CHAPTER-I

1. INTRODUCTION

The water is essential for life and there is no substitute for it as well. Therefore, living cell consist the water as predominantly. Safe drinking water is needed for healthy and productive life. The earth contains water abundantly. However, only 0.29% of the total water exists in earth and known as fresh water is useful for human consumption (Wetzel, 1983).

The safe water is defined as the water that is free from hazardous microorganism and contains the chemicals in appropriate concentrations (the concentration which do not cause acute or long-term adverse effects on human health). The safe drinking water must also be fairly clear, palatable, and odorless and should not affect the taste of food cooked in it.

Water pollution is the most serious environmental quality issues all over the world, yet the people are less aware and give little emphasis on the vital connection between water and health. Man is a common host for a variety of pathogenic microorganisms and water has been found to be vehicle for the transmission of communicable diseases. Several types of pathogenic microorganisms are found in polluted water, which lead to health hazard. Thus water may be extremely dangerous when it become the vehicle of illnesses are associated with ingestion of water containing pathogenic microorganisms i.e. bacteria, protozoa and viruses which is responsible for most mortality and morbidity in developing countries.

Arsenic is an element that is widely distributed in the earth's crust. Elemental arsenic is ordinarily a steel grey metal-like material that sometimes occurs naturally. In the environment, arsenic is combined with oxygen, chlorine, and sulfur to form inorganic arsenic compounds. Arsenic in animals and plants combines with carbon and hydrogen to form organic arsenic compounds. Inorganic arsenic compounds are mainly used to preserve wood. Organic arsenic compounds are used as pesticides, primarily on cotton plants.

Understandings are usually less harmful than the inorganic forms. Inorganic arsenic occurs naturally in soil and in many kinds of rock, especially in minerals and ores that contain copper or lead. Arsenic occurs naturally in soil and minerals and therefore it may enter the air, water, and land from wind-blown dust and may get into water from runoff and leaching. Arsenic is associated with ores mined for metals, such as copper and lead. It may change its form by reacting with oxygen or other molecules present in air, water, or soil, or by the action of bacteria that live in soil or sediment (Smedley and Kinniburgh, 2002).

Arsenic is a metalloid whose name conjures images of murders. It is ubiquitous in nature –Ranked first in 20 hazardous substances. The WHO has described the arsenic problem as the most serious global environmental health problem. Maximum permissible limit of arsenic concentration as recommended in Nepal standard for drinking water is 50 ppb. However, presence of elevated arsenic concentration in ground water used for drinking has emerged as major public health problem in Nepal. It is estimated that around 0.5 million people in Terai are living at the risk of arsenic poisoning. These above facts are known to everybody who is working on arsenic (WHO, 2002).

Nepal is rich in water resources. However, many Nepali people do not have access to piped drinking water supply even in urban areas. Western Nepal is poorly developed and thus has low water coverage. Also, marginalized communities throughout Nepal have lower accessibility to water than high caste communities due to prevailing social practices.

Arsenic contamination has been reported as the major water quality problem in many parts of Nepal especially in western Terai region. About 2 million people are using the water which contains arsenic concentrations above 0.05ppb. Therefore, specific research and programmes are required for the identification of arsenic contamination in water and arsenic affected people in arsenic risk zones of Nepal (UNICEF, 2002).

About 47% of Nepal's total population is living in Terai region and 90% of them are relying on ground water as their major source of drinking water. It has been estimated that there are about 200,000 shallow tube wells installed by different agencies in 20

Terai districts, serving 11 million people. Recently, arsenic contamination in ground water has been recognized as a public health threat to the people living in Terai (ENPHO, 2003).

Nawalparasi is one of the most arsenic affected districts in Nepal where more than 15% tube wells have been reported to contain high levels of arsenic (ENPHO, 2003). Similarly, variations in arsenic concentration have been reported in different aquifer located within 1 kilometer in the region. Therefore, it has been assumed that the difference of the arsenic concentration reported may be due to the activity of varied microbial community present in specific location of the aquifer.

Hence, the present study has been conducted to find out the role of microorganisms in leaching of arsenic in ground water. Therefore, the present study is an attempt to isolate and characterize the arsenic tolerant bacteria from the tube well water of Nawalparasi (high arsenic aquifer).

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To isolate and characterize arsenic tolerant microorganisms from ground water samples collected from high arsenic aquifers in Nawalparasi district Nepal.

2.2 SPECIFIC OBJECTIVE

- To determine arsenic concentration in the sample.
- To detect the presence of Total coliform bacteria in the water samples.
- To isolate and identify arsenic tolerant microorganism from the water samples.
- To determine the maximum arsenic tolerance level of selected water isolates of bacteria.

CHAPTER-III

3. LITERATURE REVIEW

3.1. WATER POLLUTION

Water gets polluted when its normal functions and properties are altered. It indicates the state of deviation from the quality and purity of water. It is defined as any physical, chemical or biological change in surface water or ground water that can adversely affect living organisms. Hence, consumption of polluted water is risky and responsible for many human health hazards.

Human activities and many environmental factors are responsible for the contamination of water resources when it flows away from its origin.

The detection of groundwater pollution has been reported as difficult task then the detection of surface water pollution. However, the water quality can be ensured by analyzing the water for physical, chemical and microbiological parameters.

3.2 GROUND WATER AS A SOURCE OF DRINKING WATER

The groundwater is one of the main sources of water in Nepal, which is widely used for domestic, industrial, and irrigation purpose. It is estimated that the Terai region has a potential of about 12 billion m^3 of groundwater, with an estimate annual recharge of 5.8 to 9.6 billion m^3 (the maximum that may be extracted annually without any adverse effect). The groundwater is the major source of drinking water supply in the Terai region. However, asenicosis cases detection of high concentration of Arsenic in well water in many areas and is one of the main public health problems in Nepal.

Current groundwater withdrawal is about 0.52 billion m^3 per year. The aquifers in Terai region of Nepal which contain sediments of alluvial origin are very favourable for water accumulation or water recharge beneath surface (WECS, 1999).

3.3 ASSESMENT OF WATER QUALITY

A large number of parameters signifying the quality of water have been recommended in WHO guideline. Hence, analysis of water quality parameters has been reported very helpful for the prevention of pollution of water resources and to prevent outbreak of waterborne diseases (Miller, 1988).

3.3.1 Physico chemical parameter

Temperature

Temperature of surface water is influenced by latitude, altitude. The weather and climate also influence the water temperature. The factors such as season, time of day, air circulation, and cloud cover and the flow and depth of water body also have been reported as the influential factors affecting surface water temperature. Cold water is preferable for drinking purpose since it is more palatable than warm water. The warm water temperature enhances the growth of microorganisms and has been associated to many water quality problems such as taste, odor, color and corrosion.

pH

pH is one of the most important water quality parameter. Generally, neutral to slightly alkaline pH water is required for drinking purpose. The pH also influences the water disinfection process. Hence, pH should be less than 8 for effective chlorination of drinking water. Corrosive characteristics are evident at pH values below 6.5 and low pH also causes the release of toxic metals. At pH values above 8.5, precipitation and scaling can result, which in turn are associated with taste and odor problems.

Turbidity

Particulate matter causes turbidity in drinking water. Turbidity is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted with no change in direction or flux level through the sample. High levels of turbidity can protect microorganisms from the effects of disinfections and can stimulate bacterial growth. The appearance of water with a turbidity of less than 5 nephelometric turbidity units (NTU) is usually acceptable to consumers, although this may vary with local circumstances. However, because of its microbiological effects, it is recommended that turbidity should be kept as low as possible.

Electrical conductivity

Conductivity is a measure of the ability of water to conduct an electric current. This ability depends upon the presence of ions, on their total concentration, mobility and valence and on the temperature of measurement the conductivity of distilled water ranges between 1to 5µs/cm but the presence of salts and contamination with wastewater increases the conductivity of the water.

Hardness

Hardness of water is usually expressed as the equivalent quantity of calcium carbonate. Total hardness is defined as the sum of the calcium and magnesium concentrations, both expressed as calcium carbonate in milligrams per liter.

Iron

Iron is found in natural fresh water at levels ranging from 0.5 to 50 mg per liter. In drinking water supplies iron (II) salts are unstable and are precipitated as insoluble iron (III) hydroxide, which settles out as rust coloured silt.

Table 1: Physical Properties of water

S.No.	Properties	Values/Units
1	Boiling Point	100^0C
2	Melting Point	0^0C
3	Critical Temperature	374.2^0C
4	Molar Heat of Vaporization	40.67 KJ(9.720 KCal)
5	Molar heat of Fusion	6.02 KJ (1.44 K Cal)
6	Molar Entropy of Vaporization	109 J/deg (26.1 cal/deg)
7	Viscosity at 20^0C	1.005 centipoise
8	Surface Tension at 20^0C	73dynes/cm
9	Dielectric Constant	80.54
10	Dipole Moment	1.84 debye
11	Specific Heat capacity	1 Cal/g/^{0}C
12	Heat of Evaporation	540.0 Cal/g
Adapted from**:** Sharma and Kaur, 1997-98		

3.3.2. Microbiological parameter

Pathogenic bacteria, viruses, protozoa, or helminthes' egg may contaminate drinking water by various means. Ingestion of such contaminated drinking water is the means of transmission of microorganisms causing fatal diseases, like typhoid, cholera, dysentery and hepatitis as well as many protozoal and helminthes infestations. Once the microorganisms enter the water sources, they can remain in water bodies for a long time mentioned in Table 2.

Similarly, different bacteria have different thermal death time. Some spore forming bacteria can survive at very high temperature as known as thermophiles and some are mesophiles. So the thermal death time for bacteria varies as shown Table 3. Microbial examination provides the most sensitive, although not the most rapid, indication of pollution of drinking-water supplies.

Unlike chemical or physical analysis, however, it is a search for very small numbers of viable organisms and not for a defined chemical entity or physical property. Because the growth medium and the conditions of incubation, as well as the nature and age of the water sample, can influence the spices isolated and the count, microbiological examinations may have variable accuracy.

This means that the standardization of methods and of laboratory procedures is of great importance if criteria for microbiological quality of water are to be uniform in different laboratories and internationally. International standard methods should be evaluated under local circumstances before being adopted in national surveillance programmes (Ross, 1990).

Therefore, water should be tested regularly to confirm their freedom from such contamination. However, it is impracticable to detect all of the pathogens present in contaminated water. Hence, normal practice is to detect and enumerate the common intestinal commensal bacteria present in large number in human and warm-blooded animal.

Frequent examination of the faecal pollution indicator in water remain the most sensitive and specific way of assessing the hygienic quality of water. The major faecal pollution indicators include *Escherichia coli*, Total coliform bacteria, Thermo-tolerant coliform bacteria, *faecal Streptococci* and *Clostridium perfringens* (WHO, 1997)

The presence of *E. coli* in water samples indicates the presence of faecal matter and possible presence of pathogenic organisms of human origin. *E. coli* has been used and continued to be considered as the best indicators of fecal pollution of the water since, *E. coli* are found in large numbers in the feces of human and warm-blooded animals (10^9 per gram in freshy faeces), *E. coli* are relatively easy to detect and assay and survival time and nature of *E. coli* is similar as that of the pathogens in the aquatic environment.

Methods which are used for the detection and enumeration of *E.coli* and other fecal pollution indicators include, membrane filtration (MF) technique and most probable number technique (Multiple tube dilution technique).

Table 2: Survival period of microorganism in water

Pathogen	**Survival Time**
Giardia lamblia	20 days
Entamoeba Cysts	15 days
Ascaris egg	1 year
Escherichia coli	20 days
Salmonella Typhi	30 days
Salmonella Paratyphi	30 days
Vibrio cholerae	20 days
Hepatitis A virus	60 days

Adapted from**:** Ross, 1990

Table 3: Approximate thermal death times for bacteria

Organisms	**Time (min)**	**Temperature (^{0}C)**
Escherichia coli	20-30	57
Staphylococcus aureus	19	60
Bacillus subtilis(spores)	50-200	100
Clostridium botulinum(spores)	100-330	100

Adapted from**:** Frazier and Westhoff 1978

3.3.3 Total coliform bacteria

Coliform organisms have long been recognized as a suitable indicator for drinking-water quality, largely because they are easy to detect and enumerate in water. The term “coliform organism” refers to Gram-negative, rod shaped bacteria. Coliform bacteria were regarded as belonging to the genera *Escherichia, Citrobacter, Enterobacter* and *Klebsiella*. They can be found both in faeces and the environment (nutrient-rich waters soil, decaying plant material), and also in drinking water with relatively high concentrations of nutrients.

Coliform bacteria should not be detectable in treated water supplies and if found suggests inadequate treatment, post treatment contamination or excessive nutrients. Although coliform organisms may not always be directly related to the presence of faecal contamination or pathogens in drinking water, the coliform test is still useful for monitoring the microbial quality of treated piped water supplies. According to WHO guideline value, total coliform should not be detected in 100 ml sample of all water intended for drinking purpose (WHO, 2002).

3.3.4 Thermotolerant coliform bacteria

Thermotolerant coliforms are bacteria found in the human or animal intestinal tracts. High counts of these bacteria in rivers, streams and lakes are caused by contamination from the faeces of human and other warm-blooded animals. It can be found in population ranging from zero to thousands of colonies per milliliters of water. Thermotolerant coliform bacteria are not themselves harmful but are the indicator organisms used to indicate the presence of viruses and other pathogenic organisms. According to WHO guideline, there should be zero number of thermotolerant coliform per 100 ml of water sample (APHA, 1998).

3.4. ARSENIC

3.4.1 Chemical nature of arsenic

Chemically, arsenic is intermediate between metals and nonmetals. Its properties lie, kin general, in the middle of the series formed by the family of the elements nitrogen, phosphorus, arsenic, antimony, and bismuth. Arsenic ranks about 52nd in natural abundance among the elements in crustal rocks. When arsenic is heated, it sublimes, passing directly from solid to gaseous form at 613° C (1135° F). A common form of arsenic is gray, metallic in appearance, and has a specific gravity of 5.7. A yellow, nonmetallic form also exists and has a specific gravity of 2.0. The atomic weight of arsenic is 74.9216 (Frankenberger, 2001).

3.4.2 Arsenic compounds in water:

The most important arsenic compounds in terms of drinking water supplies (ground water) are the inorganic species As(III) and As(V). These species are usually associated with low concentrations of dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA). In well-oxygenated water and sediments nearly all arsenic is present in the thermodynamically more stable pentavalent state (arsenate). In chlorinated drinking water supplies, arsenic salts are in the pentavalent state as a result of oxidation by free chlorine.

Arsenic dissolved in water can undergo either reduction or oxidation, depending upon conditions. Arsenic tends to be adsorbed by iron oxyhydroxides, aluminum hydroxides, manganese compounds, organic materials and clays. There is potential for arsenic release, when there is a fluctuation in oxidation-reduction potential and/or pH. As(III) is more mobile than As(V) because it is less strongly sorbed on most mineral surfaces than the negatively charged As(V) oxyanions (Arsenic, 2006).

3.4.3 Mode of arsenic contamination in water

Arsenic that prevails in the surface of the earth is a natural contaminant of water. So, the geological formation determines largely the level of arsenic in water and soil, which occurs as a result of leaching from rock into groundwater and possible geothermal activity. In addition, nonferrous mining and smelting operations, refining operations, wood preservative use, pesticides manufacturing sites and past use of pesticides on crops may add to the elevated concentration of arsenic in water and soil. Human beings are exposed to arsenic in variety of forms from sources such as food and water. Arsenic is transitional reactive element that forms chemical and organic complexes together with other metals, carbon and oxygen. Arsenic occurs in trivalent and pentavalent form. Inorganic Arsenic compounds are more toxic than pentavalent or zero-valent. The inorganic arsenicals are the pre-dominant contaminants in water (Smedley and Kinniburgh, 2002).

Theoretically, it is presumed that thousands of years ago, rocks rich in arsenic have been eroded from the Himalayas and other upland areas through areas through soil erosion and flood. Along with sand, gravel, silt and clay arsenic was deposited in low-lying active flood plain areas, which now make up most parts of Terai of Nepal, West Bengal of India and Bangladesh. These Arsenic-bearing sediments were buried over thousands of years, forming parts of the aquifers that are at present being tapped for water resources.

Arsenic enters human body through a variety of means. One is natural source. In nature arsenic occurs usually combined with sulfur in ores such an arsenopyrite FeAsS, orpiment As_2S_3 and regular As_4S_4. Other forms like arsenolite As_4O_6, cobaltite CoAsS, white cobalt $CoAs_2$, arsenical iron AsFe and As_4Fe_3, nickel glance NiAsS, NiAs, loallingite $FeAs_2$, and white arsenic As_2O_3 are also found (Gomez, 2001). Arsenic is released in ground water as soluble form in the following three mechanisms:

(I) Oxidation of arsenopyrites or pyrites.
(II) Reduction of oxy-hydroxides.
(III) Desorption of arsenic by phosphate.

Second important means of arsenic contamination is anthropogenic factor. Arsenic compounds enter into human body through the use of insecticides, pesticides, fungcides, rhodenticides, herbicides, defoliants, dipping agents for animals and wood preservatives. It also enters into human body by means of food chain via animal feed additives, veterinary medicines and tanning agents. Human body gets arsenic element also through contamination from

(I) Elemental arsenic used in alloys for battery leads or plates

(II) Paint materials or dyestuffs and gallium arsenide (GaAs) used electronics as substitute for silicon, or

(III) Arsenic gas in glass and silicon industries, metal smelting, galvanizing and lead plating.

Thus, surface water sources are safe for human and animal consumption as far as arsenic contamination is concerned. Water drawn from wide-brimmed dug wells may be relatively safer as the aerobic microbes in the well degrade the soluble arsenic compound to arsine ions that are quickly diffused into the air (Smedley and Kinniburgh, 2002).

3.4.4 Factors affecting arsenic concentrations in water

Arsenic release from iron oxide, from desorption or dissolution, appears to be the most common cause of regionally high arsenic concentrations. Desorption can be promoted by a high pH or the introduction of a competing adsorbent in oxic and post-oxic ground water. Iron oxide can release arsenic to ground water through dissimilatory-iron reduction involving either naturally occurring or anthropogenic (i.e. waste disposal, presence of petroleum products, etc.) organic carbon. After precipitation of amorphous iron oxide containing arsenic, the conversion to a more crystalline form releases arsenic.

The oxidation state of inorganic arsenic has an important effect on mobility. In the presence of iron oxide, As(III) is adsorbed to a lesser degree than As(V). Sulfide minerals can be a source or sink for arsenic. Oxidation of arsenic bearing sulfide minerals produces high arsenic concentrations in many parts of the country,

particularly where rocks have undergone mineralization. Except in very acidic water, where scorodite can form, the most common arsenic-bearing product is iron oxide. Precipitation of sulfide minerals, which can occur in sulfide-rich water, can remove arsenic from water (Frankenberger and Nriagu, 2002).

3.4.5 Acceptable arsenic concentration in drinking water

Arsenic free water is always very rare. In Bangladesh, the government's water quality standard for arsenic in drinking water is 50 parts per billion (ppb) or 0.05 milligram per litre (mg/L). Many countries including USA and most countries in Europe also accept this level. The WHO had initially issued a "Guideline value" for arsenic in drinking water at 50 ppb in 1984. It revised this guideline value to 10 ppb or 0.01 mg/L in 1993. However, guideline value set by WHO is not binding limits. Each country has its own national standard in the context of local or national environmental, social, economic and cultural conditions. Nepal has standardized its National Interim Arsenic Standard Limit for drinking water as 50 ppb.

3.4.6 Arsenic metabolism in human

The intrinsic atomic stability of arsenic allows relatively easy tracing and measurement of it in water and biological materials (hair, nails, urine and skin scrapings). However, clinical significance of arsenic level in biological substances is not always clear. When ingested with contaminated food or drink, the gastro-intestinal absorption varies greatly with the specific chemical form and nutritional status of the host. After absorption from intestine, the absorbed nutrient or food is the main medium of its transport.

Arsenic is deposited in some organs-bone, liver and kidney-in relatively high concentration and the metal is cumulated there for years. Most metal is excreted through renal clearance and gastrointestinal excretion. Some proportion is excreted through salivation, and perspiration, exhalation, lactation skin exfoliation and loss of hair and nails. Accumulated arsenic in liver, spleen, kidney, lungs and GI tract is relatively well cleared (Arsenic, 2006).

However, Arsenic residue in keratin rich tissues like skin, hair and nail lasts for a very long time. Arsenic is deposited in the hair surface through its excretion from sebaceous gland and arsenic becomes fixed in the hair surface for a long time resisting wash or detergents. Arsenic levels in blood, hair, nail, and urine have all been used bio-indicators of exposure. Blood arsenic is used in acute poisoning cases as an indicator of acute high-level exposure. Poor co-relation has been reported between arsenic concentration in drinking water and blood arsenic level because arsenic is cleared rapidly from the blood.

Total urinary arsenic is used as an indicator of more recent arsenic exposure. Arsenic has also been recognized during the last century as a potent human toxin and as carcinogen. In recent decades, multiple epidemiological studies have shown that there is an adequate evidence of carcinogenic risk by both inhalation and indigestion of arsenic. Arsenic is the only carcinogen whose through drinking water has been demonstrated to cause human cancer (Smedley and Kinniburgh, 2002).

The most deceptive and dangerous aspect of arsenic toxicities is its very slow and insidious development. It takes roughly about 1 to 10 years of continuous ingestion of Arsenic contaminated water to develop the overt symptoms. Onset as well as severity of chronic Arsenic poisoning depend upon the nutritional and health status of the exposed person.

The first visible symptoms are skin lesions such as melanosis and leuko-melanosis followed by keratosis, ulcer or exfoliate dermatitis and gangrene. Neurological symptoms like tingling and numbness in hands and feet, auditory nerve damage, distal weakness and even some physical disabilities are also frequently reported.

Inflammation of respiratory mucosa is related to sensorineural dysfunction. Chronic Arsenic toxicosis has also been associated greatly with elevated risk of skin cancer and possibly cancer of lung, liver, bladder, kidney and colon. Some hormonal problems associated with arsenic are related to developmental toxicity resulting in goiter, diabetes, gastro-intestinal, and liver effects (Smedley and Kinniburgh, 2002).

3.4.7 Medical management of chronic arsenic poisoning

The following are considered effective measures for medical management of chronic Arsenic toxicosis:

(I) Avoidance of further or additional exposure to arsenic contaminated water. Safer water sources to be found for the cases and exposed communities by replacing the existing contaminated sources by surface water or deep boring system or by appropriate mitigation programs for drinking water.

(II) Providing patients the supportive therapy containing high doses of vitamins, especially vitamin A and C and balanced enriched diet aids for recovery. For this reason, patients are advised to take plenty of vegetables especially leafy greens, locally available fresh fruits especially yellow fruits and nutritious food.

(III) Symptoms such as skin hardening or keratosis are treatable with simple keratolytic skin ointments.

(IV) Chelation therapy with EDTA (in acute case) and dimercaprol or BAL (in chronic case) for the treatment of recent exposure is also considered to be specific therapy for relief of systematic clinical manifestations and reduction of arsenic stores in the body, decreasing subsequent cancer risk

3.5 ARSENIC TESTING METHODS

To determine the arsenic is very difficult because of its presence remarkably in lower concentration in various states in nature. There are various methods to determine the arsenic concentration.

(I) **Arsenic testing field kit method**, which do not need any modern instruments. This method has been adopted from APHA 17^{th} edition (1989). Total arsenic i.e. As(III) and As(V) is determined by this method. Collecting the samples in

the field and bringing them back to the laboratory is unsatisfactory for reliable analysis of As(III) and As(V), due to ongoing oxidative change of the sample. There are several commercial field test kits available in the market. Some of the kits are listed as follows:

- Merck Field Kit,
- UNICEF Field Kit,
- Arsenic field test kit developed by All India Institute of Health and Public Hygiene (AIIHPH),
- NIPSOM, Field Kit,
- ENPHO Arsenic Field Kit,
- Quantofix Kit,

The principle behind most of commercial kits is basically that of **Mercuric Bromide Stain Method**. This is the semi-quantitative analysis of arsenic in drinking water, ground water and surface water. Sensitivity of field kits ranges depending on type of kits. Generally minimum detectable level in many kits is 2 ppm. The arsenic test procedures used in this methods based on the following principles:

i. As(V) is reduced to As(III) by adding KI (Potassium iodide) and $SnCl_2$ (Stannous Chloride) in the acidic environment.

ii. As(III) is allowed to react with the hydrogen ion, which would be generated by the reaction of HCl (Hydrochloric acid) and Zinc granules to form arsine gas (AsH_3).

iii. The arsine gas thus produced is passed through a column containing a roll of cotton moistened with lead acetate solution to avoid possible interference due to sulfide and antimony present in the sample water and

iv. The generated arsine produces a YELLOW-BROWN stain on mercuric bromide paper which can visibly detected.

$$As(V) \longrightarrow As(III) + H^+ + Zn\ granules \longrightarrow AsH_3 + HgBr_2 \longrightarrow yellow\ brown$$

(II) **Spectrohpotometric (Silver diethyl dithiocarbonate) method**, which needs spectrophotometer. This method has been adopted from APHA 20th edition (1998). This method is applicable to determine the total inorganic arsenic in drinking water, ground water and surface water. This method is useful to identify arsine at different pH. Minimum detectable quantity by this method is 1 ppm. The principle of this method is based on the absorbance measurement (colour intensity) of soluble colour complex formed by the reaction of arsenic with silver salt. As(III) is reduced selectively by aqueous sodium borohydride solution to arsine, AsH_3, in an aqueous medium. The generated arsine is swift through a scrubber containing a glass wool or cotton impregnated with lead acetate solution into an absorber tube containing silver diethyl dithiocarbonate and morpholine dissolved in chloroform. The intensity of red colour that develops is measured as 520 nm.

(III) **Atomic absorption spectrometric method (Hydride technique):** This is the most advanced and sensitive technique. The method has been adopted from International Standard (ISO), 1996. This method is used for determination of arsenic including organically bound arsenic in drinking water, ground water and surface water, in a concentration from 1 ppm. Higher concentration can be determined by using suitable dilution of water sample. The principle of this method is based on the atomic absorption measurement of arsenic generated by the thermal decomposition of As(III) hydride. As(III) is reduced to gaseous AsH_3 by reaction with sodium tetra hydroborate in a hydrochloric acid medium. The absorbance is determined at wavelength of 193.7 nm.

3.6. MICROORGANISM INVOLVED IN ARSENIC CYCLING

There are certain microorganisms, which use arsenic oxyanions for energy generations, either by oxidizing arsenite or by respiring arsenate. These microbes are of diverse groups and occur in a wide range of habitats. Arsenic cycling may take place in absence of oxygen and contribute to organic matter oxidation. In aquifers, these microbial reactions may mobilize arsenic from solid to aqueous phase, resulting in contamination of drinking water. Phylogenetic diversity of representative arsenic-metabolizing prokaryotes is mentioned in Appendix X. Microbes that have been

reported to found involved in arsenic cycling are from basically 3 major groups of organisms.

(I) Dissimilatory arsenate respiring prokaryotes (DARPs)

(II) Heterotrophic arsenite oxidizers (HAOs)

(III) Chemoautotrophic arsenite oxidizers (CAOs)

Dissimilatory arsenate respiring prokaryotes (DARPs)

At present there are at least 16 species in pure culture and include representative from gamma, delta and epsilon proteobacteria, low GC Gram positive bacteria, thermophillic Eubacteria and crenoarchea. Collectively these microbes are called DARPs. DARPs could be isolated from fresh water sediments, esturies, sodalake, hot spring and subsurface aquifer. DARPs are extremophiles; adopted to high temperature, pH, salinity. DARPs utilizes variety of electron donors including hydrogen, acetate, formate, pyruvate, butyrate, citrate, succinate, malate, glucose, lactate. DARPs utilizes As(V) serves as a nutrients.

The frequently reported DARPs utilizes aromatic complex compounds like benzoate and toluene. As(V) serves as nutrient to certain anaerobes by functioning as their respiratory oxidant. Such reaction is energetically favourable when coupled with oxidation of organic matter because As (V)/As(III) oxidation/reduction potential – 135mV. Metabolic diversity of DARPs are important ecological factor, because sulfur, iron and nitrate chemical species (S<-II>, FeOOH, NO_3-) interact with As in the environment (Oremland and Stolz, 2003).

DARPs reported .

- *Sulfurospirullum barsenii,*
- *Bacillus selenitrireducens,*
- *Desulfotomaculum auripigmentum,*
- *Desulfomicrfobium* spp.,
- *Sulfurospirillum arsenophilum,*
- *Citrobacter,*
- *Chrysiogenes arsenatis,*
- *Shewanella,*
- *Clostridium* spp.

These organisms posses respiratory arsenate reductase enzyme. But they need to be fully elucidate. Enzymological and immunological analysis further indicates that to respire arsenate doesn't preclude the presence of separate arsenate resistance system. Some bacteria have both respiratory and detoxifying arsenate reductase. The process of dissimilatory arsenate reduction occurring in near surface hyporheic zone greatly affects transport and speciation of arsenic in freshwater. DARPs can attack arsenate adsorbed to solid phases like ferrihydrites and alumina. 16s ribosomal DNA probe techniques is used to identify species. DARPs isolated thus far are opportunist capable of respiring electron acceptor other than arsenate.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis of amplified 16srDNA gene fragments from incubated arsenate enriched water revealed the presence of two bands. The resolved sequences of these excised bands studies have identified the bacteria as *Desulfovibrio.* Reports have confirmed that these bacteria are capable of anaerobic growth using arsenate as their electron acceptor (Oremland *et al*, 2002).

Arsenic oxidizing Prokaryotes (HAOs and CAOs)

The microbial oxidation of as to As(III) to As(V) can also impact the mobility and speciation of arsenic in the environment The process had been known for many years and than 30 strains representing at least 9 genera have been reported to be involved including alpha, beta and gamma *Proteobacteria; Deincocci;* (*Thermus* and *Crenarchaeota*).

Physiologically diverse, they include both HAOs and CAOs. Heterotrophic oxidation considered as detoxification reaction that converts As(III) encountered on cell's outer membrane into less toxic As(V), making it less likely to enter cell. CAOs couple the oxidation of arsenite to reduction of either oxygen or nitrate and use the engery derived to fix carbon dioxide into organic cellular material and achieve growth.

The arsenite oxidase of CAOs fully characterized. HAOs the oxidation of AS(III) is catalysed by a periplasmic enzyme distinct from dissimilatory arsenate reductase. Arsenite oxidation is being studied as the basis for bioremediation of system where As (III) is a pollutant, because the As(V) can be immobilized onto strong adsorbents.

In the interest of bioremediation several novel species have been isolated, both heterophic and autotrophic aerobic As(III) oxidizer from arsenic rich environment.

The main function of the HAOs and CAOs have been reported to convert As (III) to As (V). Frequently reported HAOs and CAOs are following: Alpha-*Proteobacterium*(*Rhizobium*)–StrainNT-26, *Thermophillic thermus-strain* HR13, Non photosynthetic bacterium-strain, MLHE-1, *Alcaligenes faecalis* and *Thermus aquaticus* (Oremland and Stolz, 2003).

In case of recycling in ground water arsenic metabolizing bacteria may contribute to the mobilization of arsenic from the solid phase into the aqueous phase in a subsurface drinking water aquifer. Arsenic is present primarily in the form of chemically reduced minerals like real agar(AsS), Orpiment(As_2S_3) and arsenopyrite (FeAsS). These minerals are attacked by CAOs, which results in the oxidation of As(III), as well as iron and sulfide with the concurrent fixation of CO_2 into organic matter. Construction of wells by human activity accelerates this process by providing the necessary oxidants like molecular oxygen, NO3 in agricultural region.

As(V) can subsequently be adsorbed on oxidized minerals surface like ferrihydrite aluminum. The influx of substrate organic material derived either from buried peat deposit, discharged of surface water, microbial mats promotes microbial respiration and onset of anoxia, DARPs then respire adsorbed As(V) results in release of As(III) into the aqueous phase.

Thus the studies on microorganism involved in arsenic cycling so far have confirmed the importance of microorganisms. Thus it remains to determine in our studies what type of microbes carry out such reactions in ground water of Terai regions as compared with those found in other part of the world (Oremland *et al*, 2002).

3.7 ARSENIC INVESTIGATION IN NEPAL

Presence of arsenic in groundwater in the Terai districts of Nepal was known for the first time in 1999 from the research work assisted by WHO. Since then there has been a growing concern among the Nepalese scholars towards understanding more on arsenic and its human health impacts. Few investigations that have been carried out on arsenic until now reveal that groundwater particularly in Terai districts are contaminated with arsenic.

Arsenic is recognized a serious health problem in Bangladesh and some parts of India's West Bengal, as their large populations are exposed to the potential risk of arsenicosis. Cases of arsenicosis are also reported in Thailand, Vietnam, China, Sri-Lanka and Pakistan (WHO, 1999)

Table 4: Arsenic Level at Different Districts in Nepal

S.N.	Districts	Arsenic ppb			Total Test	As(ppb) Maximum	* % of sample exceeding permissible limit of Nepal Std.	* % of sample exceeding permissible limit of WHO GV
		0-10	>10-50	>50				
1	Kailali	87	66	34	187	213	18	53
2	Kanchanpur	128	16	9	153	221	6	16
3	Bardiya	386	125	20	531	160	4	27
4	Dang	91	7	1	99	50	1	8
5	Banke	1216	474	31	1721	270	2	29
6	Kapilbastu	2246	235	91	2572	589	4	13
7	Rupandehi	1807	225	46	2078	2620	2	13
8	Nawalparasi	1492	1135	953	3580	829	27	58
9	Chitwan	86	0	0	86		0	0
10	Parsa	1862	206	52	2120	456	2	12
11	Bara	1725	240	46	2011	254	2	14
12	Rautahat	1011	1191	211	2413	324	9	58
13	Saptari	532	82	14	628	98	2	15
14	Dhanusa	157	43	9	209	106	4	25
15	Siraha	195	54	13	262	107	5	26
16	Sarlahi	345	87	13	445	93	3	22
17	Mahottari	79	10	2	91	82	2	13
18	Sunsari	303	67	2	372		1	19
19	Morang	149	22	2	173	70	1	14
20	Jhapa	462	42	1	505	79	0	9
21	Ilam		4		4			
Total		**14359**	**4331**	**1550**	**20240**	**2620**	**8**	**29**

Adapted from**:** WHO, 1999

* Exceeding the concentration limit recommended in DWQS (Drinking Water Quality Service)

3.7.1 Arsenic problem in Nawalparasi district

There are about 4119 tube wells in the Ramgram Municipality (Kunwar Village). Out of which 62% are above contamination level in WHO standard, where as in Nepal standard it is above 43%. There were about 987 tube wells installed under Nepal Red-Cross Society (NRCS), and 716 tube wells from 26 Village Development Committees (VDCs) and one Municipality were tested for arsenic contamination. Among 716 tested tube wells, 106 tube wells (14.8%) were found above Nepal Interim standard (50 ppb) and 286 tube wells (i.e. 39.9%) were found between 11 ppb to 50 ppb of arsenic concentration. Thus, about 54.7% (i.e. 392) tube wells were above WHO guideline (10 ppb) and the remaining 45.3% of tube wells were safe from arsenic contamination in the district. The analysis showed that the arsenic concentration ranges from 0 ppb to 436 ppb. The classification of tube wells into three classes of arsenic concentration –below 11 ppb, 11-50 ppb is shown in chart. The size of the chart is proportion to total number of tube wells for arsenic contamination of the corresponding VDC (ENPHO, 2003).

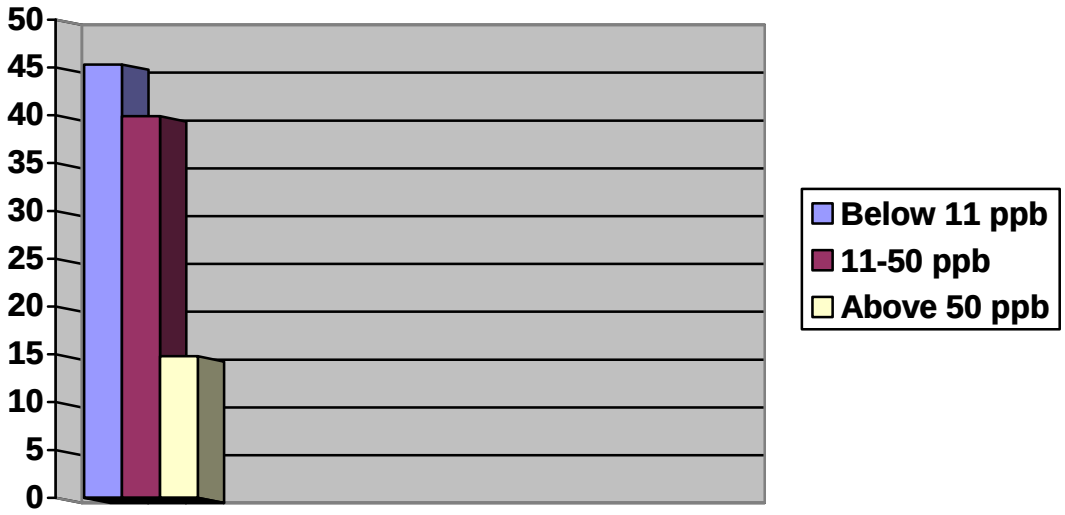


Figure 1: Classification of tube wells by Arsenic concentration

3.7.2 Arsenicosis

Arsenicosis symptoms are common problem in many areas of the Nawalparasi district. It is found in the form of melanosis and keratosis. The symptoms are seen on palm, sole and trunk of the patients. Both symptoms can be observed on spotted or disseminated form. It has been reported that the symptoms are more common among the age group of 15 to 65.Many researchers have confirmed the Arsenicosis problem by the detection of concentration of arsenic in hair and nail samples of a suspected person in the area (ENPHO, 2003).

3.7.3 Arsenic Mitigation program in Nepal

Various mitigation plan like arsenic safe tube wells, rehabilitation of dug wells, household arsenic removal filters like two gagri filters, three gagri filters and improved bio-sand filters and community level arsenic iron removal plants has been provided to users in Terai.

Two gagri filters

In this filter a black coloured arsenic removal powder is used as a coagulant and oxidant. A packet of arsenic removal powder can be used for purifying 20 liters of water.

Three gagri filters

This filter has a simple design and can be easily assembled by community. This system does not use any chemicals but uses locally available material like sand, brick and charcoal. The natural filtration process removes arsenic, iron and other unnecessary chemicals.

Improved Bio-sand filter:

This filter system removes iron, arsenic and bacteriological contamination in water without using any chemicals. This filter has been provided to few communities as pilot phase in Terai.

Arsenic iron removal plant:

This plant serves a group of households who are sharing a single tube well. It has been basically made to remove iron and also arsenic is removed with the removal of iron in water.

CHAPTER-IV

MATERIALS AND METHODS

4.1 MATERIALS

All the materials required for present work are listed in the Appendix-II

4.2 STUDY AREA AND SUBJECTS

A total of 43 water samples were collected from different tube wells of the Kunwar village, Ramgram Municipality, Nawalparasi. Large population in the village depends upon ground water for drinking purpose. Many cases of arsenicosis had been seen in the villagers.

4.2.1 Collection of samples

For physiochemical analysis, the water samples were collected in clean plastic (polypropylene) jar with polythene cap. Water samples for microbiological analysis and arsenic testing were collected aseptically in sterile 500 ml glass bottles fitted with glass stoppers. Before collecting the sample, the hand pump was operated continuously for 5/6 minutes to remove enough water. It was done to ensure that new water was drawn from aquifer depending upon depth & diameter of tube pipe. So each tube well was pumped at least 15-20 times prior to collecting the sample in standard sampler. The samples were brought to the laboratory of Central Department of Microbiology, Tribhuvan University and analyzed within 8h of sample collection.
Information regarding the arseniccosis symptoms, name of household, depth of tube wells, age of tube wells, and use of filter etc. was also collected from the consumers during sample collection.

4.3 ARSENIC TESTING OF SAMPLES

Arsenic test kit from ENPHO was used to determine the arsenic concentration in the samples.

Procedure:

The sample was acidified by adding hydrochloric acid (2% V/V). The blank was treated exactly the same way as the sample. Lead acetate soaked cotton was introduced into the glass column. Then the dried narrow glass tube was placed and mercuric Bromide ($HgBr_2$) test paper was inserted. 35ml sample was pipetted into a clean generator bottle. Then 5ml concentrated HCl, 2ml KI solution and 8 drops (0.40ml) reagent was added successively with thoroughly mixing after each addition. It was allowed for 5 min to stand for the reduction of arsenic to the trivalent state. 3gm zinc was added to generate and connect scrubber-absorber assembly immediately. All connections were fitted tightly. The reaction mixture was allowed for 30 min for complete evolution of Arsenic. The generator was warmed slightly to ensure all arsenic was released. The yellow or dark brown color was seen on mercuric Bormide paper. The length of colored portion of the paper was measured.

4.4 MICROBIOLOGICAL ANALYSIS OF SAMPLES

4.4.1 Enumeration of total coliform bacteria and *E. coli*

The total coliform in the water samples were enumerated by the membrane filter (MF) technique. 100 ml sample was filtered aseptically through sterile Millipore membrane filter (0.5Micrometer) using membrane filtration apparatus. And the filter was placed over the surface of sterile EMB agar plate. One plate was incubated at 37^0C/ 48 hours to enumerate total coliform count and another set of EMB agar plate was incubated at 44.5^0C/ 48 hours to enumerate *E. coli*. The green metallic sheen colonies were randomly picked up for IMVIC test to confirm *E. coli* on the plates.

4.4.2 Heterotrophic plate count

Water sample were diluted by serial dilution. 0.1ml the sample from each aliquot was spread on the pre-solidified sterile Nutrient Agar Plate.
The NA plates were incubated 26^0 C for 48 hours. The numbers of colonies observed after 48h were expressed as the heterotrophic plate count (CFU/ml).

4.4.3 Isolation of arsenic tolerant organism

Molten nutrient agar medium (50^0 C hot) was supplemented with 5 ml of vitamin B complex solution (250 mg/L) and 5 ml As (V) Na_2HAsO_4 [5mM] salt solution (Appendix-V). The agar medium was termed as Arsenic Tolerant Bacteria Isolation Media (AsTBIM). The water samples were streaked on the sterile plates of Arsenic Tolerant Bacterial Isolation Media (AsTBIM). All the plates were incubated at 26^0C for 48 hours. After 48 hours the plates were observed for the colonies.

4.5 IDENTIFICATION OF ARSENIC TOLERANT ISOLATES

The typical isolated colonies obtained on the Arsenic Tolerant Bacterial Isolation media (AsTBIM) were identified by Gram-staining and conventional biochemical testing (Appendix-VI)(,Appendix-IX)
Appropriate biochemical tests were performed for the confident identification of the isolates. For that, the pure colonies on the media plates were inoculated on to different biochemical media.

- Gram positive organisms were identified primarily on the basis of their response to Gram's staining, Catalase, Oxidase, growth on MSA, and coagulase tests.

- Gram negative organisms were identified on the basis of Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Triple Sugar Iron test, Urease test, Nitrate test, Sulphide production test and Gas production test.

The isolates were also tested for their survival and growth at different pH, temperature and salt concentration

4.6 DETERMINATION OF ARSENIC TOLERANCY LEVEL OF THE ISOLATES

Isolates which were tolerant to 100 ppb were subjected for determination of maximum tolerancy to the high concentration of arsenic by broth dilution technique and agar dilution technique.

Appropriate volume of stock solution of arsenic salts (10 ppm) was added in the Nutrient broth tubes and Nutrient agar medium to get 100 ppb, 200 ppb, 300 ppb, 400 ppb, 500 ppb, 600 ppb, 700 ppb, 800 ppb, 900 ppb and 1000 ppb concentration in the medium (Appendix VIII).The sets of broth medium tubes were inoculated with the log phase growth (10^6 cells per ml) of the test organism. The tubes were incubated at 26^0C for 48 hours. The tubes were checked for the turbidity and the growth of organism was noted on the sets of tube with the different arsenic concentration.

The organism which was growth positive at 1000 ppb (1 ppm) arsenic concentration as tested by broth dilution method was further tested by agar dilution technique. Appropriate volume of stock solution of arsenic salts (1000 ppm) was added in the molten Nutrient Agar Medium to get 10 ppm, 50 ppm and 100 ppm arsenic concentration in agar medium. The Agar plates with different concentration of arsenic were streaked with test organism and incubated at 26^0C for 48hrs.

Preservation of the isolates:

The obtained arsenic tolerant isolates were sub-cultured on nutrient agar slant for preservation.

Figure 2: Flow chart for enumeration of Total coliform and other Bacteria in the water samples

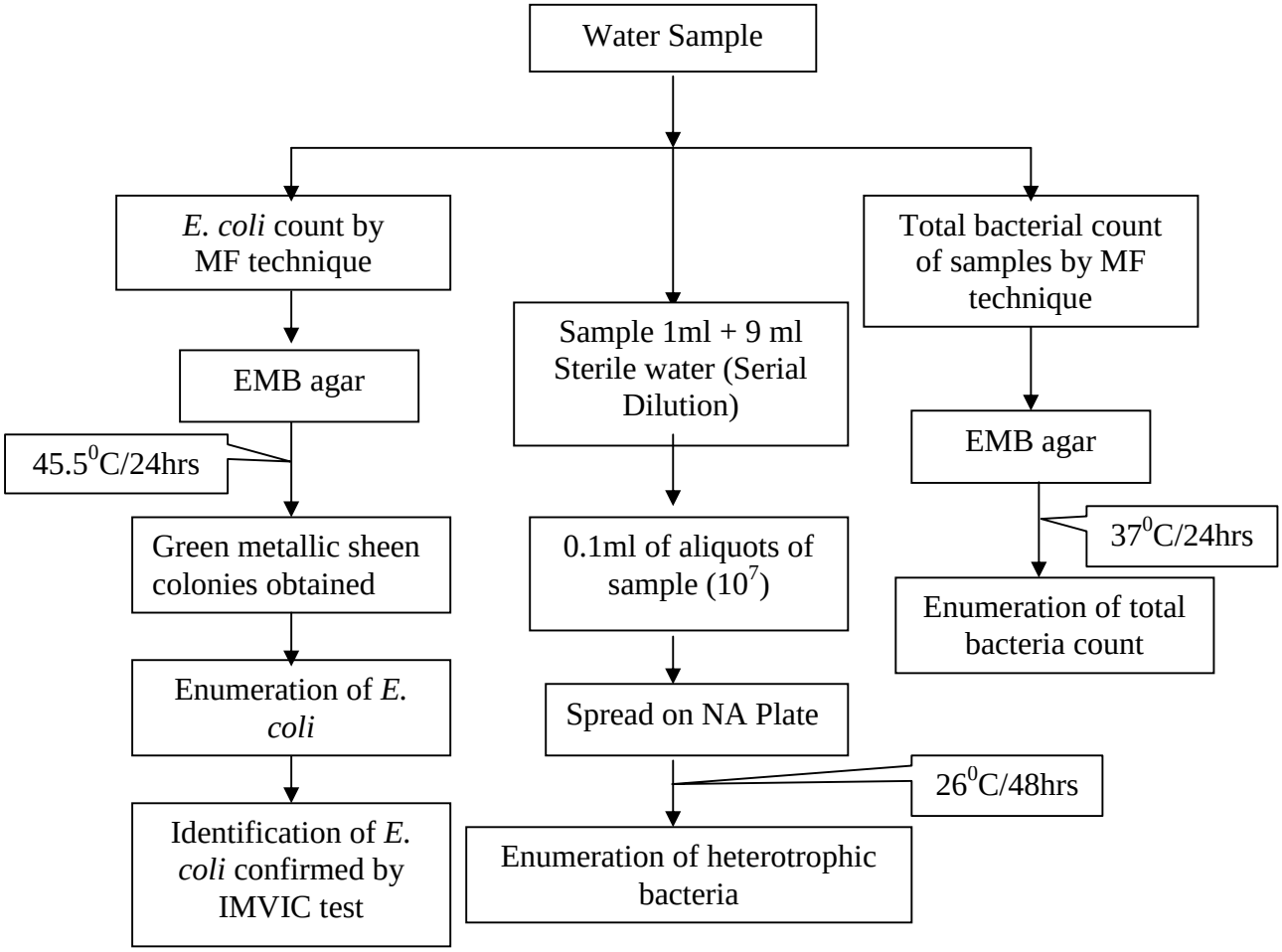
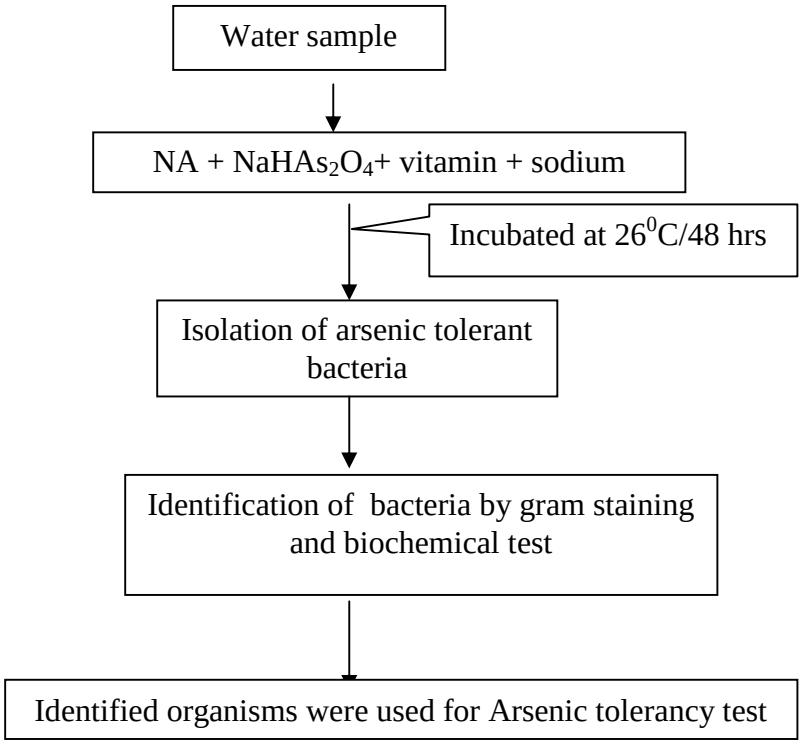


Figure 3: Flow chart for isolation of arsenic tolerant bacteria



CHAPTER-V

RESULT

This study was an attempt to isolate and characterize arsenic tolerant microorganism in ground water from high arsenic aquifer. A total 43 ground water samples were collected from different house holds of Thulokunwar and SanoKunwar of Ramgram Municipality, Nawalparasi District.

5.1 ARSENIC CONCENTRATION OF SAMPLES

In all 43 water samples analysed, highest concentration of arsenic was in the sample Tkw6. Altogather 80% of the samples exceeded the WHO guideline value of arsenic (10 ppb) and 60% of samples exceeded Nepal Interim guideline value (50 ppb).

The result of present study indicated some correlation between arsenic concentration and age and depth of tube well i.e. arsenic concentration was higher in the older tube wells than the recent one. Similarly arsenic concentration was lower in the deep tube wells than in the shallow tube wells (Table 5).

Table 5: Arsenic Concentration of Samples

Sample Code No.	Concentration of As (ppb)	Age of tube well(yr)	Depth of tube well(m)	pH of water	Coli form	No. of family member	Latitude	Longitude
Tkw1	500	20	20	8	Yes	4	27°31'.85	83°41'45
Skw2	350	6	20	7	Yes	8	27°31'.88	83°41'88
Tkw3	400	10	18	8	Yes	7	27°31'.87	83°41'45
Tkw4	50	10	18	7	Yes	25(shared)	27°32'43	83°41'08
Tkw5	30	10	18	8	Yes	6	27°32'43	83°41'08
Tkw6	620	15	20	8	Yes	4	27°31'.86	83°41'48
Tkw7	450	4	18	8	Yes	25(shared)	27°31'.89	83°41'44
Tkw8	450	7	20	8	Yes	64(shared)	27°31'.84	83°41'52
Tkw9	0	7	11	8	Yes	20	27°32'.90	83°41'37
Tkw10	10	4	34	8	Yes	20	27°31'.84	83°41'52

KEY: Tkw-Thulokunwar Village; Skw – Sanokunwar Village.

5.2 MICROBIOLOGICAL ANALYSIS

5.2.1 Total coliform count

All the samples were heavily contaminated with coliform bacteria (Table 6).

5.2.2 Presence of *E. coli*

Among the tested water samples Tkw3 had the maximum *E. coli* and Tkw6 has high total coliform count (Table 6).

Table 6: Total coliform and *E.coli* count of water sample

Sample Code No.	Arsenic Concentration (ppb)	Coliform CFU/100ml	*E.coli* CFU/100ml
Tkw1	500	$27.5X10^7$	3
Skw2	350	$22.5X10^7$	5
Tkw3	400	$25X10^7$	40
Tkw4	50	$28X10^7$	6
Tkw5	30	$22X10^7$	8
Tkw6	620	$35X10^7$	4
Tkw7	450	$20X10^7$	7
Tkw8	450	$25X10^7$	4
Tkw9	0	$25X10^7$	1
Tkw10	10	$13X10^7$	2

5.3 Heterotrophic plate count

Many samples were heavily contaminated (Table7).

Table 7: Heterotrophic plate count

Sample Code No.	Total Bacteria count (CFU/ml)
Tkw1	$65x\ 10^9$
Skw2	$45x\ 10^9$
Tkw3	$70x\ 10^9$
Tkw4	$5x\ 10^9$
Tkw5	$15x\ 10^9$
Tkw6	$85\ x\ 10^9$
Tkw7	$55x\ 10^9$
Tkw8	$15x\ 10^9$
Tkw9	$15x\ 10^9$
Tkw10	$35x\ 10^9$

5.4 ISOLATION AND IDENTIFICATION OF ARSENIC TOLERANT ORGANISM

Typical colonies from the AsTBI medium were randomly picked up for identification. At least one colony (different colored) from each growth positive sample was randomly picked up and identified on the basis of Gram staining and biochemical testing.

Table 8: List of randomly selected arsenic tolerant isolates for identification

Sample Code	**Code of Isolates**
Tkw1	N1
Skw2	N2
Tkw3	N3
$Tkw_1$6	N4
Tkw6	N5
$Tkw_1$9	N6
Tkw9	N7
Tkw10	N8

Colony Morphology characteristics of typical pigment producing isolates were shown in Table 9.

Table 9: Morphological Characteristics of Obtained Isolates

Code of Isolates	**Shape**	**Size mm**	**Colour**	**Margin**	**Surface Texture**	**Elevation**	**Consistency**	**Opacity**
N1	Circular	2	Cream	Entire	Smooth	Convex	Mucoid	Translucent
N2	Irregular	3	White	Serrated	Cotton	Convex	Nonmucoid	Opaque
N3	Round	2	Brown	Entire	Smooth	Convex	Mucoid	Opaque
N4	Circular	2	Cream	Entire	Smooth	Flat	Mucoid	Opaque
N5	Circular	2	Orange	Entire	Smooth	Flat	Mucoid	Opaque
N6	Irregular	3-4	Cream	Ciliated	Smooth	Low convex	Nonmucoid	Opaque
N7	Circular	1-2	Golden Yellow	Entire	Smooth	Convex	Mucoid	Opaque
N8	Circular	2	Greenish Cream	Entire	Smooth	Convex	Mucoid	Translucent

5.4.1 Gram's characteristics of isolated arsenic tolerant isolates

In all the isolates recovered from water samples, 40% were Gram positive cocci, 25% Gram positive rod, 25% Gram negative rod, 5% Gram negative cocci and 5% yeast and mold. This showed that Gram positive cocci were predominantly present in the ground water samples of high arsenic aquifer of Nawalparasi (Table 10).

Table 10: Gram's characteristics of arsenic tolerant organism

Code of Isolates	**Color of colony**	**Gram's character**
N1	Mucoid cream	Positive rod
N2	Mucoid white	Positive cocci
N3	Cream	Negative rod
N4	Cream	Negative cocci
N5	Orange	Positive diplo cocci in chain
N6	Cream	Positive rod
N7	GoldenYellow	Positive cocci in cluster
N8	Florescence	Negative rod

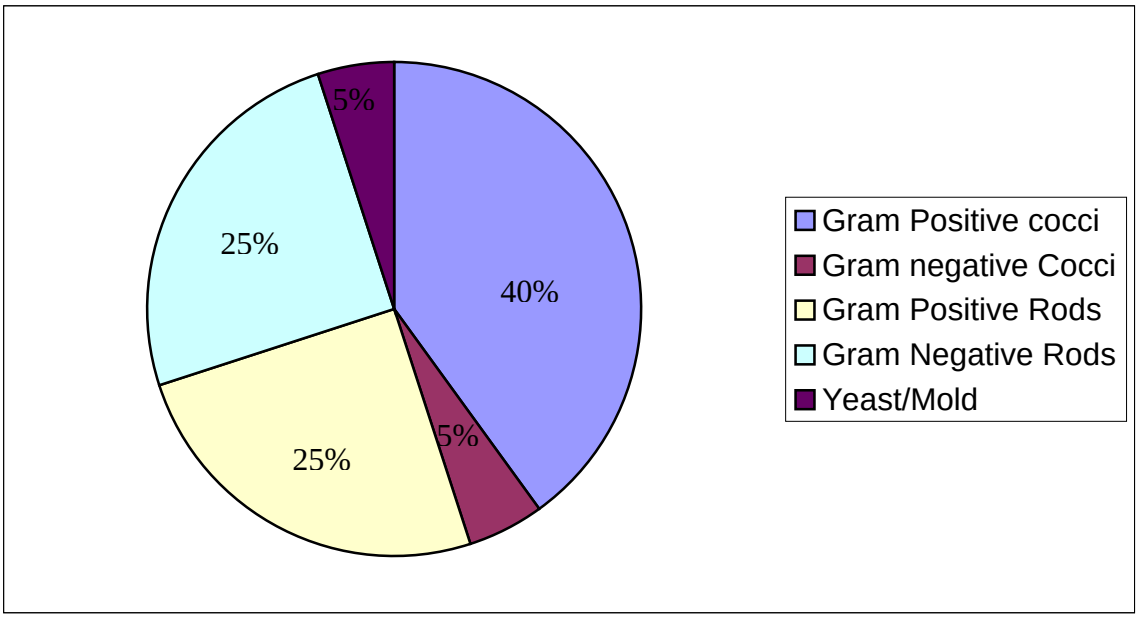


Figure 7: Gram's Staining Results

5.4.2 Biochemical testing of arsenic tolerant isolates

Randomly selected typical pigment producing arsenic tolerant isolates were identified by conventional Biochemical test method (Table 10).

Table 11: Biochemical test result of arsenic tolerant isolates

COI	**Biochemical test result**														
	Cat	**Ox**	OF	**Ur**	**Co**	**I**	**MR**	**VP**	CT	GL	N	TSI			
												S	B	H_2S	Gas
N1	+	-	F	-		-		-	-	-	+			+	
N2	+	+	O	+	-	-	-	-	+	+	+	R	R	-	-
N3	+	-	F	-		+	+	-	-	-	+	Y	Y	-	+
N4	+	-	F	-	-	-	-	-	-	-	-				
N5	-	-	F	+	-	+	-	+	-	+	-	+	+	-	-
N6	+	-	F	-	-	-	-	+	+	+	+	Y	Y	-	-
N7	+	-	F	-	+	-	+	D	-	+	+	Y	Y	-	-
N8	+	+	O	D	-	-	-	-	+	+	+	R	R	-	-

KEY: COI-Code of Isolates; Cat-Catalase; Ox-Oxidase; OF-Oxidative / Fermentative; Ur-Urease; Co-Coagulase; I-Indole test; MR-Methyl Red test; VP-Voges Proskauyer test; CT- Citrate utilization test; GL-Gelatin Liquification; N-Nitrate; TSI-Triple Sugar iron test; S- Slope; B- Butt; Y-yellow, acid; R-red alkaline; D-Different strain give different result; R-red alkaline; F- fermentative; + positive reaction; - negative reaction.

Continued….

COI	**Biochemical test result**												
	Temp ^{0}C		pH		**NaCl**		Fermentation (Sugar test)						
	45^{0}C	60^{0}C	6	9.6	6.5%	7.5%	Xy	Gl	Fr	Mn	Ma	La	Su
N1	-		-			+	+	+	+	-	+	+	-
N2		+		+	-		-	+	A	+	-	-	+
N3	+	-	+	-	+	-	A+G	A+G	A+G	A+G	A+G	A+G	A+G
N4		+			+		-	+	A+G	A+G	+	-	A
N5	+	+	+		+		-	+	+	+	+	+	+
N6	+	+		+			-	+	+	-	-	-	-
N7		+		+		+	+	+	+	-	-	A	A
N8	-	-	+	-	+	-	-	+	-	-	+	D	+

KEY: D=Different strain give different result; A+G=acid +gas; A=acid; O=oxidative; Xy- Xylose; Gl-Glucose; Fr- Fructose; Mn- Mannitol; Ma- Maltose; La- Lactose; Su- Sucrose

Continued….

COI	**Biochemical test result**						
	Medium						
	MSA media	BA	Bile aesculin agar	Cetrimide agar	MacConkey agar	EMB	Germ tube test
N1	-	+	-	-	-	-	-
N2	-	+	-	-	-	-	-
N3	-	-	-	-	+	+	-
N4	+		-	-	-	-	+
N5	-	-	+	-	-	-	-
N6	+		-	-	-	-	-
N7	+	+	+	-	-	-	-
N8	-	-	-	+	-	-	-

Legend: MSA- Mannitol Salt agar; BA- Blood Agar; EMB-Eosine Methylene Blue

Table 12: List of identified arsenic tolerant isolates

Code of Isolates	Most Probable Identified Organism
N1	Unidentified Gram positive rod
N2	*Micrococcus* spp.
N3	*Escherichia coli*
N4	Yeast
N5	*Streptococcus faecalis*
N6	*Bacillus* spp.
N7	*Staphyloccocus* spp.
N8	*Pseudomonas* spp.

5.5 Level of arsenic tolerancy of selected isolates

Identified arsenic isolates were checked for their maximum arsenic tolerancy level by broth dilution technique and agar dilution technique. Isolates were found to tolerate 1000 ppb of arsenic concentration in broth dilution technique (Table 13). Then the isolates that were tolerant to 1000 ppb of arsenic concentration were further checked for their tolerance to higher concentration of arsenic by agar dilution method. The isolates were found to tolerate very high concentration of arsenic i.e.100 ppm (Table 14).

Table 13: Arsenic tolerancy level of isolated bacteria**

Arsenic Conc. / Bacterial Isolates	100 ppb As	200 ppb As	300 ppb As	400 ppb As	500 ppb As	600 ppb As	700 ppb As	800 ppb As	900 ppb As	1000ppb As (1ppm)
Unidentified Gram positive rod	+	++	++	++	++	++	+++	+++	+++	+++
Micrococcus spp.	+	+	++	++	++	++	++	++	+++	+++
Escherichia coli	++	++	++	++	++	+++	+++	+++	+++	+++
Yeast	+	++	++	++	++	++	++	+++	+++	+++
Streptococcus faecalis	+	+	+	+	+	+	++	++	++	++
Bacillus. spp	+	+	+	++	++	++	++	++	+++	+++
Staphyloccocus. Spp	+	+	+	+	+	++	++	++	+++	+++
*Pseudomonas*spp.	+	+	++	++	++	+++	+++	+++	+++	+++

\+ Less Turbidity; ++ Moderately Turbidity; +++ High turbidity;

** tested by broth dilution technique

Table 14: Arsenic tolerancy of isolates determined by agar dilution technique

Bacterial Isolates *	**Growth on As(10 ppm)**	**Growth on As(50 ppm)**	**Growth on As(100 ppm)**
Unidentified Gram positive rod	+	+	++
Micrococcus spp.	+	++	++
Escherichia coli	+	++	++
Yeast	+	++	++
Streptococcus faecalis	++	++	++
Bacillus spp.	+	+	++
Staphyloccocus spp.	+	+	++
Pseudomonas spp.	+	++	++

* The organism selected for the tests were those shown tolerance at 1000 ppb (1ppm)

CHAPTER-VI

DISCUSSION AND CONCLUSION

6.1 DISCUSSION

Arsenic is widely distributed throughout the earth crust and it is introduced into water through the dissolution of mineral ores rich in arsenic. Elevated concentration of arsenic has been recorded from sulphide ores, subsurface ground water and hot spring. Arsenic is the oldest poison known to mankind. It has been specified as carcinogene by the International Agency for Research on Cancer (IARC).

Arsenic in ground water is now recognized and documented as a major health problem of drinking water. The impact on human health is very adverse. The most commonly reported symptoms of chronic arsenic exposure are hyper pigmentation, depigmentatrion, keratosis, peripheral vascular disorders skin cancer and numbers of internal cancer.

Drinking Water Quality Program, being the first large-scale program in Nepal on arsenic issue is also the first to provide mitigation option to arsenic affected villages. In the process of program implementation, NRCS, ENPHO, local health workers and local organizations have trained local people in conducting various mitigation activities. Various kinds of alternative options for mitigation plan like arsenic safe tube wells, rehabilitation of dug wells, household arsenic removal filters like two gagri filters, three gagri filters and improved bio-sand filters and community level arsenic iron removal plants has been provided to users (ENPHO, 2003).

The various studies have revealed the arsenic present in ground water is also due to microbial population present in the water, which can metabolize arsenic species. Arsenic cycling occurs in the region of the chemocline. Arsenate reduction is mediated by DARPs (Dissimilatory Arsenate Respiring Prokaryotes) that use released

organic matter from dying plankton to fuel their respiration. Arsenate oxidation (aerobic and anaerobic) is mediated by CAOs that also contribute to secondary production by "fixing" CO_2 into organic matters. Arsenic first enters this alkaline (pH=9.9), saline lake (~90g/litre) as a dissolved component contained in the discharge from hydrothermal springs. Arsenic, as well as other dissolved constituents, reaches high concentration because of the predominance of evaporation over precipitation in this arid region (Frankenberger, 2001).

The presence of arsenic poisoning in ground water of Terai was initially recognized in 1999 by Department of Water Supply and Sewerage (DWSS). There exists a significant contamination (>50 ppb) in shallow wells of almost all districts of entire Terai zone. As of June 2003 about 25000 wells have been tested for arsenic by several agencies (DWSS, NRCS, ENPHO, NEWAH, RWSS, FDB). Nearly 23% of the well exceeded the WHO guideline value (10ppb) and 8% exceeded the Interim Nepal Standard (50 ppb).

The most severely affected districts are Nawalparasi, Rautahat, Sarlahi, Saptari, Bara and Parsa. An estimate of the number of people in the Terai who may be using ground water containing 'high' arsenic concentration is about 3.2 million (29%) of Terai population. In our samples, about 80% have exceeded the WHO guideline value of arsenic (10 ppb) and 60% of samples have exceeded Nepal Interim guideline value (50 ppb). It was found to be very high compare to study done by other several agencies in June 2003 (DWSS, NRCS, ENPHO, NEWAH, RWSS).

Out of four thousand tube well in Ramgram Municipality (Kunwar Village) 62% are above contamination level in WHO standard where 43% are above Nepal Interim guideline value. There was vast difference in arsenic concentration in samples. It could be due to various factors like age of tube well, depth of tube well and subsurface soil sediment composition or it could be due to load of organisms too. And seasonal variation may increase or decrease arsenic concentration as July rainy season the bacterial load in the subsurface aquifer may have been flushed out.

Results of Dr. Smith Linda, USGS, Colorado, showed that arsenic concentration tested of sand crust sand 5-10 ppm and less than 5 ppm respectively. Similarly she had found arsenic concentration from 196 ppb to 1342 ppb in Kunwar village. It shows that Kunwar village is highly under threat of arsenic poisoning.

Arsenic concentration is high in certain areas but it varies greatly in wells even in close proximity. The concentration of arsenic also varies according to depth. Present study has shown that some positive correlation between arsenic concentration and age of tube well i.e. arsenic concentration increases as age of tube increases; and arsenic concentration decrease as the depth of tube well increases. 60 % of our sample showed this correlation.

In the present study, coliform bacteria in the samples were enumerated by membrane filter (MF) techniques (Baron, 1994; Manja, 2002).

All the samples were highly contaminated. Total coliform count was maximum and *E. coli* were detected in many samples. The result indicated that the water from tube wells (Nawalparasi) were contaminated by faecal coliform may be due to sewage leaking problem. Coliform presence in the sample indicated that water sample was heavily contaminated. Consumption of such water is very dangerous to health as it contains pathogenic bacteria too, which may cause disease like typhoid, paratyphoid, dysentery, cholera and hepatitis. *E. coli* presence in sample indicates faecal pollution of water. So it makes water unpotable.

Thus the ground water source must be protected and properly sealed and likely source of contamination should be prevented. Otherwise tube well water must be avoided as drinking water source and alternative source of safe drinking water must be provided by government to the villagers wells must be protected from any faecal contamination.

Heterotrophic plate count result of the sample showed the very high load of microorganism in the samples.

In the present study Arsenic tolerant microorganisms were isolated and identified. Mostly they were pigment producing bacteria. Typical colonies were picked up for morphological direct microscopic examination. Gram's staining of picked up colonies revealed 40% Gram positive cocci, 25% Gram positive rods, 25% Gram negative rods, 5% Gram negative cocci and 5% yeast.

The result based on Gram staining indicates that diverse group of organism present in high arsenic aquifer. The arsenic tolerant isolates were presumptively identified as *Microccocus* (Skw2 As conc. 350 ppb), *E. coli* (Tkw3 As 400 ppb), yeast ($TKW_1$6 As conc. 620 ppb), *Streptococcus faecalis* (TKW6 As conc. 620 ppb), *Bacillus s*pp. ($TKW_1$9 As conc. 0 ppb), *Staphyloccocus* spp. (TKW9 As conc. 0 ppb), *Pseudomonas* spp. (TKW10 As conc. 10 ppb). The study has indicated that there is no direct link between types of organism and arsenic concentration of water and also the load of organism is not responsible for high concentration of organism (Macy *et al*, 2000).

Tolerance to arsenate was tested in order to explore the potentialities of strain for use in bioremediation process and to investigate its survival in a heavily polluted environment. The organisms were found to tolerate arsenic concentration from 100 ppb to 1000 ppb (1ppm). Thus agar dilution technique is used to confirm their tolerancy level to high arsenic concentration.

Hence, tests were performed on agar plates with arsenic concentration of 10 ppm, 50 ppm and 100 ppm. The growth of isolate on plate was observed even at 100 ppm of arsenic concentration. This could be due to their special character to metabolize the arsenic. Such organism may possess plasmids which have plasmid encoded genes for arsenic resistance. Isolated strains may have enzymes like arsenate reductase enzyme or arsenite oxidase, which was making them able to grow on such high arsenic concentration.

In the present study, another sample Tkw9 we have isolated another important bacteria *Bacillus* spp. which was reported to involve in arsenic cycling (Manja, 2002). Similar to the present study Oremland *et al*, 2002 have reported the isolation of arsenic tolerant bacteria. However, in the present study no correlation has been found between arsenic concentration and load/types of microorganisms. More research needs to be done in this aspect.

The most well studied mechanism of detoxification and resistance to Arsenic is the ArsC system (Mukahopadhyay *et al*, 2002). Different but structurally related arsenate reductases have been studied in bacteria and yeast. ArsC, a small molecular mass protein (13-16 KD) mediates reduction of As(V) to As(III) in cytoplasm. The studies revealed As(III) more toxic form which could be excreted bian As(III) specific transporte, ArsB. The Ars operon in *E. coli* has been reported to have both plasmid and chromosomal loci. The four genes-arsA, arsB, arsC, arsD and arsR of plasmid R733 were reported where as arsB, arsC and arsR were of chromosomal locus. Similarly, Ars operon in p1258 of *Staphylococcus aureus* also reported to have arsB, arsC, and arsD (Oremland and Stolz, 2003).

Several different mechanisms have evolved to rid cells of arsenic. These include methyl action, and expulsion involving an As(III) specific transporter. In higher eukaryotes, glutathione reduces As(V) to As(III), which then accepts a methyl group from *S*-adenosylmethionine, producing monomethylarsonic acid (MMA) or dimetylarsonic acid (DMA). Fungi producetrimethylarsine, where as bacteria may produce MMA amd DMA. Such diverse microbes as anaerobic methanogenic Archaea and aerobic Eubacteria can also form methylated arsines (NRC, 1999).

6.2 CONCLUSION

In the present study, all the water samples were highly contaminated by coliform bacteria which indicated sewage contamination. Consumption of such water is very dangerous to health. In the present study many arsenic tolerant pigmented bacteria were isolated and identified.

Based on biochemical results, the isolates were *Microccocus* (Skw2 As conc. 350 ppb), *E. coli* (Tkw3 400 ppb), yeast ($TKW_1$6 As conc. 620ppb), *Streptococcus faecalis* (TKW6 As conc. 620 ppb), *Bacillus* spp. ($TKW_1$9 As conc. 0 ppb), *Staphyloccocus* spp (TKW9 As conc. 0 ppb) and *Pseudomonas* spp. (TKW10 As conc. 10 ppb).

The isolates were found to tolerate even 100 ppm of arsenic concentration. The isolated microbes from ground water samples of Nawalparasi were similar to those reported by various scientists abroad (Oremland and Stolz, 2003). Finally the study suggests that arsenic resistance bacteria could have vital role in biogeochemical cycling in subsurface ground water.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

On the basis of obtained analytical data and field observation it is evident that most of the ground water sources in the Kunwar Village is contaminated from arsenic and coliform bacteria. Arsenic is widely distributed throughout the earth crust and it is introduced into water through the dissolution of mineral ores rich in arsenic. A total of 43 samples of ground water from tube wells were randomly collected from Kunwar villages. The result showed that the groundwater was totally unfit for drinking as the coliform and arsenic contamination has been detected. About 80% of the sample exceeded the WHO guideline value of arsenic (10 ppb) and 60% of samples exceeded Nepal Interim guideline value (50 ppb).

1. Present study has shown correlation between arsenic concentration and age of tube well i.e. arsenic concentration increases as age of tube increases; and arsenic concentration decrease as the depth of tube well increases. 60 % of our sample showed this correlation.

2. All the samples were heavily contaminated with coliform bacteria and *E. coli*. The sample Tkw1 had the maximum *E. coli* (40) and Tkw6 has high total coliform count ($8.9x10^9$).

3. Morphological study showed various types of Arsenic tolerant bacteria were present in the samples. Most of them were pigment producing microorganism viz; 40% were Gram positive cocci, 25% Gram positive rods, 25% Gram negative rods and 5% Gram negative cocci

4. In the present study Arsenic tolerant isolates were identified as *Micrococcus*, *E.coli*, yeast, *Streptococcus faecalis*, *Bacillus.* spp, *Staphyloccocus.* Spp. and *Pseudomonas* spp. The isolates were found to tolerate very high concentration of arsenic i.e.100 ppm.

5. As the organisms were found to grow more vigorously on higher concentration of arsenic, a hypothesis could be formulated that arsenic tolerant bacteria were able to utilize arsenic as nutrient.

7.2 RECOMMENDATION

Ground water pollution is one of major factor that imposes negative impact on public health. Preventive measures to control the occurrence of unpleasurable incidences like outbreak of water borne epidemics in the Terai region of Nepal are urgently needed. Long term strategies and policies should be formulated and implemented in this regard. Strong legislation is basically needed for water pollution control mechanism. On the basis of this study some recommendations are made

1. The present study has shown high rate of coliform bacteria and *E. coli* present in ground water sample it should be immediately reported to District Health Organization, so that water treatment plant must be set up for biologically safe drinking water supply to the villagers or use of such contaminated well must be strictly avoided.

2. It is very essential that the people using ground water for drinking should be made aware about the consequences they have to bear by the consumption of such water and hence look for the ways to protect their water from contamination. Therefore, in-house water disinfection program by chlorination should be promoted.

1. Molecular mechanism of arsenic tolerancy in the isolates should be investigated further. 16s RRNA gene sequencing studies should be done to type the Arsenic tolerant isolates.
2. Studies on ability of bacteria to convert As(III) (toxic) into As(V) should be done by Atomic Absorption Spectrometric Method.
5. Further research on arsenic metabolizing bacteria with respect to the mobilization of arsenic from the solid phase into aqueous phase in a subsurface drinking water aquifer is recommended.
6. Further research on isolation of arsenite oxidizing bacteria is recommended so that they could be used as bioremedy to solve arsenic problem.

CHAPTER-VIII

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