Discussion
4. Discussion

4.1. BIOSORPTION STUDIES

4.1.1 Effect of pH on Cr(VI) and Ni(II) removal by isolated bacterial and fungal species

At an initial pH from 2-4 almost no bioaccumulation occurred in the case of both the metal for the isolated Aspergillus and Micrococcus species. Above pH 5, the percent removal for both the metals increased rapidly for both the isolated species. The low bioaccumulation capacity at pH values below 5 is attributed to the competition of hydrogen ion with metal ion on the sorption site. Thus at lower pH, due to the protonation of binding site resulting from high concentration of proton, negative charge intensity on the site is reduced which results in the reduction or inhibition for the binding of metal ion. Most of the microbial surfaces are negatively charged due to the ionization of functional group, thereby contributing to metal binding. Fungal surfaces have a negative charge on pH range 2-6. At low pH, some of the functional groups will be positive charged and may not interact with metal ions (Yan et al., 2003). At acidic pH, the predominant species of Cr (VI) are Cr$_2$O$_7^{2-}$, HCrO$_4^-$, and Cr$_2$O$_4^{2-}$ and the surface of the sorbent becomes protonated and attracts anionic species of Cr(VI) (Selvaraj et al., 2003). Similar result has been reported (Yan et al., 2003) for the removal of lead using Penicillium digitatum and Rhizopus nigricans. Low removal of nickel at lower pH range by Penicillium chrysogenum has also been reported (Tian-Wei et al., 2004). Removal of copper and lead by Micrococcus luteus (Leung et al., 2000) also showed the same trend. The increase in percent removal of metal with increase in pH from 2 to 5 is due to the strong relations of bioaccumulation to the number of surface negative charge which depends on the dissociation of functional group (Yakup et al., 2004). As
the pH increased above the zeta potential of the adsorbent, there is a reduction in the electrostatic attraction between the Cr(VI) species and sorbent surface, with a consequent decrease in percentage bioaccumulation. The rate of chromium uptake and the extent were enhanced as the pH increases up to certain pH range. Under acidic conditions, the surface of the absorbent becomes protonated (zeta potential of the adsorbent pH\text{zpc} = 5.9) and attracts anionic species of Cr (VI) (Nasseri et al., 2002). Similar results were demonstrated by *A. foetidus* and *A. carbonarius* (Prasenjit and Sumathi, 2005). At low pH the negligible removal of chromium may be due to the competition between hydrogen and metal ions. With further increase in pH, the increase in metal removal may be due to the ionization of functional groups and an increase in the negative charge density on the cell surface. At higher alkaline pH values (8 and above), a reduction in the solubility of metals may contribute to lower uptake rates. Nasseri et al., (2002) reported the removal of chromium using *A. oryzae*, where maximum removal was observed at pH 5. This pH is suitable in which the living cells of fungi and bacteria were able to grow significantly. With further increase in pH, the percent removal of metal was decreased. With increase in pH beyond 5, the chromium removal rate decreased, which might be due to the osmotic changes and hydrolyzing effect. The uptake of Cr(IV) showed a sharp increase with an increase in pH from 3.0 to 6.0 in the case of *Sargassum sp.* and *G. salicornia* which is mainly attributed to more pronounced electrostatic attraction taking place between the biosorbents and metal ions at higher pH (Shams and Darvishi, 2008). Similar results were also obtained in the case of nickel, where maximum removal is reported at pH 5.2. The removal of metals in the pH range of 4.5 to 5.5 has been reported (Hasen et al., 2000). The variation of adsorption of nickel at various pH is on the basis of metal chemistry in solution and the surface chemistry of the sorbent. The pH\text{max} where maximum removal occurs is related
to the \( pK_a \) or the first hydrolysis product of the metal. The decrease in adsorption of Ni(II) above \( pH \) 5 is due to the formation of Ni(OH)\(_2\). Substantial precipitation of nickel as nickel hydroxide occurs at high \( pH \) values. The formation of hydroxide precipitate reduces the amount of free nickel ions, which accumulates to the organism. Similar reports has been reported for Ni(II) removal using \( S. \) cerevisiae, where the outer cell wall consists of protein coat, which develops a charge by the dissociation of ionizable side groups of the constituent amino acids. The ionic state of ligands such as carboxyl, phosphate, imidazole and amino groups will promote reactions with the positively charged metal ions. At low \( pH \), the cell wall’s ligands will be closely associated with the hydronium ions \([H_3O^+]\) and restrict the approach of metal ions as a result of the repulsive forces.

Although these data could not explain why the optimal \( pH \) for the fungal isolates was lower than that for the bacterial isolates, the positive correlation between heavy metal removal and cellular growth – consistently observed regardless heavy metals, \( pH \) and temperature – strongly suggest that the observed effect of \( pH \) on the heavy metal removal was attributed mainly to biological factors(s), in particular, organism-specific physiological one(s).

4.1.2 Effect of temperature on Cr(VI) and Ni(II) removal by isolated bacterial and fungal species

The range of optimal temperature values (30-35°C) were comparable to the range of room temperature that was used when isolating the microorganisms, suggesting that the selection of these isolates might have been influenced not only with the heavy metal(s) but also with the temperature used in the isolation procedure. The temperature of the adsorption medium could be important for energy dependent mechanisms in metal removal by microorganisms.
Temperature is known to affect the stability of the cell wall, its configuration and can also cause ionization of chemical moieties. These factors may simultaneously affect the binding sites on isolated fungal and bacterial species causing reduction in heavy metal removal. Energy-independent mechanisms are less likely to be affected by temperature since the processes responsible for removal are largely physiochemical in nature (Gulay et al., 2003).

Bioaccumulation of chromium and nickel by bacterial and fungal species appears to be temperature dependent. Maximum removal of Cr (VI) and Ni (II) was observed at 35°C and 30°C for the isolated *Aspergillus* (Figures 9 -12). Similar reports have been reported in the bioaccumulation of Cr (VI) by *S. equisimilus* and *A. niger* (Goyal and Banerjee, 2003).

### 4.1.3 Kinetics of Cr(VI) and Ni(II) removal and cellular growth of the isolated bacterial and fungal species

The time-course data for heavy metal removal and cellular growth were observed for each isolate under its optimal pH and temperature conditions. When these isolates are applied in removing heavy metal from industrial wastewater, information regarding the effect of growth phase will be important in designing solid (sludge) retention time (SRT) for continuous flow completely stirred (CFCS) bioreactor, which is a general reactor type for wastewater treatment plants. In the fungal isolates, specific metal bioaccumulation (accumulative biosorption [removal] of each heavy metal per accumulative biomass) increased when cells were in stationary phases (Figures 14 and 15) for *Aspergillus* species. This trend was also observed when using the Cr-resistant bacterial isolate in removing Cr (VI) (Figure 12). However, the Ni-resistant bacterial isolate exhibited reduced bioaccumulation when cells were in stationary phase (Figure 13). Therefore, expanded SRTs (stationary phase) may be recommended using the fungal isolates in removing chromium and nickel from industrial wastewater as well as using the chromium resistant and using the Ni-resistant bacterial
isolate in removing nickel, however, a non-expanded SRT has to be designed for CFCS bioreactor so that a mid-log phase of cellular growth could be kept in the treatment system. The growth rate during the lag phase was very low because the isolated bacterial and fungal isolates was adapting with the environment. After this stage, the isolates grew in logarithmic form using the nutrients. In the third stage, the number of living and dead cells is fixed (Nasseri et al., 2002). Similar studies have been reported by Enterobacter cloacae (Koji et al., 1992), Bacillus circulans (Srinath et al., 2001).

Table 9 indicates that Micrococcus and Aspergillus sp. are more effective for the removal of Cr (VI) and Ni (II) when compared with other microbial biomass reported.

**TABLE 9**

**Comparison of the removal efficiency of isolated metal resistant microorganisms with literature**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Microorganism/ sorbent</th>
<th>Initial concentration (mg L⁻¹)</th>
<th>pH</th>
<th>% Removal</th>
<th>Time (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr(VI)</td>
<td>Aspergillus foetidus</td>
<td>5</td>
<td>7</td>
<td>97</td>
<td>92</td>
<td>Prasenjit and Sumathi, 2005</td>
</tr>
<tr>
<td>Cr(VI)</td>
<td>Distillery sludge</td>
<td>10</td>
<td>2-3</td>
<td>93</td>
<td>2</td>
<td>Selvaraj et al., 2003</td>
</tr>
<tr>
<td>Cr(III)</td>
<td>Aspergillus oryzae</td>
<td>240</td>
<td>5</td>
<td>97</td>
<td>36</td>
<td>Nasseri et al., 2002</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>Malaysian rubber-wood ash</td>
<td>20</td>
<td>5</td>
<td>65</td>
<td>4</td>
<td>Hasen et al., 2000</td>
</tr>
<tr>
<td>Cr(VI)</td>
<td>Rhizopus nigricans</td>
<td>100</td>
<td>2</td>
<td>80</td>
<td>4</td>
<td>Sudha bai and Emilia, 2001</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>Lemma minor</td>
<td>5</td>
<td>7-9</td>
<td>87</td>
<td>5</td>
<td>Nicholas et al., 2003</td>
</tr>
<tr>
<td>Cr(VI)</td>
<td>Micrococcus species</td>
<td>100</td>
<td>7</td>
<td>90</td>
<td>18</td>
<td>Present study</td>
</tr>
<tr>
<td>Cr(VI)</td>
<td>Aspergillus species</td>
<td>100</td>
<td>5</td>
<td>92</td>
<td>18</td>
<td>Present study</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>Micrococcus species</td>
<td>50</td>
<td>7</td>
<td>55</td>
<td>20</td>
<td>Present study</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>Aspergillus species</td>
<td>50</td>
<td>5</td>
<td>90</td>
<td>20</td>
<td>Present study</td>
</tr>
</tbody>
</table>
4.2. TOLERANCE IN RESPONSE TO VARYING HEAVY METAL CONCENTRATION

Initial metal ion concentration plays an important role in determining the bioaccumulative capacity of the isolated *Aspergillus* and *Micrococcus* species (Figures 16 and 17). Tolerance implies a large change in sensitivity between sets of organisms to a particular toxicant. Tolerance can be adaptive, constitutive, or induced. Adaptive tolerance is where the organism colonizing a contaminated site is less insensitive than the same species colonizing uncontaminated sites and where this change in sensitivity is caused by the selection of genes that confer enhanced insensitivity. Induced tolerance, for which there is less evidence, is where particular enzymes that cause decreased sensitivity are induced on exposure to metal ions (Meharg, 2003). Chromium resistant *Micrococcus* and *Aspergillus* species were found to be highly tolerant than the other isolates. The richness of Cr(VI) resistant strains can probably due to a number of environmental factors (Rita, et al., 2005). Studies by various researchers (Galli et al., 1994; Diaz et al., 1996) have reported that mycorrhizal fungal ecotypes from heavy metal–contaminated sites seem to be more tolerant to heavy metals (and have developed resistance) than reference strains from uncontaminated soils (Malcova et al., 2003) reported that species of *Arbuscular mycorrhizal* (AM) fungi and even various isolates of one species can differ in their sensitivity to heavy metals. (Del Val et al., 1999) isolated different fungal ecotypes from soils that received long-term applications of metal-contaminated sewage sludge, with the aim of studying the degree of tolerance and adaptation to heavy metals of AM fungi (Sharda and Alok, 2007). Although Cr(VI) inhibits microbes, there are biotic and abiotic detoxification mechanisms in soil (Avudainayagam et al., 2003). One biotic mechanism (under both aerobic and anaerobic conditions) occurs via Cr(VI) reduction to less toxic and less mobile Cr(III) (Fein et al, 2002; Wang and Shen, 1995). Cr(VI) reduction to nontoxic levels could have
been a precondition for the onset of microbial activity in the microcosms (Cindy et al., 2005). The metal resistant-plant growth-promoting bacterial (PGPB) strains *Pseudomonas sp.* and *Pseudomonas jessenii* isolated from a serpentine soil has been reported as an effective metal sequestering and growth-promoting bioinoculant for plants in metal-stressed soil (Mani and Helena, 2008). Twenty-one yeast-like microorganisms isolated from tannery effluents and from a nickel–copper mine in Argentina were selected for their multiple tolerance to the different heavy metals and highest tolerance to Cr(VI). According to morphological and physiological analysis and 26S rDNA D1/D2 domain sequences, the isolates were characterized as: *Lecythophora sp.*, *Candida sp.* and *Aureobasidium pullulans*. Resistance of the three strains to high Cr(VI) concentrations and their ability to remove Cr(VI) were assessed using YNB-glucose medium supplemented with 0.5 and 1 mM Cr(VI) (Liliana et al., 2008). The influence of different concentrations of heavy metal on the efficiency of bioremediation processes and the bacterial community composition of soil cocontaminated with heavy metal and PAH showed that the presence of different Cr(VI) concentrations did modulate the community response to phenanthrene (Agustin et al., 2008). Adaptation mechanisms differ with the time of exposure to the disturbance. In the short term, the intrinsically tolerant populations survive the pollution. In the long term, the surviving organisms may adapt to the disturbed environment using phenotypic or genetically based adaptation mechanisms (Di’az-Ravina and Baath, 1996; Oger et al., 2001). (Magyarosy et al., 2002) compared the fungal biomass grown in the presence and absence of nickel and to he reference standards in the database. The XRD data of the nickel deposited outside the cell wall and inside cells matched that of nickel oxalate dihydrate, both in the database and of the pure compound. Although individual genes coding for metal resistances have been assessed (Naz et al., 2005), a
detailed understanding of the key indigenous organisms able to tolerate heavy metal pollution is still lacking.

4.3. PROTEIN EXPRESSION IN HEAVY METAL RESISTANT ORGANISMS

Although some heavy metals are essential trace elements, most can be, at high concentrations, toxic to all branches of life, including microbes, by forming complex compounds within the cell. Because heavy metals are increasingly found in microbial habitats due to natural and industrial processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals (by either efflux, complexation, or reduction of metal ions) or to use them as terminal electron acceptors in anaerobic respiration (Anne and Elizabeth, 2003).

When compared with controls (without heavy metals), both of the *Aspergillus* sp. expressed considerable amount of polypeptide (protein) which indicates the speculation that 93kDa protein is involved in response to heavy metals and probably pervasively exists in heavy metal resistant fungi, which remains to be further examined. In *Micrococcus* species in the presence of chromium the organism express more proteins and a significant differential expression of some polypeptide was seen on 180 kDa and Ni-resistant bacterial isolate expressed below 60kDa. The bacterial isolates showed greater variation in proteome expression than for the fungal isolates (Figure 18). This was probably attributed to a higher degree of functional diversity among bacteria.

The inducible response of metal stress (Cr$^{6+}$ and Cr$^{3+}$) of *E. coli* ASU 7 was studied. The electrophoratic analysis in 12% SDS-PAGE of whole cells lysate protein was obvious that Cr$^{6+}$ induce new protein with molecular weight 23 kDa after 8 h. This group of proteins was responsible for chromate resistance
(Abskharon et al., 2008). Similar results was reported by Thacker et al., (2007) that protein with molecular weight 30 kDa induced in presence of chromium and this may possibly be associated with resistance of chromate. Similarly nickel resistant strain of *Pseudomonas fragi* (Patel et al., 2006) showed the induction of protein s with molecular weight 48 and 18 kDa proteins play a vital role in metal resistance mechanism.

Heavy metals are essential micronutrients required for a variety of processes in the cell metabolism, but they are toxic above optimal cellular concentrations. All organisms must therefore critically balance the cellular concentrations of these potentially toxic elements, as they do for non-essential heavy metals. Like other organisms, fungi can adopt two major strategies to keep a low cellular concentration of heavy metals in polluted environments: they can restrict the entry of metal ions into the cytoplasm and/or they can reduce the cytoplasmic concentrations of free metal ions (Gadd, 1993). The understanding of the molecular bases of heavy metal in microorganisms will open new technological perspectives in bioremediation, and attention has been focused on the influence of metal ions on gene expression and protein synthesis in different organisms (Mejáre and Bülow, 2001). Several intracellular polypeptides have been identified following metal induction, and play specific roles in metal tolerance. Metal-binding polypeptides such as cysteine-rich metallothioneins and phytochelatins are induced in the presence of heavy metals and reduce the concentration of free metal ions in the cell (Nedkovska and Atanassov, 1998). In the yeast *Schizosaccharomyces pombe*, a specific ABC transporter located on the vacuolar membrane was found to be responsible for compartmentation of cadmium- phytochelatin complexes into the vacuole (Ortiz et al, 2001). Other intracellular metal-induced proteins are those involved in the cell response to
oxidative stress and include enzymes responsible for glutathione biosynthesis and inactivation of reactive oxygen species (ROS) (Cakmak and Horst, 2006). Proteome analysis of the cadmium response in *Saccharomyces cerevisiae* has revealed the presence of 53 new intracellular polypeptides during acute cadmium stress, and confirmed the strong induction of antioxidant proteins (Vido et al., 2001). The presence of zinc ions induced a general increase of secreted proteins and a shift towards the release of basic proteins. It has been demonstrated that elevated concentrations of zinc ions induce a novel set of basic extracellular proteins in a heavy metal tolerant strain of the mycorrhizal species *O. maius*. Although the function of some of these proteins remains to be clarified, the results confirm the importance of antioxidant proteins as a general response to heavy metal stress, at least on some culture media, and indicate a strong up-regulation of enzymes involved in general nutrient metabolism (Elena et al., 2002).

4.4. **ISOLATION OF GENOMIC DNA**

Isolation of microbial DNA from natural environments has become a useful tool with which to study the ecological functions of certain characterized genes that encode important metabolic pathways. It also allows the tracking of genetically engineered organisms and reveals the microbial DNA diversity in microbial ecosystems. Hence isolation is initial step involved in the molecular approach in remediation of heavy metals.

4.5. **PCR AMPLIFICATION OF THE 16S sRNA AND 18S sRNA**

The current cultural methodologies for isolating and detecting specific bacterial and fungal strains from environmental samples are often hampered by the need to use selective media which often prevent viable cells from
reproducing. Polymerase chain reaction (PCR) amplifications are gaining widespread applications in a variety of studies. The significant advantages of this technique are its specificity and sensitivity. The specificity can be achieved by designing the flanking primers such that only specific DNA sequences are amplified. Sensitivity is achieved by the ability to repeatedly amplify the specific DNA sequence. Since PCR amplification does not require the culturing of the bacterial strains and since it is capable of amplifying unique sequences in the midst of a myriad of DNA sequences, it has the potential to identify specific strains. Additionally, this technique has the advantage of potentially determining whether a soil contains indigenous microorganism or not. This can be accomplished by the use of primers that are specific to targeted organisms (Surjbh et al., 1992). PCR provides a method for increasing the number of copies of a target sequence (amplifying the signal) without having to culture the organism, thereby allowing increased sensitivity in detection.

Both prokaryotic and eukaryotic microbes are so phylogenetically and biochemically diverse that even the identification of homologous proteins is not straightforward. For the analysis of natural microbial populations, in which unknown diversity must be anticipated, there are several reasons to focus on the rRNAs (Gary and David, 1995).

- The rRNAs, as key elements of the protein-synthesizing machinery, are functionally and evolutionarily homologous in all organisms.
- The rRNAs are ancient molecules and are extremely conserved in overall structure. Thus, the homologous RNAs are readily identifiable, by their sizes.
- Nucleotide sequences are also conserved. Some sequence stretches are invariant across the primary kingdoms, while others vary. The conserved
sequences and secondary structure elements allow the alignment of variable sequences so that only homologous nucleotides are employed in any phylogenetic analysis. The highly conserved regions also provide convenient hybridization targets for cloning the rRNA genes and for primer directed sequencing techniques.

- The rRNAs constitute a significant component of the cellular mass, and they are readily recovered from all types of organisms for accumulation of a data base of reference sequences.
- The rRNAs provide sufficient sequence information to permit statistically significant comparisons.
- The rRNA genes seem to lack artifacts of lateral transfer between contemporaneous organisms. Thus, relationships between rRNAs reflect evolutionary relationships of the organisms.

Ribosomal RNA genes gathered from the environment are snapshots of organisms, representatives of different types of genomes and targets for further characterization if they seem interesting or useful. The opportunities for the discovery of new organisms and the development of resources based on microbial diversity are greater than ever before. Molecular sequences have finally given microbial biologists a way to define their subjects, through molecular phylogeny. The sequences are also the basis of the tools that will allow microbial biologists to explore the distribution and roles of the organisms in the environment.

4.6. MAGNETIC BEAD CATCH HYBRIDIZATION PCR AMPLIFICATION OF THE 16S SRNA AND 18S sRNA

Classical methods for DNA isolation are either column-based techniques or include precipitation and centrifugation steps with toxic organic solvents.
having the disadvantage of being time consuming, difficult to automate or not useful for downscaling to small sample volumes. Through the magnetic bead technology these main limitations can be avoided (Taylor et al. 2000; Amagliani et al. 2006). This makes the sample preparation fast and highly adaptable to automation processes. Additionally, hybrid capture using magnetic particles, which relies on selective isolation of target DNA by hybridization to oligonucleotide probes linked to magnetic nanoparticles, allows the replacement of time consuming cultural enrichment steps with specific nucleic acid sequence enrichment. This would decrease the total detection time, increasing PCR sensitivity, and removing most of the inhibitors of the amplification reaction and excess of non target DNA. The MCH-PCR protocol finally excludes the generation of false PCR amplification products, which may arise from mispriming in the presence of humic or phenolic compounds in the soil samples (Steffan and Atlas, 1988). With the high stringency hybridization during magnetic capture, the majority of nontarget DNA, which could have served as incorrect annealing sites for the primers, is removed. The magnetic beads have several characteristics that make them well suited for solid phase sequencing. First, the use of a magnet facilitates liquid handling and the needs for centrifugal steps are avoided. Second, the low density of the beads makes it unnecessary to mix the beads during sequencing. Third, the beads are of equal size (monodisperse), which gives uniform kinetics in the magnetic fields. Finally, low or little interference with the enzymes used for sequencing is observed (Thomas et al., 1991).

4.7. TERMINAL RESTRICTION LENGTH POLYMORPHISM (T-RFLP)

A variety of culture-independent methods have been developed to carry out comparative analyses of microbial communities and to relate community
composition to environmental parameters. Use of a culture-independent method requires a trade-off between phylogenetic resolution and sample throughput. T-RFLP has demonstrated its utility as a community fingerprint method for comparisons of bacterial community composition between environments or treatments (Angela et al., 2003). T-RFLP analysis has gained widespread applications in various aspects of microbiology ranging from disease monitoring for the determination of plant-microorganism interactions. Microbial evolution with respect to various kinds of environmental stresses have been studied with T-RFLP analysis, where in soil samples with their constitutive microbial communities were subjected to a pre-determined stress (qualitative and quantitative) for a certain length of time and resulting fluctuation of microbial diversity was analyzed. Biological remediation of hazardous pollutants by environmental release of degradative microorganisms has a major constrain of stringent monitoring for the mis-effects on the existing microbial flora. With T-RFLP it has become conveniently feasible to perform such monitoring studies. T-RFLP offers, the possibility of rapidly studying several samples makes it most widely applicable technique for the future and offers the characterization of the unseen microbial diversity and its dynamics (Pandey et al., 2007).

T-RFLP has been proved as a powerful tool for the investigation of bacterial community profiles and for characterizing how environmental factors such as heavy metals drive community changes (Osborn et al., 2000). As a monitoring tool, however, it can be affected by experimental and analytical difficulties (Ann et al., 2008).

The present study demonstrates the adaptation of microbial communities to toxic e!ects of metal-contaminated soils. The 16S rRNA and 18S rRNA T-
RFLP provides complementary data on the response of microbial community to the elects of toxic metals. T-RFLP also provides a population fingerprint based on different molecules, which can be used to detect changes in the community structure. In addition, T-RFLP analysis provides an estimate of the species richness (Riina et al., 2004).

4.8. PHYLOGENETIC ANALYSIS

Knowledge of microorganisms in the environment in the past depends mainly on studies of pure cultures in the laboratory. Rarely are microbes so captured. Studies of several types of environments estimate that more than 99% of organisms seen microscopically are not cultivated by routine techniques (Amann et al., 1995). With the sequence-based taxonomic framework of molecular trees, only a gene sequence, not a functioning cell, is required to identify the organism in terms of its phylogenetic type. The occurrence of phylogenetic types of organisms, "phylotypes," and their distributions in natural communities can be surveyed by sequencing rRNA genes obtained from DNA isolated directly from the environment. Analysis of microbial ecosystems in this way is more than a taxonomic exercise because the sequences provide experimental tools—for instance, molecular hybridization probes—that can be used to identify, monitor, and study the microbial inhabitants of natural ecosystems (Hugenholtz and Pace, 1996).

The tree can be considered a rough map of the evolution of the genetic core of the cellular lineages that led to the modern organisms (sequences) included in the tree. The time of occurrence of evolutionary events cannot be extracted reliably from phylogenetic trees, despite common attempts to do so. Time cannot be accurately correlated with sequence change because the
Discussion

evolutionary clock is not constant in different lineages. This disparity is due to the fact that lines leading to the different reference organisms are not all the same length; these different lineages have experienced different extents of sequence change. Nonetheless, the order of occurrence of branchings in the trees can be interpreted as a genealogy, and intriguing insights into the evolution of cells are emerging (Woese, 1987).

Figures 32 and 33 are phylogenetic trees based on small-subunit (SSU) rRNA sequences of the organisms represented. The construction of such a tree is conceptually simple (Swofford et al., 1996). Pairs of rRNA sequences from different organisms are aligned and the differences are counted and considered to be some measure of "evolutionary distance" between the organisms. There is no consideration of the passage of time, only of change in nucleotide sequence. Pair-wise differences between many organisms can then be used to infer phylogenetic trees, maps that represent the evolutionary paths leading to the modern-day sequences. The trees in figures are largely congruent with trees made using any molecule in the nucleic acid-based, information-processing system of cells. On the other hand, phylogenetic trees based on metabolic genes, those involved in the manipulation of small molecules and in interaction with the environment, commonly do not concur with the rRNA-based version (Doolittle and Brown, 1994; Palmer, 1997) for reviews and discussions of phylogenetic results with different molecules. Incongruities in phylogenetic trees made with different molecules may reflect lateral transfers or even the inter mixings of genomes in the course of evolution. Some metabolic archaeal genes, for instance, appear much more highly related to specific bacterial versions than to their eucaryal homologs; other archaeal genes seem decidedly eukaryotic in nature; still other archaeal genes are unique. Nonetheless, the recently
determined sequence of the archaeon *Methanococcus jannaschii* shows that the evolutionary lineage archaea is independent of both eucarya and bacteria (Bult et al., 1996). Evolutionary distance in this type of phylogenetic tree, the extent of sequence change, is read along line segments.

Several studies have shown that metal stress results in decreased microbial diversity and activity (Maliszewska et al., 1985) using plate count procedures, found that As(V), the dominant As species in soils (Cullen and Reimer, 1989, Pongratz 1998, Turpeinen et al., 1999), stimulated the proliferation of certain groups of microorganisms in soil resulting in a shift of the community to comprise only a few tolerant species. However, (Speir et al., 1999) As(V) did not inhibit overall microbial respiration or decrease the microbial biomass in soil. In contrast, Cr(VI) was found to inhibit soil biological properties, such as phosphatase and sulfatase activities and decreased microbial biomass (Speir et al., 1995). Although the extent of inhibition caused by Cr(VI) diminished with time, the differences were generally much smaller than the observed decline in extractability of Cr(VI) (Speir et al., 1995). The experiment of (Speir et al., 1995), however, lasted only for 100 days and therefore the results gave only short-term information of the recovery of soil microbial activity after exposure to Cr(VI) contamination. Copper contamination reduced bacterial diversity (Smit et al., 1997) and relatively large differences were found in amplified ribosomal DNA restriction analysis (ARDRA) between clean and Cu-contaminated soils. In long-term field sites, however, Cu-resistant bacteria, which produced a large quantity of exopolymers, were suggested to have reduced the toxicity of Cu in soil and thus enhanced the growth of other microbes as well as various plants (Kunito et al., 2001). This could lead to an
increase in the diversity of ecological functions at Cu-contaminated sites with time.

In the heavy metal polluted area, the long aging period increases the binding of heavy metals to soil, and decreases the available fractions. Ambient soil properties affect the soil microbial biomass more than the heavy metal pollution because of low metal bioavailability. However, heavy metal pollution tended to impact on the microbial functional composition: the tolerance of microbial community to metal stress in the polluted site seemed to be enhanced. Results with Cu suggests that if heavy metal pollution occurred in soil with low microbial biomass due to low nutrient, low pH and low moisture, a high ecological risk would be predicted because the inherent microbial community in such an area will be vulnerable to metal pollutants (Takafumi et al., 2006).

Correlation analysis studies in case of soluble Pb has shown a significant negative relationship with Nei' gene diversity of subpopulation. It was assumed that soluble Pb may be responsible for the reduced genetic diversity of the Arthrobacter population. The genetic differentiation of microbial populations was consistent with the changes of environmental factors, particularly heavy metals (Zhang et al., 2007).

Species diversity and the structure of microbial communities in soils are thought to be a function of the cumulative selective pressures within the local environment. Shifts in microbial community structure, as a result of metal stress, may have lasting negative effects on soil ecosystem dynamics if critical
microbial community functions are compromised. It was hypothesized that the microbial communities native to the soils would initially be unique to each site, but would converge on a microbial community with similar structure and function, as a result of metal stress. Amendment of the soils with metal-salts resulted in a decrease in microbial activity and biomass, as well as shifts in microbial community structure and function at each site. Soil microbial communities are sensitive to changes in soil pH as a result of metal-salt amendment; however, the magnitude of these pH-associated effects varied between soils. Microbial communities from each site will not converge on a structurally or functionally similar community following metal-salt amendment, indicating that other factors may be equally important in shaping microbial communities in soils. Among these factors, soil physiochemical parameters like organic matter and soil pH, which can both influence the bioavailability and toxicity of metals in soils, may be critical (Anderson et al., 2008). The microbial community diversity is attributed primarily to the change in vegetation type rather than in organic matter content (Dong et al., 2006).