

151. Ebie Y, Matsumura M, Noda N, Tsuneda S, Hirata A, Inamori Y. Community analysis of nitrifying bacteria in an advanced and compact Gappei-Johkasou by FISH and PCR-DGGE. Water Sci Technol. 2002;46:105-11.

152. Collins G, Kavanagh S, McHugh S, Connaughton S, Kearney A, Rice O. Accessing the black box of microbial diversity and ecophysiology: recent advances through polyphasic experiments. J Environ Sci Health Tox Hazard Subst Environ Eng. 2006;41:897–922.

153. Kotsyurbenko OR, Chin KJ, Glagolev MV, Stubner S, Simankova MV, Nozhevnikova AN. Acetoclastic and hydrogenotrophic methane production and methanogenic populations in an acidic West-Siberian peat bog. Environ Microbiol. 2004;6:1159-73.

154. Jardillier L, Boucher D, Personnic S, Jacquet S, Thenot A, Sargos D. Relative importance of nutrients and mortality factors on prokaryotic community composition in two lakes of different trophic status: microcosm experiments. FEMS Microbiol Ecol. 2005;53:429-43.

155. Cho JC, Tiedje JM. Quantitative detection of microbial genes by using DNA microarrays. Appl Environ Microbiol. 2002;68:1425–30.

156. Fodor SP, Rava RP, Huang XC, Pease AC, Holmes CP, Adams CL. Multiplexed biochemical assays with biological chips. Nature. 1993;364:555–56.

157. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995;270:467-70.

158. DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science. 1997;278:680-86.

159. Zhou J, Thompson DK. Challenges in applying microarrays to environmental studies. Curr Opin Biotechnol. 2002;13:204–7.

160. Wu L, Thompson DK, Li G, Hurt RA, Tiedje JM, Zhou J. Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl Environ Microbiol. 2001;67:5780-90.

161. Rhee SK, Liu X, Wu L, Chong SC, Wan X, Zhou J. Detection of genes involved in biodegradation and biotransformation in microbial communities by using 50-mer oligonucleotide microarrays. Appl Environ Microbiol. 2004;70:4303–17.

162. Tiquia SM, Wu L, Chong SC, Passovets S, Xu D, Xu Y. Evaluation of 50-mer oligonucleotide arrays for detecting microbial populations in environmental samples. Biotechniques. 2004;36:664-70.

163. Wu L, Thompson DK, Liu X, Fields MW, Bagwell CE, Tiedje JM. Development and evaluation of microarray-based whole-genome hybridization for detection of microorganisms within the context of environmental applications. Environ Sci Technol. 2004;38:6775–82.

164. Wu L, Liu X, Schadt CW, Zhou J. Microarray-based analysis of sub nanogram quantities of microbial community DNAs by using whole-community genome amplification. Appl Environ Microbiol. 2006;72:4931–41.

165. Loy A, Kusel K, Lehner A, Drake HL, Wagner M. Microarray and functional gene analyses of sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal co-occurrence of recognized genera and novel lineages. Appl Environ Microbiol. 2004;70:6998–9.

166. El Fantroussi S, Urakawa H, Bernhard AE, Kelly JJ, Noble PA, Smidt H. Direct profiling of environmental microbial populations by thermal dissociation analysis of native rRNAs hybridized to oligonucleotide microarrays. Appl Environ Microbiol. 2003;69:2377-82.

167. Chandler DP, Jarrell AE, Roden ER, Golova J, Chernov B, Schipma MJ. Suspension array analysis of 16S rRNA from Fe− and $SO_{(4)2}$− reducing bacteria in uranium-contaminated sediments undergoing bioremediation. Appl Environ Microbiol. 2006;72:4672-87.

168. Zhou J. Microarrays for bacterial detection and microbial community analysis. Curr Opin Microbiol. 2003;6:288-94.

169. Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J. Microarray applications in microbial ecology research. Microb Ecol. 2006;52:159–75.

170. Taroncher-Oldenburg G, Griner EM, Francis CA, Ward BB. Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl Environ Microbiol. 2003;69:1159–71.

171. Steward GF, Jenkins BD, Ward BB, Zehr JP. Development and testing of a DNA macroarray to assess nitrogenase (nifH) gene diversity. Appl Environ Microbiol. 2004;70:1455-65.

172. Denef VJ, Park J, Rodrigues JL, Tsoi TV, Hashsham SA, Tiedje JM. Validation of a sensitive method for using spotted oligonucleotide DNA microarrays for functional genomics studies on bacterial communities. Environ Microbiol. 2003;5:933–43.

173. Voordouw G, Voordouw JK, Karkhoff-Schweizer RR, Fedorak PM, Westlake DW. Reverse sample genome probing, a new technique for identification of bacteria in environmental samples by DNA hybridization, and tts application to the identification of sulphate-reducing bacteria in oil field samples. Appl Environ Microbiol. 1991;57:3070-8.

174. Rudi K, Skulberg OM, Skulberg R, Jakobsen KS. Application of sequence specific labeled 16S rRNA gene oligonucleotide probes for genetic profiling of cyanobacterial abundance and diversity by array hybridization. Appl Environ Microbiol. 2000;66:4004–11.

175. Castiglioni B, Rizzi E, Frosini A, Sivonen K, Rajaniemi P, Rantalal A. Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. Appl Environ Microbiol. 2004;70:7161-72.

176. Small J, Call DR, Brockman FJ, Straub TM, Chandler DB. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. Appl Environ Microbiol. 2001;67:4708-16.

177. Loy A, Kusel K, Lehner A, Drake HL, Wagner M. Microarray and functional gene analyses of sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal co-occurrence of recognized genera and novel lineages. Appl Environ Microbiol. 2004;70:6998-09.

178. Adamczyk J, Hesselsoe M, Iversen N, Horn M, Lehner A, Nielsen PH. The isotope array, a new tool that employs substrate-mediated labelling of rRNA for determination of microbial community structure and function. Appl Environ Microbiol. 2003;69:6875–87.

179. Saleh-Lakha S, Miller M, Campbell RG, Schneider K, Elahimanesh P, Hart MM. Microbial gene expression in soil: methods, applications and challenges. J Microbiol Methods. 2005;63:1–19.

180. Hamamura N, Olson SH, Ward DM, Inskeep WP. Microbial population dynamics associated with crude oil biodegradation in diverse soils. Appl Environ Microbiol. 2006;72:6316-24.

181. Surridge AKJ. Denaturing gradient gel electrophoresis characterisation of microbial communities in polycyclic aromatic hydrocarbon and polychlorinated biphenyl contaminated soil. Ph.D. Thesis, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa; 2007.

182. Chikere CB. Bacterial diversity and community dynamics during the bioremediation of crude oil-polluted soil. Ph.D. Thesis. Microbiology Department, University of Port Harcourt, Rivers State, Nigeria; 2010.

183. Delmont TO, Robe P, Cecillon S, Clark IM, Constacias F, Simonet P, et al. Accessing the soil metagenome for study of microbial diversity. Appl Environ Microbiol. 2011;77:1315-24.

184. Beloqui A, Pita M, Ploaina J, Martinez-Arias A, Golyshian OV, et al. Novel poly phenol oxidase mined from a metagenome expression library of bovine rumen. Biochemical properties, structure analysis and phylogenetic relationships. J Biol Chem. 2006;281:22933-42.

185. Ferrer M, Golyshina OV, Chernikova TN, Khachane AN, Reyes-Duarte D, et al. Novel hydrolase diversity retrieved from a metagenome library of bovine rumen microflora. Environ Microbiol. 2005;7:1996-2010.

186. Suenaga H, Ohnuki T, Miyazaki K. Functional screening of a metagenomic library for genes involved in microbial degradation of aromatic compounds. Environ Microbiol. 2007;9:2289-97.

187. Brennerova MV, Josefiova J, Brenner V, Pieper DH, Junca H. Metagenomics reveals diversity and abundance of meta cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air sparging bioremediation. Environ Microbiol. 2009;11: 2216-27.

188. Suenaga H, Koyama Y, Miyakoshi M, Miyazaki R, Yano H, Sota M, et al. Novel organization of aromatic degradation pathway genes in a microbial community as revealed by metagenomic analysis. ISME J. 2009a;3:1335-48.

189. Suenaga H, Mizuta S, Miyazaki K. The molecular basis for adaptive evolution in novel extradiol dioxygenases retrieved from the metagenome. FEMS Microbiol Ecol. 2009b;69:472-80.

190. Miller TR, Delcher AL, Salzberg SL, Saunders E, Detter JC, Halden, R.U. Genome sequence of the dioxin-mineralizing bacterium *Sphingomonas wittichii* RW1. J Bacteriol. 2010;192:6101-02.

191. Iwai S, Chai B, Sul WJ, Cole JR, Hashsham SA, Tiedje JM. Gene-targeted-metagenomics reveals extensive diversity of aromatic dioxygenase genes in the environment. The ISME J. 2010;4:279-85.

192. Suenaga H. Targeted metagenomics: a high-resolution metagenomics approach for specific gene clusters in complex microbial communities. Environ Microbiol. 2012;14:13-22.

193. Chikere CB, Okpokwasili GC, Surridge AKJ, Cloete TE. Molecular approach to aerobic biodegradation of crude oil. Proceedings of Biotechnologies for Improved Production of Oil and Gas in the Gulf of Guinea (BIPOG3) 1st International Conference, Workshop and Exhibition, Abuja. 2009c;1-6.

194. Siggins A, Gunnigle E, Abram F. Exploring mixed microbial community functioning: recent advances in metaproteomics FEMS Microbiol Ecol. 2012;80:265-80.

195. Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG. Characterization of hydrocarbon degrading microbial populations in contaminated and pristine Alpine soils. Appl Environ Microbiol. 2003;69:3085-92.

196. Widada J, Nojiri H, Omori T. Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation. Appl Microbiol Biotechnol. 2002b;60:45-59.

197. Margesin R, Hammerle M, Tscherko D. Microbial activity and community composition during bioremediation of diesel-oil contaminated soil: effects of hydrocarbon concentration, fertilizers and incubation time. Microb Ecol. 2007;55:259-69.

198. van Beilen JB, Funhoff EG. Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol. 2007;74:13-21.

199. Muangchinda C, Pan Sri R, Wongwongsee W, Pinyakong O. Assessment of polycyclic aromatic hydrocarbon biodegradation potential in mangrove sediment from Don Hoi Lot, Samut Songkram Province, Thailand. J Appl Microbiol. 2013. DOI: 10.1111/jam.12128 (accepted article).