PREVALENCE OF MICROORGANISMS IN INFLUENT AND EFFLUENT OF MEMBRANE BIOREACTOR (MBR)

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This thesis is dedicated to my Parents who have meant and continue to mean so much to me, who have been so close to me that I found them with me whenever I needed

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Reenum Anwar

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LIST OF A BBREVIATIONS

EPA	Environmental Protection Agency
EC	Electrical Conductivity
DO	Dissolved Oxygen
TSS	Total Suspended Solids
TDS	Total Dissolved Solids
TP	Total Phosphates
COD	Chemical Oxygen Demand
rRNA	Ribosomal Ribonucleic Acid
EMB	Eosin Methylene Blue
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
WASA	Water and Sanitation Agency
WHO	World Health Organization
APHA	American Public Health Association
CFU	Colony Forming Unit

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ABSTRACT

The population growth and rapid urbanization, as well as climate change and the indiscriminate use of freshwater has lead to the era of water resources dwindling and water scarcity problem worldwide. Therefore, most researchers have shown great interest in treatment, recycling and reuse of wastewater particularly in agricultural activities. In general, wastewater contains beneficial features such as nutrients that is required for plants growth and development. The present study was conducted at NUST H-12 to evaluate performance efficiency of membrane bioreactor by cultivating green vegetables. Selected vegetables were Lettuce and Spinach. The studied water quality parameters were; Chemical oxygen demand (COD), Total suspended solids (TSS), Total dissolved solids (TDS), Electric conductivity (EC), pH, Total phosphates (TP), Dissolved oxygen (DO), Temperature, Total coliforms and Fecal coliforms. The system proposed was found to have produced satisfactory results where the average removal efficiencies of COD, TSS, and TP were 87, 74.3 and 75% respectively. HPC in both lettuce and spinach samples were exceeding the quality standard of 10^3 CFU/g provided by International Commission on Microbiological Specifications for Food (ICMSF) and 2.3 log CFU/g by WHO. *Salmonella* count in lettuce and spinach grown in wastewater was 6.86 and 5.88 log CFU/g whereas the MBR treated wastewater vegetables had *Salmonella* count 2.20 and 2.18 log CFU/g. Predominant species of parasite were *A. lumbricoides*, *T.trichiura* and *Hookworm. A. lumbricoides* was the most dominant specie. *Acinetobacter johnsonii*, *Pseudomonas monteilii*, *Pseudomonas putida*, *Raoultella ornithinolytica*, *Aeromonas hydrophilla* and *Aeromonas veronii* were the predominant genera of bacteria in wastewater and MBR treated wastewater. Principal species identified in spinach and lettuce were *Escherichia coli*, *Shigella flexneri* and *Salmonella enterica.*

CHAPTER 1

1. INTRODUCTION

Water is very essential for life and although it covers approximately 70% of the terrestrial crust area, only a small portion of the water is actually attuned with terrestrial life forms (Shiklomanov, 1993). Stress on water resources increases because of economic development, population growth and due to change in climatic conditions. It is expected that in some countries of the world water demand increases twice than human population. (FAO, 2013).

The global water withdrawal suffered a 6.3 fold increase, rising from less than 600 km^3/year, at the beginning of the 20^{th} century, to more than 3800 km^3/year by the start of the 25^{th} century. 70% of this withdrawal is due to irrigation practices (FAO, 2013). Therefore, the utilization of freshwater has been surpassing the minimum recharge levels, resulting to the depletion of groundwater and desiccation of water streams. It is projected that in next 60 years more than 50 percent population of the world will face water scarcity (WHO, 2006). In this context, to reduce the water stress/ scarcity wastewater reuse is a possible option (Niemczynowicz, 1999; WHO, 2006). Besides reducing the use and abstraction of freshwater, reuse of wastewater will also have great contribution in minimizing the effluent discharge into freshwater ecosystems (Bixio *et al*., 2006). So if we consider wastewater as a resource rather than a waste product then this help us in reducing water stress over fresh water resources.

Wastewater is the product of the various human activities such as domestic, industrial and commercial activities. The composition of wastewater is determined by various factors, which includes the lifestyle of resident's and living standards, the amount of domestic and commercial effluents, or even the construction design of the sewer system (Henze *et al*.,

2008). Discharge of untreated wastewater result in several basic health and environmental risks, therefore to minimize the impacts, wastewater treatment technologies shall be established (Tchobanoglous *et al*., 2003). Due to complexity of sewerage network, cluster of houses, the centralized wastewater treatment option is very expensive and is not feasible in large populated areas due to complexity of sewerage network (Metcalf *et al*., 2003). In this regard, it is better to adopt on-site wastewater treatment options based on the environment, situation and locality (Metcalf *et al*., 2003).

Pakistan is losing 4% of its economy due to bad water supplies and sanitation. Only 72 % and 34% population in urban and rural areas have proper access to water and sanitation respectively. There is a need for an on-site domestic wastewater treatment solutions that treats wastewater to the Pakistan Environmental Protection Agency (PEPA) and National Environmental Quality Standards (NEQS).

Treated urban wastewater is primarily composed of dissolved organic matter, particulate and inorganic substances like N, P, K, Na, Ca, Mg, Cl and B, also containing microorganisms, including viruses, pathogens and antibiotic resistant bacteria (Varela *et al*., 2013). Additionally, recalcitrant, toxic or bio accumulative chemicals (e.g., trace metals, natural or semi-synthetic compounds and xenobiotics) are usually present, although demonstrating minor components, often designated as micro pollutants or micro contaminants (Henze *et al*., 2008; Metcalf *et al*., 2003). Given such complexity, the comprehensive chemical and biological classification of treated wastewater is necessary to evaluate its quality, without detailed characterization of wastewater it is very difficult to the estimate the negative effects that arise from its reuse. Wastewater reuse recommendations established by the State of California, the US Environmental Protection Agency (EPA) and the World Health Organisation (WHO) (Aquarec, 2006; WHO, 2006) have the background of the permissible guidelines recommended in countries such as Portugal, USA, Spain, Cyprus, Italy, Israel, Australia, Jordan, France, China, Kuwait,

Oman, Saudi Arabia and Germany. Detailed analysis of the treated wastewater before its reuse is required to compliance with these recommended guidelines. Although revised policies and guidelines cover different uses of wastewater (e.g. aquifer recharge, industrial reuse, irrigation and impoundments) the main focus of our discussion is on the reuse of wastewater for agricultural irrigation. In general, standards are based on the evaluation of microbiological and physicochemical parameters.

Several countries like USA, Jordan and Spain establish different guidelines for the wastewater reuse for irrigation of crops. The recommended values are for raw-consumed crops are different from other crops which are used after further processing (processed food) or used as fodder or energy crops. Physicochemical classification of wastewater includes the assessment of several properties such as suspended solids (SS), turbidity (Nephelometric Turbidity Units (NTU), acidity (pH), sodium absorption rate (SAR), salinity, electrical conductivity (EC), chemical oxygen demand (COD), organic load, biological oxygen demand (BOD) and nutrients. Besides these parameters, some regulations are also recommend by USA, Mexico, Italy, and Oman for the determination of potentially toxic agents such as organic contaminants and metals. The microbiological categorization of wastewater is primarily focused on the presence of parasitic agents and potential pathogens, and is usually based on the enumeration of nematode eggs and faecal indicators. The reuse of wastewater not only cause disturbance in physiochemical properties of soil through the presence of some toxic compounds and pathogens but also has potential effect on soil fertility and productivity some guidelines also aim at preventing these potential effects (Aquarec, 2006; EPA, 2012; WHO, 2006).

Now a days attention has been given to the different wastewater treatment technologies to overcome the emerging challenges, as the sustainable reuse of wastewater for irrigation or the removal of different types of contaminants. In particular pesticides, pharmaceutical products and disinfectants are foresighted in most of the discussions

around wastewater treatment and quality (Michael *et al*., 2013; Pal *et al*., 2014; Rivera-Utrilla *et al*., 2013). A recent literature review by Norton-Brandão *et al*. (2013) offers a detailed overview of wastewater treatment technologies commonly used for the treatment of wastewater reused for irrigation purposes, making a comparison based on the parameters like salinity, nutrients, pathogens and heavy metals. The use of treatment technologies like filtration, sedimentation or disinfection processes such as UV, chlorine dioxide, ozone are somehow suitable for wastewater treatment used for irrigation, depending upon the quality of raw wastewater and application demands (Norton-Brandão *et al*., 2013). **Membrane bioreactor (MBR) systems** are very efficient in removing microorganisms and also high removal rate of heavy metals are also achieved by using MBR. Other processes such as ponds, phytoremediation plants, disinfection oxidants may also achieve good removal rates of microorganisms, but their removal rates are not so efficient to achieve the adequate levels of other parameters, in particular salinity. The choice of suitable methods for the wastewater treatment should be cost effective and also produces the effluent of high quality which can be easily used for irrigation (Norton-Brandão *et al*., 2013).

In this respect, now a days, binding quality criteria should include also the absence of emerging contaminants as pharmaceuticals or antibiotic resistant bacteria. Since the implementation and maintenance costs and the environmental impacts cannot be ignored, sometimes it may be challenging to achieve an ideal compromise.

1.2 THE PRESENT STUDY

In the present study, water samples were collected from the Membrane Bioreactor installed at National University of Sciences and Technology (NUST) and analyzed for changes in the physicochemical and microbiological parameters as a result of treatment. Plants were also grown using three types of water i.e. wastewater, treated and tap water.

Membrane filtration and Heterotrophic plate count (HPC) were performed to evaluate bacterial growth. Predominant species were isolated and identified.

1.3 OBJECTIVES OF STUDY

The research had the following objectives:

a) Identification and isolation of parasites (helminths & nematodes) and potential pathogens.

b) Comparison of influent and effluent of membrane bioreactor by cultivating the green vegetables (lettuce & spinach).

CHAPTER 2

2. LITERATURE REVIEW

2.1 WATER AVAILABILITY

85% of the world population lives in the driest part of the planet. 783 million individuals lack access to clean water and about 2.5 billion lack access to proper sanitation (Anwar *et al*., 2010). Global increase in population growth over the next 40 years, is projected to be 2–3 billion which results in increasing food demand of almost 70% by 2050. Half of the world population is currently living in urban areas and their numbers are increasing day by day. Main reason behind this high migration rate toward urban areas is because urban areas have better living facilities as compared to rural areas (EPA, 2012). By 2030, food demand is expected to increase by 50 percent due to expected increase in population which is 70% by 2050 (Bruinsma, 2009), while there is 60% rise in energy demand from renewable energy resources .

These issues are interrelated as increasing pressure on agricultural sector, will significantly increase both energy and water consumption, which leads to increased stress on water resources and also competition for water between water-using sectors also increased. Water availability is estimated to decline in many regions. Future global agriculture consumption only is expected to rise 19 percent by 2050, and in the absence of any technological improvements or policy intervention it will become even greater (World Bank 2002).

Fresh water used for the irrigation and for the production of food is one of the greatest cause of pressures on freshwater resources. Agriculture alone is responsible for 70 percent of global freshwater withdrawals (90% in case of fast-growing economies) (Anderson, 2010).

Economic growth and individual wealth are shifting diets from predominantly starch-based diets to meat and dairy, which needs more water. For the production of 1 kilogram of rice, 3,500 L of water is required approximately, 15,000 L for 1 kilogram of beef, and 140 litres for a cup of coffee (Toze, 2006). Over the past 30 years this dietary shift has great impact on water consumption, and is expected to increase till the middle of the 20th century (FAO, 2004). About 65% of Africa is arid or semi-arid and in sub-Saharan Africa more than 300 of the 800 million people live in extreme water scarcity means that they have less than 1,000 m^3 per capita (FAPRI, 2005).

Water stress will rise in central and southern Europe, by 2070 as predicted by IPCC, this will ultimately increase the number of people affected by water stress from 28 million to almost 44 million. Summer flows are likely to drop in southern Europe and in some parts of Eastern and central Europe by almost 80%. Mediterranean hydropower potential is expected rise by 20–50% around 2070 but in Europe's it is expected to drop by an average of 6% (Alcamo *et al*., 2007).

2.2 WATER AVAILABILITY IN PAKISTAN

Due to continuous increase in population and depletion of fresh water resources, Pakistan is directed towards a situation of water scarcity and famine threat (Fig 2.1). Per capita water available for irrigation in 1951 was 5260 m^3 per year. Water availability has reduced to 1100 m^3 per capita in 2006. 1,000 m^3 per capita per year is the minimum amount of water requirement for Pakistan to avoid being a "water scarce country" (UNIDO, 2000).

In 2012 Pakistan have reached the phase of "acute water shortage", where individual fight for single drop of water. There is a need to create fresh storages, by building more dams to overcome the lost capacity and save Pakistan's agricultural

economy from total disaster, and to produce more food grains for rapidly increasing population (Ashfaq *et al*., 2010).

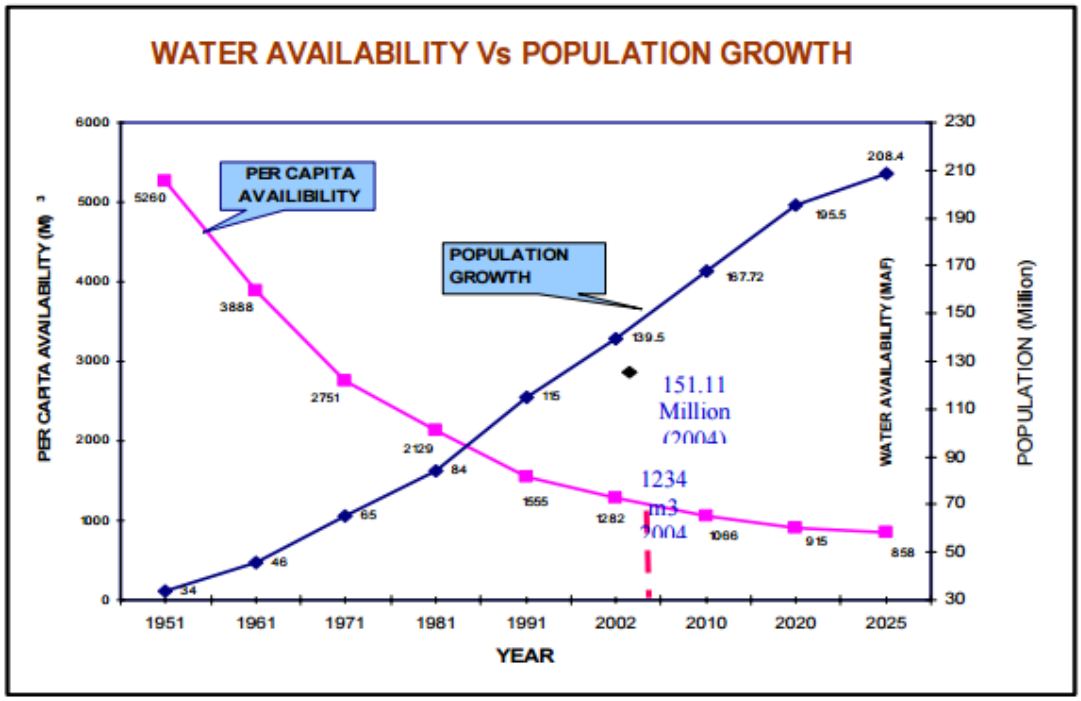


Fig. 2.1 Water Availability and Population Growth

According to report of Water Sector Investment Planning, Pakistan will face a shortage of 12 million tons in annual crop production in the year of 2012-2013 which is only 32% of estimated target. Such a large scale loss cannot be compensate only by improving farming practices and technology. The irrigation supplies scenario, in the year 2012-2013, was very disastrous, and it will become more disastrous year-by-year thereafter (Baig *et al*., 2011).

Total wastewater discharge calculated on the basis of 1998 population census, for 14 major cities of Pakistan, is around 1.93×10^7 m^3 h^{-1} (FAO, 2002). According to most recent estimation (PWSS, 2002) that average amount of wastewater production in Pakistan is 962,345 million gallons which includes 288,326 million gallons from industrial activities and 674,009 million gallons from municipality. Tanneries, petrochemicals, food processing, sugar industries, refineries and textile are responsible for most of the water pollution in Pakistan. According to some estimations around 2,500 million gallons of

wastewater is being released to nearby water bodies each day (UNIDO, 2000). Paper, textile, cement, fertilizer, sugar and polyester industries are responsible for the production of 80% of the total industrial effluents (Pak-SCEA 2006).

Table 2.1 Sector Wise Estimation of Wastewater Production in Pakistan

Sr. No.	Source	Volume	
		$10m^3y^{-1}$	**Percent %**
1	**Industry**	395	6
2	**Agriculture**	1,036	16
3	**Urban residential**	1.628	25
4	**Rural residential**	3.059	48
5	**Commercial**	266	8
	Total	6,414	100

Source: (Pak-SCEA 2006)

2.2.1 Wastewater Treatment

Domestic wastewater in Pakistan is mainly composed of effluent from households and human waste either directly discharged to a sewerage systems, in a drain or nullah, water bodies or in a nearby sport fields or an internal septic tank. Typically, domestic wastewater is not exposed to any treatment process and except Islamabad and Karachi no other city have any biological treatment plant, and even in these cities only a small proportion of their wastewater is being treated before disposal. Despite that all the installed treatment plants are working at their full installed capacity, it is estimated that only 8% of urban wastewater is probably treated in municipal treatment plants. The treated wastewater then flows into open drains, and there are no provisions for reuse of the treated wastewater for agriculture activities or other municipal uses (WAPDA, 2005).

Only a small fraction i.e. 8% of wastewater in Pakistan is treated through water treatment process to a basic level only. Most of the treatment plants in Pakistan are not

even functional. In Pakistan wastewater is only treated to primary level, secondary and tertiary treatment plants of wastewater are not available in Pakistan. In Pakistan wastewater treatment plants are present only in major cities i.e. Lahore, Islamabad and Karachi in some cases these have been built without the completion of associated sewerage networks, and the plants are often either under-loaded or abandoned (Pak-SCEA, 2006).

2.2.2 Wastewater Use/Disposal

Urban centres are responsible for the major cause of water pollution in Pakistan. Typically, water drains and nullahs accumulate and transport wastewater which then flows into irrigation canals, streams and rivers. Some sewage collection systems, normally discharge wastewater to the closest water body, although there are collection levels estimated to be greater than 50% nationally and less than 20% are in rural areas. According to some estimates, the untreated wastewater is directly applied to crops for irrigation of approximately 32,500 ha in Pakistan (Ensink *et al*., 2004). Expected total amount of sewage water i.e. 0.146×109 m^3/yr is directly dumped into water bodies, mainly in canals and the amount of wastewater directly consumed for agricultural irrigation is 0.876×109 m^3/yr. Vegetables, rice, cotton and fodder are the crops which are usually irrigated with wastewater. Vegetables are irrigated with wastewater after three weeks, cotton after one week and fodder after three weeks. In Faisalabad the amount of Nitrogen, Potassium and Phosphorous applied through wastewater irrigations in area of 0.40 m ranged from 117-195, 108-249 and 7-21 kg ha^{-1}, respectively. The magnitude of Nitrogen and Potassium are quite appropriate for any crop while that of Phosphorous is considerably low and would need to be enhanced. Since Phosphorous present in sewage is 100 % soluble, so it become easily available to the crops and its availability is usually greater than Phosphorous which is applied through synthetic manures. Some researchers found out that sewage contains very high amount of nutrients so in some cases Nitrogen and Phosphorous contents exceeded the recommended quantity actually needed for the growth of crops (Ensink *et al*.,

2002). Excess amount of nutrients in irrigated water may sometime become problematic if it don't coincides with plant needs (Murtaza *et al*., 2010). Sometimes overdose of Nitrogen may result in over-fertilization and may also cause enhance weed growth, unnecessary growth, increase lodging and thus result in reduction of crop yield (Asano *et al*., 1987). Moreover, due to the presence of excess nutrients lead to eutrophication in local water bodies and thus deteriorates the quality of water and aquatic life. Excess Nitrogen in many crops (potatoes, tomatoes, citrus and grapes) damage the quantity and yield of crops (Bouwer *et al*., 1987). Because of high nutrient contents of wastewater it is preferred by farmers over water from other supplies. Reuse of wastewater has numerous positive socio-economic impacts for the users. Land irrigation with partially treated wastewater is considered as an economical method for the disposal of wastewater for a very long time (Salgota *et al*., 2006). In Haroon Abad (Pakistan), the area flooded with sewage water has a higher value as compared to the land flooded with canal water, and the rents of land irrigated with wastewater were on average four and a half times more than those which are irrigated with canal water (Hussain *et al*., 2001). Following are some socio-economic impacts of wastewater irrigation (1) potential yield losses, (2) depreciation in market value of land, (3) loss of soil productive capacity and (4) additional cost of using nutrients and soil reclamation measures. After a research presented by Ensink *et al*. (2004), the focus of many investigators of the nation has shifted on this area which was totally ignored in the past. Further investigation on this aspect of wastewater reuse inspire farmers to minimize the use of synthetic fertilizers even they are well aware about some negative impacts of sewage water irrigation on physio-chemical characteristics of soil moreover to contamination in food chain and health associated risks.

2.3 WASTEWATER REUSE IN IRRIGATION

Untreated and treated wastewater is widely used for agricultural irrigation because it provides all the nutrients necessary for crop growth and also provides adequate moisture

required for plant growth. Crops irrigated with wastewater produce high yields and thus reduce the need of chemical fertilizers, which is economically beneficial for the farmers. Wastewater is valuable resource but the reuse of wastewater also have some negative effects. Due to high nutrient contents in wastewater we can use it as a natural fertilizer for crops. Wastewater reuse have many positive impacts on the communities and municipalities and also is a very accessible to farmers as compared to canal or river water. However, there is a need to identify and evaluate the negative impacts of wastewater reuse on humans and ecosystem (Cristina *et al*., 2015).

2.4 MICROBIOLOGICAL GUIDELINES FOR WASTEWATER REUSE

Pathogenic microorganisms such as bacteria, fecal coliforms, viruses and helminths eggs are present in wastewater. Sufficient quantity of pathogenic microorganisms in wastewater is responsible for many diseases in humans because they are parasites and human body act as a source of host for them. High health risk is associated with nematodes because of their parasitic nature as compared to bacteria which poses a very low risk for infections (Ramzan *et al*., 2013). Viruses exhibit the lowest risk. WHO develop guidelines for irrigation that inhibit the transmission of infectious diseases (Bixio *et al*., 2006).

Restricted irrigation: The criteria for restricted irrigation is that less than one intestinal nematode egg should be present in a litre of wastewater. Restricted irrigation is meant for those crops which are in Category A in this category those crops are irrigated with wastewater which are not directly consumed by humans (cotton and sunflower) and also recommended for the irrigation of processed crops like wheat, oats and barley and Category B includes fruit trees, pastures and fodder crops.

Unrestricted irrigation: In unrestricted irrigation not more than one nematode egg/L plus not more than 1000 Cfu/100 ml of bacteria should be present in irrigated wastewater. Unrestricted irrigation is meant for those crops which are in Category C they are directly

consumed by humans or eaten in raw forms like raw vegetables (spinach, lettuce, cucumber etc.) and can also be used for the irrigation of sport grounds and public parks (Asano and Pettygrove, 1987).

Table 2.2 Recommended Microbiological Quality Guidelines for Wastewater use in Agriculture

Category	Reuse Conditions	Exposed Groups	Intestinal Nematodes (Arithmetic mean no. of eggs per litre)	Faecal Coliforms (geometric mean no. per 100ml)
A	Irrigation of crops likely to be eaten uncooked, sport fields and public parks	Workers, consumers, public	≤ 1	≤ 1000
B	Irrigation of cereal crops, industrial crops, fodder crops, pastures and tress	Workers	≤ 1	No standard recommended
C	Localized irrigation of crops in category B if exposure of workers and public doesn't occur	None	Not applicable	Not applicable

Source: (Asano and Pettygrove, 1987)

2.5 CHEMICAL GUIDELINES FOR WASTEWATER REUSE

Most of the guiding principles deals with microbiological quality of wastewater used for irrigational purpose, the reason behind dominance of microbiological aspect is that microbiological aspects is that they have immediate effect on human health. Chang *et al* (1996), found that, there are no proper chemical guidelines for wastewater reuse and only few quality standards were established for wastewater reuse and they usually deal with the microbiological aspect of wastewater irrigation. Mostly the manuals and guidelines e.g. US.EPA, 1992) only deals with wastewater reuse for irrigation purposes do not address the health and safety problems associated with the introduction of poisonous chemical pollutants into the ecological system through wastewater irrigation (Murtaza *et al*., 2008).

2.6 WASTEWATER COLLECTION AND TREATMENT CONCEPTS

Wastewater treatment system approaches maybe categorized into two concepts. The first one is centralized wastewater treatment, in which the entire area is connected with each other and the collection system is centralised and wastewater through this network reaches a nearby treatment plant if available and gets treated. The second approach is called decentralized or cluster approach. In which the collection system is not centralized and if the treatment is to be made it could only be possible to install on-site wastewater treatment solution to get treatment. Typically centralized wastewater collection system is quite costly and require larger size pipes and big infrastructure. Whereas, the decentralized system treats wastewater from individual households or cluster of houses (Tchobanoglous *et al,* 2004). Centralized systems for collection and treatment involves larger volumes of wastewater, thus this network due to its concept, is very costly and may be applicable to larger cities with developed economies. The construction of centralized treatment is not recommended for cities or countries having low income due to its cost (Asano *et al*., 1987).

Decentralized systems are recommended and suitable for countries with low income since it is very economical and affordable than centralized systems. Decentralized systems consists of modified septic tanks with baffles, conventional septic tanks or any other on-site treatment system for houses or cluster of houses. This system require regular check-up for operation and maintenance. The decentralized systems are becoming more popular with time due to their low cost and sustainability (Tchobanoglous *et al*., 2003).

2.7 WASTEWATER TREATMENT TECHNOLOGIES

Treatment technologies for recycling of wastewater includes large number of options. Membrane treatment technologies are considered as vital elements of advanced wastewater

reuse and reclamation schemes and are a part of number of water treatment schemes worldwide e.g. for artificial recharge of groundwater, for industrial processes as well as for indirect potable reuse. Membrane bioreactors (MBRs) are a combination of membrane process like ultrafiltration or microfiltration with activated sludge treatment (Melina *et al*., 2010).

2.7.1 Membrane Bioreactors

Membrane bioreactors (MBR) is a combination of activated sludge treatment with a membrane which separates liquid from solid. The membrane used in MBR are micro-filtration or ultra-filtration membranes which eradicates the need of further tertiary filtration process. The membranes are usually submerged in the aeration tank however in some cases a separate membrane tank is used for this purpose). One of the main benefits of a MBR system over conventional activated system (CAS) processes is that it efficiently overcomes the issues associated with poor settling of sludge. The technology permits bioreactor operation with considerably higher mixed-liquor suspended solids (MLSS) concentration than CAS systems, which are limited by sludge settling (Judd, 2006). The MBR system is usually operated at CAS in the range of 2,000–3,000 mg/L and MLSS is in the range of 8,000–12,000 mg/L. The high concentration of biomass in the MBR process allows effective removal of both insoluble, particulate and soluble biodegradable materials at higher loading rates. Thus increased Sludge Retention Times (SRTs) usually exceeding 15 days ensure complete nitrification, even in extremely cold weather. In MBR systems there is the option of autonomous selection of hydraulic and sludge retention time (HRT and SRT), which allows a more firm control over all operational parameters. Efficient treatment of wastewater in MBR is because of high sludge concentrations in the bioreactor. The retention of activated sludge containing solids and macromolecules in combination with long sludge age prolongs the time of contact between sludge and critical classes of substrates. This will help in the production of specific, microorganisms having slow

growth which are responsible for the removal of biodegradable pollutants present in wastewater.

Table 2.3 MBR Removal Efficiency and Effluent Quality

Parameters	Units	Removal Efficiency (%)	Effluent Quality
TSS	mg/L	>99	<2
Turbidity	NTU	98.8-100	<1
COD	mg/L	89-98	10-30
BOD	mg/L	>97	<5
NH_3-N	mg/L	80-90	<5.6
N_{TOT}	mg/L	36-80	<27
P_{TOT}	mg/L	62-97	0.3-2.8
Total Coliforms	CFU/mL	5-8 log	<100
Faecal Coliforms	CFU/mL	----	<20

Source: (Judd, 2006)

The operational and building cost of a MBR is usually very high as compared to the other conventional wastewater treatment, however, due to increasing popularity and wider acceptance of the technology in industrial sector the significant decrease in cost has been observed. MBR systems are very useful for water-reuse applications because they produce very small footprints and high quality effluent (Judd, 2006; Verstraete, 2005).

Pre-treatment system has to be installed for the prevention of clogging of membranes by some fibres or from some other material. Pre-filtration is done with the grid distance of maximum 3 mm. Flux decline has been usually observed during filtration in most membrane filtration processes this is mostly caused due to fouling of membrane. Main issue associated with the operation of an MBR is membrane fouling. Main reasons behind membrane fouling are the type of membrane used, module configuration, by hydrodynamic conditions and due to the presence of higher molecular weight compounds, which produces as a result of microbial metabolism or introduced into the sludge bulking process (e.g. poly-electrolytes) (Melina *et al*., 2010).

2.7.1.1 Advantages and Disadvantages of Membrane Bioreactors

The main advantages of MBR technology as compared to conventional activated sludge systems are:

a) Decreased sludge production (option for high sludge age)
b) Production of high effluent quality as a result of membrane filtration
c) Lower sensitivity to contaminant peaks
d) Smaller footprint and smaller reactor volume as a consequence of higher MLSS concentration and loading rate (option for low to moderate sludge age)

The main disadvantages of MBRs are:

a) Frequent monitoring of membrane and proper maintenance
b) Less efficient oxygen transfer caused by high MLSS concentrations
c) Limitations imposed by pressure, temperature, and pH requirements to meet membrane tolerances
d) Membranes sensitivity to some chemicals
e) Quite expensive to install and operate
f) Treatment of remaining sludge is questionable (Judd, 2006).

2.7.1.2 Impact of MBR Technology on Removal of Microorganisms

The microfiltration membranes used in MBRs have proven to achieve constantly high removal rates for microorganisms such as Fecal coliforms, viruses, Total coliforms and even bacteriophages. The reported log removal rate varies between 6–8 log and 3–5 log scales for bacteria and viruses. MBR effluents were found to be compliant with the EU Bathing Water Directive (EC/160/75) including parameters such as, *Streptococcus faecalis* as *Salmonella spp*. Coliphages, Total coliforms, and Fecal coliforms. The major reason behind the frequent selection of MBRs for water treatment is the satisfactory microbiological removal and the production of high quality effluent with the introduction of any disinfectant which prevent the growth of microorganisms when this water is

introduced in distribution or storage networks. With the addition of residual chlorine MBR effluent will be quite acceptable for many wastewater reuse applications. The main problem with MBR is continuous monitoring which is very important for the proper maintenance of MBR systems (Tanzania *et al*., 2014).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 STUDY SITE

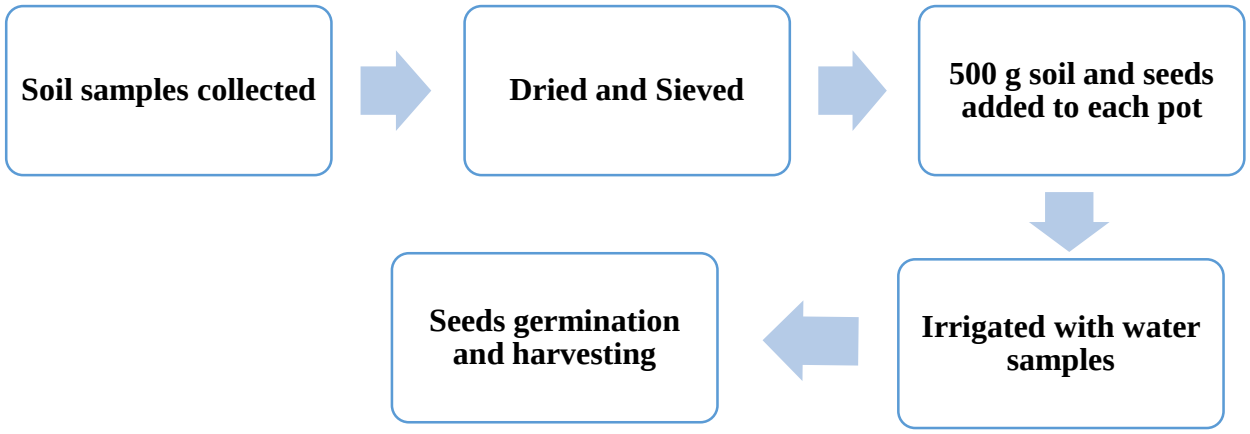
New campus residential area of National University of Sciences and Technology, Pakistan was selected as the study site. Semi-Pilot Scale MBR plant was installed in residential area of NUST for the treatment of domestic wastewater.

3.2 EXPERIMENTAL SETUP

Soil was collected from National Agriculture Research Centre (NARC) and air dried for 24 hrs. Soil was sieved and then 500 g soil was added to 36 pots. NARC certified seeds of Lettuce and Spinach were added to pots. Pots were labelled and irrigated with wastewater, MBR treated water and tap water. Seeds were irrigated on daily basis for 3 months. Plants started germinating after one week and were harvested after germination.

Fig. 3.1 Lab Scale Experimental Setup

Fig. 3.2 Flow Sheet for Experimental Setup



3.3. SAMPLING

3.3.1. Preparation of Glassware

Sterile leak proof 250 ml Schott glass bottles were used for water sampling. All sampling bottles were thoroughly washed with detergent, rinsed with distilled water and then autoclaved at 121°C, 15 psi for 15 mins and then oven dried at 105°C for one hour. Following this treatment bottles were tightly capped and wrapped.

3.3.2. Sample Collection, Transportation and Storage

Samples were collected from the Semi-Pilot Scale MBR treatment plant having 30 L capacity installed in NUST residential area. Treated and untreated wastewater samples were collected in sampling bottles. Samples were analyzed within one hour of their collection or stored in refrigerator at 4°C and analyzed within 4 hrs. All the collection, transportation and storage procedures were carried out as prescribed in the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

3.4. WATER QUALITY ANALYSIS

Temperature, pH, Dissolved oxygen were measured on site using HACH 156 pH meter. All the analysis were performed as per the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

3.4.1. Analysis in Laboratory

Conductivity, turbidity, total dissolved solids (TDS), total suspended solids (TSS), total phosphate and chemical oxygen demand (COD) were measured in the laboratory within four hours of sample collection. WTW series pH/ Cond 720 meter and HACH 2100N turbidity meter were used for measuring conductivity and turbidity meter respectively. Total Phosphate was measured using portable spectrophotometer HACH DR 2010.

3.4.2. Microbiological Analysis of water

3.4.2.1. Spread Plate Count

3.4.2.1.1. Preparation of Agar Plates

For the enumeration of heterotrophic plate counts (HPC), 20 g nutrient agar was mixed in 1 L distilled water and autoclaved at 121°C and 15 psi for 15 mins. Molten agar was then poured in autoclaved petri plates and incubated at 37°C for 48 hrs to observe sterility.

3.4.2.1.2. HPC Enumeration

Heterotrophic plate counts for water samples were analyzed using spread plate count technique as per standard procedures (APHA, 2012). 0.5 ml of the sample was spread plated onto sterile agar plates. The plates were then incubated at 37°C for 24 hrs and counted with colony counter (560 Suntax Colony Counter).

3.4.2.2 Membrane Filtration

For the detection of Coliforms and Fecal Coliforms membrane filtration technique was used. Flasks were autoclaved having 100 ml water. 10 ml of sample was collected with the

help of sterile pipette and added to first flask. Then 10 ml sample was taken from second flask and added to third. Same procedure was repeated for other flasks.

Sartorius membrane filters of pore size 0.45 μm were passed by serial dilutions and placed on EMB agar plates for 24 hrs. Plates were incubated for 24 hrs at 37˚C and colonies of fecal coliform were counted by using colony counter (Singh and McFeters, 2012).

3.4.3. Isolation of Bacteria

3.4.3.1. Streak Plate Technique

Pure cultures of bacteria were obtained from treated and untreated wastewater by sample purification through streaking on nutrient agar plates. Significant number of bacterial colonies were observed in untreated wastewater as compared to treated wastewater. Samples were analyzed to identify the presence of predominant bacterial communities.

3.4.4. Identification

For the identification of isolated bacterial strains following morphological, physiological and biochemical tests were performed (Table 3.1)

Table 3.1. Tests for identification of bacteria

Morphological	Physiological	Biochemical
Colony Morphology	Optimum temperature	Oxidase, Catalase

Source: (Pelczar, 1957)

3.4.4.1. Morphological Identification

Colony morphology of the isolated strains were observed to identify and characterize them. All physiological and morphological identification were performed as per Bergey's Manual

of Determinative Bacteriology (Holt *et al*., 1994). Following morphological characteristics were usually observed as reported by (Pelczar, 1957).

Table 3.2. Morphological characteristics of bacteria

Morphological Characteristics	Description
Size	Small, large, punctiform
Margins	Entire, curled, lobate, undulate, filiform
Texture	Creamy, dry, mucoid
Color	Yellow, orange, off white, pale yellow
Form	Rhizoid, circular, filamentous, irregular

Grams staining was used to identify cell morphology. Prepared slides were observed under 100X oil immersion with a light microscope. Cells were identified as gram positive or gram negative cocci, bacilli or cocco-bacilli.

3.4.4.2. Biochemical Characterization

Different biochemical tests following standard procedures were carried out to identify bacterial strains. These are mentioned below:

3.4.4.2.1. Oxidase Test

Strips of filter paper were taken and loop full of inoculum of a 24 hrs fresh culture was placed on one paper. On the inoculum one drop of 1% N, N-dimethyl-p-phenylenediamine dihydrochloride solution was added. Appearance of blue or purple color within seconds indicated the presence of enzyme cytochrome oxidase and hence oxidase positive test.

3.4.4.2.2. Catalase Test

Inoculum from a 24 hrs fresh culture was placed on a clean glass slide using a sterilized wire loop. A drop of 3% hydrogen peroxide was then added to it. Bubble formation confirmed catalase positive test and thus presence of enzyme catalase which breaks hydrogen peroxide into molecular oxygen and water. This enzyme is produced by bacteria to neutralize toxic forms of oxygen.

3.4.4.2.3. Growth on Differential Media

Bacterial isolates were streaked on **EMB** and **MacConkey agar**. **EMB** is a selective and differential agar which inhibits the growth of gram positive bacteria and allows differentiation between organisms that do and do not ferment lactose. Lactose fermenting bacteria give coloured colonies on EMB agar while non-fermenting bacteria give colourless colonies.

Isolated pure cultures were streaked on **EMB agar** and plates were placed in an incubator at 37°C and results were noted after 24 hrs. Rapid fermenters appeared as dark colonies with metallic sheen which indicated presence of faecal coliform, while less fermenting showed brown-pink colonies and non-fermenters appeared as colourless colonies.

MacConkey agar is used for the isolation and differentiation of gram negative rods from gram positive ones by selective growth of gram negative bacteria. Strong lactose fermenters result in the formation of pink boundaries around the colonies while weak fermenters appear pink without boundaries and non-fermenters appear colourless. Bacterial cultures were streaked on agar plates and results were noted after incubation at 37°C for 24 hrs**.**

3.4.5 Helminth Egg Count in Wastewater

1 litre of raw or partially treated wastewaters and 3 litre of final treated effluents was collected. Samples were allowed to sediment for 1-2 hrs. 90 % of supernatant was

removed using siphon or suction pump. Sediment was then transferred to centrifuge tube and centrifuged at 1000 rpm from 15 mins. Detergent solution was added to rinse the sediment. Sediment was removed and all the sediments were transferred to one tube and re centrifuged for 15 mins at 1000 rpm.

Pellet was suspended in equal volume of acetoacetic acid buffer. Then ethyl acetate was added equal to the volume of pellet and thoroughly mixed with vortex mixer. Again sample was centrifuged for 15 mins. Sample was separated into layers which includes non-fatty, heavy debris and helminth egg layer. Final volume of the pellet containing helminth eggs was recorded and the rest was poured off. Then pellet was suspended in zinc sulphate solution and again mixed with vortex. Volume of the pellet was recorded. Minimum of 1.5 ml is required to fill the chambers of McMaster slide. Aliquot was removed with the help of Pasteur pipette and transferred to McMaster slide for examination. McMaster slide was placed on flat surface for 5 mins before examination. Slide was placed under microscope and examined under 40x and 10x magnification (Ayres *et al*., 2005)

Formula for calculating total number of eggs per litre:

$$N = AX/PV$$

Where:

N = number of eggs per litre of sample

A = number of eggs counted in the McMaster slide or the mean of counts from two or three slides

X = volume of the final product (ml)

P = volume of the McMaster slide (0.3 ml)

V = original sample volume (litres)

(If a single-chamber McMaster slide is being used, P = 0.15 ml)

3.5 MICROBIOLOGICAL ANALYSIS OF VEGETABLES

3.5.1 Coliform Count

To determine the coliform count in vegetables each part of plant i.e. roots, shoots and leaves were washed with phosphate buffered saline solution. Serial dilutions were prepared. Filtrate was passed through filter assembly and filter paper was placed on Eosine methylene blue agar plate. Plates were incubated for 24 hrs at 37˚C. Light pink and dark pink colonies were counted (Downes *et al*., 2001).

3.5.2 Spread Plate Count

25 g of vegetable sample was removed aseptically with the help of sterile scalpel and vigorously shaken for 3 mins in 225 ml of sterile 0.1% (w/v) bacteriological peptone water. Serial dilutions were prepared. 0.1 ml of the sample from serial dilutions were then spread-plated on a suitable agar medium. After incubation at 30 to 32° C for 24 hrs colonies were counted (APHA, 2012) (Biniam *et al*., 2010).

3.5.3 Isolation of Bacteria

For the isolation of *Salmonella* and *Shigella* spp. 25 g vegetable samples were added to 225 ml buffered peptone water, shaken vigorously and the suspension incubated at 37°C for 24 hrs for the metabolic proliferation and recovery of cells.

1ml of culture was then transferred into the tubes containing 10 ml of Selenite broth and Tetrathionate broth. Selenite broth was incubated at 37°C for 24 hrs and Tetrathionate broth was incubated at 43°C for 48 hours. After secondary enrichment, culture from each enrichment broth was separately streaked on plates of MacConkey Agar, Salmonella-Shigella (SS) Agar and Xylose Lysine Desoxycholate (XLD) medium.

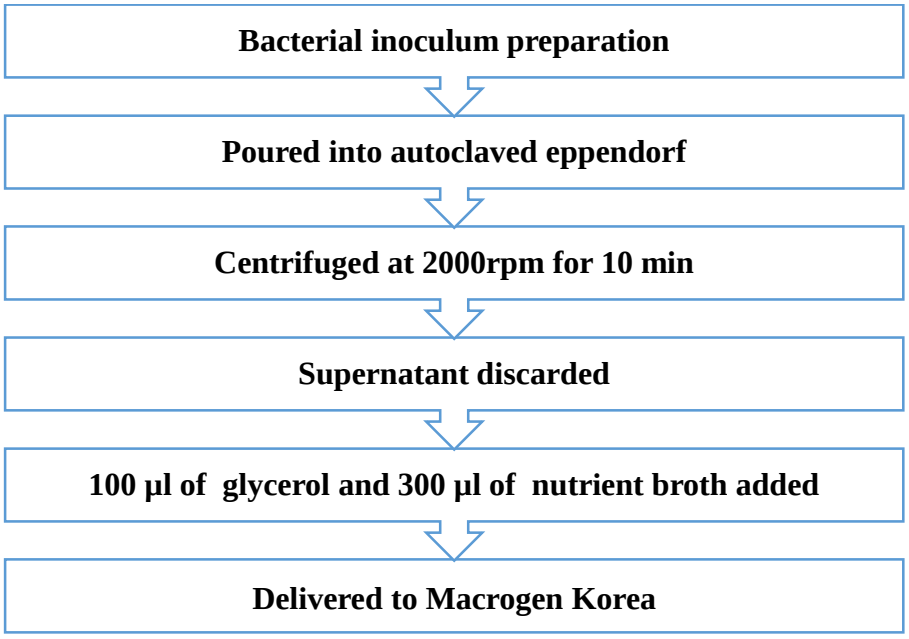
Characteristic colonies from each medium were then picked, purified and tested biochemically (Josiah *et al*., 2015).

3.6 Molecular Characterization

3.6.1 16S rRNA Gene Sequencing

Bacteria isolated from wastewater and plants (Lettuce and Spinach) were further preserved for gene sequencing. Bacterial isolates were wiped gently with distilled water with help of glass rod and the inoculum was added to eppendorf tubes. Tubes were centrifuged for 10 mins to separate supernatant from bacterial culture. Supernatant was removed. For sample preservation 1 ml of 50% glycerol and 3 ml of 30 % nutrient broth were added to eppendrof tubes and preserved at -20 ºC. For further 16S rRNA sequencing the preserved isolates were sent to Genome Analysis Department Macrogen Inc. Korea.

Fig. 3.3. Sample Preparation for 16S rRNA Gene Sequencing



3.6.2 Phylogenetic Analysis

A phylogenetic tree is an evolutionary tree having a branching diagram or "tree" which shows the evolutionary relationships among various species or other entities of biological origin, their phylogeny based upon similarities and differences in their physical or genetic characteristics. The taxa joined together in the tree are implied to have

descended from a common ancestor (Brinkman, 2001). Phytogenic tree may be rooted having same ancestor or unrooted having unknown ancestors. In a phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of the descendants and unrooted trees illustrate only the relatedness of the leaf nodes and do not require the ancestral root to be known or inferred (Olena Morozova, 2008).

After 16sRNA gene sequencing, sequences were processed through BLAST nucleotide search from databases of National Center for Biotechnology Information (NCBI). Then using FASTA low quality sequences were removed. Resulted sequence was run in MEGA4 software for the construction of phylogenic tree. It demonstrates the phylogenetic connection and linkage of identified bacterial strains with strain selected from GenBank.

3.7 DETERMINATION OF HELMINTH EGGS

100 g of raw vegetable sample was weighted in sterile plastic bags and samples were washed with 0.85% NaCl and the washing water/saline was left for sedimentation to take place for about 24 hrs

The supernatant was discarded and 5 ml of the remaining water was centrifuged at 2000 rpm for 5 mins. The supernatant was discarded and the residue carefully collected. The samples were gently agitated by hand in physiological saline solution containing lugol iodine for the distribution of the cysts and eggs and then were examined in lugol stained through light microscopy (Al-Megrin *et al*., 2010).

3.8 FOR EXTRACTION OF NEMATODES

Roots were washed, chopped into 1-2-cm segments and placed in a 150-ml beaker with 50 ml tap water. Twenty millilitres of chlorine bleach was added to give 1.5% NaOC1 and the root tissue pieces were allowed to remain in this solution for 5 mins with occasional agitation. Following the NaOCI treatment, the root segments were rinsed with

tap water (30-45 sec) and allowed to soak in tap water for 15 mins for remove residual NaOCl.

The material was then drained and transferred to a beaker containing 30 ml of water to which had been added 1 ml of stain (750 ml distilled water, 250 ml acetic acid and 3.5 g acid fuchsin). This solution was further heated to boiling for about 30 sec. After cooling to room temperature, excess stain was removed by rinsing in running water. The root, material was then placed in 20-30 ml of glycerin acidified with a few drops of 5N HC1, heated to boiling, and cooled. The root segments were then pressed between glass plates or microscope slides for observation (Ayres *et al*., 2007).

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 TREATMENT PERFORMANCE OF MBR

MBR system analyzed for its treatment performance. The system was found to have produced satisfactory results where the average removal efficiencies of COD, TSS, and TP were 87, 74.3 and 75 % respectively. Table 4.1 represents the treatment performance results for each water quality parameter tested in this study compared with National environmental quality standards (NEQS) Pakistan environmental protection agency (Pak-EPA) standards for inland disposal and irrigation standards. The effluent concentrations for some parameter analyzed were found below the permissible national environment quality standards (NEQS).

Table 4.1 Treatment Performance of MBST

Parameters	Unit	Influent	Effluent	Removal Efficiency %	NEQs	Irrigation Standards
pH		7.50 ± 0.2	7.02 ± 0.1	----	6.0 - 9.0	6.5 - 8.5
Temp	˚C	26.9 ± 2.9	26.3 ± 2.8	----	40ºC	----
DO	mg/L	1.79 ± 0.4	4.12 ± 0.7	----	----	----
COD	mg/L	247.6 ± 26.7	33.0 ± 10.6	87	150	<150 mg/L
EC	µS/cm	913.9 ± 82.3	683.4 ± 37.9	----	----	>2500 µS /m unacceptable
TDS	mg/L	584.9 ± 41.1	231.7 ± 18.9	60.3	3500	----
TSS	mg/L	32.3 ± 5.13	8.33 ± 2.8	74.2	200	<100 mg/L
TP	mg/L	32.3 ± 5.13	4.54 ± 1.7	75	----	----
Total Coliform	CFU/mL	----	----	87	----	<1000/100 mL
Faecal Coliform	CFU/mL	----	----	85	----	<200/100 mL

4.2 GERMINATION AND SEEDLING GROWTH

Data presented in Table 4.2 (a) and 4.2 (b) revealed that seed germination was seriously affected by varying composition of applied irrigated water. Greater the concentration of nutrients in wastewater higher the rate of seeds germination. Maximum germination rate (96 and 98 %) was noted in the seeds irrigated with wastewater whereas minimum germination was observed in control (68 and 72 %). The observed germination percentage of Lettuce and Spinach seeds were 96% and 98% respectively.

4.2 (a) Germination and Seedling Growth Lettuce (cm)

	Germination (%)	**Seedling Growth (cm)**		
		Radical	**Plumule**	**Vigour Index**
Control	68	1.43 ± 0.32	2.17 ± 0.26	244.8
Untreated Wastewater	96	1.97 ± 0.18	3.01 ± 0.75	479
Treated Wastewater	82	1.81 ± 0.27	2.85 ± 0.25	382.1

4.2 (b) Germination and Seedling Growth Spinach (cm)

	Germination (%)	**Seedling Growth (cm)**		
		Radical	**Plumule**	**Vigour Index**
Control	72	1.61 ± 0.34	2.82 ± 0.84	318.9
Untreated Wastewater	98	1.89 ± 0.44	3.69 ± 0.61	546.8
Treated Wastewater	87	1.82 ± 0.16	3.05 ± 0.75	423.69

Data presented here also depicted that there was a decrease in length of radical in particular and plumule in general when irrigated with control (tap water). It was observed that in case of untreated wastewater the plumule and radical length was significantly higher. The results revealed that untreated wastewater had positive impact on seed germination. Polluted water at low concentration does not inhibit the seedling growth but at very higher concentration germination of seeds and seedlings growth will be affected. Other researcher also reported that waste water contain some essential organic compound which increase

growth of crop ((Nagada *et al*., 2006). Nath *et al*., 2009 also suggested that sewage sludge is common manure and can be used for crop and other plants growth due to presence of important organic matter. The use of domestic wastewater in plant nourishment would be beneficial alternative resource to fresh water

4.2.1 Plant Growth

The root length of the saplings of Spinach and Lettuce after the treatment with municipal tap water, treated wastewater and untreated wastewater was 6.88 ± 1.4, 8.08 ± 0.9, 9.42 ± 0.4 and 7.4 ± 1.3, 9.04 ± 1.0 and 10.2 ± 0.7 cm respectively (Fig 4.1).

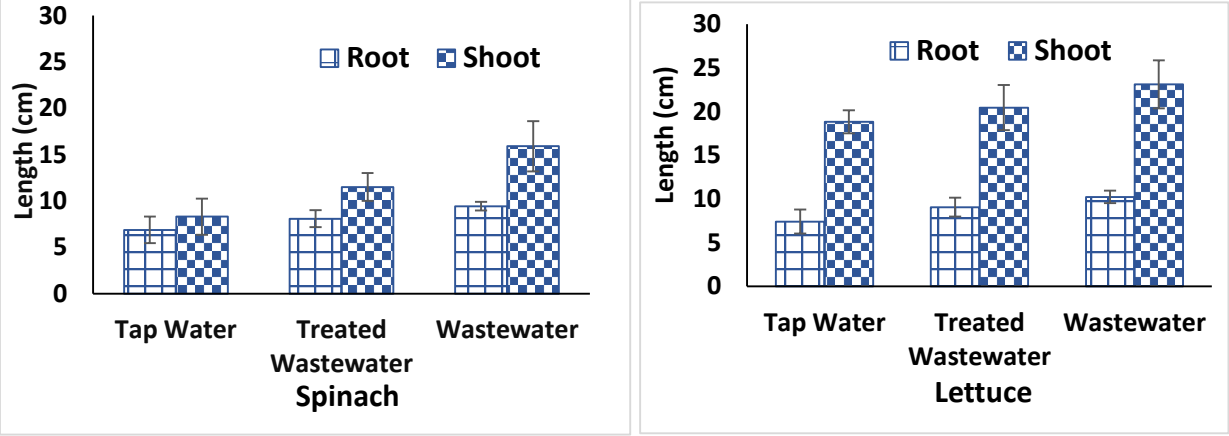


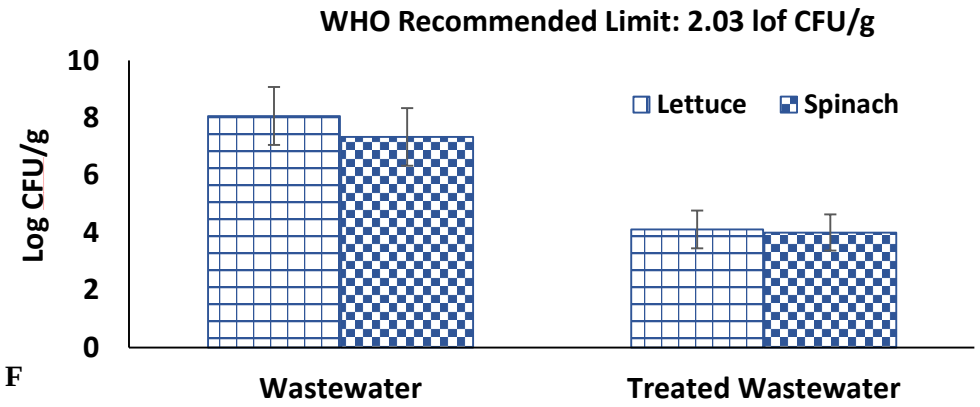
Fig. 4.1 Plant Growth (Tap, Treated and Wastewater)

Wastewater has high level of Na which is responsible for increasing growth parameters in treated plants. Also wastewater contains all essential nutritional elements such as N, P, K which are necessary for the growth of plants (Prabhakar *et al*., 2004). High level of organic matters present in wastewater improves soil physical condition. Due to high concentration of macro and micro-elements plant growth become (Keller *et al*., 2002). Increasing root and aerial parts prolin, water soluble carbohydrates and catalase were observed in wastewater treated plant in comparison with control (Ben-Ghadelia *et al*., 2001).

4.3 MICROBIOLOGICAL QUALITY OF LETTUCE AND SPINACH

4.3.1. Total Heterotopic Plate Count in Samples

The total heterotrophic plate count observed in lettuce irrigated with treated and untreated wastewater was 4.11 and 8.06 log CFU/g. In spinach the observed count was 4.00 and 7.33 log CFU/g for treated and untreated wastewater (Fig 4.3).



F

ig. 4.2 Microbiological Quality of Lettuce and Spinach (HPC)

Mean heterotrophic plate count in lettuce and spinach treated with raw wastewater was significantly higher as compared to those treated with treated wastewater. HPC in both lettuce and spinach samples were exceeding the quality standard of 10^3 CFU/g provided by International Commission on Microbiological Specifications for Food (ICMSF) and 2.3 log CFU/g by WHO.

Torsvik *et al*., 2002 studied that high bacterial load in green vegetables (4.95 – 7.77) $\log_{10}$ CFU/g when irrigated with partially treated wastewater. One of the main sources of pathogenic micro-organisms contamination in vegetables is the use of untreated wastewater another important source of contamination is the use of water from those water supplies which are contaminated with sewage water (Abougrain *et al.* 2010).

4.3.2 Total *E.coli* Count in Samples

High *E.coli* counts were observed in lettuce roots, shoots and leaves as compared to spinach. Samples irrigated with wastewater were highly contaminated with *E.coli* as compared to those irrigated with treated wastewater. Total bacterial load recorded in lettuce root, shoot and leaves irrigated with wastewater was 7.72, 6.62 and 5.21 log CFU/g respectively. Similarly the bacterial load observed in root, shoot and leaves of spinach was 7.62, 6.85 and 5.26 log CFU/g as shown in Fig 4.3.

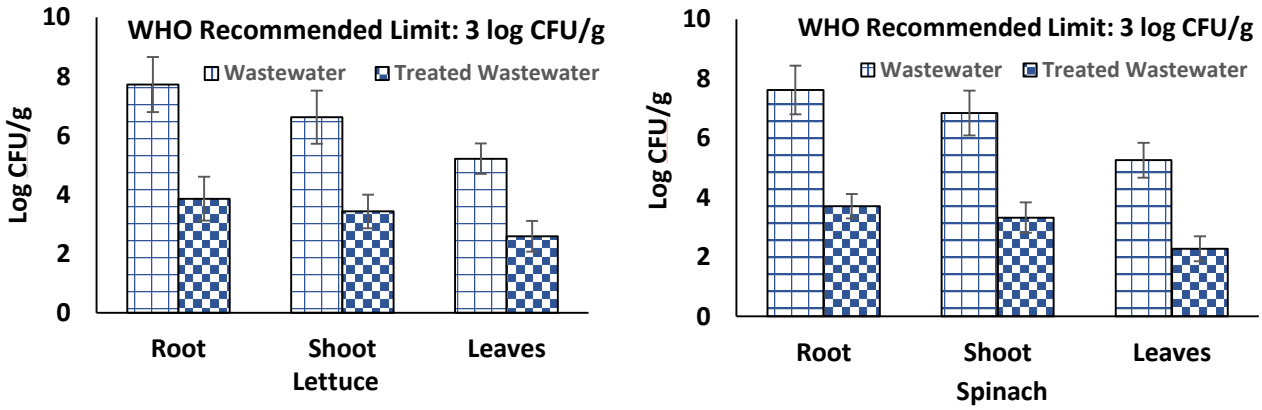


Fig. 4.3 Microbiological Quality of Spinach and Lettuce (*E.coli*)

Indicator bacterial load was significantly less in vegetables irrigated with treated wastewater.

The high risk of acquiring infectious diseases is associated with consumption of vegetables contaminated with indicator bacteria. The occurrence of such indicator microorganisms is an indication of the contamination of the vegetables with faecal matter derived from humans and other animals (Andereson, 2003). According to World Health Organization (WHO, 2014) recommendation biologically treated effluent should be used for the irrigation of raw vegetables. Effluent should be disinfected to achieve a coliform level of not more than 100 coliform per 100 ml in 80% of the samples. The data further showed that all the bacterial counts recorded in this study exceeded the recommended levels by

WHO and International Commission on Microbiological Specifications for Food (ICMSF) standards (2.03 log CFU/g).

4.3.3 *Salmonella* Count in Samples

Significant concentration of *Salmonella* was present in lettuce and spinach samples. The data in Figure 4.4 shows the *Salmonella* contamination in lettuce and spinach samples irrigated with wastewater and treated wastewater. Samples of untreated wastewater were more contaminated than samples grown in MBR treated wastewater. *Salmonella* count in lettuce and spinach grown in wastewater was 6.86 and 5.88 log CFU/g whereas the MBR treated wastewater vegetables had *Salmonella* count 2.20 and 2.18 log CFU/g.

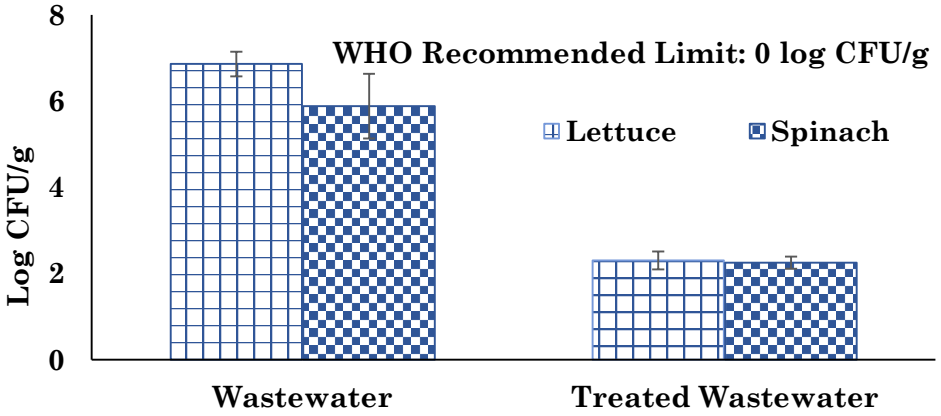


Fig. 4.4 Microbiological Quality of Lettuce and Spinach (*Salmonella)*

In the present study, excess microbiological count as compared to standard were recorded for most of the vegetables. WHO recommended limit for *Salmonella* in vegetables is 0 log CFU/g. The basic source of microbial contamination in vegetables is because of the water used for irrigation. The pathogens of major concern on fresh vegetables and fruits are those of intestinal origin; *Salmonella, Shigella*, *Escherichia coli.* Although most of these pathogens are known to die off rapidly in soil and water, their survival may be increased and regrowth is possible when sufficient organic water and moisture is present (Biniam *et al*., 2010).

4.3.4 *Shigella* Count in Samples

Results showed that all samples were contaminated with varying level of *Shigella* counts. Mean *Shigella* count recorded in lettuce and spinach grown in untreated wastewater was 6.27 and 5.86 log CFU/ml whereas the mean count in vegetables irrigated with MBR treated wastewater was relatively low as compared to untreated wastewater. Observed *Shigella* count in MBST treated spinach and lettuce was 1.65 and 1.78 log CFU/g (Fig 4.5).

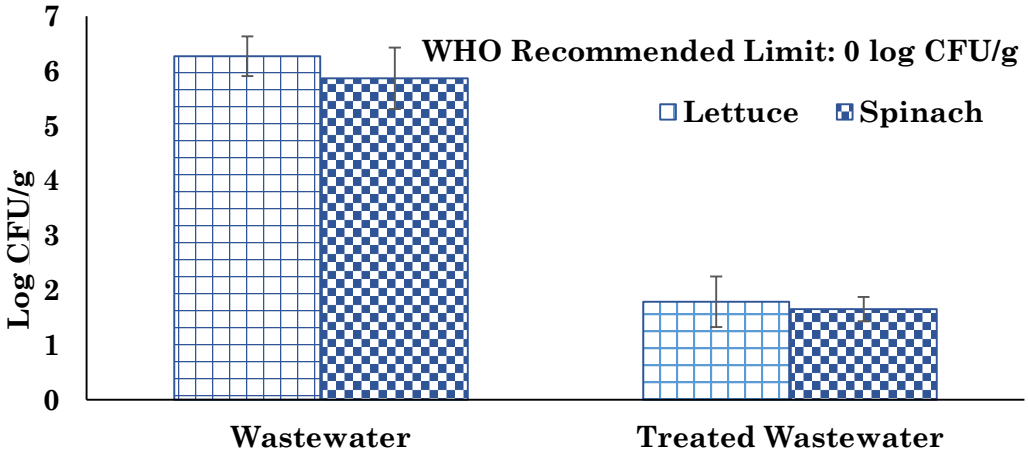


Fig. 4.5 Microbiological Quality of Lettuce and Spinach *(Shigella*)

Shigella is mostly associated with chicken, raw vegetables, dairy products and poultry. Contamination of these foods is usually through the faecal-oral route and is most commonly due to faecally contaminated water (Gomez-Govea *et al*., 2012). Bacterial numbers in all the samples exceeded both the WHO and ICMSF recommended levels making it risky for consumption in the raw state.

4.4 HEMLINTH EGG COUNT

Result indicates that significant quantity of helminth eggs were present in both MBR treated water and in wastewater. Predominant species of parasite were *A. lumbricoides*, *T.trichiura* and *Hookworm. A. lumbricoides* was the most dominant specie. Recommended limit for helminth egg is ≤ 1 egg/ litre. High level of parasite contamination was observed in lettuce leaves due to large surface area. Observed *A. lumbricoides*, *T.trichiura* and

Hookworm count in lettuce irrigated with wastewater was 18, 2.5 and 5.5 eggs/L whereas spinach treated with wastewater had parasitic count 13.5, 0.5 and 4.5 eggs/L respectively as shown in Fig. 4.6.

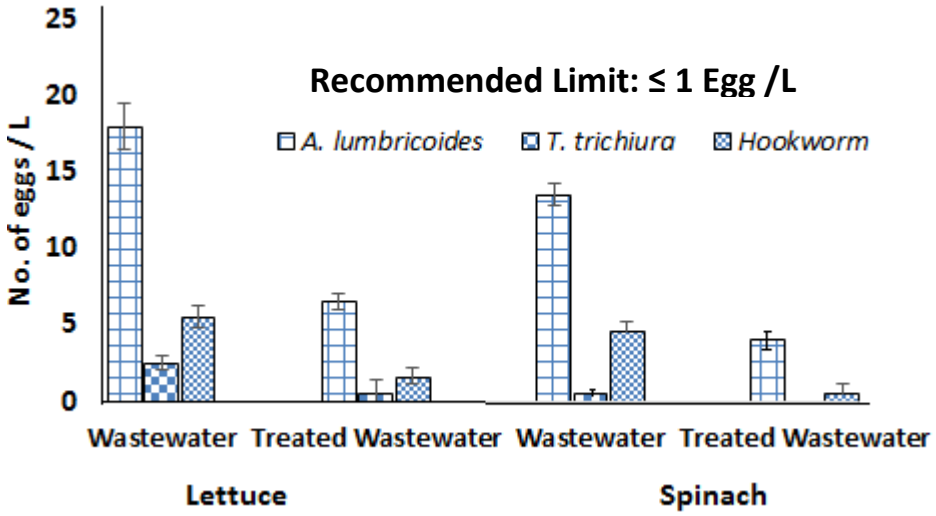


Fig. 4.6 Helminth Egg Count in Spinach and Lettuce

The parasitic count observed in treated wastewater was relatively less in spinach and lettuce but it still exceeds the recommended standard. *A. lumbricoides, T.trichiura* and *Hookworm* observed in lettuce and spinach irrigated with wastewater and treated wastewater was 6.5, 0.5, 1.5 and 4, 0, 0.5 eggs/L respectively.

Al-Binali *et al.* (2006) evaluated the annual risks of rotavirus and Ascaris infections for consumers of lettuce irrigated with the different water qualities after post-harvest handling and of farmers using different irrigation water qualities. The assessment revealed a high risk of Ascaris and rotavirus infections above the TR levels for farmers using different irrigation water quality and also the much larger number of consumers of irrigated lettuce. Many epidemiological studies have revealed an excess of parasitic infestations associated with raw water reuse in irrigation.

Uga *et al*. (2009) whose work shows that contamination was high in leafy vegetables followed by root and fruity vegetables. A high prevalence of *A. lumbricoides* contamination of raw vegetables has been reported by Kozan *et a*l. (2005).

4.5 IDENTIFICATION OF BACTERIAL SPECIES

4.5.1 Bacterial Species Isolated from Wastewater and Plants

From wastewater and MBR treated water 6 different strains were obtained R1-R-6 while from plants 3 different species were obtained as R7-R9. *Acinetobacter johnsonii*, *Pseudomonas monteilii*, *Pseudomonas putida*, *Raoultella ornithinolytica*, *Aeromonas hydrophilla* and *Aeromonas veronii* were the predominant genera of bacteria in wastewater and MBR treated wastewater. Presence of these species may be the reason of higher bacterial counts in wastewater. Principal species identified in spinach and lettuce were *Escherichia coli*, *Shigella flexneri* and *Salmonella enterica.* Isolated species along with their accession numbers are presented in Table 4.3

Table 4.3 Predominant Species

Predominant Species		**Accession numbers**
1.	*Acinetobacter johnsonii*	KT445980
2.	*Pseudomonas monteilii*	KT445981
3.	*Pseudomonas putida*	KT445982
4.	*Raoultella ornithinolytica*	KT445983
5.	*Aeromonas hydrophilla*	KT445984
6.	*Aeromonas veronii*	KT581978
7.	*Escherichia coli*	KT581977
8.	*Shigella flexneri*	KT581976
9.	*Salmonella enterica*	KT581975

Isolated bacterial strains were studied for their form, color, opacity, elevation, margin and surface. Table 4.4 represent colony and cell morphology of bacterial isolates from wastewater and plants respectively. Only one isolated strain was gram +ive and rest of other isolated strains were gram –ive.

Table 4.4. Characterization of Isolated Species

Characteristics	Observations								
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**
Colony Morphology	Shiny, slight raised, mucoid, entire margins	Shiny, slight raised, mucoid, entire margins	Translucent, smooth, convex, pale or yellow or reddish yellow	Oval, rough, wavy, convex, margins, mucoid, pigmented green	Glistening, circular, pinpoint, entire, yellow to translucent	Translucent. Smooth, creamy white	Shiny, round, flat, undulate, umbonate, mucoid, white translucent	Circular, convex, smooth, moist, grey white	Circular, convex, irregula, outgrowth from margins, grey white
Cell Morophology	Gram -ive	Gram -ive	Gram -ive	Gram -ive	Gram -ive	Gram -ive	Gram+ive	Gram - ive	Gram -ive
Motility Test	+	-	+	+	+	-	-	+	-
Oxidase	-	-	+	+	+	-	+	+	-
Catalase	+	+	+	+	+	+	+	-	+
Growth on EMB	+ (Green sheen)	+ (Pink-purple)	+ (Colorless)	-	-	+ (Brown, dark centered)	-	-	+ (Blue)
Growth on MacConkey	+ (Pink)	- (Red)	- (Yellow) -	-	-	+ (Pink)	-	-	–
Oxidation/ Fermentation	Facultative anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Facultative anaerobic	Aerobic	Aerobic	Aerobic

4.5.2 Selection of Strains for Gene Sequencing

After detailed analysis of isolated strains through morphological and biochemical characterization strains were selected for further 16S rRNA gene sequencing. It was performed at Genome Analysis Department Macrogen Inc. Korea.

The strains were screened and noise was removed manually. Strains were identified through BLAST search (Morozova *et al*., 2008)) available at National Center for Biotechnology Information (NCBI) databases revealing 99% similarity to different bacterial species. Schloss in 2004 market the limit of 97% for identification of species (Schloss, 2004).

A phylogenetic tree, constructed through MEGA 4 program demonstrates the phylogenetic relatedness and linkage among identified strains, shown in Figure 4.7.

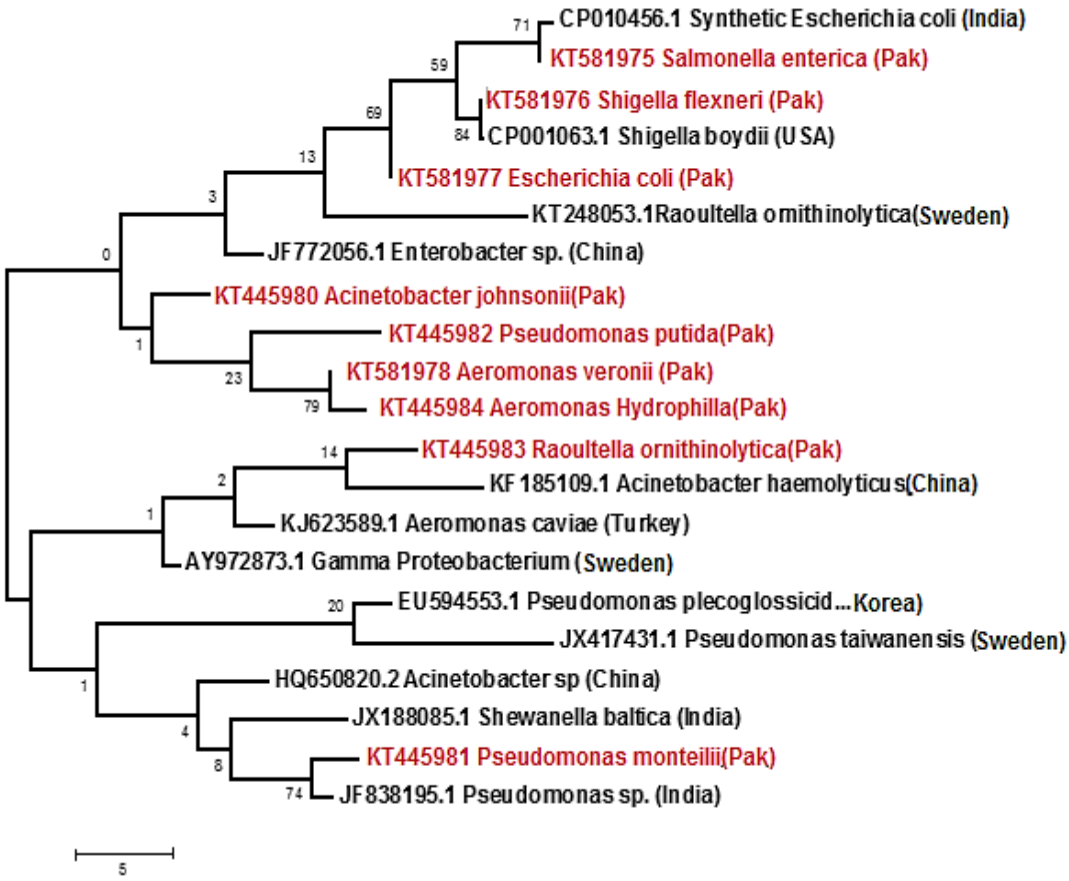


Fig. 4.7 Phylogenetic Tree Demonstrating the Relatedness and Linkage of Bacterial Strains

In this figure 4.7 amount of genetic change is represented by horizontal lines. The horizontal lines represent the evolutionary shift over time. The larger the branch length in the horizontal direction, the greater the amount of change. The bar at the bottom of the figure provides a scale for this. In the above phylogenetic tree the line segment with the number '5' shows the branch length that represents an amount genetic change of ‘5’. The units of branch length are usually nucleotide substitutions per site that is the number of changes or 'substitutions' divided by the length of the sequence (although they may be given as % change, i.e., the number of changes per 100 nucleotide sites). The vertical dimension in this figure has no meaning and is used simply to lay out the tree visually with the labels evenly spaced vertically. The vertical lines therefore simply tell you which horizontal line connects to which and how long they are irrelevant (Roberto, 1993).

Chapter 5

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

1. The system was found to have produced satisfactory results where the average removal efficiencies of COD, TSS, and TP were 87, 74.3 and 75% respectively.
2. HPC in all the lettuce and spinach samples were found to exceed the permissible WHO limit of 2.3 log CFU/g, the possible reason of it might be use of untreated wastewater for irrigation and treated wastewater.
3. Total bacterial load observed in lettuce root, shoot and leaves irrigated with wastewater was 7.72, 6.62 and 5.21 log CFU/g respectively. Similarly the bacterial load recorded in root, shoot and leaves of spinach was 7.62, 6.85 and 5.26 log CFU/g.
4. MBR treated wastewater vegetables had *Salmonella* count 2.20 and 2.18 log CFU/g whereas the *Salmonella* count in lettuce and spinach grown in wastewater was 6.86 and 5.88 log CFU/g.
5. *Shigella* count observed in lettuce and spinach grown in untreated wastewater was 6.27 and 5.86 log Cfu/g whereas the mean *Shigella* count in vegetables irrigated with MBR treated wastewater was relatively low as compared to untreated wastewater.
6. *Salmonella enterica, Shigella flexneri, Escherichia coli, Acinetobacter johnsonii*, *Pseudomonas monteilii*, *Pseudomonas putida*, *Raoultella ornithinolytica*, *Aeromonas hydrophilla* and *Aeromonas veronii* were the predominant genera of bacteria in wastewater and MBR treated wastewater.
7. Principal species identified in spinach and lettuce were *Escherichia coli*, *Shigella flexneri* and *Salmonella enterica.*
8. Predominant species of parasite identified were *A. lumbricoides*, *T.trichiura* and *Hookworm.* Helminth egg count in vegetables also exceeded the limit.

5.2.1 RECOMMENDATIONS

Following recommendations are proposed for further research:

1. Final treated effluent should be disinfected through UV disinfection method before reuse to prevent bacterial contamination.
2. Levels of contamination by Cryptosporidium and Giardia in wastewater should be estimated.
3. *Listeria monocytogenes* contamination in vegetables grown in wastewater should be studied.
4. Comparative studies should further be conducted to ascertain the bacterial and trace metal contamination levels in the wastewater
5. Great attention should be paid in using contaminated water for production of vegetables for the public health perspective. The general public should be made aware to use vinegar or salt solution to wash any raw vegetable prior to consumption.

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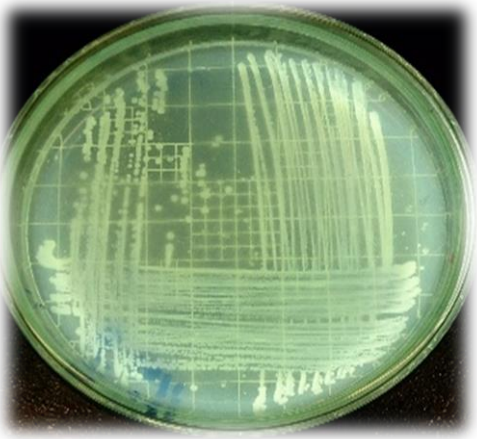
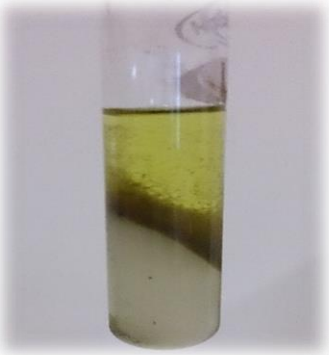
ANNEXURE-I



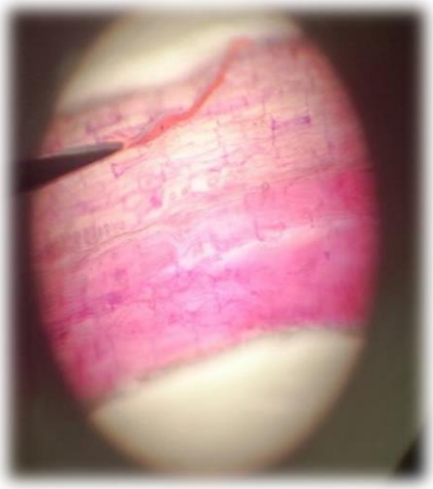
Isolated strain *Pseudomonas monteilii*

Germination Test for Lettuce



Isolation of Helminth Eggs

Staining of Spinach Roots for Nematode Extraction



Microscopic Observation of Jevunile Nematode