# EFFECTS OF HEAVY METALS ON MICROBIAL BIOMASS AND MICROBIAL ACTIVITY IN A WETLAND SOIL

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## *CERTIFICATE*

The research work embodied in this dissertation entitled " *Effects of Heavy Metals on Microbial Biomass and Microbial Activity in a Wetland soil*" is done by me under the supervision of Prof Brij Gopal in partial fulfilment of the requirements for the degree of Master of Philosophy. The work has been carried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted in part or full for any degree or diploma to any university.

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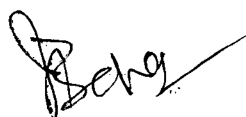

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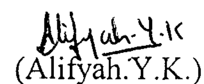


(Alifyah.Y.K.)

# *INTRODUCTION*

Soil is one of the components that maintain the structure and function of both natural and managed ecosystems. Soil is a complex, dynamic and heterogeneous environment that provides a habitat for a wide variety of microorganisms (Nannipieri, 1990). The main function of soil microorganisms is to decompose the soil organic matter into simple organic compounds that results in the mobilization and immobilization of major nutrient elements such as carbon, nitrogen, phosphorous and sulfur. The fertility of natural soils depends significantly on the rate of turnover of soil organic matter, mediated by soil microorganisms. Agents that retard the growth of soil organisms, or change the quality and quantity of soil organic matter (either fresh inputs or the soil organic matter itself), can damage the functioning of the natural ecosystems, either in the short term or over much longer periods (Brookes, 1995). With the degradation of many ecosystems in the world and the lack of knowledge of soil microbial communities, increasing awareness concerning the importance of soil microorganisms in natural ecosystems has emerged (Yao et al., 2003).

Pollution of soils by heavy metals from industrial, agricultural and domestic sources is a major environmental problem. Heavy metals have been given much attention because they are persistent and non-biodegradable in nature. They become irreversibly immobilized within different soil components such as humic substances, particulate organic matter, iron and aluminium oxides and hydroxides, and clay particles. They remain in soil for several thousands of years (Valsecchi et al., 1995). Heavy metal pollution affects adversely the various parameters related to plant growth as well as cause changes in size, composition, and activity of the microbial community (Giller et al., 1998). Inhibition of the microbial activity by heavy metals may result in persistence in soil of potentially decomposable and mineralizable compounds with subsequent effects on fertility, nutrient cycling, and soil structure (Sparling, 1985).

## *CHARACTERISTIC FEATURES OF WETLANDS AND WETLAND SOILS:*

Wetland is a generic term and it encompasses a wide variety of wet habitats such as bogs and fens, pocosins, marshes, swamps, floodplains, mangroves and estuaries (Tiner, 1999). They occupy nearly 6% of the earth's surface. These areas are saturated or flooded with water

that can be fresh or saline, lotic or lentic and permanent or transient. The presence of excess water in wetlands is the driving force for determining the nature of soil development and the types of plant and animal communities that live in the wetlands (Kent, 2001).

*WETLAND SOILS:*

Wetland soils are often described as hydric soils. The term "hydric soils" was coined by U.S Fish and Wildlife service and defined as "the soils that in its undrained condition is saturated, flooded, or ponded long enough during the growing season to develop anaerobic conditions that favour the growth and regeneration of hydrophytic vegetation" (Cowardin et al., 1979; Tiner, 1999).

Extended flooding and waterlogging significantly influences soil forming processes resulting in a set of unique and recognizable soil properties such as absence of oxygen, low redox potentials, accumulation of organic matter, production of methane, hydrogen sulfide, and nitrogen gases and formation of grey colored subsoil horizons (Tiner, 1999).

In wetlands oxygen is introduced into the soils by diffusion from the overlying water column, from photosynthetic oxygen, by diffusion and mass flow from the atmosphere through plants into the root zone and by fluctuations in water table depth (Reddy et al., 2000).

The diffusion coefficient of oxygen through water is 10,000 times slower than through air. Therefore, gas exchange between water in the pores and the soil is extremely low than from air filled pores to the soil (Tiner, 1999). Thus, diffusion of atmospheric oxygen is curtailed in flooded or saturated soils, which induces biological and chemical processes that change the soil from an aerobic and oxidized state to an anaerobic or reduced state. This shift in aeration status of the wetland soil is usually accompanied by declining redox potentials (Fig. 1). Redox potential is a measure of the degree of wetness or intensity of soil anaerobic conditions. Analogous to pH (which measures $H^+$ activity), redox potential measures electron activity in the soil. Redox potential ranges from +700 to – 300 mV in a wetland soil (Reddy et al., 2000, Craft. 2001).

Anaerobic bacteria are important agents in the formation of soil properties associated with repeated and prolonged flooding. These microorganisms are well adapted to low oxygen conditions and derive their metabolic energy from oxidation of organic matter to support growth, metabolism, and reproduction (Tiner, 1999). The rate of organic matter decomposition is however lower in the absence of oxygen than when oxygen is present. This results in an

accumulation of organic matter in the wetland soils because of a high rate of production relative to the rate of decomposition (Reddy et al., 2000).

| ---------------Anaerobic condition---------------------------------------- ------Aerobic-------- | | | | SOIL CONDITIONS |
|---|---|---|---|---|
| Highly reduced | Reduced | Moderately reduced | Oxidized | REDOX CONDITION |
| $CO_2$ | $SO_4^{-2}$ | $Fe^{+3}$, $Mn^{+4}$, $NO_3^-$ | oxygen | ELECTRON ACCEPTOR |
| Anaerobic microorganism | | Facultative | Aerobic | MICROBIAL METABOLISM |
| -200 | 0 | +200 +400 | +600 | |
| REDOX POTENTIAL Eh (millivolts) | | | | |

Figure1 Schematic diagram showing relationship between soil hydrologic conditions, redox potential and metabolic activities of microorganisms in wetlands (Source: Reddy et al., 2000).

Anaerobic metabolism increases the concentration of reduced compounds which are often soluble in water and hence readily bioavailable. Oxidation – reduction reactions govern many of the chemical processes occurring in saturated soils and sediments. These redox reactions are mediated by anaerobic soil microorganisms. Organic matter is a major source of electrons and when it is oxidized, the electrons released are used for reducing reactions. In case of anaerobic soils, the major electron acceptors are nitrate, iron, manganese, sulfate, and carbon dioxide. These get reduced to form $N_2$, $Fe^{2+}$, $Mn^{2+}$, $H_2S$, and $CH_4$. Chemical reduction processes affects the availability of nutrients and plant toxins in the soil. The reduction processes may therefore have a profound effect on plant composition as well as on the soil chemical and morphological processes (Craft, 2001). In wetland soils, the limiting factor is oxygen rather than organic matter, which governs the type and metabolism of microorganisms.

### *MICROORGANISMS IN WETLAND SOILS*

Microorganisms such as bacteria and fungi are key agents in regulating accumulation of organic matter in soils. They take part in the transformation of a variety of chemical species and are instrumental in the elemental cycles of nature such as carbon, nitrogen, phosphorous and sulfur (Craft, 2001).

Bacteria are important in wetland soil environment. They decompose dead and decaying organic matter and convert complex organic compounds to simple organic compounds. These simple organic compounds are then utilized as substrates by other microorganisms for more complete utilization of organic matter. In this process, they release major essential elements such as carbon, nitrogen, phosphorous and sulfur from their unavailable organic forms to available forms to be utilized by other higher organisms for their growth and metabolism. The activity and growth of bacteria therefore sustains the metabolism of other living organisms and maintains the productivity of the whole ecosystem (Fig. 2).

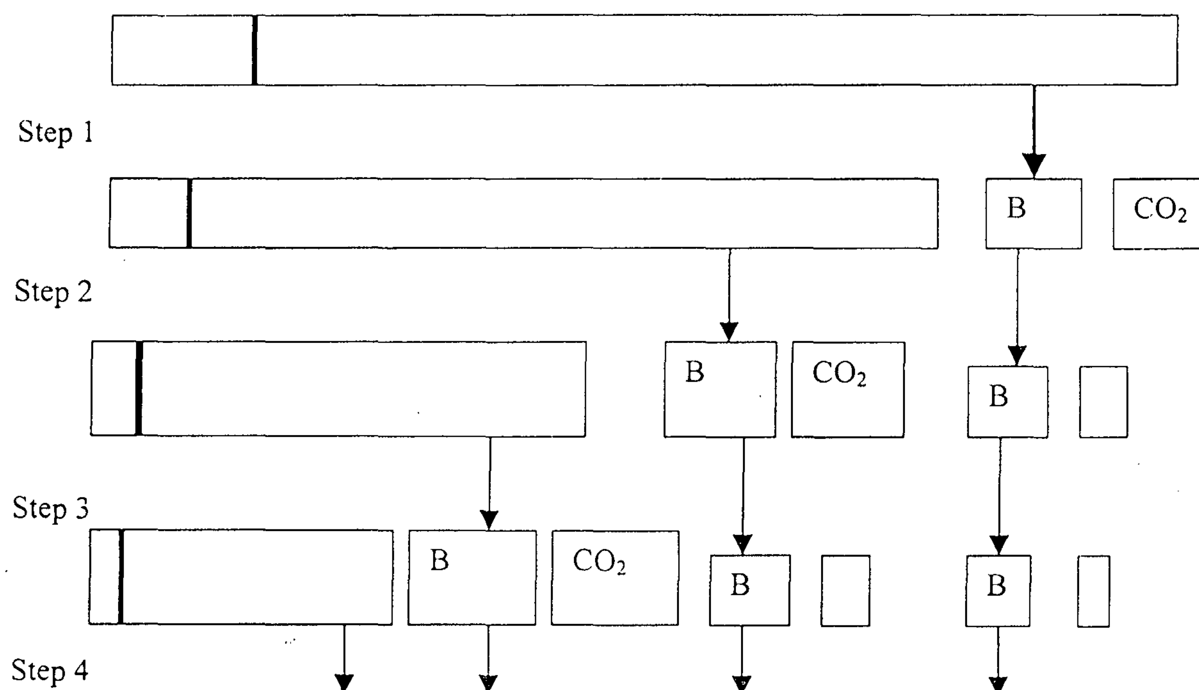



Figure 2. Schematic diagram showing the various stages in microbial decomposition process.
Step 1 – Decay of readily mineralizable carbon and partial conversion to carbon dioxide and biomass (B).
Step 2 - Cellulose and other carbohydrate utilized with further reduction in weight. Part of biomass reutilized and a part of the biomass newly formed.
Step 3 – Decomposition of cellulose continues with a further decline in biomass.
Step 4 – The remaining carbon in soil stabilizes to form humus. Microbial biomass formed in soil serves as a store of nutrients and participates in nutrient cycling.
(Source: Stevenson, 1986)

Based on their requirement for oxygen, bacteria are classified into aerobic bacteria, facultative anaerobic bacteria, and obligate anaerobic bacteria. Oxygen is toxic to all obligatory anaerobic microorganisms. Many anaerobic microorganisms are rich in flavin enzymes, quinones and iron-sulfur proteins. They react spontaneously with oxygen to yield hydrogen

peroxide, superoxide and hydroxyl radicals. Since most anaerobic microorganisms lack peroxidase, catalase and superoxide dismutase enzymes, which destroy the reactive oxygen species, essential cell components are damaged upon exposure to oxygen (Stams et al., 2003). Kirby et al (1981) have discovered an enzyme superoxide dismutase from the obligatory anaerobe *Methanobacterium bryantii*. This provides support to the evidence that methanogenic bacteria, which are strict anaerobes, survive in dry and aerobic conditions (Peters & Conrad, 1996).

Aerobic respiration and anaerobic metabolism are mutually exclusive processes in wetland soils. Submergence or flooding of soils with water results in the development of two distinct layers in wetland ecosystems.

1. a thin oxidized surface layer at the soil-water interface which is dominantly inhabited by aerobic soil microorganisms that decompose any fresh inputs of organic matter using oxygen as the electron acceptor. The microbial activity is rapid and is usually accompanied by complete mineralization of organic carbon compounds to carbon dioxide.
2. an underlying bulk of reduced soil layer in which facultative and obligate anaerobic bacteria survive. They decompose organic matter using oxidized inorganic compounds such as nitrate, sulfate, ferric, manganic, or organic metabolites as electron acceptors.

Even though the deeper layers of the wetland soil remain reduced, this thin oxidized surface layer is often very important in the chemical transfer and nutrient cycling in wetland soils (Reddy et al., 2000). The decomposition of organic matter in submerged soils differ from that in a well-drained soil in two aspects – it is slower and the end products are different.

Further, anaerobic soils or sediments contain a paucity of soil animals that play an important role. They fragment the organic matter in soil such that bacteria, fungi, and other microorganisms can degrade it. The absence of soil animals particularly the bacterial grazers from wetland soils results in the accumulation of bacterially derived organic compounds. Thus, organic matter accumulates in wetlands as a result of reduced fragmentation and decomposition (Craft, 2001).

Besides oxygen, organic matter decomposition is regulated by other factors that include temperature and pH of the soil, quality of organic matter, availability of nutrients and terminal electron acceptors (Craft, 2001). The nature of organic matter in soils being complex, numerous species of microorganisms are involved in its decomposition. The microorganisms

first decompose the available readily mineralizable carbon such as sugars and amino acids. Some of the carbon is converted to carbon dioxide, some is incorporated into microbial tissues and some is converted into stable humus. When all of the readily mineralizable carbon is utilized, microorganisms target more resistant fractions. Also, in each stage of decomposition, a part of the biomass in particular the dead biomass is reutilized (Stevenson, 1986).

The readily mineralizable form of carbon is therefore readily available to microorganisms and any change in this form of carbon is reflected in changes in microbial activity and growth.

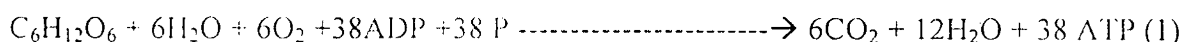
Organic carbon in any ecosystem is usually stored in two forms.

1. living forms of storage of organic carbon-vegetation and microbial biomass.
2. non-living forms of storage of organic carbon- dead plant tissues, litter and soil organic matter.

In wetlands organic carbon is mostly stored in the non-living forms. Detritus based food chain is therefore dominant over grazing based food chain in such systems. (Collins et al., 2001).

*AEROBIC RESPIRATION IN FLOODED SOIL*

The energy needs of the soil microorganisms are met by respiration where organic matter is oxidized and free energy is liberated during the process. In aerobic respiration, molecular oxygen is the ultimate electron acceptor. The oxidation of organic substrates by oxygen to carbon dioxide and water is shown below



$$C_6H_{12}O_6 + 6H_2O + 6O_2 + 38ADP + 38\ P \text{ ------------------------} \rightarrow 6CO_2 + 12H_2O + 38\ ATP \quad (1)$$

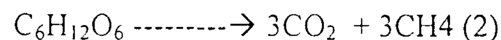
The availability of oxygen significantly influences the redox potential of the environment as well as the energetic situation of the microorganisms. The oxidation of NADH ($E_0'$ = -320 mV) coupled with oxygen reduction to water ($E_0'$ = +818 mV) has a free energy change ($\Delta G^0$) of – 220 KJ/mol whereas the same reaction coupled with $CO_2$ reduction to $CH_4$ ($E_0'$ = -244 mV) yields a free energy of merely –15 KJ/mol (Brune et al., 2000). Aerobic decomposition produces 38 moles of ATP for every one mole of glucose metabolized as compared to only 2 moles of ATP by anaerobic decomposition. Aerobic decomposition therefore yields more energy than anaerobic decomposition.

The aerobic heterotrophs actively metabolize the organic matter in soil shortly after the flooding. Organic matter for the heterotrophic microorganisms in the oxidized layer of flooded soil are supplied from either the anaerobic zone in the reduced layer by diffusion or from the above ground plant and animal biomass, and root exudates.

There are other groups of aerobic bacteria that derive their energy by oxidizing inorganic compounds and use carbon dioxide as their carbon source. These are chemoautotrophic bacteria, which include nitrifying bacteria, ferrous iron and manganese oxidizing bacteria, sulfur oxidizing bacteria, methane oxidizing bacteria or hydrogen oxidizing bacteria. Aerobic conditions also prevail along the root surfaces of aquatic emergent macrophytes, which promote aerobic microbial activity in the bulk of anaerobic wetland soils (Yoshida, 1975).

*FERMENTATION IN FLOODED SOIL*

Fermentation is an energy yielding oxidation-reduction reaction in which organic metabolites serve as terminal electron acceptors. There are many types of fermentation bacteria including those belonging to the genus *Bacillus, Clostridium* (*C.butyricum, C.pasteurianum, C. celulovorans, C. thermocellum), and Lactobacillus* (Lovely 1991). Other common species are *Acetovibrio, Cellulolythicans, Eubacterium cellulososolvens,* and *Rumenococcus* species (Sorokin, 1999; Craft, 2001). During fermentation, complex organic compounds are broken down into simple organic compounds such as formate, acetate, and ethanol (Ponnamperuma, 1972). These compounds serve as substrates for other facultative and obligate anaerobic bacteria including nitrate reducers, iron and manganese reducers, sulfate reducers and methanogenic bacteria (Craft, 2001). Fermentation of organic matter involves successive actions of four populations of microorganisms that degrade complex organic compounds into simple organic compounds (Yoshido 1975).



$$C_6H_{12}O_6 \text{ ---------}\rightarrow 3CO_2 + 3CH4 \quad (2)$$

1. *Hydrolysis:* the process of hydrolyzing biological polymers into oligomers and monomers using hydrolytic enzymes. These enzymes are extra cellular enzymes secreted by heterotrophic bacteria, and fungi. The products of hydrolysis serve as substrates for other microorganisms. Enzyme hydrolysis is generally considered the rate-limiting step in organic matter decomposition.

2. *Acidogenesis:* The process of producing volatile fatty acids, organic acids , and alcohols from monomer compounds or intermediary compounds (formed by hydrolysis) by a facultative or obligate anaerobic bacteria.
3. *Acetogenesis:* The process of producing acetate and hydrogen from previous metabolites by a syntrophic bacteria or homoacetogenic bacteria. Acetogenic fermentation requires the participation of bacteria belonging to the genus *Clostridium (C. Aceticum, C.magnum), Acetobacterium (A.woodii), Acetogenicum (A.kivui), Bacillus (B.methylotrophica)* (Sorokin, 1999).
4. *Methanogenesis:* constitutes the terminal step of organic matter decomposition in anoxic soils. Methane is produced from simple one-carbon compounds like $CO_2$, acetate, methyl alcohol, and methylamines by methanogenic bacteria.

Fermentation plays a very important role in anaerobic mineralization (Ehrlich 1993). Many anaerobic mineralizers are very restricted in the substrates that they can attack and thus depend on fermenters to produce these substrates from complex organic compounds (Fig. 3)..

*ANAEROBIC RESPIRATION IN FLOODED SOIL*

Anaerobic respiration is a biological energy yielding, oxidation-reduction reaction in which an inorganic compound other than oxygen serves as an electron accepto.. Beneath the thin oxidized surface layer anaerobic conditions prevail which promote the growth and activity of facultative and obligate anaerobic microorganisms. The facultative anaerobic bacteria use nitrate, manganic ions, ferric ions, sulfate, and carbon dioxide as electron acceptor and reduce them to molecular nitrogen, manganous and ferrous compounds, sulfide and methane respectively. They couple oxidation of organic matter to carbon dioxide with reduction of these inorganic oxidized compounds in soil to yield energy for their growth and metabolism (Yoshida, 1975). There are various reduction processes, which occur sequentially in wetland soils. They are nitrate reduction, iron and manganese reduction, sulfate reduction, and methane reduction. The sequential reduction of the various electron acceptors in wetland soils depends upon the abundance and physiology of respective microorganisms, the availability of electron acceptors, and redox potential. The electron acceptors enter wetlands through both internal and external inputs. The internal inputs include oxidation of reduced chemical species such as $NH_4^-$, $H_2S$, Fe (II), and Mn (II) that diffuse from anaerobic to aerobic zone in soil. External inputs of electron acceptor include atmospheric oxygen diffusion, sulfate from tidal exchanges, nitrate from surface runoff and precipitation. The respiration processes in which these electron

acceptors are used are separated either in space or time. This is due to differences in energy yield of the reactions. According to Gibb's free energy equation

$\Delta G = n F \Delta E^0$ (7)

where, $\Delta G$ = Gibb's free energy of reaction

n = number of electron transferred during oxidation reaction

F = faraday constant

$\Delta E^0$ = redox potential of electron accepting reaction- redox potential of electron donor reaction.

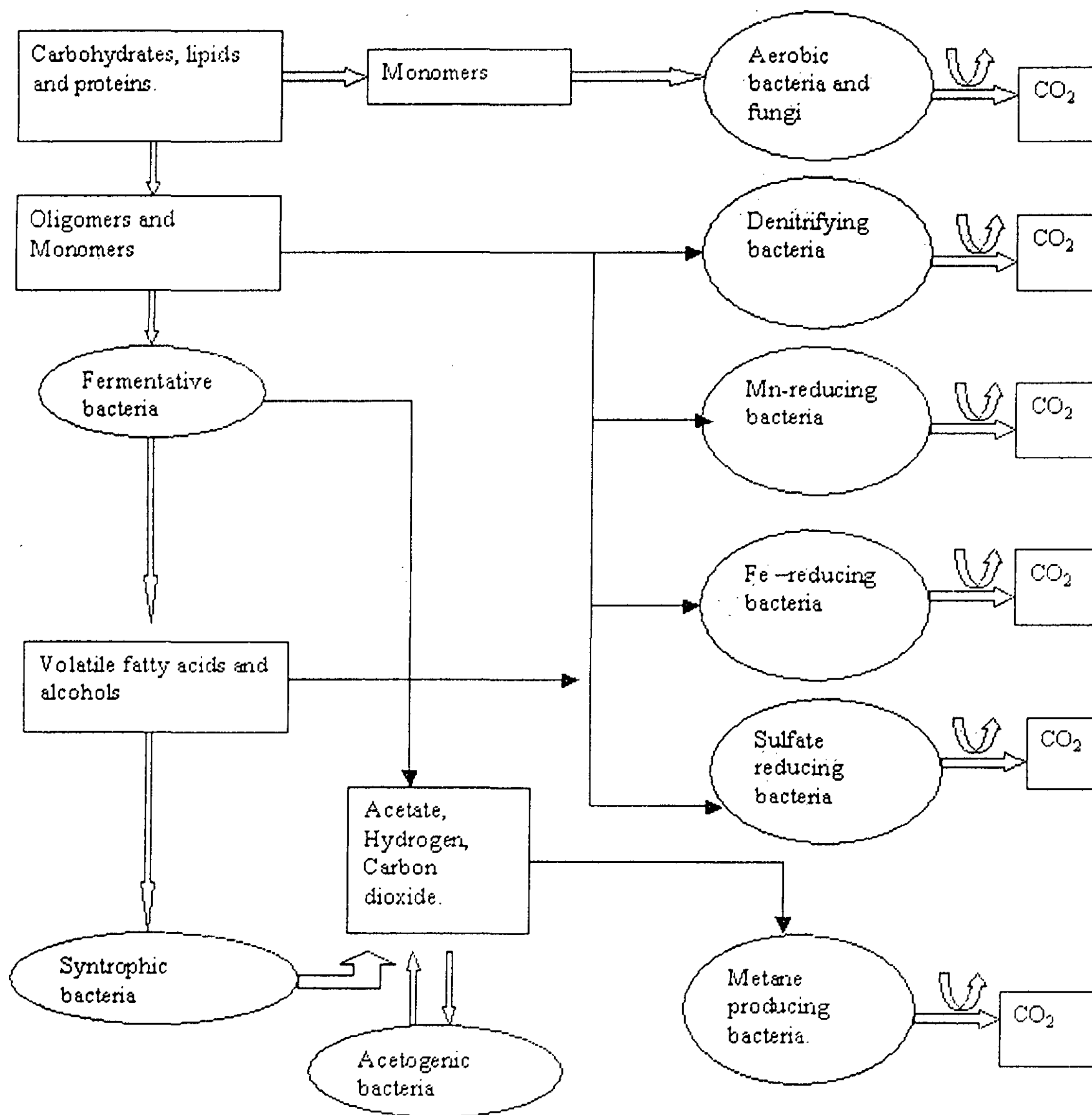



Figure 3 Schematic diagram showing various pathways of organic carbon decomposition in wetland soils. (Source: Westerman, 1993)

The amount of energy available to the organism for performing the reaction depends primarily on the difference between the redox potential of electron accepting and redox potential of electron donor reaction (Stams et al., 2003). This implies that electron acceptors with the highest redox potential will be reduced first. The successive microbial changes are usually accompanied by a stepwise chemical and biochemical reduction of organic and inorganic substrates in soil, lowering of soil redox potential and a change in pH of the soil system (Peters & Conrad, 1996).

***Methane production:***

Methane is the end product of the anaerobic decomposition of organic matter. The gas escapes in large amounts from flooded soils, marshes, lake muds, anoxic lake and ocean waters, sewage disposal units, and from the stomachs of ruminants, accompanied by usually smaller amounts of carbon dioxide and hydrogen (Ponnamperuma, 1972).

Methane is produced by a specialized group of microorganisms – the methanogens. Methanogens are a morphologically diverse yet physiologically coherent group of organisms. Methanogens taxonomically belong to the archaeal kingdom of Euryarchaeota. They are classified in five orders (Balch et al., 1979; Boone et al., 1993)

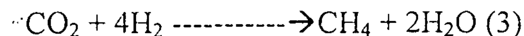
1. *Methanobacteriales*
2. *Methanococcales*
3. *Methanomicrobiales*
4. *Methanopyrales* *and*
5. *Methanosarcinales*

Methanogenic bacteria are obligate anaerobic bacteria, which produce methane at very low redox potentials of <-150mV. Methane bacteria are highly substrate specific. They utilize acetate, $H_2$, $CO_2$, formate and other C1 compounds such as methanol, methyl thiols, and methylamines as energy substrates. These substances may be derived from the breakdown of carbohydrates, proteins, or fats.

Methanogenic bacteria produce methane by three different pathways

- Carbon dioxide reducing pathway
- Methanotrophic pathway
- Aceticlastic pathway

In carbon dioxide reducing pathway, carbon dioxide serves as the substrate for methane.



$$CO_2 + 4H_2 \text{-----------}\rightarrow CH_4 + 2H_2O \ (3)$$

In methanotrophic pathway, methanol, methylamines, and methyl thiols serve as substrates. Here, one of the methyl moieties is reduced to methane and the other is oxidized to $CO_2$ to release reducing equivalents for reduction reactions.

In aceticlastic pathway, acetate serves as one of the major substrate of methanogenic bacteria. The ability to use acetate as a substrate for methanogenesis and growth is restricted to the genera *Methanosarcina and Methanosaeta* (Thauer, 1998; Deppenmeier et al., 1996).

The process of methane production in methanogenic bacteria is coupled to the generation of a primary proton ion gradient and a primary sodium ion gradient. These electrochemical gradients are used to drive ATP synthesis via electron transport phosphorylation in methanogenic bacteria (Thauer et al., 1998). The enzymes, which catalyze key processes in energy conservation mechanisms in methanogenic bacteria, contain metals such as nickel, cobalt, molybdenum, and zinc in their active sites (Deppenmeier et al., 1992; Gartner et al., 1993; Abbanat et al., 1991; Borner et al., 1991). Metals are therefore required by methanogenic bacteria in appropriate amounts to sustain the activity of these enzymes.

In the presence of sulfate, iron and nitrate methanogenic activity ceases because methanogens have a higher threshold and relatively lower affinity for acetate and hydrogen. They face competition from other bacteria for these substrates (Lovely, 1991). Methane formation is ecologically important because it helps in the decomposition of large amounts of organic matter sedimented in lakes (Ponnamperuma, 1972). Methanogenic bacteria metabolize the end products of a large number of organisms (Fermentative, acetogenic, syntrophic bacteria), which decompose the organic matter anaerobically. If methanogenic activity ceases, these end products would accumulate and become potentially toxic to those bacteria, which produce them (Walichnowski & Lawrence, 1982). Methane plays a very important role in carbon cycle in freshwater sediments. From their study Rudd and Hamilton (1978) showed that 36% of the total carbon input into an artificially eutrophied lake was recycled by methane oxidation. This suggests that if methanogenic activity were inhibited in freshwater wetland ecosystems by toxic or harmful chemicals, then nutrient cycling within the system would be adversely affected.

*HEAVY METAL POLLUTION AND WETLANDS*

Intensive industrialization and urbanization has resulted in widespread pollution of nearly all ecosystems in the world. Although natural ecosystems are subjected to natural perturbations such as fire, earthquakes, and floods, they usually recover from such effects on a successional basis (Edwards, 2002). However, pollutants like heavy metals are persistent and have long-term effects so that recovery from their effects may be slow or limited. Heavy metals are known to affect adversely the metabolic activities of microorganisms in soils. There are several heavy metals, which are essential for the growth, reproduction, and survival of the living organisms while others have economic, industrial or military uses (Duxbury, 1985). When heavy metals reach high concentrations in the environment, they become toxic to plants, animals and microorganisms. They are readily taken up by plants and soil microorganisms from the environment and have the potential to accumulate in their tissues. For these reasons, heavy metals have recently been given much attention. Soils serve as a principal sink for heavy metals in the environment.

Table 1 Source of Heavy Metals in Soil

| Sources of heavy metals | Details | References |
|---|---|---|
| Industrial sources | Wastewater discharges from several industries-Cr plating, electrical industries, iron and steel production, mining and smelting operations, and ore cleaning operations. Use of industrial products and their disposal are a major source. | Sun & Shi, 1998. Banin et al 1981. Brimblecomebe,1994. Sposito et al., 1982. |
| Domestic and Municipal sources | Household effluents and drainage water,commercial waste water (car washes, dental uses, and other enterprises),disposal of sewage sludge, and solid wastes on land, application of sewage sludge on agricultural fields, and storm water runoff. | US EPA 1986. Sorme nd Lagerkvist, 2002. |
| Agricultural sources | Us of fertilizers and pesticides (fungicides) on agricultural fields, application of irrigation water, and animal manures are also sources of heavy metals. | Valsecchi et al., 1995. Han et al., 2003. |
| Other sources | Atmospheric deposition, and traffic related emissions | Banin et al., 1981. Sposito et al., 1982. |

The heavy metals from these several sources (Table 1) eventually enter wastewaters that are directed without any pre-treatment of the wastes into the rivers and streams. This results in widespread pollution within the wetland systems that occupy the banks of rivers and streams. The fate of heavy metals in wetland soils depends upon the metal specific properties

and the physical and chemical properties of the soil such as pH, redox potential, organic matter content, clay mineralogy, and cation exchange property.

### *TOXICITY OF HEAVY METALS*

Heavy metals are toxic to living organisms when present in high concentrations in the environment (Dai et al., 2004, Giller et al., 1998, Baath, 1989).

The influence of heavy metals on photosynthesis and other physiological processes in plants is quite well established. Lot of literature exists which investigate the toxic effects of heavy metals on plants and animals (Wang et al., 2003). Since heavy metals accumulate within the living tissues of plants, animals and microorganisms (Yim et al., 1999). A number of laboratory scale studies have shown that many emergent wetland plants especially Typha (cattail) and Phragmites species accumulate metals to higher concentrations (Peltier et al., 2003).

Microorganisms are no different in this respect and heavy metal exposure has since the last century been known to affect microbial growth and survival). Heavy metals affect the growth, morphology and metabolism of microorganisms in soil (Fliessbach et al., 1994; Giller et al., 1998). They affect the folding of proteins and denature them, and destroy the structural arrangement of phospholipids in cell membranes (Leita et al., 1995). The toxic effects of most heavy metal ions are expressed when they enter a living cell. Heavy metals enter living cells by two types of uptake mechanisms in bacteria.

- A fast and unspecific type of uptake mechanism driven only by the chemiosmotic gradient across the cytoplasmic membrane of bacteria.
- A slow and highly specific uptake mechanism driven not only by chemiosmotic gradient but also by the energy released through ATP hydrolysis (Nies, 1999).

Since most metal ions such as copper, nickel, zinc, and manganese are accumulated by fast and unspecific type of uptake system that is constitutively expressed, the metal ion is transported into the cytoplasm despite its high concentration. Hence, heavy metal ions are toxic (Nies, 1999). Within the cells heavy metal cations (especially $Hg^{+}$, $Ag^{+}$, and $Cd^{+2}$) bind to sulf-hydryl groups and inhibit the activity of sensitive enzymes. There are some heavy metal cations, which interact with the physiological ions for instance $Cd^{+2}$ with $Zn^{+2}$ and $Ca^{+2}$; $Ni^{+2}$ and $Co^{+2}$ with $Fe^{+2}$; $Zn^{+2}$ with $Mg^{+2}$ thereby inhibiting the function of the respective physiological cations. Heavy metal cations also cause a considerable oxidative stress by releasing hydroperoxide radicals (Kachur et al., 1998, Nies, 1999). Copper toxicity is based on

the production of hydroperoxide radicals and on interaction with the cell membrane (Suwalsky et al 1998). Finally heavy metal oxyanions (chromate and arsenate) interfere with the metabolism of structurally related non-metals (sulfate and phosphate) and their eventual reduction leads to the production of radicals (Nies, 1999).

There is undoubtedly a natural variability in inherent tolerance within populations of bacteria to metals. In short-term laboratory scale studies, microorganisms are subjected to very high concentrations of heavy metals in a short span of time. Therefore those organisms, which possess metal resistant genes or acquire them by transformation mechanisms, are selected. While in long term field experiments microorganisms are subjected to low concentrations of heavy metals (for instance long term sewage sludge application to land) which may result in subtle changes in competitive abilities, gradually leading to changes in community structure with an associated risk of loss of functional diversity (Giller et al., 1998).

## *SIGNIFICANCE OF MICROBIAL PARAMETERS IN EVALUATING HEAVY METAL POLLUTION*

Microbial parameters as indicators of soil pollution assume significance over plant related parameters because microorganisms respond rapidly to any change in the environmental conditions due to pollution and serve as an early warning of stress in the environment. Microorganisms are in intimate contact with the soil microenvironment and therefore are ideal monitors of soil pollution (Brookes, 1995). A number of soil microbiological parameters have been employed in national and international monitoring programs (Yao et al., 2003). The use of microbial parameters in evaluating heavy metal pollution in soils fall into two main groups;

*Microbial Activity*: Microbial activities may reflect the functions of total (respiration) or specific (nitrification) group of microorganisms in the soils. The most commonly studied microbial activities affected by inorganic or organic pollutants include; C- mineralization, N-mineralization, $CO_2$ production and enzyme activities (Vig et al., 2003).

*Microbial population size*: It reflects the abundance and diversity of microorganisms in soil measured at the single organism level (plate count method), at the functional group level (community structure by phospholipid fatty acid analysis) or at the whole population level (microbial biomass estimation).

Recently, specific activities of microbial populations have been recommended as a biological indicator of heavy metal stress (Anderson & Domsch, 1989; Insam et al., 1996). The

specific activities of microbial populations are derived from combinations of both activity and biomass measurements for instance metabolic quotient ($qCO_2$) or specific respiration rate.

*MINERALIZATION OF SOIL ORGANIC MATTER:*

Mineralization of soil organic matter has a fundamental role in soil fertility because it releases nutrients that will be available to plants, thus sustaining productivity of the ecosystem. It also prevents accumulation of organic matter to levels that might limit primary production (Stevenson, 1986). The measurement of mineralization of soil organic carbon in the presence of heavy metals reflects the possible damage of such potentially toxic materials to the physiological functions of the soil.

Mineralization of organic carbon compounds to carbon dioxide is commonly known as soil respiration. Soil respiration is one of the most well studied parameter with respect to the effects of heavy metals on microbial activities in soils (Baath, 1989). However, in flooded wetland soils mineralization of soil organic carbon refers to the production of carbon dioxide and methane by the degradation of soil organic matter by both aerobic and anaerobic microorganisms.

Numerous studies have been conducted on the influence of heavy metals on carbon dioxide evolution from metal contaminated soils. The results from these studies show considerable disparity. Several studies have reported a decrease in carbon dioxide evolution from soils contaminated with heavy metals (Doelman & Haanstra, 1984; Brookes & Mc Grath, 1984; Brookes et al., 1986; Baath, 1989; Hattori, 1991&1992; Insam et al., 1996; Rost et al., 2001; Dai et al., 2004). In contrast several other studies have reported an increase in carbon dioxide evolution from metal contaminated soils (Chander & Brookes, 1991; Leita et al., 1995; Valescchi et al., 1995: Aceves et al., 1999).

Such conflicting results can be explained by the fact that some microorganisms succumb to the toxicity of heavy metals and their dead cells serve as labile substrate for the more resistant microorganisms in soil finally leading to more carbon dioxide being released from organic carbon mineralization (Giller et al., 1998). Further, based on Odum's theory on bioenergetics of Ecosystem Development, an increase in the respiration rate of soil serves as an early sign of stress in the ecosystem. According to this theory, if damage is caused to the ecosystem by natural or man-made disturbances, repairing such damages require diverting energy from growth and production to maintenance (Odum, 1985).

In a recent study by Rost et al (2001), a marked reduction in the carbon dioxide production and nitrogen mineralization due to an increasing metal contamination in soil was observed. They attributed these effects of zinc on soil microbial activities to the inhibition of polysaccharide decomposing enzymes such as cellulases. Metal ions like copper and zinc may inactivate enzyme reactions by complexing the substrate, by blocking the active sites of the enzymes, thereby modifying the active conformation of the enzymes, and by reacting with the enzyme substrate complex. However, Renella et al. (2002) points to the fact that heavy metals added as soluble salts to the soils would have caused metal concentrations in soil solution to be comparatively large. Thus microorganisms in soils are exposed to large concentrations of heavy metals in biologically active forms, which explains the decline in carbon dioxide production observed in their study upon metal contamination.

The effects of heavy metals on soil respiration also differ with the type of soil and the different chemical forms of metal added to soil. When Cadmium was added to sandy loam soil in insoluble forms as carbonate and oxide it was less toxic to cellulose decomposition than when Cadmium was added as sulfate and chloride (Khan & Frankland, 1984; Vig et al., 2003).

In one study (Doelman & Haanstra, 1984), the inhibitory effects of heavy metals (Cd, Cr, Cu, Pb, Ni, and Zn) on soil respiration rate were investigated in five Dutch soil types. The measurements were taken both immediately after the addition of heavy metals and approximately 18 months later. They observed that the inhibitory effects of heavy metals on soil respiration partially decreased with time. Evidence from field suggests that under long term metal stress there is a change in the genetic structure of the soil microbial community, without there necessarily being an increase in metal tolerance (Giller et al., 1998). Long-term exposures to metals enable adapted microbial community to proliferate (Shi et al., 2002). In stark contrast, when large concentrations of heavy metals are added to soils, they constitute a sudden, drastic disturbance to the soil environment and any microbial response that is immediately measured pertains to the disturbance

*Methanogenesis:*

In freshwater sediments, methanogenesis predominates due to limited sulfate content. nickel, cobalt, and molybdenum are essential elements for certain methanogens. Jones et al (1982) amended freshwater sediments with 0.06ppm, Ni or Co or 0.096ppm Mo and observed that methanogenesis was not stimulated except for some surface sediment samples. However, the addition of about 1900ppm Mo resulted in a 60% to 80% decrease in methane production. Contrary to this when adequate sulfur was added to these sediments with a nitrogen and carbon

dioxide atmosphere, stimulation of methanogenesis was observed. Similar increase in methane production was also recorded from salt marsh sediments spiked with molybdate by Capone et al (1983). They suggested that inhibition of sulfate reducers by molybdate might have resulted in stimulation of methane production. The chlorides of nickel, cadmium and copper as well as zinc sulfate, lead sulfide, and mercuric sulfide caused short-term inhibition, but showed no significant long-term effects. The effects on methane production were found to vary with the type of metal and its chemical form (Duxbury, 1985).

*MICROBIAL BIOMASS:*

The soil microbial biomass forms the living part of soil organic matter. It constitutes the total mass of microorganisms including dead and living cells in soil particularly those cells that have volumes of less than $5000\mu m^3$ (Brookes, 1995).

Microbial biomass acts as a labile reservoir of major plant nutrients such as carbon, nitrogen, phosphorous, and sulfur. Microbial biomass plays a very important role in wetland carbon cycle. The organic carbon that has been fixed through photosynthesis by phytoplankton and aquatic macrophytes is decomposed by bacteria without the involvement of higher trophic level organisms. Although microbial biomass constitutes only 1-4% of soil organic matter, yet most of the ecosystem production passes through the microbial component. Microorganisms derive energy for their growth and maintenance from organic carbon decomposition in soil, thereby allowing the cycling of major nutrients within the wetland system (Collins et al., 2001). Microbial processes are limited by the availability of organic carbon substrate. The substrate availability in microbial habitats fluctuates throughout the year influenced by abiotic factors. The dynamics of microbial biomass turnover is affected most by sudden changes in the physico-chemical environment such as water and redox regime in the soil as well as by toxic environmental pollutants such as heavy metals, pesticides, and recalcitrant organic matter in soil. According to Powlson and Jenkinson (1976) microbial biomass is a much more sensitive indicator than total soil organic matter to changing soil conditions. It detects changes in soil quality and fertility long before they are detected by changes in soil organic matter. It also reflects changes in microbial growth and activity due to environmental pollutants such as heavy metals in soils.

Heavy metals affect the size of microbial populations by two ways. One is by producing direct toxic effects i.e., by killing and or biochemically disabling the organisms. The

other way is by decreasing the availability of organic substrates. Thus, the decreased energy available to the microoganisms also results in a smaller population size (Brookes. 1995).

Microorganisms differ in their sensitivity to metal toxicity. Development of tolerance in microorganisms and gradual shifts in community structure are few of the mechanisms to compensate for the loss of sensitive microbial populations.

Results from laboratory eco-toxicological studies suggest that changes in community structure occur parallel with a decrease in the soil microbial biomass (Frostegard et al., 1993; 1996). Heavy metal toxicity suppresses the growth of metal-sensitive microorganisms and their death enables the growth and proliferation of metal resistant microorganisms, which eventually leads to a shift in population composition (Fliesbach, 1994; Kelly et al., 1999). Analysis of soils contaminated with heavy metals from different sources such as Cu and Zn in animal manures (Christie and Beattie, 1989), run-off from timber treatment plants (Yeates et al., 1994), past applications of Cu containing fungicides (Zelles et al., 1994; Filser et al., 1995), metal contaminated sewage sludge (Chander & Brookes, 1991,1993,1995; Fliesbach et al., 1994, Rost et al., 2001, Brookes & McGrath, 1984) and metal contaminated waste disposal sites (Kuperman and Carreiro, 1997) shows a decrease in microbial biomass carbon even at low to moderate heavy metal loadings. The effect of heavy metals on soil microbial biomass is seen even after a long gap. Brookes & Mc Grath (1984) observed a decline in microbial biomass after 20 years since sludge was last applied on agricultural soils. Similarly Insam (1996) and Baath (1991) also observed a decrease in microbial biomass carbon from a long-term metal contaminated soil. According to Fliesbach et al (1994), this decrease in microbial biomass in metal contaminated soil is due to inefficient biomass synthesis. The efficiency of biomass synthesis is calculated as the increase in microbial biomass carbon as a function of total amount of carbon applied with sewage sludge. In the presence of heavy metals, the ability of microorganisms to decompose organic substrates is severely affected. Lesser amount of organic carbon is incorporated into the microbial biomass of the total organic carbon in soil.

Chander & Brookes (1995) investigated the short term and long term effects of additions of sewage sludge on soil microbial biomass carbon. The amount of biomass carbon increased on a short-term scale (up to 4 week incubation) while it decreased upon further incubation (up to 64 week incubation) irrespective of sludge type or application rate. The increase in biomass was the result of an increase in the availability of readily mineralizable organic carbon. When this pool of organic carbon was exhausted, it resulted in a decline of soil microbial biomass carbon in sludge treated soils.

In another study, Yao et al (2003) investigated the toxic effects of heavy metals on soil microbial community in a sequence of heavy metal polluted paddy soils around a smelter. There was a consistent decrease in the microbial biomass carbon and nitrogen with increasing metal concentrations. There was a decline in the ratio of microbial carbon to soil organic carbon observed in metal contaminated soils, due to adverse effects of heavy metals on microbial form of carbon. Since microbial processes are inhibited there is a continuous build up of organic matter in soil. The metabolic quotient on the other hand increased consistently along the gradient of heavy metal concentrations and showed a positive significant correlation between heavy metal content and metabolic quotient ($qCO_2$). Thus, metabolic quotient serves as an important indicator of soil quality and is closely related to soil pollution.

Extensive literature is available on the effects of heavy metals on microbial biomass and microbial activity in well-drained upland soils. Most of these studies have been conducted in developed countries. Research in this area is still in its infancy in developing countries like India. Though pollution is widespread in India, very few studies have monitored the microbial response to these pollutants. Heavy metals have gained much attention but studies emphasizing the effects of heavy metals on soil microbial populations and their activities are few. Earlier works in India have been done on rice soils while no literature is yet available on a study conducted in natural wetland soils. Therefore, this preliminary study is carried out in natural freshwater wetland sediments to investigate the effects of heavy metals on microbial biomass and microbial activity over a certain incubation period.

## *OBJECTIVES OF THE STUDY*

The objective of the present study is "***to investigate the effects of heavy metals at two different concentrations on the microbial activity-carbon dioxide production and methane evolution from soil and on microbial biomass in soil***".

# MATERIALS AND METHODS

## SAMPLING AREA:

This study was made on lake Bhalsawa, a natural freshwater wetland located on the northern outskirts of Delhi. The lake is roughly rectangular in shape and extends from north to south. There is a boundary wall, which separates the lake area from the adjoining areas. Recreational activity is present along the eastern shore of the lake and a dairy farm is located along the western shore of the lake. Fishing is quite common in the lake. There is a municipal landfill site located about 500m along the west of the lake.

## SAMPLE COLLECTION AND PREPARATION:

Soil samples were collected randomly from different sites at the northern end of the lake. The area is relatively undisturbed and dominated by *Scirpus littoralis.* A 20 cm×20 cm quadrat was used to demarcate the area on the soil. Soil samples were collected from the surface to a depth of 20cm with the help of a khurpi into a polyethylene bag. The soil samples were immediately brought to the laboratory in an ice-chest. Soil samples were air dried on blotting sheets for 72hrs. The air-dried soil was subsequently crushed, grounded in a pestle mortar and sieved through a 2mm seive. The sieved soil samples were stored in polyethylene bags at 4°C for further use.

## EXPERIMENTAL STUDY:

The heavy metals selected for this experiment were copper and nickel.
The experiment was carried out using 250ml conical flasks (Borosil). Thirty grams of soil was weighed into each conical flask. The soil was flooded with double distilled water adding 1.25 ml of water for every gram of soil (Mishra et al, 1999). Salts of copper chloride and nickel chloride were dissolved separately in distilled water to obtain solutions of 20ppm and 40ppm concentrations each. There were four treatments for this experiment

- Control set: In this set, heavy metals were not added to the soil.
- Cu-20 set: In this set copper solution of 20 ppm was added to the soil
- Cu-40: In this set copper solution of 40 ppm was added to the soil
- Ni-20 set: In this set, nickel solution of 20 ppm was added to soil
- Ni-40 set: In this set nickel solution of 40 ppm was added to the soil.

Three replicates were taken for each treatment. A blank set was also prepared containing glass beads in place of soil sample. In four sets of soils heavy metal solution was added in place of distilled water. Both experiments were conducted for 9 days. After the initial reading, observations were made after 24hrs (1day) and then alternate days up to the ninth day. A total of 90 samples were incubated for 9 days. On each sampling day, 15 samples were analyzed for the following four parameters

1. to determine of the amount of $CO_2$ produced from soil.
2. to determine the amount of methane evolved from soil.
3. to determine the readily mineralizable carbon content
4. to determine the microbial carbon content

### *MICROBIAL ACTIVITY:*

Microbial activity in soil is determined in terms of the amount of $CO_2$ and $CH_4$ produced from the soil (Stotzky, 1965). Carbon dioxide is produced as a result of both aerobic and anaerobic respiration of microorganisms in soil while methane is produced as a result of anaerobic respiration only. However, some carbon dioxide is also produced during the process of fermentation by anaerobic microorganisms from soil.

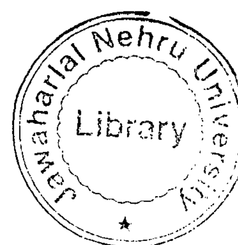
### *METHOD TO ESTIMATE $CO_2$ PRODUCTION FROM SOIL:*

The amount of $CO_2$ produced from the incubated soils is estimated by volumetric method (Stotzky, 1965). In this method, a strong alkaline solution of sodium hydroxide solution is used which absorbs $CO_2$. For this experiment, 10 ml glass vial containing 5 ml of 0.25N of sodium hydroxide solution was hung in each of the conical flasks. A blank set was also included in this estimation. This was done to correct for any error due to the amount of carbon dioxide present in the air trapped inside the conical flasks.

At the end of each incubation period, the remaining unused sodium hydroxide (NaOH) solution was then titrated against 0.25 N hydrochloric acid (HCl) using phenolphthalein as indicator. The colour change at the end point was from dark pink to colourless.

The decrease in the volume of sodium hydroxide solution was determined to calculate the amount of carbon dioxide evolved from soil (in terms of mg) as given by the following equation.

$$CO_2 - C\ (mg) = (B - V)\ N\ E$$



*METHOD TO ESTIMATE METHANE EMISSION FROM SOIL:*

Methane gas was drawn from each of the samples with the help of an air tight hypodermic syringe fitted with a 19 gauge $1^{1}/2$ inch long bevel, luer lock, stainless hypodermic needle into the rubber cork. The sample was injected into pre-evacuated 10 ml glass vials. These glass vials were sealed with a n-butyl rubber septum and an aluminum cap and were stored in the refrigerator at $4^{0}$C before analysis.

*Methane analysis:*

Methane in the air samples was samples were analyzed on a Perkin Elmer ASXL Gas Chromatograph equipped with a Flame ionization detector and a Porapaq Q column. The column used was 6m long with a mesh size of 80/100. Nitrogen was used as a carrier gas at a flow rate of 30ml/min. The oven temperature was maintained at 45°C, injector at 80°C, and detector at 150°C. Under these conditions, the retention time of methane was 0.65 min. The gas chromatograph was calibrated before each set of measurement using 108, 54, 36 μl of methane/ml in $N_2$. A one ml capacity gas tight Hamilton syringe (1001 LTN, ga.22/51mm/pst 2) fitted with a 2-inch × 51mm long, point style 2 needle was used for methane analysis on Gas Chromatograph. One ml of gas sample was drawn from each vial and injected directly through the injection port into the packed column of the Gas Chromatograph. The concentration of methane in the gas samples was determined by correlating the peak area obtained for each sample with that of the standard curve.

*Standard Curve:*

A standard gas mixture (108ppm in air) obtained from EDT Research, London supplied by Nucon Engg Ltd, New Delhi, was used to prepare standard curve. The concentration of 54 and 36 ppm was prepared by diluting standard gas mixture in $N_2$ (vol/vol) in an airtight glass vial in a 1:2 and 1:3 ratios respectively. A total of three concentrations (108, 54, 36) were used to prepare the standard curve.

*Calculation of the methane emission:*

The amount of methane evolved was calculated as μg/g from the concentration of methane obtained in the gas sample.

Total volume of conical flasks with rubber cork (litres) = $V_1$

Volume of soil + water (litres) = $V_2$

Volume of headspace (litres) = $V_1$-$V_2$

Concentration of methane in one ml of gas injected into GC = $X_1$ppm

Here ppm = μL/L

Total concentration of methane in $V_1$- $V_2$ ml of headspace = $X_1$ μL/L × ($V_1$-$V_2$)

= $X_2$μL

Since, 16 μg of methane occupies a volume of 22.4 L under standard conditions of temperature and pressure.

Amount of methane emitted from soil (μg) = ($X_2$ μL * 16)/22.4 = $X_3$ μg

Actual amount of methane emitted from soil (μg) = sample treatment – blank

*Rate of methane emission:*

The rate of methane emission was calculated using the following formula

Rate of methane emission = ($X_3t_2$ – $X_3t_1$) / ($t_2$-$t_1$) * W

Where, $t_2 - t_1$ = time interval between two dates of sampling

$X_3t_2 - X_3t_1$ = amount of methane produced from the soil in terms of μg between two dates of sampling.

W = weight of soil (g)

## *CARBON ANALYSIS:*

### *READILY MINERALIZABLE CARBON:*

Readily mineralizable carbon (RMC) is defined as the amount of carbon incorporated into or easily degradable or labile form of organic carbon compounds. It was determined by extracting the soil samples with 0.5M potassium sulfate solution (Vance, 1987; Inubushi et al, 1991; Mishra et al, 1999; Mishra et al, 1997; Veroney & Peters, 1993). At the end of each incubation period, 10 gms of wet soil was weighed from each sample set into a clean, oven dried 250 ml conical flask. Soil was extracted with 40ml of 0.5 M potassium sulfate solution, added in a ratio of 1part soil to 4 parts 0.5M potassium sulfate (w/v) and the mixture was shaken vigorously for 30 min. The soil suspension was then filtered through Whatman no 5 filter paper and the extract was stored in bottles at 1-2°C for one week. A white precipitate that formed (presumably $CaSO_4$) on storage in a few samples was dispersed by ultrasonication.

Organic carbon in the extracts was determined by digesting the filtered extract (8ml) with 66.7mM potassium dichromate (2ml), mercuric oxide (70mg) and a mixture (15ml) of 2 parts of sulfuric acid (97%) and one part of phosphoric acid (88%).

The mixture was boiled gently under reflux for 30min, allowed to cool and then diluted with 50ml double distilled water, added through the condensor as a rinse. The excess dichromate remaining in the extract was determined by back titration with Ferrous ammonium sulfate (33.3mM) prepared in 0.4M sulfuric acid. The indicator used was 25mM 1,10,phenanthroline ferrous sulfate complex solution (supplied by Merck ltd). The colour changes from yellow through dark green to maroon red.

The acidified Ferrous ammonium sulfate was standardized against 66.7mM potassium dichromate (cold blank) before conducting titration of the samples.

The amount of dichromate consumed is that remaining in a blank digestion with 8ml potassium sulfate (hot blank) less that remaining in the digest of the extract. Extractable carbon is calculated assuming that 1ml of 66.7mM potassium dichromate is equivalent to 1200μg of carbon.

*Calculation of readily mineralizable carbon:*

Volume of solution in extracted soil

V = FW – DW + EV

Where,

V = volume of solution in the extracted soil (ml)

FW = soil fresh weight (g)

DW = soil dry weight (g)

EV = extractant volume (ml)

Mass of extractable carbon (μg/g) = EC × V/DW

Where, EC = extractable carbon in control sample in μg/ml extractant.

*MICROBIAL BIOMASS CARBON:*

Microbial biomass carbon is the amount of carbon (μg) incorporated into the microbial cells. Microbial biomass is measured by numerous methods in aerobic soils such as fumigation-incubation, fumigation-extraction, substrate-induced respiration, ATP content. Of these various recently developed methods, fumigation-extraction is the most widely preferred

method for the estimation of microbial carbon in waterlogged or anaerobic soils (Vance, 1987; Inubushi et al, 1991).

*Chloroform Fumigation extraction method:*

Chloroform fumigation kills most soil microorganisms and destroys their membranes and cell walls. Chloroform acts as an effective biocide when compared with other commercial fumigants such as methyl bromide or methyl isothiocyanate because they are difficult to remove from soil after fumigation and are more toxic than chloroform. It is essential to remove ethanol from chloroform before use (Jenkinson & Powlson, 1976b) as commercially available chloroform contains ethanol as a stabilizer. The ethanol was removed from chloroform by shaking the analar grade reagent 3 times with 5% concentrated sulfuric acid and five times with double distilled water. The chloroform was then dried over anhydrous potassium carbonate and redistilled. The purified reagent was finally stored over anhydrous potassium carbonate in the dark for a few weeks.

*Fumigation of the Samples:*

At the end of each incubation period, 10 g of soil was weighed from each of the sample sets into a clean 50ml beaker. To each of the soil samples, 5ml of ethanol free chloroform was added. The fumigation was done in large desiccator lined with moist filter paper. The samples along with a beaker containing 50 ml ethanol free chloroform were placed in the desiccator. The dessicator was sealed tightly with silica gel and incubated in the dark at $25^0$C for 24 hrs.

After fumigation, the soils were placed in open trays for a few hours to evacuate chloroform from the soil. The soil samples were then extracted with 40ml of 0.5M potassium sulfate solution, followed by vigorous shaking for 30 min. The soil suspension was then filtered through Whatman no5 filter paper and the extract was collected in bottles.

The extracts were digested by wet chromic acid method (Vance, 1987) as described earlier for readily mineralizable carbon analysis (RMC). The procedure for Readily mineralizable form of carbon and Microbial carbon are similar except that fumigation is conducted for microbial form of carbon analysis. A fumigated sample includes carbon extracted from the soil as well as the microorganisms while an unfumigated sample includes only carbon present in the soil. The latter serves as a good measure of the readily mineralzable form of carbon. Microbial carbon is therefore determined by subtracting the extractable carbon of an unfumigated set from a fumigated set

*Calculation of Microbial biomass carbon:*

The volume of the solution in extracted soil was calculated in the same way as shown earlier for RMC calculation.

Mass of extractable carbon in fumigated soil = $EC_F \times V/DW$

$EC_F$ = extractable carbon in fumigated sample in μg/ml of extractant.

V = volume of solution in extracted soil

DW = dry weight of soil (g)

Microbial biomass carbon in soil = $E_C \times 2.64$

The factor of 2.64 is used assuming a $K_{EC} = 0.38$

Where, $E_C$ = (mass of extractable carbon from fumigated soil) – (mass of extractable carbon from an unfumigated soil.

## *ANALYSIS OF FIELD SOIL SAMPLES*

The soil samples brought from lake Bhalsawa were analyzed for total soil organic carbon, total nitrogen, and total concentrations of heavy metals. Five replicates were taken for each of the above analysis and results were expressed as mean of all five values.

### *SOIL ORGANIC CARBON:*

The oxidisable soil organic matter was determined by dichromate digestion (Walkley Black, 1935). 0.5 g of soil was digested in 500ml conical flasks with 10ml of 1M potassium dichromate and 20ml of sulfuric acid. The flasks were left undisturbed for 3 hours and then 200ml of double distilled water along with 10ml phosphoric acid was added to it. The flasks were allowed to cool. The mixture was then titrated against 0.4M Ferrous ammonium sulfate using 25mM 1,10 phenanthroline ferrous sulfate complex solution as indicator. The endpoint is a colour change from yellow through dark green to maroon red. The ferrous ammonium sulfate solution was standardized prior to titration against blank.

Percent oxidizable organic carbon = $(B - S) \times 0.3 \times M/$ weight of soil (g)

Where, M = concentration of Ferrous ammonium sulfate

B = ml of ferrous ammonium sulfate against blank

S = ml of ferrous ammonium sulfate against sample

*TOTAL NITROGEN:*

0.5 g of 0.15mm soil was digested in digestion tubes with 1g of catalyst mixture and 3ml of concentrated sulfuric acid. The digestion tubes were heated gently until frothing ceased. The heat was then slowly raised to $350^0$C for $2^1/2$ hours until the digest cleared. After digestion, the tubes were allowed to cool and the digestion mixture within the tubes was shaken vigorously with double distilled water and clear supernatant liquid was decanted to make up the volume of 50ml in a volumetric flask.

An aliquot of the soil digest (20ml) was distilled with 15ml of 40%NaOH in a Kjeldahl distillation apparatus. Approximately 25ml of distillate was collected in 5ml of boric acid mixed indicator solution. The distillate was finally titrated against 0.01N hydrochloric acid. The colour changes from deep blue to pink at the end point (Hesse, 1971).

The amount of total nitrogen present in soil sample was calculated using the following formula

$$\%N = \frac{(S - B) \times N \text{ of HCl} \times 1.4 \times V}{v \times S_w}$$

S = ml of acid used with the sample

B = ml of acid used with blank

V = ml of total digest

v = ml of digest distilled

$S_w$ = dry weight of soil (g)

*TOTAL HEAVY METAL ANALYSIS:*

The total concentrations of heavy metals such as nickel, copper, lead, and zinc already present in soils were determined by acid digestion (Agemian & Chan, 1976). 0.5 g of air-dried soil sieved through 80-micron mesh was digested in Teflon bombs with 4ml concentrated nitric acid, 1ml perchloric acid, and 6ml hydrofluoric acid. The mixture was digested at $150^0$C for 4-5 hours. The soil digest clears upon digestion. The soil digest is rinsed with 15ml of double distilled water and then it is poured into a 100ml volumetric flask containing 4.8g of boric acid. The volume of the digest mixture is made up to 100ml. It is left undisturbed for 15 days and then it is filtered through a membrane filter paper. The filtrate is collected in 100ml plastic bottles and stored at $4^0$C. The filtrate was later analyzed on Atomic Absorption Spectrometry (Shimadzu ,model no.AA-6800).

*Calculation of Heavy metal concentration:*

Concentration of heavy metals in one ml of filtrate injected into AAS = $y_1$ppm

Concentration of heavy metal in 100 ml filtrate = $y_1$ppm × 100

= $y_2$ppm.

## *STATISTICAL ANALYSIS*

The data were statistically analyzed for a two-factor analysis of variance (ANOVA) using Microsoft EXCEL 2000.

# *RESULTS*

## *SOIL PHYSICOCHEMICAL PARAMETERS*:

Table 2 shows the soil physicochemical characteristics that were analyzed before the start of the experiment. The soil is alkaline and mineral in nature.

Table 2 *Soil Physicochemical Characteristics*

| S.No | Physicochemical parameter | Measurements |
|---|---|---|
| 1 | Ph | 8.00 |
| 2 | Conductivity, mS/cm | 541.5 |
| 3 | Soil organic carbon, % | 0.811 ± 0.065 |
| 4 | Total Nitrogen, % | 0.032± 0.01 |
| 5 | Total Heavy metals | |
| | Total copper, ppm | 28.625 |
| | Total nickel, ppm | 21.8 |
| | Total lead, ppm | 17.425 |
| | Total zinc, ppm | 67.225 |
| 6 | Organic matter, % | 1.398 ± 0.11 |

The heavy metal amended soil in the four treatments therefore contained a total concentration of 46.8ppm or 71.8ppm of nickel and 53.625ppm or 78.625ppm of copper.

## *CARBON DIOXIDE EVOLUTION FROM SOIL:*

The average amount of carbon dioxide evolved from soil (mg/g) increased continuously with incubation time in all the treatments (Figs. 4 & 5). Carbon dioxide evolution from soil (mg/g) varied significantly with time in all the treatments during the incubation. The average carbon dioxide evolution was higher in the control than other metal treatments for the first three days of incubation while it was higher in the metal treatments than control treatment for the remaining days of incubation. There was a significant difference in the response of carbon dioxide evolution observed to the presence of copper or nickel at a higher concentration of 78.625ppm or 71.8ppm respectively in soils. It was also observed that when the concentration

of copper in soil was raised from 53.625ppm to 78.625ppm, the carbon dioxide evolution showed a significantly different response when compared with control (28.625ppm). This response was equally insignificant with respect to the increase in concentration of nickel in soil (Table 7).

Though the amount of carbon dioxide evolved from copper 20 treatment was higher than that evolved from copper 40 treatment, there was no significant effect of the increasing concentration of copper or nickel on carbon dioxide evolution.

The rate at which carbon dioxide evolved from soil was calculated for 1, 3, 5, 7, and 9 days of incubation in terms of mg/g/day and gm/m2/day. The rate of carbon dioxide evolution increased up to the third day of incubation and then it decreased till the seventh day of incubation. However there was a slight increase in the rate of carbon dioxide evolution on the ninth day of incubation (Figs. 6 &7; Table 3). This trend was observed in all the treatments during the incubation. The maximum rate of carbon dioxide evolution observed on the third day of incubation expressed in both mg/g/day and g/m2/day respectively was 0.12 & 31.686 for control, 0.122 & 29.822 for copper 20, 0.121 & 29.449 for copper 40, 0.125 & 30.568 for nickel 20, 0.124 & 30.195 for nickel 40 treatments. The average rate of carbon dioxide evolution from soil varied significantly over days of incubation and was least affected by the different heavy metal treatments applied at different concentrations of 20 and 40ppm (Table 8).

## METHANE PRODUCTION FROM SOIL

The average amount of methane emitted from soil (expressed as μg/g soil) increased for the first five days of incubation in all the treatments and then it decreased till the end of incubation. The maximum amount of methane was released on the fifth day of incubation. The values include 0.25503μg/g, 0.394849μg/g, 0.298851μg/g, 0.316862μg/g from control, copper 40, nickel 20, and nickel 40 treatments respectively. In case of copper 20treatment maximum amount of methane i.e., 0.26154μg/g was released on seventh day of incubation (Figs. 8 &9). Methane emission from soil varied significantly with different heavy metal treatments. The effect of nickel applied at 20 and 40ppm concentrations affected significantly the methane production from soil towards the end of incubation. It was higher in nickel 20treatment than in nickel 40 treatment. Similarly the effect of copper treatment was also significant when concentration was raised to 78.625ppm from 53.625ppm towards the end of incubation.

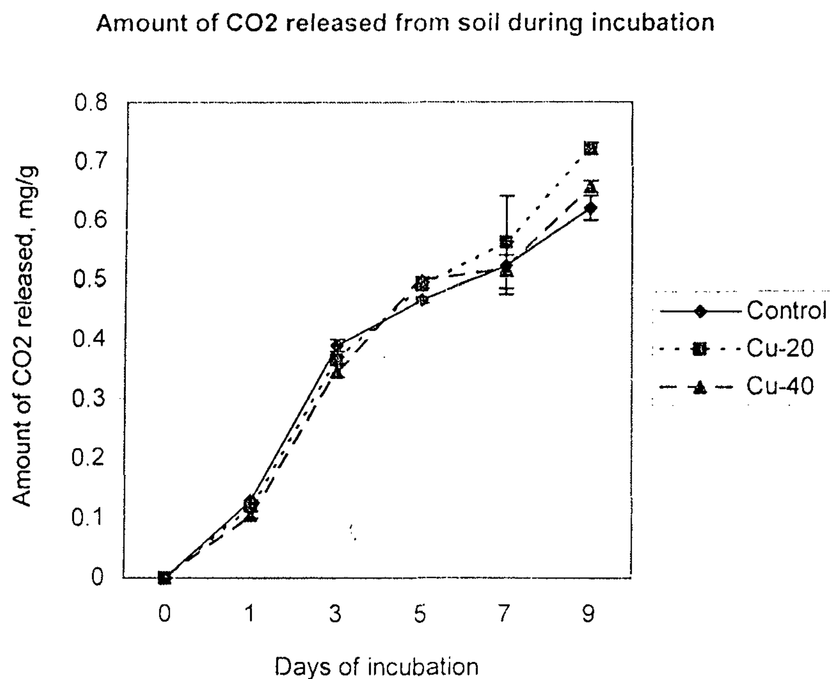



Figure 4 Change in carbon dioxide evolution from soil treated with copper during the study.

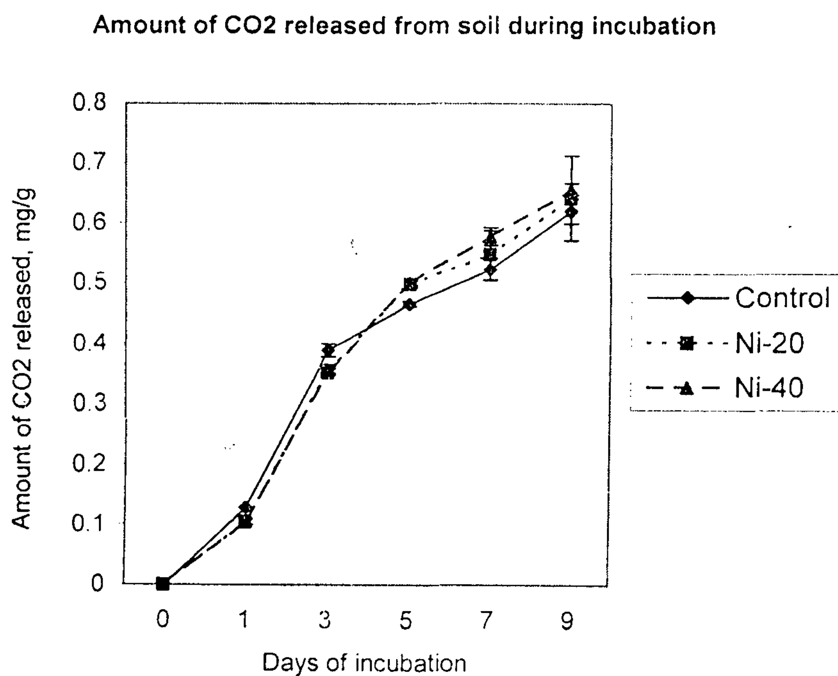



Figure 5 Change in carbon dioxide evolution from soil treated with nickel during the study.

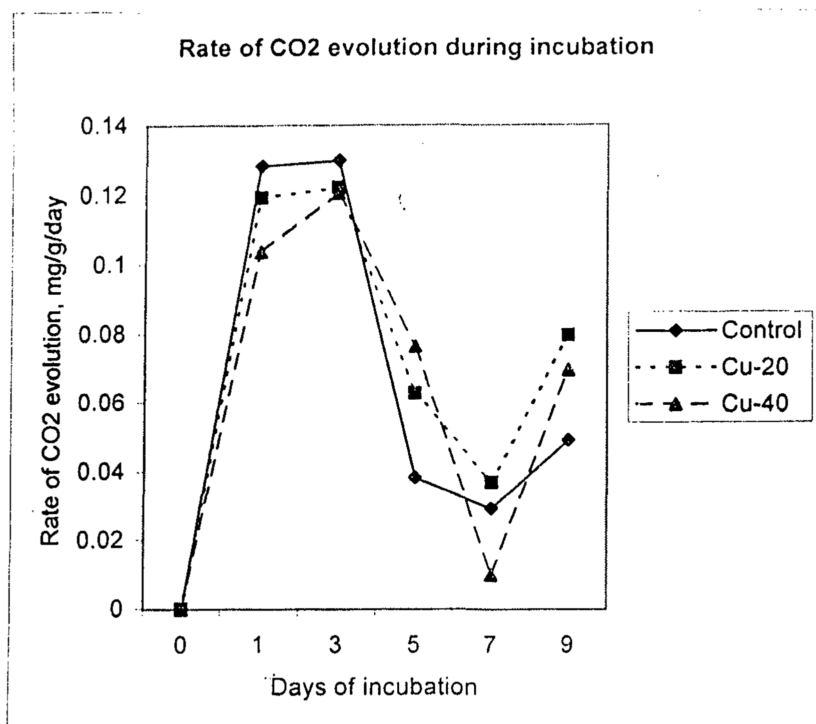



Figure 6 Rate of carbon dioxide evolution from soil treated with copper during the study.

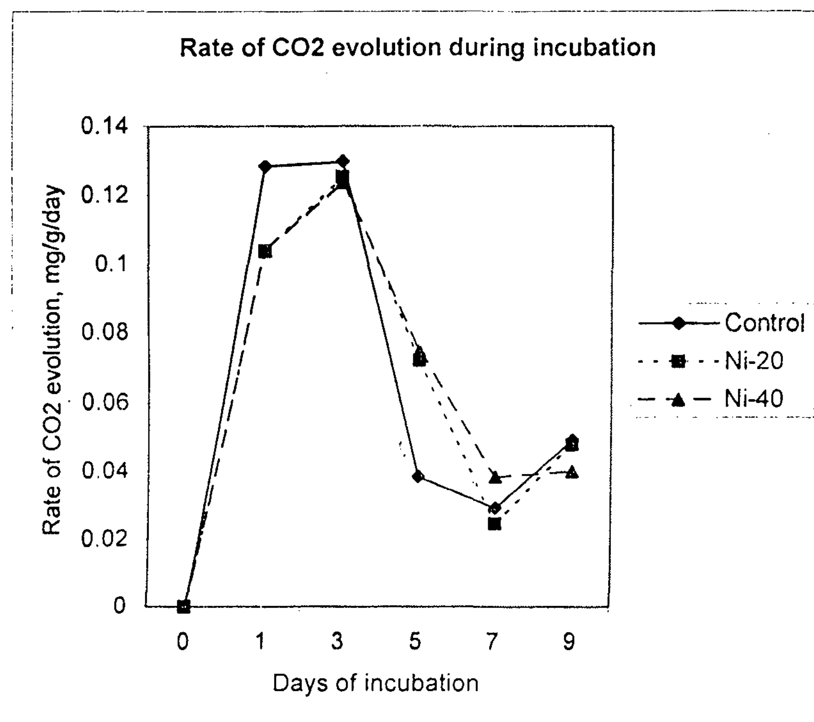



Figure 7 Rate of carbon dioxide evolution from soil treated with nickel during the study.

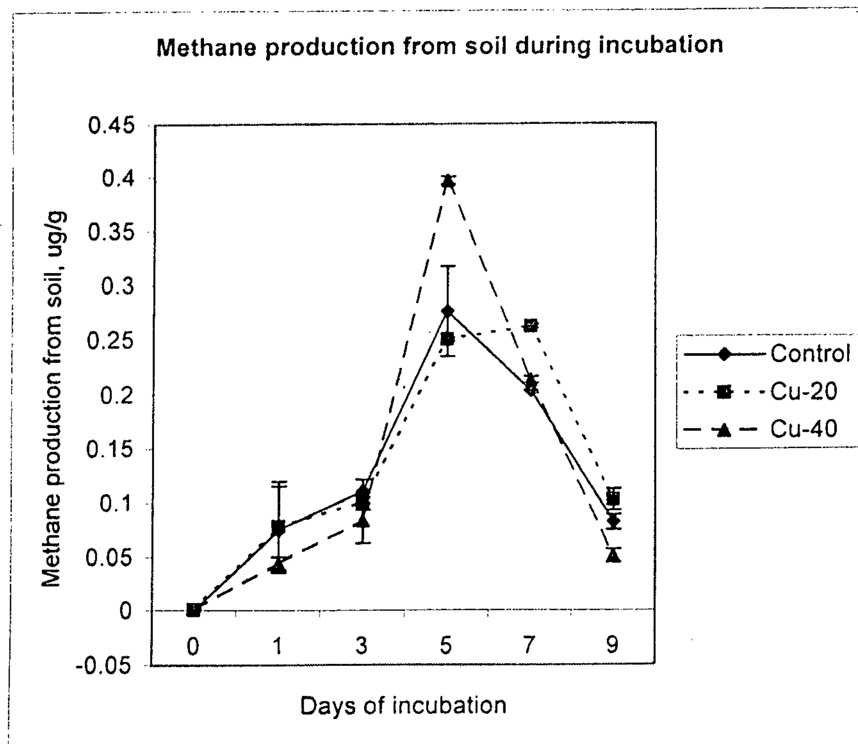



Figure 8 Change in methane production from soil treated with copper during the study.

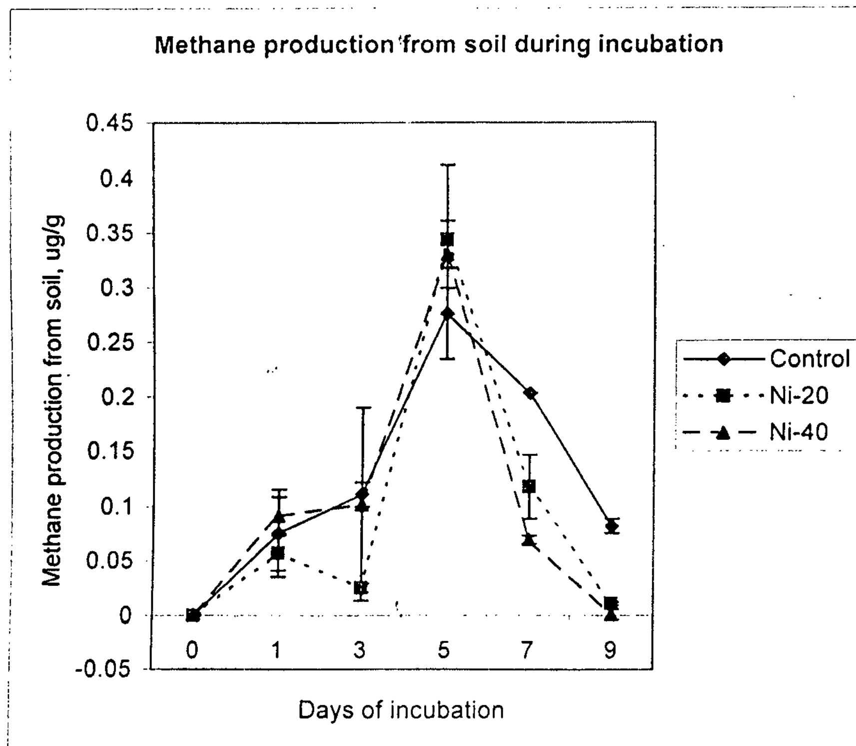



Figure 9 Change in methane production from soil treated with nickel during the study.

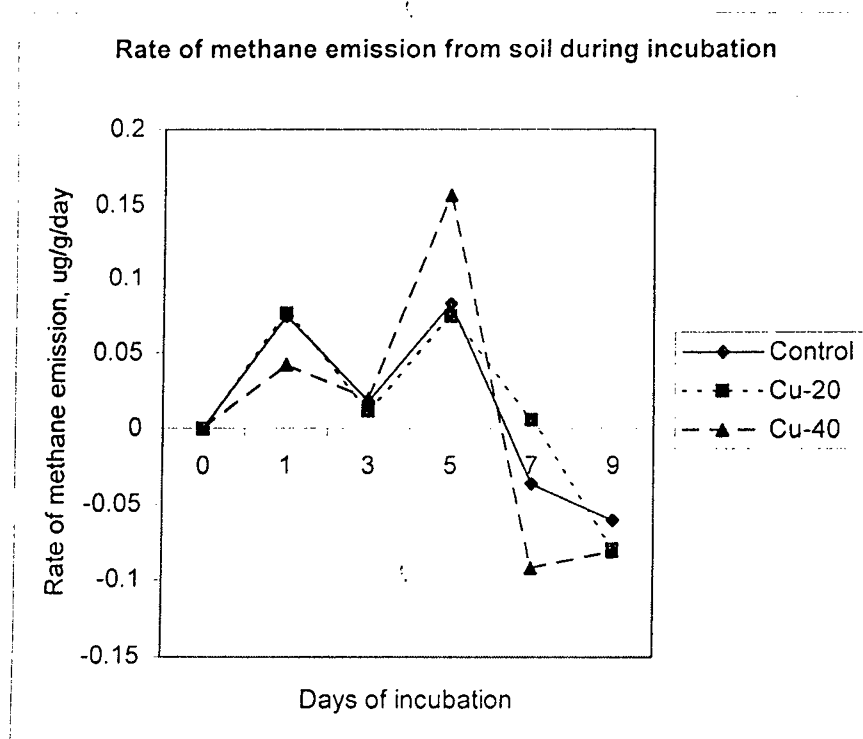
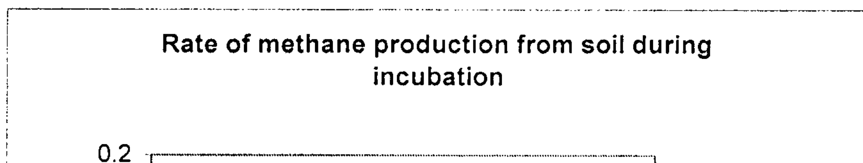



Figure 10 Rate of methane emission from soil treated with copper during the study.



Methane emission from soil was affected significantly in response to nickel contamination when compared with control (21.8ppm). But it did not show any significant difference in its response to copper treatment at different concentrations (Table 9).

The results of ANOVA also showed that the presence of copper or nickel in soil at a concentration of 53.625 and 46.8ppm respectively elicited a highly significant response of the methane emitted from soil against control. Though the response was significant when copper and nickel were present at a higher concentration of 78.625ppm and 71.8ppm respectively but it was less significant than the response measured at the lower concentration.

The rate of methane emission from soil expressed in terms of µg/g/day and mg/m2/day was calculated for 0, 1, 3, 5, 7, and 9 days of incubation. The rate of methane emission reached a peak on fifth day of incubation for all the treatments. Then, it decreased and even became negative towards the end of incubation. The maximum average rate of methane emission from soil observed on fifth day of incubation was 0.07705µg/g/day and 19.0225 mg/m2/day for control, 0.0786 µg/g/day and 19.1819 mg/m2/day for copper 20, 0.1663 µg/g/day and 40.5795 mg/m2/day for copper 40, 0.1388 µg/g/day and 33.8748 mg/m2/day for nickel 20, and 0.0783 µg/g/day and 19.10765 mg/m2/day for nickel 40 treatments (Figs. 10 &11; Table 4).

There was no significant effect of copper or nickel treatments observed on the rate of methane emission when applied at both the concentrations of 20 and 40ppm. But the rate of methane emission varied significantly with incubation time. Even the interaction between the different heavy metals and methane emission rate over days was significant (Table 10).

*READILY MINERALIZABLE CARBON*

The readily mineralizable carbon content (expressed as both µg/g soil and g/m2area) varied with time among all the treatments during the incubation (Figs. 14 &15; Table 6). In case of control treatment, the average amount of readily mineralizable carbon content decreased on the first day of incubation from its initial amount in the soil and again increased to reach a maximum value of 209.2029µg/g or 51.046g/m2 on the fifth day of incubation. Then, it decreased on seventh day and again rose on the ninth day of incubation, thereby showing fluctuations over days of incubation.

In soils treated with copper applied at 20ppm, the readily mineralizable carbon content decreased from its original content in soil till the third day of incubation and then the carbon content suddenly rose to reach a maximum value of 189.2031µg/g or 46.1656g/m2 on seventh day of incubation after which it declined again.

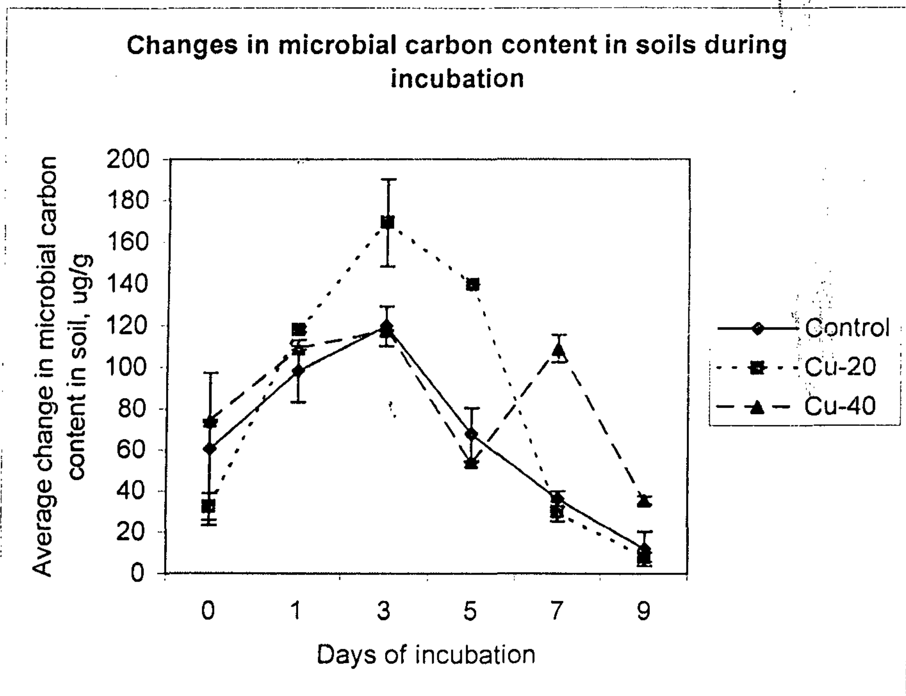



Figure 12 Change in microbial carbon content in soils treated with copper during the study.

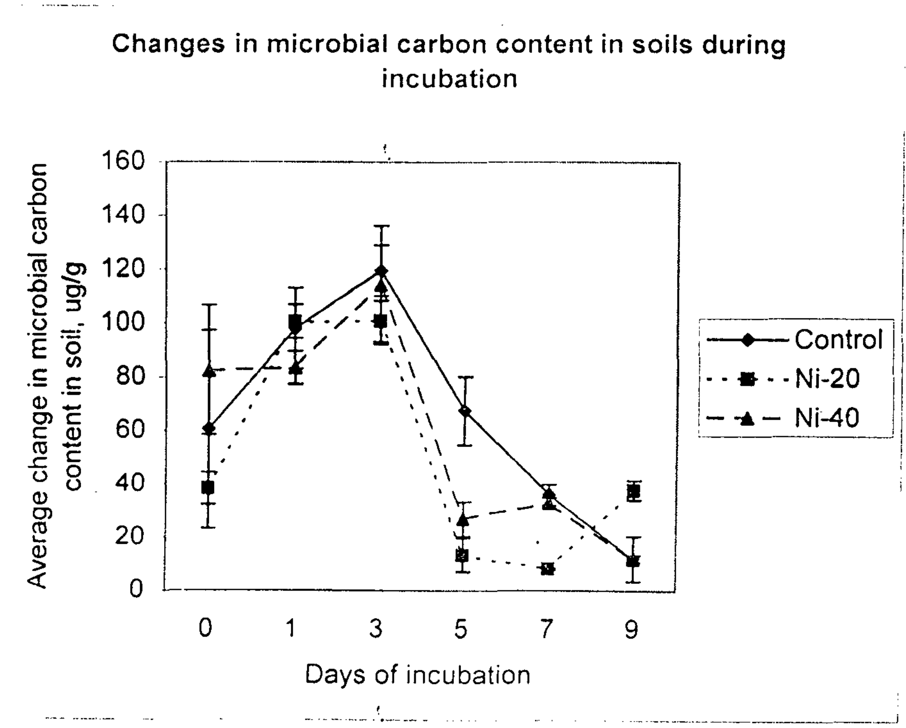



Figure 13 Change in microbial carbon content in soils treated with nickel during the study.

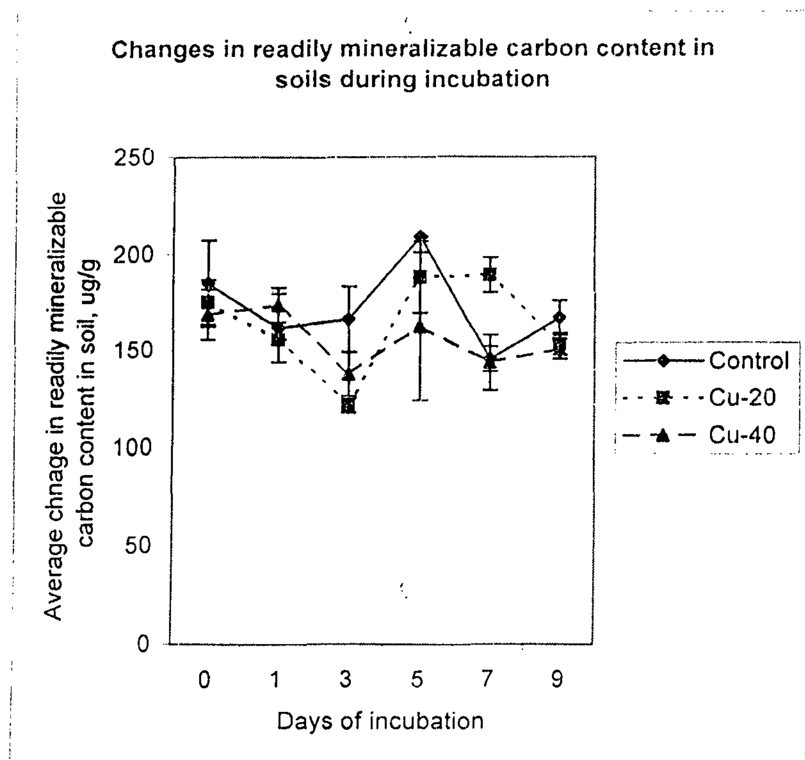



Figure 14 Change in readily mineralizable carbon content in soils treated with copper during the study.

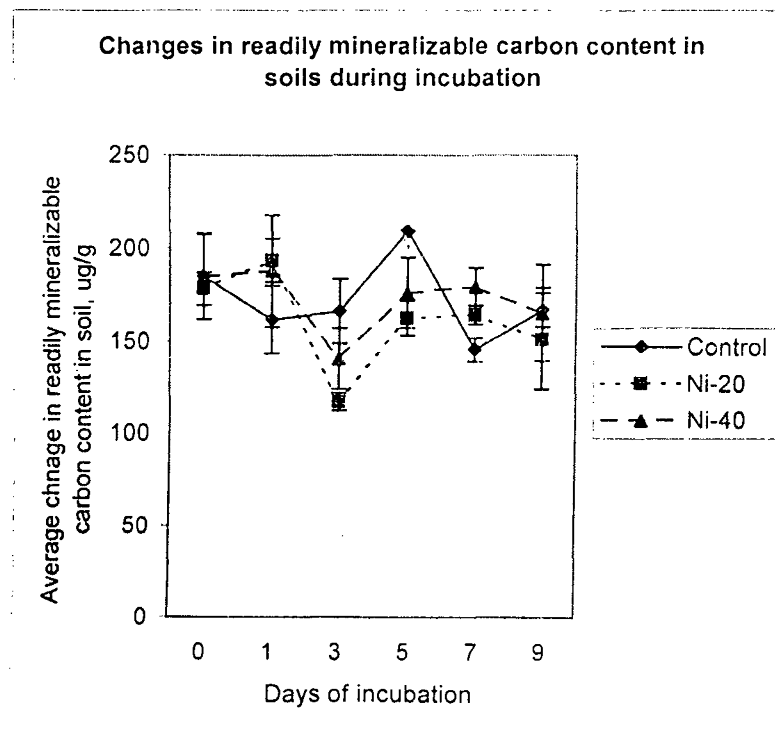



Figure 15 Change in readily mineralizable carbon content in soils treated with nickel during the study.

The readily mineralizable carbon content fluctuates to a large extent with incubation time in copper 40 treatment. It increased on the first day and then decreased to rise again to attain a maximum value of 162.5453μg/g or 39.661g/m2 on fifth day of incubation. This rise in carbon content was followed by a decline on seventh day after which it again increased to 150.5096μg/g or 36.724g/m2 on ninth day of incubation.

Similarly in nickel 20 treatment, the readily mineralizable carbon content increased on first day and then decreased on third day. Then, it increased again to attain a maximum value of 162.5453μg/g or 40.0378g/m2 on seventh day of incubation after which it again declined. A similar trend was observed for soils treated with nickel applied at 40ppm. The lowest values of readily mineralizable carbon content were observed on third day of incubation for all the treatments except control. The values include 121.313μg/g or 29.6g/m2 for copper20, 137.669μg/g or 33.59g/m2 for copper 40, 116.847μg/g or 28.51g/m2 for nickel 20 and 140.589μg/g or 34.304g/m2 for nickel 40 treatments.

The readily mineralizable carbon content was relatively higher in nickel 40 treatment than in nickel 20 treatment. Although the effect of nickel treatment at the concentrations of 46.8 and 71.8ppm was insignificant, the effect of copper treatment at the concentrations of 53.625ppm and 78.625ppm elicited a highly significant response when compared with control containing 21.8ppm and 28.625ppm respectively for copper and nickel. Further, it was also observed from the ANOVA results that there was a significant difference in the response of readily mineralizable carbon content to the presence of either copper or nickel in soil (Table 11).

*MICROBIAL CARBON*

The microbial carbon content in soil is calculated in terms of μg/g as well as g/m2 area (Figs. 12 & 13; Table 5). The initial concentration of microbial carbon in soil varied among all the treatments. The microbial carbon content in control treatment increased continuously and reached a maximum value on the third day of incubation and thereafter it decreases till the end of incubation. A similar trend is observed in soils treated with copper at 20ppm. However, the microbial carbon content in soils treated with copper solution of 40ppm shows a different trend. There was a rise in microbial carbon content for the first three days of incubation after which it declined to increase again on seventh day and then again it decreased on ninth day of incubation. A similar trend was observed in soil treated with nickel solution of 40ppm. The

microbial carbon content was high in copper 20 treated soil for the first five days of incubation and then it decreased than the microbial carbon content in copper 40 treated soil.

In soils treated with nickel at a concentration of 20ppm, the microbial carbon content increased until the third day of incubation. Then, it decreased until the seventh day and again on ninth day the microbial carbon content increased in soil. Nickel contamination of soil results in a decline in microbial carbon content when compared with control. Though the amount of microbial carbon in Ni 40 treatment is higher than that in Ni 20 treatment except on first and ninth day of incubation, there is no significant effect seen with respect to the increasing concentration of nickel on microbial carbon content in soil.

The maximum microbial carbon content observed on third day of incubation expressed in both μg/g and g/m2 respectively is 119.569&29.1747 for control, 169.354& 41.3225 for copper 20, 117.6254&26.1015 for copper 40, 100.719&14.2087 for nickel 20, and 114.2824& 20.2955 for nickel 40 treatments.

The presence of copper at concentrations of 53.625 and 78.625ppm and nickel at concentrations of 46.8ppm & 71.8ppm in the soil had a significant influence on the microbial carbon content when compared with the control containing 28.625ppm of copper and 21.8ppm of nickel. However, the response of the microbial carbon content was highly significant to the presence of copper or nickel at lower concentrations than at higher concentrations in soil against control (Table 12).

*In order to observe the significance of the different treatments and incubation time on various parameters such as carbon dioxide evolution and methane emission from soil, the amount of readily mineralizable carbon and microbial carbon content in soil in the present experiment, a two factor Analysis of Variance (ANOVA at $p < 0.05$) was performed. The results of ANOVA showed that incubation time had a significant influence on all parameters in soil. However, there is a mixed trend of significance observed with respect to the effect of different treatments on various parameters of soil.*

Table 3 Average rate of carbon dioxide evolution from soil (g/m2/day)

| Treatments | Days of Incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| Control | 0 | 31.3133 | 31.6859 | 9.3196 | 7.082 | 11.9289 |
| Cu-20 | 0 | 29.0767 | 29.8222 | 15.2838 | 8.946 | 19.3844 |
| Cu-40 | 0 | 25.3489 | 29.449 | 18.6389 | 2.423 | 16.9613 |
| Ni-20 | 0 | 25.3489 | 30.5677 | 17.5206 | 5.964 | 11.5561 |
| Ni-40 | 0 | 25.3489 | 30.19498 | 18.2661 | 9.319 | 9.6922 |

Table 4 Average rate of methane emission from soil (mg/m2/day)

| treatments | Days of Incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| Control | 0 | 18.13095 | 4.308145 | 20.19383 | -8.93651 | -14.7607 |
| Cu-20 | 0 | 18.70364 | 2.78447 | 18.1674 | 1.442091 | -19.4263 |
| Cu-40 | 0 | 10.41465 | 4.857697 | 38.20309 | -22.4659 | -19.749 |
| Ni-20 | 0 | 13.77396 | -3.8982 | 38.89544 | -27.5955 | -13.0216 |
| Ni-40 | 0 | 21.95447 | 1.20962 | 27.90968 | -31.8112 | -8.37101 |

Table 5 Average change in microbial carbon (g/m2) in soils treated with heavy metals.

| treatments | Days of Incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| Control | 14.7246 | 23.93678 | 29.17475 | 16.4529 | 8.78046 | 2.88965 |
| Cu-20 | 5.28695 | 28.78075 | 41.32255 | 34.07355 | 7.27915 | 1.91305 |
| Cu-40 | 18.02579 | 26.6281 | 26.10148 | 13.12388 | 26.51192 | 8.61543 |
| Ni-20 | 9.353865 | 24.5468 | 14.20863 | 3.257265 | 1.9809 | 9.14855 |
| Ni-40 | 23.15074 | 20.39255 | 20.2955 | 6.5589 | 8.034335 | 2.88095 |

Table 6 Average concentration of Readily mineralizable carbon (g/m2)

| Treatments | Days of Incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| Control | 45.101 | 39.47067 | 40.53 | 51.046 | 35.46203 | 40.72767 |
| Cu-20 | 42.747 | 37.973 | 29.6 | 45.906 | 46.16557 | 37.33047 |
| Cu-40 | 41.15733 | 42.38567 | 33.59133 | 39.661 | 35.05373 | 36.72433 |
| Ni-20 | 43.50167 | 47.18567 | 28.51033 | 39.66133 | 40.0378 | 36.88593 |
| Ni-40 | 45.12167 | 45.79933 | 34.304 | 42.96567 | 43.6873 | 40.30337 |

## *Analysis of Variance (ANOVA Two factor with replication) Tables*

Table 7 Test showing significance of the treatments vs time on average amount of $CO_2$ (mg/g) evolved from soil

| No. of Treatments | F value | Pvalue | F critic |
|---|---|---|---|
| 1 Treatment-1 | 6.2883 | 0.004547 | 3.259 |
| 2 Days of Incubation | 1166.72 | 9.50E-39 | 2.477 |
| 3 Interaction | 3.89 | 0.0012 | 2.106 |
| 4 Treatment 2 | 1.2808 | 0.29 | 3.259 |
| 5 Days of Incubation | 1255.84 | 2.55E-39 | 2.477 |
| 6 Interaction | 2.513 | 0.0208 | 2.106 |
| 7 Treatment-3 | 3.1199 | 0.0563 | 3.259 |
| 8 Days of Incubation | 742.007 | 3.06E-35 | 2.477 |
| 9 Interaction | 2.374 | 0.02813 | 2.106 |
| 10 Treatment-4 | 5.4426 | 0.00861 | 3.259 |
| 11 Days of incubation | 3418.77 | 4.05E-47 | 2.477 |
| 12 Interaction | 9.485 | 1.79E-07 | 2.106 |

Table 8 Test showing significance of the treatments vs time on average rate of $CO_2$ evolution from soil (mg/g/day)

| No. of Treatments | F value | Pvalue | F critic |
|---|---|---|---|
| 1 Treatment-1 | 1.1217 | 0.3368 | 3.259 |
| 2 Days of Incubation | 79.6708 | 1.91E-18 | 2.477 |
| 3 Interaction | 2.1735 | 0.04325 | 2.106 |
| 4 Treatment-2 | 0.09527 | 0.909351 | 3.259 |
| 5 Days of Incubation | 246.8103 | 8.51E-27 | 2.477 |
| 6 Interaction | 4.8589 | 0.000196 | 2.106 |
| 7 Treatment-3 | 1.2829 | 0.2896 | 3.259 |
| 8 Days of Incubation | 76.2035 | 3.96E-18 | 2.477 |
| 9 Interaction | 1.5175 | 0.1733 | 2.106 |
| 10 Treatment-4 | 0.07843 | 0.9247 | 3.259 |
| 11 Days of Incubation | 267.0738 | 2.14E-27 | 2.477 |
| 12 Interaction | 9.9766 | 9.68E-08 | 2.106 |

Table 9 Test showing significance of the treatments vs time on average amount of methane emitted from soil (ug/g)

| No of Treatments | Fvalue | Pvalue | Fcritic |
|---|---|---|---|
| 1 Treatment-1 | 2.0106 | 0.1487 | 3.259 |
| 2 Days of Incubation | 688.557 | 1.16E-34 | 2.477 |
| 3 Interaction | 29.0937 | 2.67E-14 | 2.106 |
| 4 Treatment-2 | 5.1236 | 0.01101 | 3.259 |
| 5 Days of Incubation | 112.956 | 5.77E-21 | 2.477 |
| 6 Interaction | 5.1026 | 1.27E-04 | 2.106 |
| 7 Treatment-3 | 46.7823 | 9.74E-11 | 3.259 |
| 8 Days of Incubation | 577.8239 | 2.62E-33 | 2.477 |
| 9 Interaction | 33.0269 | 3.63E-15 | 2.106 |
| 10 Treatment-4 | 5.2136 | 1.03E-02 | 3.259 |
| 11 Days of Incubation | 126.0561 | 9.04E-22 | 2.477 |
| 12 Interaction | 6.4209 | 1.38E-05 | 2.106 |

Table 10 Test showing significance of the treatments vs time on average rate of methane emission from soil (ug/g/day)

| No of Treatments | Fvalue | Pvalue | Fcritic |
|---|---|---|---|
| 1 Treatment-1 | 1.2187 | 0.3075 | 3.259 |
| 2 Days of Incubation | 194.053 | 5.56E-25 | 2.477 |
| 3 Interaction | 14.60405 | 6.83-10 | 2.106 |
| 4 Treatment-2 | 0.4874 | 0.6182 | 3.259 |
| 5 Days of Incubation | 100.622 | 4.01E-20 | 2.477 |
| 6 Interaction | 5.1423 | 0.000118 | 2.106 |
| 7 Treatment-3 | 2.0726 | 0.14062 | 3.259 |
| 8 Days of Incubation | 188.353 | 9.31E-25 | 2.477 |
| 9 Interaction | 18.3065 | 2.80E-11 | 2.106 |
| 10 Treatment-4 | 0.3238 | 0.7254 | 3.259 |
| 11 Days of Incubation | 98.34 | 5.89E-20 | 2.477 |
| 12 Interaction | 5.3017 | 8.93E-05 | 2.106 |

Table 11 Test showing significance of the treatments vs time on the readily mineralizable carbon content in soil (ug/g).

| No of Treatments | Fvalue | Pvalue | Fcritic |
|---|---|---|---|
| 1 Treatment-1 | 5.3993 | 0.008897 | 3.259 |
| 2 Days of Incubation | 10.08679 | 4.26E-06 | 2.477 |
| 3 Interaction | 4.2567 | 0.000601 | 2.106 |
| | | | |
| 4 Treatment-2 | 2.711023 | 0.08003 | 3.259 |
| 5 Days of Incubation | 8.92641 | 1.40E-05 | 2.477 |
| 6 Interaction | 3.3986 | 0.003264 | 2.106 |
| | | | |
| 7 Treatment-3 | 3.79632 | 0.031916 | 3.259 |
| 8 Days of Incubation | 18.28228 | 5.24E-09 | 2.477 |
| 9 Interaction | 7.514 | 2.61E-06 | 2.106 |
| | | | |
| 10 Treatment-4 | 4.6914 | 0.01546 | 3.259 |
| 11 Days of Incubation | 5.2089 | 0.001068 | 2.477 |
| 12 Interaction | 1.8515 | 0.08619 | 2.106 |

Table 12 Test showing significance of the treatments vs time on microbial carbon content in soil (ug/g)

| No of Treatments | Fvalue | Pvalue | Fcritic |
|---|---|---|---|
| 1 Treatment-1 | 8.7492 | 0.002216 | 3.255 |
| 2 Days of Incubation | 75.881 | 1.86E-11 | 2.773 |
| 3 Interaction | 14.6113 | 1.08E-06 | 2.4117 |
| | | | |
| 4 Treatment-2 | 4.1236 | 0.0335 | 3.555 |
| 5 Days of Incubation | 46.948 | 1.05E-09 | 2.773 |
| 6 Interaction | 3.377 | 0.01199 | 2.4117 |
| | | | |
| 7 Treatment-3 | 22.4044 | 1.30E-05 | 3.555 |
| 8 Days of Incubation | 82.9038 | 8.72E-12 | 2.773 |
| 9 Interaction | 12.1327 | 4.38E-06 | 2.4117 |
| | | | |
| 10 Treatment-4 | 10.9272 | 7.82E-04 | 3.555 |
| 11 Days of Incubation | 41.4694 | 2.92E-09 | 2.773 |
| 12 Interaction | 4.1115 | 4.51E-03 | 2.4117 |

LEGEND TO TABLES - 7,8,9,10,11 & 12:

At significance level – 0.05
Treatment 1 – Control, Copper 20, Copper 40
Treatment 2 – Control, Nickel 20, Nickel 40
Treatment 3 – Control, Copper 20, Nickel 20
Treatment 4 – Control, Copper 40, Nickel 40

# *DISCUSSION*

In the present study, the soil had a total concentration of copper and nickel close to the threshold concentration in an unpolluted soil (US EPA Sediment quality standards). After addition of heavy metal solution, the concentration in the soil was 53.625ppm and 78.625ppm of copper chloride and 46.8ppm and 71.8ppm of nickel chloride. The soils were therefore moderately polluted and heavily polluted when compared against the US EPA Sediment quality standards.

## *CARBON DIOXIDE EVOLUTION FROM SOIL:*

Though average carbon dioxide production from mineralization of organic carbon in soil varied significantly among the metal treatments, the results show that the presence of toxic metals copper and nickel affect the mineralization of organic carbon by microorganisms in soil. Though the effect is not significantly seen in the rate of carbon dioxide production but is evident in the cumulative amount of carbon dioxide released into the headspace from the soil. Copper was known to be a toxic element that affects the carbon dioxide production from soil. Earlier studies by Baath (1989), Hattori et al (1991, 1992) report that heavy metal pollution at low levels of contamination has little effect on carbon dioxide production than heavy metals at higher levels of contamination. This is evident by the lower amount of carbon dioxide released in copper 40 treatment as compared with the amount of carbon dioxide released in copper 20 treatment. These observations of copper treatments are significantly different from one another and against control (at $p<0.05$).

The maximum rate of carbon dioxide production observed on the third day of incubation coincides with the maximum microbial carbon and minimum readily minerlizable carbon content observed in soil. This suggests that during the initial period of incubation there is a higher activity of aerobic microorganisms in soil. They utilize the organic carbon in soil and mineralize it to carbon dioxide. They also assimilate a substantial portion of the organic carbon into their cellular material as microbial biomass. Then, there is a decline in the rate of carbon dioxide production observed, which probably might be due to the shift in soil oxidation-reduction conditions from an oxygen rich aerobic to an oxygen deficit anaerobic condition. Thus the facultative and obligate anaerobic microorganisms replace the aerobic microorganisms in soil. The slight increase in carbon dioxide production observed towards the

end of incubation is explained by the fact that carbon dioxide may be released during anaerobic respiration or fermentation.

*METHANE PRODUCTION FROM SOIL*

The average methane production continuously increased during the five days of incubation. However the rate of methane production declined on the third day of incubation, which might be attributed to the activity of methanotrophic bacteria. They usually reside at the soil-water interface and consume methane that is emitted from the anoxic layers of the soil. The existence of both aerobic and anaerobic micro sites therefore enables the co-existence of aerobic and anaerobic microorganisms in soil. After five days of incubation, the average methane production from soil continued to decrease until the end of incubation. Similarly, the average rate of methane production declined and even became negative towards the end of incubation.

This implies that certain processes consume methane. Although aerobic oxidation of methane by methanotrophic bacteria is a well-known process, anaerobic oxidation of methane by microorganisms in soil is a recent discovery (Zehnder & Brock, 1980; Davis & Yarbrough, 1966; Mason, 1977). Recently Zehnder and Brock (1980) have shown that some methane-forming bacteria are able to oxidize methane anaerobically. Many methanogenic bacteria produce carbon dioxide from oxidation of methane while some bacteria like Methanosarcina produce acetate from methane oxidation. Methane is oxidized under anaerobic conditions through a coupled two-step mechanism. The methanogenic bacteria in the first step activate methane and form intermediates like acetate and methanol. These compounds in the second step are subsequently oxidized to carbon dioxide by a non-methanogenic population, which utilizes manganese, iron, and sulfate as electron acceptor. If these intermediate products accumulate in soil then methane activation by methanogenic bacteria shifts into an endergonic range. Therefore these intermediates are kept at low concentrations by organisms of the second step. This explains the increase in the rate of carbon dioxide production observed on the ninth day of incubation in all the treatments.

Nickel treatment were considered to enhance the methane production from soil because nickel was known to be a constituent of cofactor $F_{430}$ of methyl coenzyme M, reductase which is involved in the terminal step of methane formation from $C_1$compounds (Sprott & Shaw, 1987; Thauer, 1998; Kong, 1993; Deppenmier et al., 1996). However, the effect of nickel treatment on methane production was insignificant for the first five days of incubation.

Thereafter, the amount of methane released declined drastically and the effect of nickel at both the concentrations of 46.8ppm and 71.8ppm was significant when compared against control (21.8ppm). Recently Kruger et al (2003) have extracted a nickel compound from the microbial mats in the Black sea. These mats catalyze anaerobic oxidation of methane rather than carry out methanogenesis. The nickel compound that was extracted displayed a similar absorption spectrum as the nickel cofactor $F_{430}$. Although nickel is known to be a constituent of cofactor $F_{430}$ of methyl coenzyme M reductase, it is thought to play an important role in anaerobic oxidation of methane. It is therefore possible that the presence of nickel in soil may promote methane oxidation by the different groups of microorganisms anaerobically.

Mishra et al (1999) reported a positively significant relationship between readily mineralizable carbon content and methane production in a flooded alluvial soil treated with different heavy metals. Similar observations were also reported in the present study where the estimate of readily mineralizable carbon content was lower in metal treated soil as compared with that estimated in control. The average methane production was also observed to be lower in metal treated soils than in control except for the fifth day of incubation where a high amount of methane released is reported for copper 40, nickel 20, and nickel 40 treatments. Since readily mineralizable carbon serves as a ready source of energy and carbon for methanogenic and other microbial consortia, the heavy metals might be affecting the microbial processes in turn influencing the turnover of readily mineralizable carbon.

*CARBON BUDGET FOR EVERY GRAM OF SOIL:*

Data on carbon budget calculated for each day of incubation for all the treatments and (given in tables 13,14,15,16, & 17) show that the total organic carbon of the system increases for the first three days of incubation and then it begins to decline until the end of incubation. The total organic carbon of the system refers to and includes the organic carbon that is mineralized to carbon dioxide and methane, the readily mineralizable form carbon and microbial form of carbon.

Table 13 Carbon dynamics (per gram wetland soil) under control treatment during the study period

| Parameters | Days of incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| $CO_2$ production (ug of carbon) | 0 | 34.9 | 106 | 126.6 | 142.5 | 169.2 |
| $CH_4$ production (ug of carbon) | 0.000515 | 0.0562 | 0.0827 | 0.2069 | 0.1519 | 0.06119 |
| Readily mineralizable carbon content (ug) | 184.842 | 161.765 | 166.106 | 209.203 | 145.336 | 166.917 |
| Microbial carbon content (ug) | 60.346 | 98.1005 | 119.569 | 67.432 | 35.985 | 11.843 |
| Total | 245.189 | 294.822 | 391.757 | 403.442 | 323.973 | 348.021 |

Table 14 Carbon dynamics (per gram of wetland soil) under Copper 20 treatment during the study period:

| Parameters | Days of incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| $CO_2$ production (ug of carbon) | 0 | 32.5 | 99.2 | 133.3 | 153.3 | 196.6 |
| $CH_4$ production (ug of carbon) | 0.000993 | 0.0585 | 0.0756 | 0.1873 | 0.1961 | 0.0767 |
| Readily mineralizable carbon content (ug) | 175.194 | 155.625 | 121.313 | 188.132 | 189.203 | 152.994 |
| Microbial carbon content (ug) | 32.315 | 117.954 | 169.354 | 139.642 | 29.832 | 7.840 |
| Total | 207.509 | 306.137 | 389.943 | 461.261 | 372.531 | 357.510 |

Table 15 Table 3 Carbon dynamics (per gram wetland soil) under copper 40 treatment during the study period.

| Parameter | Days of incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| $CO_2$ production (ug of carbon) | 0 | 28.3 | 94.2 | 135.8 | 141.2 | 179.2 |
| $CH_4$ production (ug of carbon) | 0.000819 | 0.0328 | 0.0627 | 0.2975 | 0.1594 | 0.03803 |
| Readily mineralizable carbon content (ug) | 168.678 | 173.711 | 137.669 | 162.545 | 143.663 | 150.509 |
| Microbial carbon content (ug) | 73.877 | 109.132 | 117.625 | 53.987 | 108.655 | 35.309 |
| Total | 242.556 | 311.176 | 349.557 | 352.629 | 393.677 | 365.056 |

Table 16 Carbon dynamics (per gram wetland soil) under nickel 20 treatment during the study period.

| Parameter | Days of incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| $CO_2$ production (ug of carbon) | 0 | 28.3 | 96.6 | 135.8 | 149.2 | 175 |
| $CH_4$ production (ug of carbon) | 0.00017 | 0.0425 | 0.0185 | 0.2576 | 0.0880 | 0.00795 |
| Readily mineralizable carbon content (ug) | 178.285 | 193.383 | 116.847 | 162.545 | 164.089 | 151.172 |
| Microbial carbon content (ug) | 38.336 | 100.603 | 100.719 | 13.350 | 8.118 | 37.494 |
| Total | 216.621 | 322.328 | 314.185 | 311.953 | 321.495 | 363.674 |

Table 17 Carbon dynamics (per gram wetland soil) under nickel 40 treatment during the study period.

| Parameter | Days of incubation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 |
| $CO_2$ production (ug of carbon) | 0 | 28.3 | 95.8 | 136.6 | 157.5 | 179.2 |
| $CH_4$ production (ug of carbon) | 0.001 | 0.0685 | 0.0759 | 0.2475 | 0.0519 | 0.00047 |
| Readily mineralizable carbon content (ug) | 184.926 | 187.703 | 140.589 | 176.091 | 179.046 | 165.177 |
| Microbial carbon content (ug) | 82.511 | 83.575 | 114.282 | 26.878 | 32.927 | 11.807 |
| Total | 267.438 | 299.647 | 350.747 | 339.817 | 369.525 | 356.184 |

The increase in the total organic carbon of the system suggests that microorganisms in soil are actively decomposing complex organic matter such as cellulose, lignin, and humic particles and converting them into readily mineralizable form of carbon. A substantial portion ≥ 50% of the decomposable products are assimilated into the biomass of microorganisms. From the remaining, a major portion is mineralized to carbon dioxide and methane with the rest constituting readily mineralizable form of carbon. Mineralization of complex organic matter is mediated by aerobic microorganisms, which produce oxygenase enzymes that results in the formation of oxygen reactive species that diffuse into large complex organic molecules and break bonds inaccessible for the larger enzyme molecules. After three days of incubation, the death of microorganisms in soil results in the release of microbial carbon into the soil. The toxic effects of copper and nickel on soil microbial populations was evident by a very sharp decline in microbial carbon observed in copper 20, nickel 20, and nickel 40 treatments when compared with that in control treatment. Chander and Brookes (1991, 1993) reported a decrease in microbial biomass carbon with increase in heavy metal contamination in soils treated with metal enriched sewage sludge. They found copper and zinc to be more toxic than nickel to soil microorganisms. The results of the present study in contrast show that nickel and

copper are toxic to microbial biomass in soil. Similar observations were also reported in a field experimental study (Brookes and Mc Grath, 1984) that copper and nickel enriched sewage sludge when applied to agricultural fields adversely affected the microbial biomass in soil while zinc and chromium rich sewage sludge had a little effect on soil microbial biomass carbon. The toxic effects of copper and nickel were observed after three days of incubation when microbial biomass carbon content had reached a peak in soil. This implies that the metabolically active microorganisms in soils are more sensitive to heavy metals toxicity (Ohya et al., 1998; Landi et al., 2000). During the same time, it was observed that the rate of carbon dioxide production was higher in copper and nickel treated soil than in the control soils, which suggests that microorganisms are under heavy metal stress. They enhance their activities to break down organic carbon to release carbon dioxide with little carbon being incorporated into their cells. Thus, less microbial biomass was produced per unit of organic substrate input in the presence of toxic heavy metals. Such similar observations were reported in earlier field studies for instance Chander and Brookes, (1991, 1993); Shi et al., (2002); Leita et al., (1995); Brookes and Mc Grath, (1984); Valescchi et al., (1995); and Giller et al., (1998).

The microbial carbon released is transformed into more resistant fraction or least degradable fraction of organic carbon in soil, which is not measured during the analysis. Recent investigations in marine sediments have revealed the ability of bacteria to consume labile dissolved organic matter within 48hrs to form refractory dissolved organic matter (Brophy et al., 1989; Stoddergger et al., 1998; Ogawa et al, 2001). However, physico-chemical reactions such as abiotic condensation reactions or humification processes are also important in the formation of refractory dissolved organic matter in soils or sediments (Harvey et al., 1983). Since the formation of refractory dissolved organic matter in the present study has been rapid (within 2 days), it has to be a biological process.

## *CONCLUSIONS*

It is concluded from the present study that copper and nickel are toxic to the growth and activity of microorganisms in soil. Their toxic effects are observed after three days of incubation when the microbial carbon shows a sudden and an abrupt decline as compared with the control. The carbon dioxide production from organic carbon mineralization in soil was also affected by copper treatment. The effect was more pronounced at the higher concentration of copper. Methane production was inhibited by the presence of nickel. However, the different concentrations of nickel showed no significant difference in the microbial activity.

# *SUMMARY*

Microorganisms are ubiquitous. Their metabolic activities are essential to the proper cycling of nutrients, which indirectly affects the growth, and functions of all other living organisms in an ecosystem. The toxic pollutants in the environment adversely affect the growth and activity of microorganisms. One group of such pollutants is that of heavy metals which are persistent and non-biodegradable. They are released from various sources and tend to accumulate in soils. Considering the potential toxicity of heavy metals and the important role of microorganisms in nature, this study was aimed at investigating the effects of copper and nickel at two different concentrations on the microbial biomass and activity in a wetland soil under flooded condition.

Samples of soils (30 g) collected from a local wetland near Delhi were placed in 250 ml flasks, flooded with heavy metal solutions (20ppm and 40ppm), and incubated at $35^0$C for 9 days. One set with only distilled water served as control. Carbon dioxide evolution from soil was estimated by absorbing the gas in 0.25N sodium hydroxide placed in a vial hanging in each flask. Methane evolution was estimated by analyzing the air inside the flasks by Gas Chromatography. Readily mineralizable carbon and microbial carbon were determined by standard methods. The four parameters were analyzed every alternate day for 9 days after taking an initial reading and another after 24-hours.

The results show that copper and nickel are toxic to the microbes and their activity. However, different concentrations of copper and nickel were ineffective in eliciting a significant response. In other words, the range of concentrations selected for my study was not large enough to obtain significant effects. Copper was the only metal found to affect carbon dioxide evolution differently when applied at 20ppm and at 40ppm. In all other parameters studied, the response was insignificant. Further, this study was limited by its short duration of 9 days, and the long term responses of the microbes may be different.

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