MICROBIOLOGICAL ANALYSIS OF MEMBRANE BASED SEPTIC TANK EFFLUENT AND ITS REUSE

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I dedicate this thesis to my beloved parents and teachers

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List of Abbreviations

MBST	Membrane based septic tank
API	Analytical profile index
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
EMB	Eosin methylene blue
HRT	Hydraulic retention time
MF	Microfiltration
TP	Total phosphorous
WFMF	Woven fibre microfiltration membrane module
PCR	Polymerase chain reaction
ADH	Arginine dehydrolase
LDC	Lysine decarboxylase
ODC	Onthinine decarbolyxase
CIT	Citrate utilization
TDA	Tryptophan deaminase
VP	Voges–proskauer
GEL	Gelatin liquefaction

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Abstract

With declining trend in global water resources wastewater reclamation and reuse has gained considerable importance worldwide. The present study was conducted with the objective to reuse domestic wastewater treated by using membrane technology for crop irrigation. Membrane based septic tank was designed (MBST) by submerging a woven fiber microfiltration membrane module (WFMF) to treat domestic wastewater. To evaluate the performance efficiency of MBST, effluents were characterized for microbiological and physical parameters. Removal of organics (COD), turbidity, nutrients deduction (P and N) and indicator bacteria (*Escherichia coli*) removal efficiency was about 73, 96, 48 and 88 % respectively. Three crops *Triticum aestivum* (wheat), *Coriandrum sativum* (coriander) and *Mentha arvensis* (mint) were irrigated with treated MBST effluents under controlled conditions. All the crops were also grown using untreated wastewater (after primary settling) and tap water for comparative study. Upon maturity, the roots, shoots and leaves of these plants were aseptically removed for microbiological analysis. Two bacterial strains *Escherichia coli* and *Salmonella sp.* were isolated from MBST treated effluents and crops which were irrigated with these effluents. These bacterial strains were identified using biochemical test, 20E API kit and 16S rRNA gene analysis. A remarkably positive effect was observed on removal of bacterial load ($p<0.001$) and helminthes eggs from effluent treated by MBST. While on the other hand increase in germination percentage, dry biomass, root and shoot length and reduction in bacterial strains was observed in crops irrigated by MBST discharge in comparison with untreated wastewater. The current study revealed that wastewater treated with MBST with UV disinfection may be used to meet the irrigation requirements and better yield.

Chapter 1

1. Introduction

1.1 Problem statement

Demand for water resources is increasing greatly in Pakistan. According to World Bank, from a water stressed country, Pakistan has become a water scarce country. Noticeably, the per capita water availability in Pakistan has fallen since independence and could fall to less than 1,000 cubic meters by 2012 and countries below this per capita water availability are listed as water short country. Pakistan will also join the list of countries with severe water scarcity by 2025. According to Pakistan Institute of Development Economics, no less than 38.5 million Pakistani's lack access to safe drinking water. Pakistan's major water consumption sector is agriculture with approximately 96 % water demand. Given the growing concerns about water availability in Pakistan now it's time to think about solutions that will help to reduce water shortages other than constructing huge dams and reservoirs that appear futile in current social and political context. Reuse and decentralization concept of wastewater treatment is very essential now a day for meeting human demands for water and sanitation in both developed and developing nations. In this regard, membrane technology is considered as one of the solution for wastewater treatment and its on-site reuse. Due to stringent water quality criteria for reuse effluent quality should be of high level. Membrane based septic tank system is one of its applications. In this system, submerged membrane filtration system is placed directly inside the septic tank. It removes suspended solids, BOD reduction, pathogens and even nitrogen and phosphorus from septic tank waste water. So, the current study is designed to examine the efficiency of reclaimed water for irrigating crops.

More than 70 % of fresh water consumption is devoted to agricultural activities (FAO, 2010). According to global estimation, around 10 % of the populace utilize wastewater feed crops (WHO, 2013). Nonetheless, the recycling of waste water for irrigation purpose may release the demand pressure on the scarce freshwater. Wastewater reserves, simultaneously enhances the food production capacity of households.

Evidently, the increasing world population, specifically in urban and peri-urban regions of the developing economies, requires a sustainable plan for food availability amidst

environmental protection strategies (Samuel *et al*., 2013). Generally, sewage water provides an alternative water source in areas faced with water scarcity. Arguably, the abundance of minerals in wastewater contains results in increased crop yields without the use of fertilizer (Shende and Chakrabarti, 1987). However, wastewater also contains a number of chemical substances and microbiological loads from household. As the demand for vegetables in and around urban areas is high throughout the year, the need for irrigation water is also stressed. Faced with scarcity of irrigation water, the farmers rely on wastewater sources for irrigating the farms.

On one hand, the reliance on wastewater irrigation has fundamentally reduced urban food insecurity and has provided livelihood sustenance, on the other hand, this activity has stretched staunch public health issues because of risks associated with usage of untreated wastewater not only to the vegetable consumers but also the farmers (Howard *et al.,* 2004). Therefore, interest in wastewater reuse technologies and its possible outcomes is gaining momentum now a day.

1.2. Need for research

In the thickly populated developing countries like Pakistan, centralized system of water treatment prevails in which sewer pipes run from each house to a central collection point where the raw water is treated and then typically discharged into some surface water like a river or lake, or spray irrigated onto land. These systems are expensive to build and require trained certified operators to run them.

Ironically, not more than 1 % of domestic and industrial wastewater receives treatment in Pakistan (Joshi, 2004). A wastewater treatment plant in Islamabad has three phases, however, only one is currently functional. Furthermore, Karachi has two dripping filters, where sewage is screened and sedimentation is performed. Also, the screening and grit removal systems built in Lahore are dysfunctional for long. Moreover, the wastewater treatment plant constructed in Faisalabad merely provides the primary water treatment and the concept of wastewater treatment is virtually non-existent in the rural areas of the country resulting in surface and groundwater pollution (Whang *et al*., 2008).

This is indicative of the mismanagement of wastewater collection and treatment system in the country. Notably, 1.6 million residents of Rawalpindi city generate wastewater, an approximate of 70 MG daily. However, RWASA collected only 35 % of wastewater, while

65 % has been disposed off into open drains that eventually pour into Nalluh Lai (Kanwal *et al.,* 2012). Moreover, the on-site sanitation system fulfilling the requirement of temporary dumping and treatment has been developed in the areas lacking sewage coverage (Karak and Bhattacharyya, 2011). Therefore an alternative plan of decentralized wastewater treatment is suggested in Pakistan.

1.3. Conventional septic tank

Septic tank works over principle of flotation and sedimentation and clear water overflows to pubic sewer or leach field if public sewer system is not available. As per model building and zoning regulations, enforced in Punjab, it is mandatory to have a septic tank in each building (Samuel *et al.,* 2013).

In absence of a centralized sewage treatment plant, septic systems are used to treat and dump wastewaters generated in the household. The wastewaters may contain pathogenic microorganisms and pollutants that pose a threat not only to humans, but also to the environment, hence, it must requires treatment. Therefore, the septic systems may provide with a permanent solution for the preliminary treatment and dumping of waste produced (Yates, 1985).

Commonly, the septic tank provides an on-site anaerobic preliminary treatment of wastewater. Yet, the inherent flaw in the design has raised questions on its performance despite the long operated HRT (Beal *et al.,* 2005).

Owing to the low installation and maintenance costs connected with conventional septic system, the households prefer a system based on a septic tank that involves soil absorption system for on-site disposal of wastewater. This system may function for years if proper installation in suitable soil is ensured. Yet, this conventional septic system of on-site treatment merely provides the primary treatment leaving out the biological degradation thus, questioning its effectiveness (Gill *et al.,* 2009).

1.4. Membrane based septic tank

Membrane based septic tank system is able to remove suspended solids (SS), chemical oxygen demand (COD) and even nitrogen (N) and phosphorus (P) from domestic wastewater (Gill *et al.,* 2009). Effluent of the membrane septic tank may be used for

horticulture, landscaping and any other non-potable purposes. Yet, the inherent risks associated with the consuming crops irrigated with such water are not clearly understood.

1.5. Treated water irrigated crops

Numerous economic benefits are attached with the on-site wastewater treatment and reuse scheme. Moreover, it not only address the issue of water wastage but also releases the increased demand pressure for irrigated water. Nonetheless, the practice of treated wastewater for the purpose of irrigation significantly reduces the sewage disposal costs and leads to efficient water utilization. However, the underlying health and environment hazards associated with wastewater irrigation are grossly ignored. Vegetables and edibles may become infected with "pathogenic organism during growth, harvest, postharvest handling or distribution" (Mcmahon and Wilson, 2001). Increasingly, the transfer of these pathogenic organisms such as *faecal coliforms, E.coli* and eggs of some helminthes such as *Ascarislumbricoides, Trichuristrichura, Hymenolepisdiminuta, Fasciola hepatica and Strongyloides* whose resistant eggs may be found in the wastewater and are debatably connected with the usage of untreated wastewater for crop irrigation (Amoah *et al*., 2007; Mara *et al*., 2007).

Not only this, the farmers and people involved in farm activities, if exposed long to untreated wastewater, may develop helminth infections and parasitic diseases (Samuel *et al*., 2013). It has also been reported that watering of salad crops with untreated wastewater initiated surplus disease (e.g. *Shigellosis* in England) in those who utilized them (Frost *et al*., 1995). There exist various opportunities for attachment and transmission of pathogenic microorganisms on vegetables (e.g. lettuce) in the field, during harvesting, handling and marketing, particularly when an infected product is bare to water or is damaged (Takeuchi *et al*., 2001). Agronomic factors including the selection of crop type, irrigation method along with the cultural harvesting practices have a strong influence on transmission of disease (Carr, 2005).

It is pertinent to note that the wastewater may appear harmless, yet it may possess heavy metals, pathogens, toxic chemicals and other hazardous elements that may not only pollute the soil but also contaminate the crop (Eriksson *et al*., 2002). Essentially, if wastewater usage for irrigation purpose is to be undertaken for recycling of water resource, extensive

research is required to understand the inherent potentials and risks associated with the practice.

Crop quality results were compared to a control group of crops grown under tap water irrigation in identical conditions, and the comparative microbial risk of consuming the wastewater irrigated crops is presented in the final sections of the report.

1.6. Objectives of Study

The aim of the study was to compare the membrane based septic tank effluent with the untreated wastewater in order to relate it with the performance of the membrane based septic tank so the following objectives were established.

- Monitoring of growth response of selected crops (wheat, coriander and mint) for untreated, treated and tap water.
- Analysis and enumeration of indicator microorganisms, potential pathogens and helminthes to establish the microbiological quality of reclaimed water.

Chapter 2

2. Review of literature

2.1 Wastewater reuse in agriculture

The era is marked with increase food insecurity and water scarcity. The emphasis on treatment and reuse of water resources has gained attention of the researchers. Amidst food insecurity, most of the water resource is used for agriculture sector and water recycling, thus, may provide an attractive alternative for supplementing freshwater supplies.

Conventionally, wastewater irrigation is regarded as an efficient end-use for reclaimed wastewater as it may contain nutrients essential for plant growth, thus surpassing the need for artificial fertilizers. This practice of wastewater irrigation is less common in developing countries like Pakistan, but, is being increasingly utilize by several municipalities as an emerging substitute for irrigation purpose.

In the poverty stricken areas across the globe, where farmers have restricted access to freshwater and fertilizers, the practice of wastewater irrigation has been widely adopted. However, the wastewater used for irrigation in these areas is usually untreated and has dire consequences for public health and environment. This has called for extensive attention for exploration of the dynamics involved in health issues, environmental concerns and their link with raw/untreated wastewater reuse in agriculture sector.

The pathogenic microorganisms contained in wastewater may cause health diseases, if intake in the form of crops or inhaled by means of aerosols produced during spraying activities. This may lead to a widespread outbreak of foodborne diseases and illness in humans as well as animals.

Meanwhile, wastewater holds nutrients essential for healthy plant activity, and holds natural fertilization potential for crops. All the vital macro and micronutrients including nitrogen, phosphorous, potassium, calcium and magnesium for the plant growth are present in municipal and domestic wastewater. Wastewater may supplement or either replace fertilizers commercially being use by farmers. Reuse of wastewater in agriculture benefits the environment in positive terms, as it permits these valuable nutrients to be averted from water to plants, rather than release into the water streams as pollutants. In developed countries where reuse of wastewater is properly regulated and streamed according to

quality guidelines to confirm that the nutrient reutilizing prospective of wastewater may be utilize by reducing health concerns related to its reutilization (Finley *et al*., 2009).

Various studies that includes microbial parameters, reported the presence of high *Salmonella* count in kitchen sink and dish water effluent (Eriksson *et al*., 2002; Lazarova *et al*., 2003). In household wastewater, Ottosson, (2003a) outlines the full spectrum of microorganisms potentially hazardous and provides an outline for evaluating the health concerns they may pose. Faecal bacteria, *Campylobacter*, *Salmonella*, *Legionella*, Enteric viruses (especially *Rotavirus*), and *Protozoa*, including *Giardia* and *Cryptosporidium* are the identified pathogenic organisms (Ottosson, 2003b). Measurement of individual pathogen while testing the water quality is a very expensive procedure and practically non-feasible. In microbial studies mostly *faecal* bacteria, normally the *E.coli* group or *Escherichia coli* in particular, investigated for determining the water quality as it acts as an indicator for the presence of pathogenic microorganisms. However, some researchers are of the view that the estimation of *fecal-coliforms* or *Escherichia coli* to determine the pathogenic level may directed to overestimation of pathogen count in samples (Finley *et al*., 2009).

To reduce the negative impacts associated with wastewater, treatment before use is strongly recommended. For wastewater, treatment systems may vary with their complexity of treatment method, and location and it should be intended according to the wastewater source, quality, and reuse patterns.

Reused wastewater may be possibly applied to the household requirement include toilet flushing and garden irrigation. For these two reuses, enteric pathogens are identified being the cause of most significant hazard posed by the direct contact of residents with recirculated water (Gerba and Smith, 2005)

Tierney *et al.*, (1977) established the connection to crop quality and investigated that the application of wastewater induce *Poliovirus* which ultimately directed to an enlarged population of enterovirus on the radish top sand lettuces surface and by Al-Ghazali *et al.*, (2000) who pointed *Listeria* species on the alfalfa and parsley plants by the sludge cake applications.

Gale, (2005) investigated the translocation of *Salmonella*, *Listeriamonocytogenes*, *Campylobacters*, *Escherichia coli* O157, *Cryptosporidium parvum*, *Giardia*, and

enteroviruses from plant's root, grown in the soil irrigated by wastewater. The connection between the pathogens induced into agricultural soils (by means of wastewater and sewage sludge reuse, and potentially wastewater irrigation) and the real pathogenic contamination of edible crops is not a matter of generality. It depends on many factors, illustrated below.

2.2. Transmission of pathogens

Among all the possible risks posed by wastewater and its reuse, the infection of plants and soil irrigated by highly contaminated reclaimed water, offers the significant risk to humans (Christova *et al*., 1996; Ottosson, 2003b). Armon *et al.*, (1994) make a direct connection between the quality of reused effluent and plant contamination. Soil contamination is hazardous even when the edible portion of crops are not contaminated, especially in the case of home gardening as the likely possibility of human connection with soil is more.
Jiang *et al.*, (2002) investigated that *Escherichia coli* O157:H7 in warm soil, may survive up to 231 days. In the microbial analysis of crops studies, the determination of microbial population of soil is important especially when the possibility for infection of root, stem, and fruit crops be investigated. Because underground plants are in direct exposure, irrigated by highly contaminated water, therefore their signs of bacterial contamination will high. Indeed, Rosas *et al*., (1984) reported the isolation of upto 94 % of the *faecal coliforms* from root section of the wastewater-irrigated plants.

2.3. Pathogen transmission and irrigation method

Microbial contamination in agriculture has direct link with irrigation method. Research reported the direct transmission of pathogenic microorganisms from irrigated water to the above ground plant surface (Gerba and Smith, 2005). However the vascular systems of plants are sterile. Therefore, the only route for the transmission of pathogenic microorganisms from the irrigated water to crops is the direct surface contact (Mills *et al*., 1925). To reduce the health risks associated with it, avoid the direct transmission route, by installing the underground irrigation method, which provide water under the soil surface. Sadovski *et al*., (1978) reported that pathogen level may be reduced to nearly undetectable level by installing the drip irrigation method.

Enriquez *et al*., (2003) found significantly low transmission rates by employing the underground irrigation method in turf grass irrigated with bacteriophage-seeded water.

2.4. Faecal indicator organisms

Faecal bacteria commonly known as *E.coli*, particularly use as indicator bacteria to determine the level of pathogenicity.

Fewer pathogens in water may be detected reliably, and few may not be identified at all (WHO, 1989).The water, in which pathogenic microorganisms be detected may not be regarded as safe, however low the concentration.

Escherichia coli or more precisely thermotolerant *coliforms* are measured to satisfy the standards suggested for indicator organisms. These are:

- present in the humans faeces and warm-blooded animals;
- determine by simple methods;
- and resistant in natural waters; and
- removal by water treatment

2.5. Safe use of wastewater and excreta in agriculture and aquaculture

Human excreta has widely been used as fertilizers because of its rich organic nature. South East Asian and African countries have reportedly been using human faecal matter to fulfil their fertilizing needs in the fields of agriculture and aquaculture. Septic tanks and public toilets serve as main sources from where faecal sludge is collected, from where this sludge is applied directly (without treatment) or only given primary treatment through storage. Use of this kind of wastewater has rapidly established especially in arid and seasonally arid areas. This natural fertilizer has allowed farmers to subside the use of chemical fertilizer and induced a shift from inorganic to organic farming.

Countries like USA and Saudia Arabia apply advanced wastewater treatment (filtration, disinfection) prior to its application.

There is no doubt that human waste contributes significantly to enhance food production because of its high nutrient content for that its application has been expanding even in the urban fringes of developing nations. In some areas where wastewater is employed untreated or public concerns are not considered lead to the recovering of pathogens among the consuming populations. Havelaar *et al*., (2001) reported that by applying raw faecal sludge

farmers along with their families as well as consumers get more vulnerable to disease transmission. Wastewater reuse guidelines, shown in Table-2.1, were first published by WHO (Hespanhol and Prost, 1994; WHO, 2013).

For both unrestricted and restricted irrigation, intestinal nematode egg guidelines was introduced because of epidemiological concerns. A elevated grade of helminthes removal was then required, particularly as there were some data demonstrating that when treatment of wastewater ensured rates of infection would be very low. The level was set at ≤1 egg per litre, equivalent to a removal efficiency of up to 99.9 % (Havelaar *et al*., 2001).

A bacterial guideline of ≤1000 *faecal coliforms* per 100 ml was recommended for unrestricted irrigation (category A). Epidemiological evidence, particularly from outbreaks, indicated the transmission of bacterial infections such as cholera and typhoid through use of untreated wastewater. It was thought that transmission was less likely to occur through treated wastewater, considering the degree of bacterial removal achievable through treatment and the relatively high infectious dose for some bacterial infections (Bartone *et al*., 1985; Oragui *et al*., 1987; Polprasert *et al*., 1983).

No bacterial guideline was recommended for restricted irrigation (category B) as there was no epidemiological evidence for the transmission of bacterial infections to farm workers when wastewater was partially treated.

Public health safety measures were also considered. They included:

- crop selection
- wastewater application measures
- human exposure control

The theme of the above mentioned management practice revolves around one aim and that is the reduction in exposure of pathogenic organisms. The idea is laid on the principles of hurdling the pathogens movement from the wastewater to the worker, and the precautions illustrated act as obstacles to infectious agents movement whereas by treatment removal of the pathogens achieves. Drip irrigation of wastewater would lead to reduction in contamination of low growing crops whereas by protective gear would allow the farm workers to work in a healthier manner. Amalgamation of protective measures at individual and at advanced levels are encouraged (Havelaar *et al*., 2001).

Table-2.1: Microbial guidelines for wastewater practice in agriculture (WHO, 2013)

Cat.	Reuse conditions	Exposed group	Intestinal Nematodes (/Litre)	Faecal coliforms (/100ml)	Wastewater treatment expected to achieve required quality
A	Irrigation of crops likely to be eaten uncooked, sports fields, public parks	Workers, consumers, public	≤1	≤1000	A series of stabilization ponds designed to achieve the microbiological quality indicated, or equivalent treatment
B	Irrigation of cereal crops, industrial crops, fodder crops, pasture and trees	Workers	≤1	None set	Retention in stabilization ponds for 8-10 days or equivalent helminthes removal
C	Localized irrigation of crops if category B exposure of workers and the public does not occur	None	n/a	n/a	Irrigation technology demanded pre-treatment, but not lower than sedimentation at primary level

Pakistan is already declared as water-stressed country and the prevailing situation lead it towards the water scarcity (Briscoe and Qamar, 2006).One of the options to meet rising water demand is water reclamation and reuse for non-potable purposes (Anderson, 2003). There are two basic approaches generally use for the treatment of wastewater: centralized and the decentralized treatment (Elmitwalli *et al*., 2003; Zeeman and Lettinga, 1999).

The centralized systems usually assemble large quantity of wastewater for treatment to facilitate larger communities, which require big infrastructure (Fisher, 1995; Massoud *et al*., 2009). While, decentralized systems are the onsite treatment systems installed in separate homes and blocks (Gikas and Tchobanoglous, 2009; Crites and Tchobanoglous, 1998).

Cluster systems, which may be either centralized or decentralized, aid more than a single household and reach up to 100 homes and more (Jones *et al*., 2001; Massoud *et al*., 2009). Opposite to the on spot treatment, in cluster systems, network of pipes is needed, cluster systems are suitable in areas with dense population or that have deprived soil having adverse topography.

2.6. Centralized wastewater treatment

Advanced gathering and treatment methods in huge quantities of wastewater tangled in centralized wastewater treatment systems (Massoud *et al.*, 2009). In small rural communities, construction of centralized system, will result in load of loans for the populace (Parkinson and Tayler, 2003).

2.7. Decentralized wastewater treatment

Decentralized wastewater management is a system for dumping of wastewater from individual homes, colonies, communities at, or near the point of waste generation (Massoud *et al*., 2009).

A decentralized system collects wastewater from one or several houses in the same area. The wastewater is partially treated within the system and then either discharged into the soil for final treatment or conveyed to a small wastewater treatment plant. A septic system or on-site wastewater treatment system is an example of a decentralized system for a single home. Larger septic systems are sometimes used for a group of homes or a small business. Another type of decentralized system is used by communities or larger commercial developments.

These systems use a septic tank to trap large solids, then use a “package plant” (a small treatment plant that contains multiple treatment processes) to treat the wastewater. The treated wastewater or effluent is discharged below ground through soil absorption field, drip irrigation, or sometimes spray irrigation. There is a growing interest in these types of

systems because they are typically less expensive to install or operate than large centralized systems.

Several advantages are associated with on-site wastewater treatment. This system will fulfil our future infrastructure needs in affordable range.

On-site recycled water allow the ground water to recharge. Decentralized system avoids the pollution potential associated with conventional system that occasionally occurs when fault place in central sewage treatment systems and they fail (Hedberg, 1999; Luostarinen *et al.,* 2007)

2.8. Septic tank

Anaerobic wastewater treatment systems are considered sustainable and appropriate for on-site treatment (Zeeman and Lettinga, 1999) because of their energy efficiency, which requires small foot print.

First stated application of septic tank was in France in 1860 that was a 'box' placed between the home and the, which produced a clean effluent and reduced the amount of solids entered to the soil. In the United States, domestic septic tank was first used in 1883 which have two-section tank design. After that the use of septic tank use increases rapidly in most parts of world (Butler and Maccormick, 1996).

The septic tank in a conventional on-site wastewater disposal system offers only primary treatment with little biological degradation. The soil absorption field obtains significant load of suspended solids. These suspended solids have high harmful bacterial load and pathogens, they obstruct the pores of the innate soil, eventually affecting the system functionality. To avoid the surface water contamination by conventional septic tank maintains (100-feet) minimum distance between both.

2.8.1 Advantages and disadvantages

2.8.1.1. Advantages

- Uncomplicatedness, consistency and economical
- Easily maintainable
- Nutrients recycling
- Long life up to twenty years

2.8.1.2 Disadvantages

- Sitting restrictions for septic systems instalment involves soil texture and absorbency, bedrock and groundwater elevations
- Conventions relating to set-backs from effluent drainage, pipe lines must be consider
- Limitations on the character of incoming wastewater must be consider during project
- Inadequately installed systems may present nitrogen, phosphorus, organic matter, and
- Bacterial and viral pathogens in the neighbouring environment (Wagner *et al*., 2002).

2.9. Introduction to membrane technology

Membrane is an intentally introduced blockage that act as a selective barrier and allows specific objects to pass through its pores. In water and wastewater treatment systems membrane may be differentiated into four groups on the basis of filtration. Microfiltration (MF), ultra-filtration (UF), and nano filtration (NF), reverse osmosis (RO).

Membrane based treatment systems are increasingly use for water and wastewater treatment membrane technology has immense use in industrial countries and growing economies like China (Massoud *et al*., 2009).

Table-2.2: Membrane filtration and pathogen removal (USEPA, 2003)

Membrane Filtration	Size (µm)	Removal
Micro filtration (MF)	0.1	Removes suspended or colloidal particles and may retain bacteria
Ultra filtration (UF)	0.01	Removes organic macro molecules and ability to remove viruses
Nano Filtration (NF)	0.001	May remove dissolved contaminants and renders water soft
Reverse Osmosis (RO)	0.0001	Designed to remove dissolved contaminants

In principle, it is also striking for the TC and DC as it introduce blockage for regulating hygiene hazards and its modular construction allows implementation on all possible scales (Churchouse and Wildgoose, 1999). Research and development of membrane systems aimed specifically for the DC remains limited to isolated cases and is often not published in the available literature (Parkinson and Tayler, 2003;Wilderer and Schreff, 2000). Details of membrane filtration, their sizes and pathogen removal are given in Table-2.2.

2.9.1. WFMF membranes

In most real applications, a membrane will eventually become fouled. Operational strategies such as scouring, sub-critical flux operation, back flushing etc. reduce the rate of fouling, but will not prevent the eventual fouling of the membrane. The ability to be cleaned and recover permeability after being fouled is a critical aspect of the technical viability of any membrane technology. It is usually possible to find a concoction of chemicals that will remove any given fouling layer. However, if chemical cleaning may be avoided the applicability, economics and environmental impact of the technology will improve greatly. It will also make the technology more sustainable in developing economies, where regular access to chemical cleaners may not be guaranteed. In previous investigations into WFMF, a major advantage was that the system never required a chemical clean. Mechanical agitation (e.g. pulsing) or drying was sufficient to remove the fouling layer. This is probably because the WFMF system does not have "pores" that may be penetrated by foullants as in a conventional rigid membrane. It may filter the water by removing suspended matter, dissolved matter and all pathogens as shown in Figure-2.1.

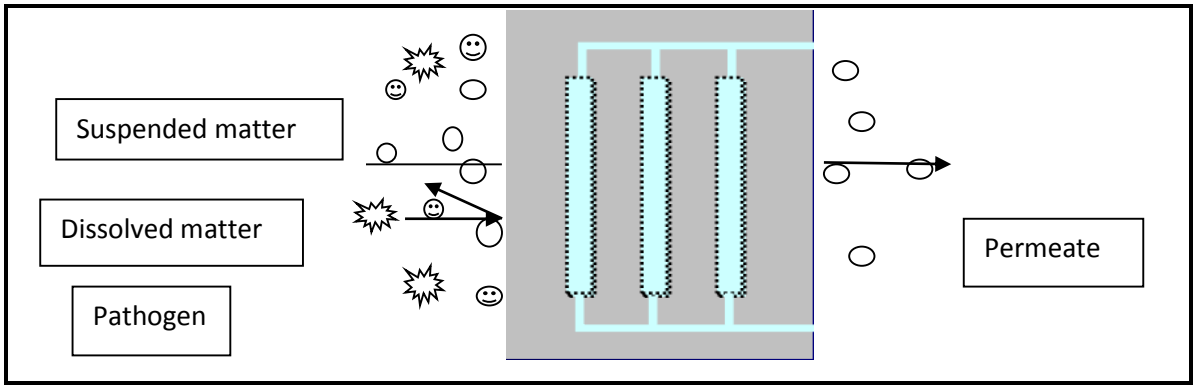


Figure-2.1: Membrane filtration

2.9.2. Membrane based septic tank (MBST)

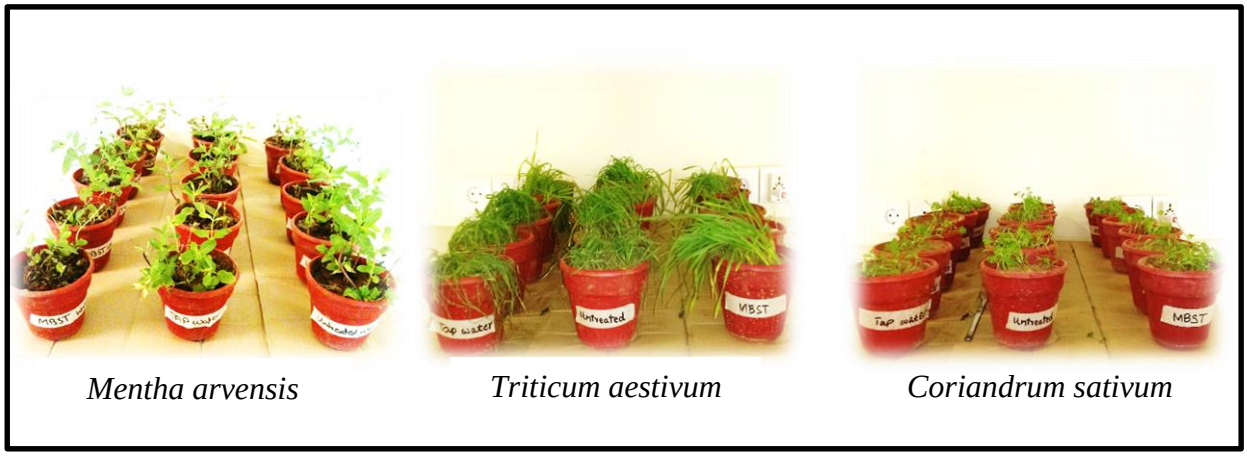
Conventional wastewater treatment techniques take more space and are less efficient, therefore it is important to evaluate advanced wastewater treatment technologies which produce reusable water in comparatively less time. But there is need of extensive research in the field of advance wastewater treatment to make them economically viable.

3. Methodology

3.1 Experimental setup

A pilot scale membrane based septic tank was studied. The system was optimized having submerged woven fibre microfiltration membrane module with dead end mode, outside in flat spiral sheet having 1-3 µm pore size with $1m^2$ effective surface area. Two membranes (M1 and M2) were on-stream during the study period having 1 (10 mm wire-mesh module casing) and 3 (1 mm wire-mesh modified module casing) permeate outlets respectively. Domestic wastewater was fed into the septic tank using a membrane flux control system. Ideally membrane flux should be equal to the inflow of the system but due to the membrane fouling limitation the system was operated at different flux rates. A peristaltic pump used to draw permeate was adjusted with the filtration (10 min) and relaxation (1 min) mode.

The total of fifty-four (54) pots consisted of three (3) plant species, each plant species having 6 replicas. Seeds and vegetative growth were used to personify the following plant species: *Triticum aestivum* (wheat), *Coriandrum sativum* (coriander) and *Mentha arvensis* (mint).



Mentha arvensis *Triticum aestivum* *Coriandrum sativum*

Figure-3.1: Plant species grown by using untreated, MBST effluent and tap water

Untreated (after primary settling) wastewater was collected from the domestic wastewater inlet before entering into the compartment of membrane based septic tank, and treated water was taken from the membrane based septic tank permeate. Tap water was taken as control. The physico-chemical and microbiological characteristics associated with

untreated (after primary settling), treated (through membrane based septic tank) and tap water used for crop irrigation in current studies is sighted in Table-4.1.

3.2 Sample collection

MBST effluent and untreated wastewater was collected from the membrane based septic tank installed at IESE, SCEE, NUST in sample bottles (autoclaved at 121 ^{o}C and 15 psi for 15 min) and used for irrigating plants on an alternative day. Meanwhile, the MBST effluent along with untreated wastewater and tap water was subjected for microbial analysis. After maturation phase plant samples were collected by using aseptical scissors.

3.3 Isolation of bacterial strains from membrane based septic tank and plants

Serial dilution technique was performed as per standard procedure. After preparation of dilution and mixing of the test tubes 0.1 ml of sample was taken and plated onto nutrient agar plates. Spread plate technique was performed to plate the sample and allowed to grow in the incubator for 24-48 hours at 37 °C. Colony counting was done after 24 hours of incubation.

Irrigation water and vegetable samples were analysed quantitatively for the determination of total bacterial count, *E.coli, Salmonella* and Helminth eggs. Vegetable sample (10 g) was weighed into 180 mL of phosphate- buffered saline and rinsed vigorously. The water resulting from the rinsing was used for the determination of total bacterial count and *E.coli* analysis. The membrane filtration (MF) technique was used to analyse these parameters. 10 mL of each of the samples (irrigation water and solution) were separately filtered through a 0.45 m pore size membrane filter. The filter was then placed on EMB agar plate for the detection of *E.coli* respectively. Incubation was then done at 37 °C ± 0.5 °C for the determination of *E.coli*, for 16-24 hours. Colonies were counted using a colony counter.

3.4. Purification of bacteria

Maximum possible bacteria were marked on the basis of their morphological characteristics such as shape, size and color and isolated on fresh agar plates. Selected colonies were single colony streaked. Streak plate technique was performed to isolate the colonies. Plates were incubated for 24-48 hours at 37 °C. Colonies were streaked for 3-5

rounds or more till assured of having obtained a pure colony. Each pure colony was stored in the refrigerator for further use.

3.5 Isolation of *Escherichia coli*

Eosin methylene blue agar (EMB) was utilize for the detection of desired microorganism from membrane based septic tank effluent and irrigated crops. EMB plates were plated with treated water and plant samples upto 10^{-4} dilution.

3.6 Isolation of *Salmonella*

Salmonella was enumerated and isolated from the MBST effluent and irrigated crops by using standard method. It is a five day procedure. Weigh out 10 g of crops samples with a sterile wood spatula, put it into an erlenmeyer flask and soaked under 90 mL buffered peptone water to obtain 1 part sample +9 part buffer and incubated for 24 hours at 37 ºC. Add 1 mL of incubated sample with a pipette into 10 ml tetrathionate broth, and 0.1 ml of incubated sample into salenite broth, incubated both at 37 ºC for 24 hours and plated on BGA and XLD agar plates and incubation was done. Enumerate the black centered colonies from BGA agar plates, picked and streaked on nutrient agar plates. On XLD agar plate a typical *Salmonella* colony surrounded by a reddish zone was picked and recorded. Further confirmation was done by means of API 20E kit.

3.7 Morphological characterization

3.7.1 Colony morphology

Single colonies were studied for their color, shape, size, margin, elevation, texture etc. to observe the characteristics of the isolated strains.

3.7.2 Cell morphology

Gram staining was performed as per standard method for all the isolates of the wastewater sample.

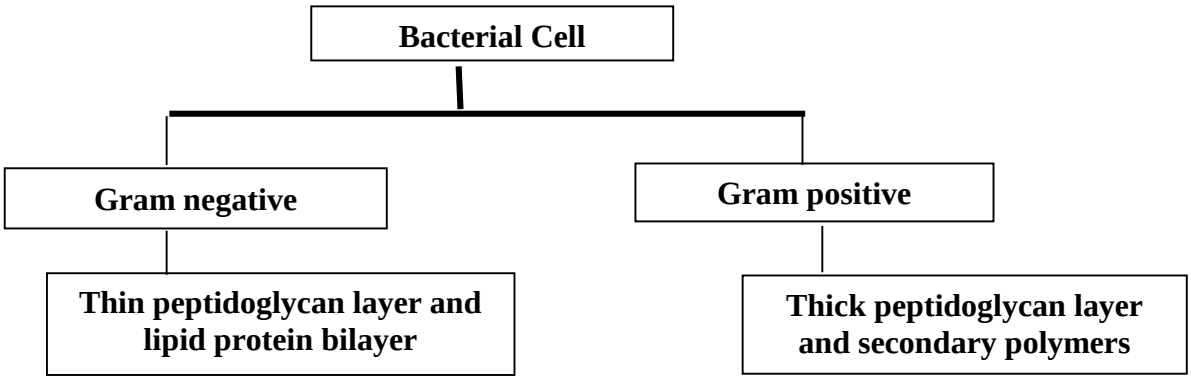


Figure-3.2: Bacterial cell staining

3.8. Analytical profile index (API)

API 20E (Biomeurix, Canada) is a test kit used for the identification of enteric and other non-fastidious bacteria. It comprises of a plastic strip that has 20 mini cupules in it. Each cupule contains a specific medium for biochemical characterization. For performing the test a saline suspension (0.85 % NaCl) was prepared and autoclaved. Saline suspensions were formed for fresh colonies; the suspensions were added in the cupules of API strips till the end except for citrate utilization (CIT), voges–proskauer (VP) and gelatin liquefaction (GEL), where the capsule was filled completely. A drop of mineral oil was added in the cupules filled to neck to avoid drying out. The strip was covered with the lid provided and placed in incubator overnight. Color changes were noted and results was recorded (Table-3.3). In the entire carbohydrates test, fermentation is shown by acid production and is indicated by yellow color. Few cupules have to be provided with reagents, supplied by the manufacturer. TDA reagent is added into TDA cupule. James/Kovacs reagent was added to IND while VP1 and VP2 were added to VP. The test was allowed to develop for a few min and results were recorded.

An additional oxidase test was performed to develop seven digit code required for API web software. In order to perform this test tryptic soy agar plates were prepared. Colonies were grown and 1 % *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride was added. A color change to purple was noted as positive while no color change as negative result.

Table-3.1: API result indicator color

Cupule Medium	Results	
	Positive	**Negative**
O-Nitrophenyle-B-D-galactoside	Light yellow to yellow	Colorless
Arginine dehydrolase (ADH)	Light to dark red	Yellow
Lysine decarboxylase (LDC)	Light to dark red	Yellow
Onthinine decarbolyxase (ODC)	Light to dark red	Yellow
Citrate utilization (CIT)	Blue green to Blue	Pale green to yellow
Hydregen sulfide (H2S)	Black	Grey to colorless
Tryptophan deaminase	Deep red	Brown
Indole (IND)	Pink	Colorless/pale green/yellow
Voges–proskauer (VP)	Red/Pink	Colorless/ slight pink
Gelatin liquefaction (GEL)	Goes Black	No change
Glucose (GLU)	Yellow	Yellow green, green, blue
Mannitol	Yellow	Yellow green, green, blue
Inositol	Yellow	Yellow green, green, blue
Sorbitol	Yellow	Yellow green, green, blue
Rhamnos	Yellow	Yellow green, green, blue
Sucrose	Yellow	Yellow green, green, blue
Melibiose	Yellow	Yellow green, green, blue
Amygdaline	Yellow	Yellow green, green, blue
Arabinose	Yellow	Yellow green, green, blue

3.9 DNA extraction from *Escherichia coli* and *Salmonella*

The pure cultures of *Escherichia coli* and *Salmonella* were obtained from the American type culture collection center (ATCC). *Escherichia coli* O157:H7 strain EDL 933(ATCC 43895) and *Salmonella typhimurium (*ATCC 14028), the cultures were handled as per manufacturer's instructions. The PCR instrument was optimized initially with pure cultures followed by drinking water samples for detection of *Salmonella* and *Escherichia coli*.

DNA extraction, PCR procedure, BLAST procedure for primers, primers dilution, PCR optimization of four parameters (i.e. temperature, $MgCl_2$ concentration, template DNA

amount and primer concentration), development of PCR reaction mixture protocol, enrichment of samples, agarose gel electrophoresis, sensitivity analysis of PCR instrument and detection of microbes from water and plant samples were studied.

DNA of *Escherichia coli* and *Salmonella* was extracted by using kit method and its description is as under:

3.9.1 Extraction using kit

Prepease kit (Affymetrix, Canada) and the provided protocol was used for DNA extraction. Steps of DNA extraction are as under:

1. Addition of 0.24 mL of homogenization buffer after preparation of bacterial suspension followed by vortex mixing.
2. 0.2 mL of chloroform/isoamyl alcohol and 0.8 mL of protein precipitation buffer was added.
3. Centrifugation was done at 13,000 rpm for 4 min and 0.88 mL of supernatant was transferred to new vial with 0.62 mL of isopropanol.
4. It was mixed followed by centrifugation at 13,000 rpm for 4 min. DNA precipitated out.
5. The pellet was washed with 70 % ethanol followed by centrifugation at 12000 rpm for 2 min.
6. Supernatant was aspirated and DNA pellet was dried followed by addition of 50-300 µl of DNA resuspension buffer.
7. The vial was vortex mixed and stored at -4 °C.

3.9.2 DNA extraction for detection of *Escherichia coli* and *Salmonella*

Soil DNA Isolation Kit (Norgen, Canada) was used for nitrifying bacteria. This kit is provided with the ability to remove all traces of humic acid content such as manure and is therefore best suited for isolation of DNA from soil and wastewater samples. Major steps of DNA extraction are mentioned in the Annexure B.

3.10 Selection of primer

Primers (Affymetrix, Canada) used for detection of selected species are listed in Table-3.4. PCR amplification was carried out in 25 µL reaction mixture containing 1.25 unit of taq polymerase (Bio basic, Canada) with manufacturers reaction buffer and 25 Mm $MgSO^{4-}$,

10 μM of each primer (Table-3.2) and 2.5 mM of dNTPs. The PCR mixture was placed in PCR (9600 TE thermocycler, Taiwan) for amplification.

It was run at an initial denaturation of 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 1 min, annealing, elongation at 72 °C and a final extension of 10 min at 72 °C. Primers selected for amplification of bacteria important in wastewater treatment were as under.

Table-3.2: Primers and target genes for selected bacterial species

Primers	**Sequence (5'-3')**	**Target Species**	**Annealing temp (ºC)**
Int-F	GACTGTCGATGCATCAGGCAAAG	*Escherichia coli*	67.9
Int-R	GGAGTATTAACATTAACCCCAGG		
SIRA2-F	GCCGTACTAACGCCGTTGAC	*Salmonella typhimurium*	63.5
SIRA2-R	TAGCGATAGCTGTTCACCGT		

3.11. PCR amplification

For amplification of DNA template a complete mixture was formulated that comprises of the following ingredients, purpose of each ingredient is presented in Table-3.3.

Table-3.3: PCR ingredients and their purpose

Ingredients	**Purpose**
Taq DNA polymerase	Amplifies the DNA
Mg ions	Serve as cofactor
Buffer	Keep conditions optimised
dNTP	Nucleotides to be added
DNA template	Sample DNA
Primers	Specific binding to DNA template

Steps vary for different microorganisms and enzymes of bacteria however a general range of a PCR cycle has been provided in Table-3.4.

Table-3.4: Steps of a PCR reaction

Step	Temperature (°C)	Time (min)
Denaturation	92-95	2:00-5:00
		00:30-1:00
Annealing	Varies	1:00-3:00
Elongation	72	1:00-3:00
Final Extension	72	7:00-10:00

After the reaction was completed gels were prepared for the amplicons and results were recorded in the form of a picture.

3.12. Helminthes detection

Helminth eggs were counted by using modified Bailenger method given by USEPA. About 50 g of each crop part washed in 1 L sterile distilled water. MBST effluent and the washed water placed for over 2 hours for settling period. 90 % of the supernatant was removed from each sample (MBST effluent and the crops) by using sterile pipette and the sediment after transferring to several tubes was centrifuged for 15 min at 1500 rpm. After that sediment was subjected to recentrifugation in a single tube (collected from all the tubes). Acetoacetic buffer (pH 4.5) was used, in equal volume for the suspension of the pallet formed. Added ether (2 volumes) in a pallet and vortex the mixture. The mixture was then again centrifuged at 1000 rpm for next 15 min. Volume of the pellet was recorded. 5 volumes of $ZnSO_4$ solution were used, and the pellet was suspended in it. The mixture was vortex again before transferring to McMaster slide. The slide was then viewed under a microscope for the enumeration of helminthes eggs at 40X magnification.

Equation- N = AX/PV

Where N = number of eggs per litre of sample,

A = the mean of counts from the 3 slides,

X = volume of the final product (mL),

V = original sample volume (L) and

P = volume of the McMaster slide (0.15 mL).

3.13. Growth and biomass analysis

The root systems of sample plants were carefully excavated by auger method ensuring minimum breakage. Further, these were washed thoroughly and completely exposed. However, their dimensional structure as well as finer roots and nodules present on them kept undisturbed. Each root system, so obtained after washing, was spread on a blotting paper to get its original morphology. Root and shoot were separated, their fresh biomass were recorded. All observations related to root, shoot growth and nodulation were recorded on five of the randomly selected plants from each treatment / replication and thus, the mean value for each trait was obtained taking into the average of five replications.

Dry biomass of both root and shoot were obtained after drying the plant (root and shoot separately) samples in hot air oven at 65+5 ^{o}C to constant weight. Ratio of root and shoot were calculated based on length, lateral spread and dry biomass for each species. In order to establish comparative root architectural pattern, root system of one healthy representative plant was arranged on a graph paper. Then, border line was drawn for each rootlet accordingly complete rough outline was developed. This was photocopied on an A4 sheet and traced further on a transparent paper and fed into computer through digital camera.

3.14. Detection of endophytes

For the detection of endophytes plants were harvested followed by the separation of roots and shoots. Plant surface was tender by placing them in 3 mL of 0.9 % NaCl (180 rpm for 30 min) and then washed by using sterilized distilled water for 2 min. Plant material was sterilized with 70 % ethanol. Treat sterilized plant material further with 1 % NaOCl and washed with sterilized distilled water. Surface sterilized roots or shoots were homogenized by using pestle and mortar in 12 mL NaCl solution (0.9 %, w/v). The homogenized sample was agitated for 1 hour at 30 ^{o}C. After settling of solid material, serial dilutions upto 10^{-3} were spread on nutrient agar and then incubated for 24 hours.

3.15. Analytical methods

Untreated wastewater and MBST effluent were analyzed by following standard methods (APHA *et al.,* 2012). COD and phosphate was determined by using closed refluxed and Hach spectrophotometery methods respectively. Turbidity was measured by using turbidimeter.

3.16. Statistical analysis

Arithmetic mean and standard deviations were computed by using R-statistics software (version 3.1.0 for windows). Total bacteriological load and indicator organism (*E.coli*) load on crops and MBST effluent along with untreated wastewater were normalized using log factor transforming the original data before analyzing. Single-factor ANOVA, used to gauge the difference ($p < 0.001$) between crops and waters applied.

Chapter 4

4. Results and discussions

4.1. Removal efficiency of membrane based septic tank

Membrane based septic tank effluent is a mixture of suspended and dissolved organic matter along with living and dead bacterial population. The internal process, of septic tank is similar as anaerobic process, settling of solids, the anaerobic conversion of organic matters and accumulation or digestion of sludge (Zeeman and Lettinga, 1999). Water from septic tank, which has soluble substances, is discharged out. Settled sludge will be stabilized by anaerobic digestion. The solid that are not decomposed remain in the tank in the form of sludge. So the performance efficiency regarding the effluent may be measured by organic matter removal and reduction in bacteriological load.

Table-4.1: Percentage removal efficiency of membrane based septic tank

Total Bacteriological Load (%)	*E.coli* removal (%)	COD removal (%)	Phosphate removal (%)	Turbidity removal (%)
84.2	88.4	73.0	48.6	96.0

4.2 MBST performance parameters

4.2.1 COD removal efficiency

Organic matter removal is the key point in wastewater treatment. The removal is generally measured in terms of COD and TOC. The average COD removal percentage of membrane based septic tank effluent was 73 % as shown in Table-4.1. In MBST the removal of COD is only due to filtration that's why the removal efficiency in term of COD is low. Figure-4.1 shows the detail performance of system in term of COD removal of wastewater. Ideally it should be 100 % which is not achievable as the membrane has pore size of 1-3 µm. COD removal rate may be increased up to 95 % by reducing the pore size. Effluent produced by nano-filtration was of high quality with high elimination of soluble organic matter (>90 %) and ionic species (50 %). It may be concluded that direct dense membrane

filtration is a favourable candidate for efficient treatment of wastewater for unrestricted reuse. Ramon *et al*., (2004) reported that the membrane with a molecular weight cut off of 0.2 kDa was able to achieve an organic removal rate of 93 %, showing that the pore sizes of the membranes have an important impact on the organic removal efficiency.

Our results were similar to the findings by Shin *et al*., (1998), who also confirmed that wastewater was low in soluble organic carbon. However, they were contradictive to the findings of Jefferson *et al*., (2001), who claimed that wastewater is relatively low in suspended solids and it has a greater proportion of soluble organic carbon.

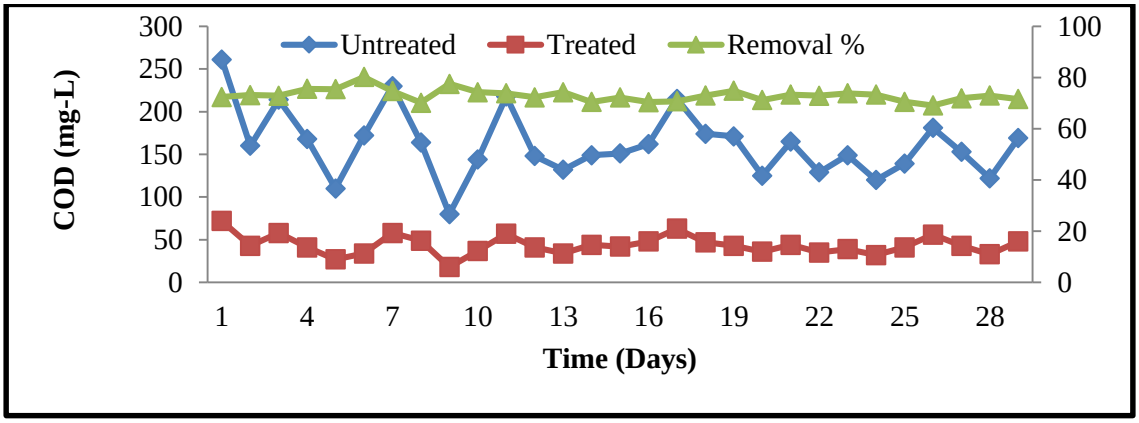


Figure-4.1: Percentage removals of COD in untreated and treated water through membrane based septic tank

4.2.2 Phosphate-phosphorous (PO_4^{3-}-P) removal

In advanced wastewater treatment the treatment efficiency of certain nutrients like ammonia nitrogen and phosphorous is also monitored in the treated effluent along with conventional COD removal. In this study MBST was installed in an institution with the objective that the influent wastewater contained more nutrients then other organic matter as wastewater contains more urinal part then manure.

Phosphorous removal efficiency of MBST was 48.6 % (average) shown in Table-4.1.The mean total concentration of phosphorous found in feed water and resulting permeate is 17.34 mg/L and 9.06 mg/L respectively. Figure-4.2 clearly exhibit that MBST performance in terms of nutrient removal is low, MBST permeate mostly maintains low soluble nutrient removal as phosphorous from the feed water may pass though the membrane and remain in permeate (Khan *et al*., 2013).

The average TP concentration in Elmitwalli and Otterpohl, (2007) study was 9.8 mg/L, of which the orthophosphate concentration and particulate phosphorus concentration constituted 8.0 and 1.8 mg/L, respectively. Compared with the study of Elmitwalli and Otterpohl, (2007), the septic tanks had no influence on the removal of phosphorus. This may be explained by the fact that particulate phosphorus in wastewater constitutes less than 30 % of the TP, and is largely in colloid form resulting in ineffective removal by sedimentation in the septic tanks. Due to the uses of phosphorus containing detergents, the phosphorus concentration in wastewater is present at similar levels compared to the entire municipal wastewater. Our results are further supported by the (Li *et al*., 2008) who reported that the average TP in permeate was 6.7 mg/L.

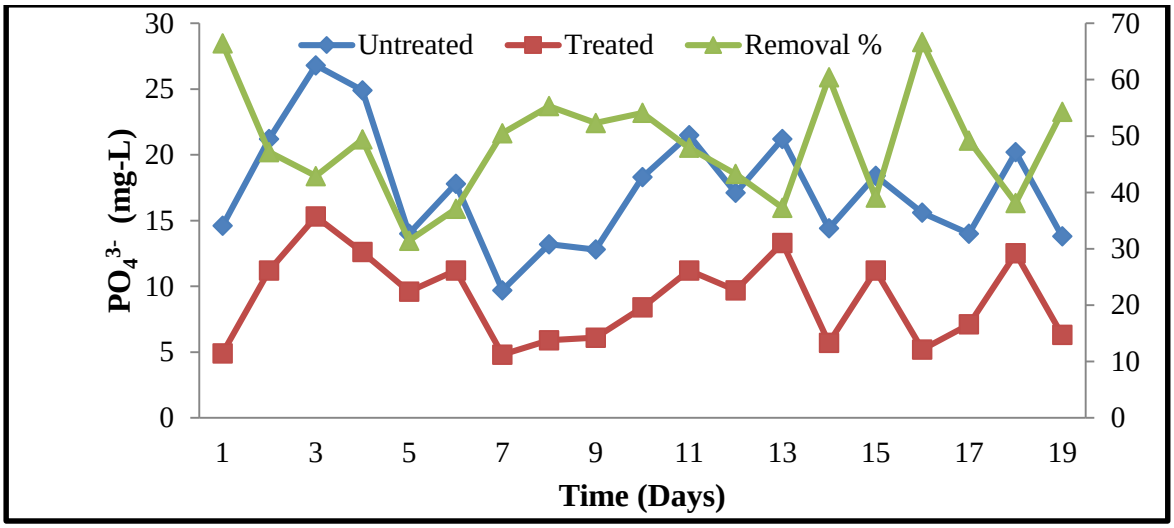


Figure-4.2: Percentage removal of phosphorous in untreated and treated water through membrane based septic tank

4.2.3 Turbidity removal

As may be viewed from the data spotted in the Figure-4.3 there is considerable variability shown in the effluent quality in the concentration of suspended matter measured in terms of turbidity, average rate of turbidity deduction was 199 to about 7.3 NTU (about 96 %) as shown in Table-4.1. This change in concentration in the effluent quality seems to be an inherent characteristic of MBST to remove the impurities. The turbidity reduction of up to 100 %, by employing membrane has been recorded (Ahn *et al*., 1998; Ramon *et al*., 2004). Turbidity was reduced from 140 NTU in the feed water to less than 1 NTU in permeate. Due to the exclusion of urine in wastewater, permeate was colorless (Li *et al*., 2008).

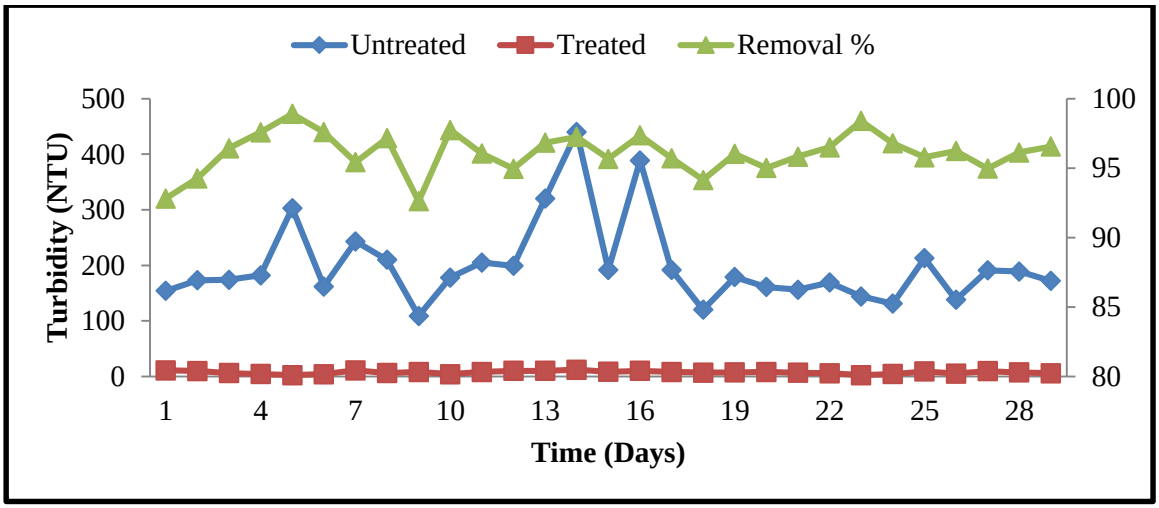


Figure-4.3: Percentage removal of turbidity in untreated and treated water through membrane based septic tank

4.3 Removal of total bacteriological load and *fecal coliforms*

1-3 µm membrane submerged in septic tank was capable of removing 84.2 % total bacteriological load and 88 % *E.coli* removal as shown in Table-4.1, Table-4.3 shows the 1 Log deduction of bacteriological load present in feed water.

In membrane having pore size 0.0062 µm *Escherichia coli* could not be existed, as *Escherichia coli* bacterium has a size of 0.5 µm in width by 2 µm in length. Due to the lager membrane pore size used in this study, it is therefore not surprising that permeate was not free of *Escherichia coli* (Li *et al*., 2008).

4.4 Crop irrigation by using membrane based septic tank

Results revealed that crops irrigated by using membrane based septic tank effluent gave significantly higher yields and increase the plant growth as compared to crops fed by using tap water, although crops treated with untreated wastewater produce the highest growth, but there is very slight difference between the results shown by untreated wastewater and membrane based septic tank permeate in terms of growth and plant biomass. And this slight difference is indicated towards the low nutrient removal associated with membrane based septic tank. As observed previously, watering with wastewater enhance plant growth and mass, as compared to crops fed with tap water only (Salukazana *et al*., 2006). Table-4.2 presented the plant growth and yield as indicated by root and shoot length and dry plant biomass respectively.

Table-4.2: Mean yield and growth of plants under untreated wastewater, MBST effluent and tap water

Parameters	*Triticum aestivum*			*Coriandrum sativum*			*Mentha arvensis*		
	Untreated Water	Treated Water	Tap Water	Untreated Water	Treated Water	Tap Water	Untreated Water	Treated Water	Tap Water
Germination %	97.77	88.88	62.22	91.1	84.44	71.10	……	……	……
Root length	15.33 ±1.05	14.3 ±0.72	9.65 ±1.13	11.03 ±0.65	10.43 ±0.45	7.25 ±0.75	19.96 ±0.25	19.25 ±0.64	15.55 ±0.83
Shoot length	18.65 ±0.66	17.81 ±0.82	13.08 ±0.85	12.93 ±0.72	12.33 ±0.53	9.93 ±0.57	25.63 ±0.64	23.93 ±0.78	16.01 ±0.41
Dry biomass root	97.5 ±3.44	89.83 ±3.65	65.83 ±3.76	68.33 ±1.96	66.16 ±1.47	55.66 ±1.86	118 ±0.63	112.5 ±2.07	91.66 ±2.80
Dry biomass shoot	108.16 ±3.04	100.6 ±3.93	74.5 ±1.87	81.83 ±2.63	73.83 ±1.60	66.83 ±1.47	128.16 ±2.48	122.33 ±2.58	100 ±2.28

The projected supply-demand gaps pertaining to plant nutrients are wider in South Asia, where fertilizer use is rapidly increasing (FAO, 2010). These gaps in fertilizer demand and supply may be partly offset with nutrients in wastewater (Sato *et al*., 2013). Reclaiming nutrients in wastewater improves the soil quality (Khatun and Amin, 2011).

4.5 Microbiological examination

Total bacteriological load (average) on irrigated water and the crops are spotted in Table-4.3 in logarithmic form. The untreated wastewater and the crops irrigated with it, recorded high level of contamination as compared with water collected from MBST (effluent) and tap water and crops irrigated with them. MBST effluent and crops dwell by utilizing it, has shown more than 1 log reduction ($p<0.001$) of bacterial load. Mean level of the bacteriological load on root part of each plant *Triticum aestivum*, *Coriandrum sativum* and *Mentha arvensis* dwell by using MBST effluent has shown 5.9, 5.7 and 5.9 $\log_{10}$ CFU.g^{-1} higher load than other plant parts, because of direct contact with irrigated water (shown in Table-4.3). Previously discussed in WHO guidelines, means of wastewater application could reduce the contamination if other plant parts not come in contact (Havelaar *et al*., 2001).

Table-4.3: Bacteriological loads on water and plants irrigated with untreated wastewater, membrane based septic tank effluent and tap water

Irrigating Waters	Bacteriolo-gical load on water	*Triticum aestivum*			*Coriandrum sativum*			*Mentha arvensis*		
		Root	**Shoot**	**Leaf**	**Root**	**Shoot**	**Leaf**	**Root**	**Shoot**	**Leaf**
Untreated wastewater	9.4	5.94	5.65	5.42	5.79	5.55	5.29	5.99	5.87	5.69
MBST effluent	8.6	4.56	4.43	4.2	4.40	4.24	4.1	4.7	4.5	4.2
Tap water	2.7	2.5	2.4	2.3	2.5	2.3	2.2	2.7	2.4	2.3

Units for Bacterial load is in $\log_{10}$ CFU.g^{-1} and CFU.mL^{-1}

4.6 Bacteriological characterization of MBST wastewater and irrigated plants

4.6.1 Colony count and cell characteristics

The extent and nature of activity in an effluent and its consequential effects on plants is found out by taking into the account the total number of bacteria and types of bacteria present in it. The CFU/mL, found out by spread plate technique indicates that the bacterial activity was greatest in influent that in turn introduce into the membrane based septic tank and the plants irrigated by utilizing it. Untreated wastewater followed by MBST and then tap water. Thus it may be anticipated that the plants dwell by utilizing untreated wastewater showed high level of contamination as compared to the plants dwell by up taking MBST treated water. The result indicates the performance of MBST in terms of microbial count. The colony count for all types of water and plants irrigated are spotted in Table-4.3.

6 bacteria were isolated from MBST effluent among which 2 were studied for their transmission into the plant parts after their identification through API 20E. Each isolate was given a code according to its origin like MBN-1, PWR1, PCR1, PMR1 and so on. The results for cell morphology, colony morphology for all isolates and API of the gram negative isolates are given in Annexure A and discuss here.

The gram staining results showed that gram-negative rod-shaped bacteria were dominating. Results are supported by the previous findings that gram negative bacteria are

phylogenetically more diverse than gram positive bacteria and this may be used as an explanation of their dominance in biological wastewater treatment.

4.6.2 Isolation of *Escherichia coli*

Escherichia coli was isolated from MBST effluent only that dark blue black, mucoid colonies with green metallic sheen on eosin-methylene blue (EMB) agar. The isolated colony was further identified by API 20E kit and the seven digit code (5144552) generated also confirmed the isolate to be *Escherichia coli.*

4.6.3 Isolation of *Salmonella typhimurium*

Salmonella typhimurium was also isolated from MBST effluent only that red colonies with black centre on XLD agar plates. Which were further confirmed by using BGA agar and *Salmonella- Shigella* (SS) agar plates. The isolated colony was further identified by API 20E kit and the seven digit code generated (4504552) also confirmed the isolate to be *Salmonella typhimurium.*

4.6.4 Analytical profile index (API) 20E identification

Normally Bergey's manual is used for identification of activated sludge however various commercial products are also being applied for identification (Jiang and Morgan, 2001). API 20E system may be applied for identification of dominating microorganisms i.e. gram negative bacteria. API web software used confirmed many isolates. The isolates of the effluent dominated by gram negative were identified as *Enterobacteriaceae*. *Enterobacteriaceae* includes various pathogenic bacteria such as *E.coli, Salmonella, Shigella, Yersina* along with many harmless symbiotic bacteria. Results are supported by the previously conducted researches that pathogenic organisms of concern associated with wastewater reuse include *E.coli, Salmonella, Shigella, Legionella,* and enteric viruses (Finley *et al*., 2009; Rose *et al*., 1991). API identification of isolates with their generated codes are given in Table-4.4.

Table-4.4: API 20E results for isolates of MBST and plants sp.

MBST	Targeted Plants
Escherichia coli (5144552)	*Escherichia coli* (5144552)
Salmonella typhimurium (4504552)	*Salmonella typhimurium* (4504552)
Klebsiella pneumonia (5215773)	
Proteus mirabilis (0736000)	
Serratia plymuthica (1200063)	

After identification from API when these bacteria were studied for their sizes from literature then it reveals that isolated bacteria sizes are smaller than the pore size of membrane (1-3 μm) as reported earlier, that's why they flow out and crosses the membranous barrier. Among all isolates *E.coli* has been found to be the ideal indicator organisms to point the existence of these pathogens as reported earlier by (Ottosson, 2003a). *Salmonella typhimurium* was also found in secondary-treated wastewater where the cells of bacteria lose cultivability but retain viability and the potential to revert to the metabolically active and infectious state (Oliver *et al*., 2005).

Klebsiella pneumonia is the clinically most important specie of genus *Klebsiella,* has the bacterium size ranges from 0.3~1.0 μm in diameter, 0.6~6.0 μm length long (Rees *et al.,* 1998). *Proteus mirabilis* also isolated from treated wastewater, as it has a bacterium size of 1-2 μm.

Proteus mirabilis instead of producing pathogencity is known for its role in promoting plant growth, it lowers the harmful impact of heavy metals present in wastewater and increases plant growth rate.

Islam *et al*., (2014) reported that it decreases the oxidative injuries caused by Zn in plant roots and shoots and it significantly enhanced the activities of catalase, guaiacol peroxidase, superoxide dismutase and ascorbic acid and lowered the proline accumulation in Zn stressed plants.

Proteus vulgaris strain isolated from wastewater also belongs to the family *Proteus*, it is a chemoheterotroph bacterium. And the size of individual cells varies from 0.4~0.6 μm by 1.2~2.5 μm. Yu and Lee, (2013) reported that either synthetic or biological/bacterial indole

of *Proteus vulgaris* could increase the growth of cabbage plant. He reported that the vigor index and fresh weight of the seedlings were increased by 39.9 and 32.6 %, respectively when the seeds of cabbage were bacterized with *Proteus vulgaris* cells (1x 10^7 CFU/mL).

4.7 Transmission of *E.coli* from membrane based septic tank treated water into plant parts

All sampled vegetables showed more or less similar adaptivity to the treated water. However, treated water has relatively high counts than the vegetables. All vegetables showed similar trend in transferring of *Escherichia coli* bacteria. Each vegetable has relatively high count in its root section as compared to the edible part except mint probably because of larger surface area accessible for the microorganisms. High counts in root section indicates the direct method of watering plants as contamination level would reduce through drip irrigation (Havelaar *et al*., 2001). Figure-4.4 - 4.6 clearly illustrates that treated water and vegetables breached the line drawn by WHO and International Commission on Microbiological Specifications for food. Mean level of *Escherichia coli* bacteria in both water and vegetables has exceeded the recommended limit of 3 $\log_{10}$ CFU/100mL and CFU/g respectively. Vegetables contamination of *Escherichia coli* bacteria emanate primarily through treated wastewater used for watering vegetables. Among targeted vegetables wheat falls in restricted irrigation for which there is no guideline limit from, WHO available in case of *Escherichia coli* contamination. Detection of *Escherichia coli* through PCR was used to culture dependent techniques. Thermocycler conditions were achieved for the amplification of microbes, mentioned earlier. The optimal annealing for *Escherichia coli* was 66-71.1 ºC, shown in Table-4.6.

From represented results in Figure-4.4 - 4.6, it is anticipated that *Escherichia coli* transfuse from MBST treated wastewater into plants. Results obtained are supported by the Solomon *et al.*, (2002), who demonstrated in his study that the transmission of *E.coli* to salad plants from infected manure combined into the soil. Indicated that *E.coli* has the ability of leeching into the roots of mature lettuce plants and may be translocate to the edible portions of the plant.

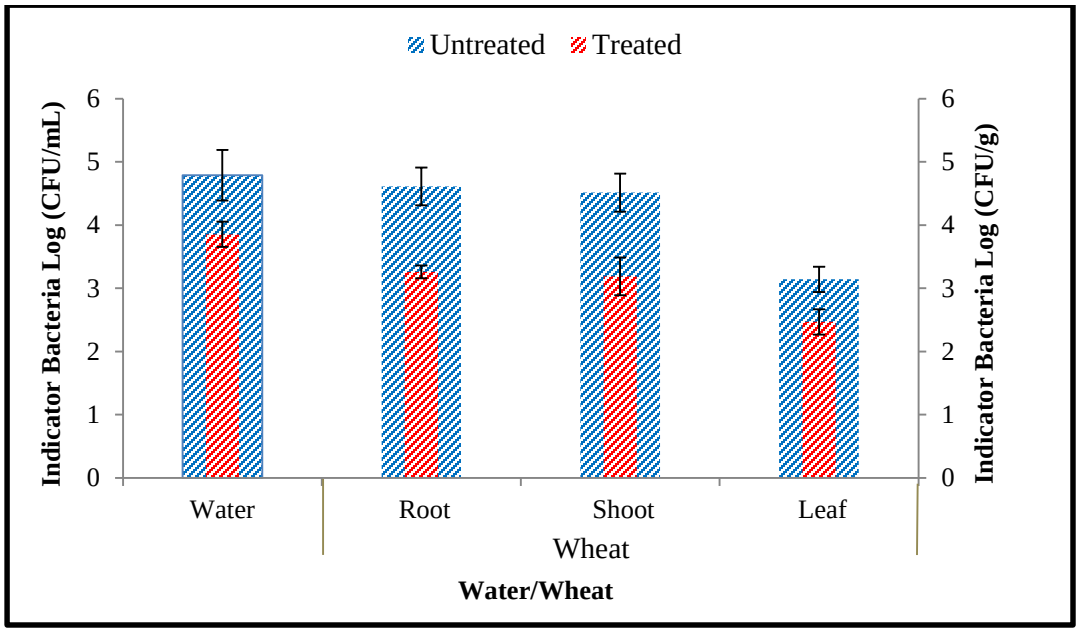


Figure-4.4: Quantification of *Escherichia coli* in wastewater and wheat

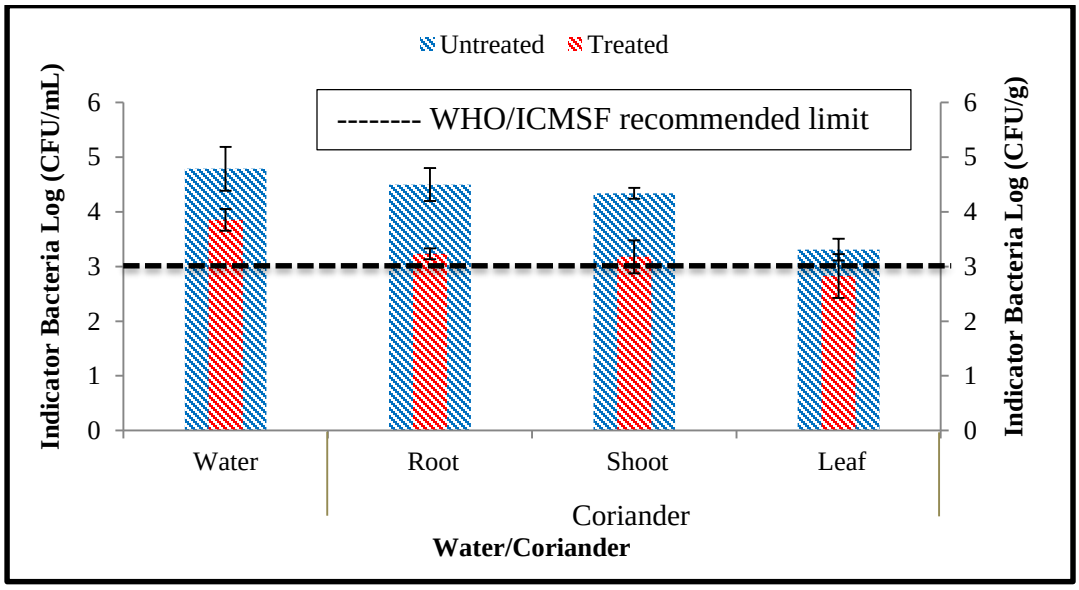


Figure-4.5: Quantification of *Escherichia coli* in wastewater and coriander

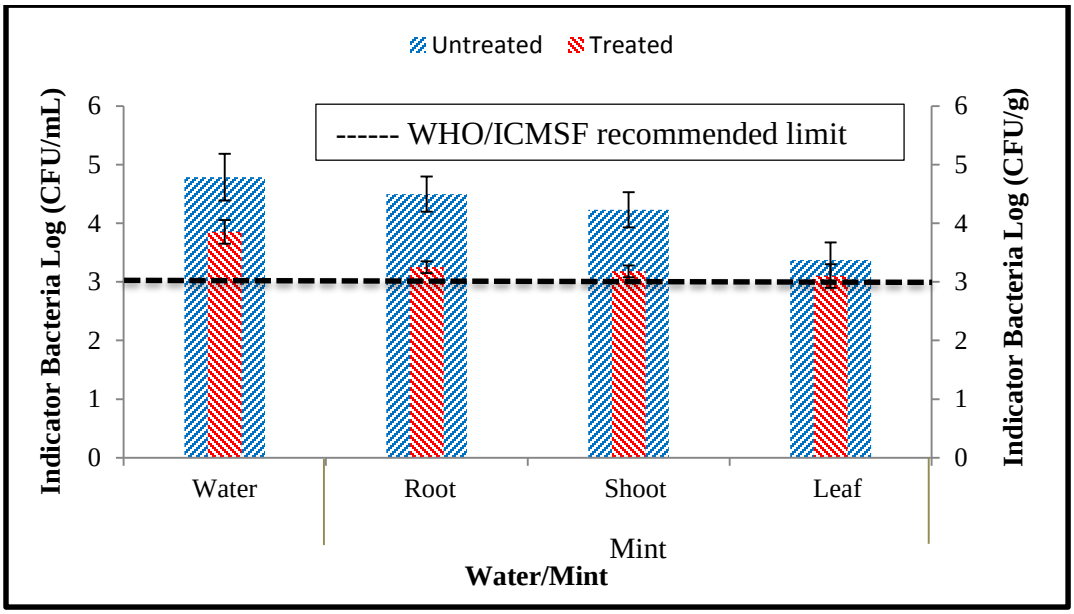


Figure-4.6: Quantification of *Escherichia coli* in wastewater and mint

Chalmers *et al*., (2000) also reported that direct contact between the leaves and an infected source is not necessary for the organism to become transported into edible lettuce tissue. *E.coli* associated with infected manure or irrigation water may be transmitted from the root system into the edible portion, particularly by the plant vascular system (Kudva *et al*., 1998).

Ackers *et al*., (1998) reported that under normal circumstances, even a low level of infection, could pose a major human health risk, since the infective dose of *E.coli* is less than 1000 cells. *E.coli* may survive for extended periods in manure and water (Solomon *et al*., 2002).

Our study is further supported by the Armon *et al.*, (1994) and Rosas *et al*., (1984), who demonstrated that using wastewater for crops irrigation have found higher bacterial counts on crop portions than developed underground or near the surface of the soil. However, it shows contradiction with Gerba and Smith, (2005) along with Mills *et al.*, (1925) as, they reported that the plants has sterile vascular systems, therefore the principal transmission route of pathogens from water to crop is the direct contact. However, it is asserted from the obtained results that by lowering the contact level of contaminated wastewater could reduce the bacterial count but it is not the only transmission route.

None of the water sample taken from MBST met the international standards for the guideline limit for *fecal coliform* bacteria in unrestricted irrigation of crops likely to be eaten raw: 10^3 to 10^5 (WHO, 2013). Hence the water may easily be used for the restricted irrigation as the wheat plant fulfills the international standards. Abakpa, (2013) observed in his study that the counts of *fecal coliform* in the water and irrigated vegetables exceeded the 1000 CFU/100 mL guideline for water used in fresh produce and recommended that these waters are not suitable for human consumption and irrigation of vegetable and salad crops without prior treatment.

4.8 Transmission of *Salmonella typhimurium* from MBST treated water into plant parts

In case of treated wastewater the effluent concentration of 3 $\log_{10}$ CFU/100mL *fecal-coliform* convinced the complete removal of pathogens and low level of viruses. Therefore the correlation between the *Escherichia coli* concentration and pathogen organisms exist. High concentration of *Escherichia coli* indicated the presence of *Salmonella* as depicted in Figure-4.7, selected as a pathogen microorganism associated with wastewater along with *Shigella* and *Legionella* (Ottosson, 2003a; Rose *et al*., 1991). Figure-4.7 shows the presence of 73.33 % presence of *Salmonella* in membrane based septic tank effluent 80 % in root section of mint and wheat and 60 % in coriander. Edible portion of mint and coriander shows the 60 and 20 % of prevalence. Detection of *Salmonella* through PCR was done for the confirmation of species. The optimal annealing temperature for *Salmonella* was 61-63.9 ºC, shown in Table-4.7.

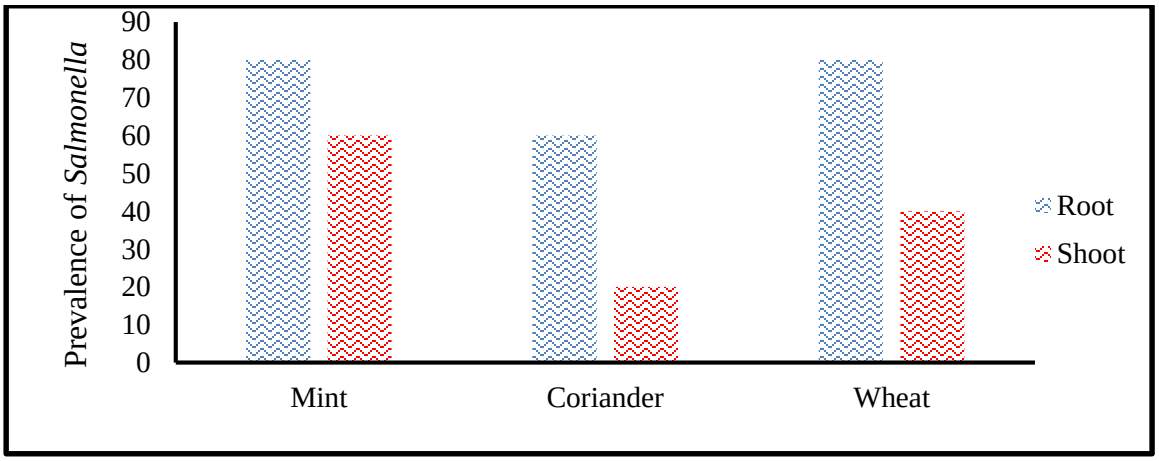


Figure-4.7: Prevalence of *Salmonella typhimurium* in MBST effluent and plants

Pathogen estimation in treated effluent may give information complementary to classical indicators in order to establish the level of reclamation to achieve.

With the epidemiological concern, one very important matter is the presence of *Salmonella* strains in treated water. Municipal wastewater having undergone an activated sludge process continued to bear *Salmonella*: the raw water yielded an MPN of 266.7/100 mL and the treated water 45/100 mL, representing a reduction of only 83 %. This reveals a considerable risk of the transmission of salmonellosis (Howard *et al*., 2004).

Espigares *et al*., (2006) reported that *Salmonella* is easily transmitted by water, and so water disinfection constitutes a key preventive mechanism.

Our results are further supported by the Iniguez *et al*., (2005) and Schikora *et al*., (2008) who demonstrated that *Salmonella* were found to form biofilm-like structures on the surface of roots, preferentially colonizing regions around emerging lateral roots and wounded tissues. Barak *et al.*, (2011), Golberg *et al*., (2011) and Kroupitski *et al*., (2009) have reported that the possible entry points of bacteria is the inner layers of leaves. And it was postulated that trichomes are preferential colonization sites. The formation of biofilms of *Salmonella* on leaves was also reported Schikora *et al*., (2012). By contrast, it was shown by the Kroupitski *et al*., (2009) that *Salmonella* translocate through stomata in order to penetrate lettuce leaves.

In the same year, another research pointed that *Salmonella* strain MAE110 has the ability to translocate within tomato (*Solanum lycopersicum*) plants, contaminated distal, non-infected leaves and fruits without noticeable symptoms and only marginally reducing plant growth (Gu *et al*., 2011).

4.9. DNA extraction

For genomic DNA extraction of *Escherichia coli* and *Salmonella typhimurium* kit method was used. Results were recorded as pictures using UV illuminator. It was observed that although lesser DNA concentration is achieved from kit extraction but it was free from all debris. Because of lesser debris, DNA did not degrade early and amplification was achieved easily. The gel picture saved for kit DNA extraction is given in Figure-4.8(a).

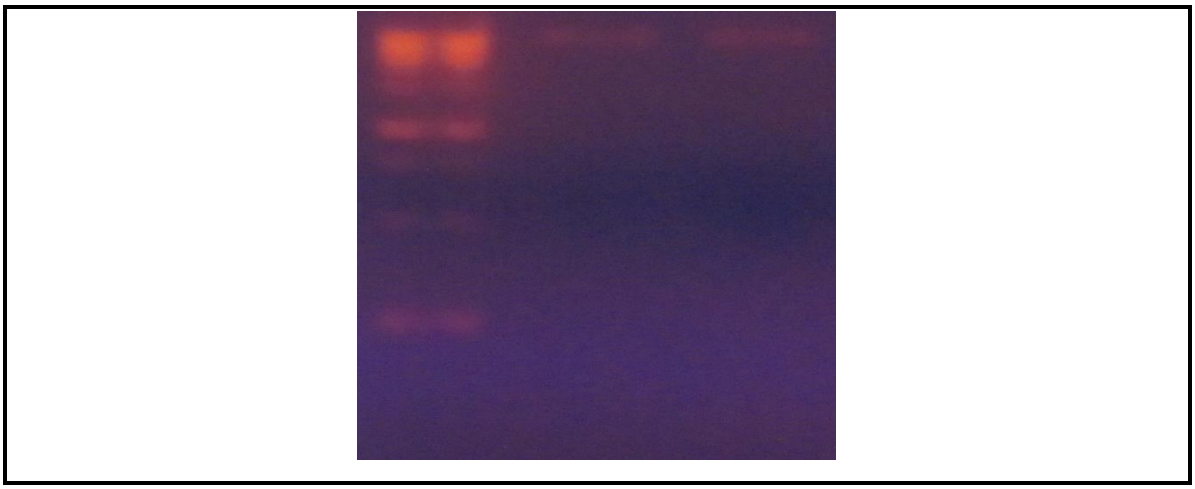


Figure-4.8 (a): Agarose gel picture of DNA of *E.coli* and *Salmonella typhimurium*

4.10. PCR amplification

Various PCR mixtures combination and concentration as well as temperature variations were used for *Escherichia coli* and *Salmonella typhimurium* bacteria. The final concentration and quantity that proved to be helpful is given in Table-4.5.

Table-4.5: PCR mixture composition used for amplification

Reagents	Final Concentration	Quantity (µL)
10x Taq reaction buffer	10x	2.5
Magnesium Sulphate	20mM	2.5
dNTP	10µM	2
Primer, Forward	10µM	1
Primer, Reverse	10µM	1
Taq DNA polymerase	1U	0.3
Template DNA	> 10ng/µl	3
PCR Water		12.7
Total Volume		25

The PCR mixture is subject to PCR thermocycler conditions and amplification may only be achieved at a specific condition. The amplification conditions for both bacteria's studied are discussed hereafter.

4.11.1 *Escherichia coli*

The DNA spin kit extraction of all the samples taken from both plants and MBST effluent were subjected to PCR. The thermocycler PCR condition for *Escherichia coli* is given in Table-4.6.

Table-4.6: PCR thermocycler condition for *Escherichia coli*

Steps	Temperature (ºC)	Time (min)
Denaturation	95	5
Annealing	66-71.1	00.30
Elongation	72	1
Final Extension	72	10

N = 35

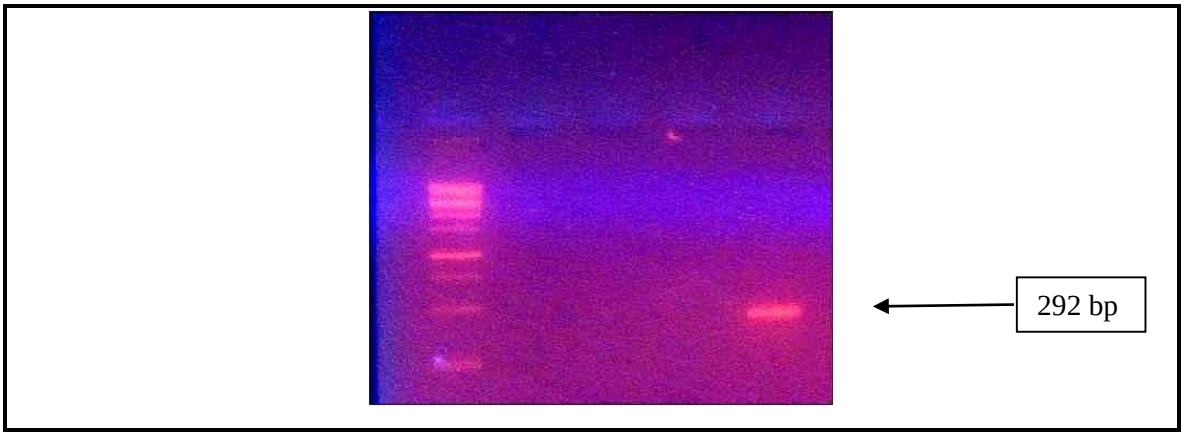


Figure- 4.8 (b): Agarose gel picture of amplification of *Escherichia coli*

4.11.2 Salmonella typhimurium

Table-4.7: Thermocycler PCR condition for *Salmonella typhimurium*

Steps	Temperature (ºC)	Time (min)
Denaturation	95	10
Annealing	61-63.9	00:30
Elongation	72	1
Final Extension	72	10

N=40

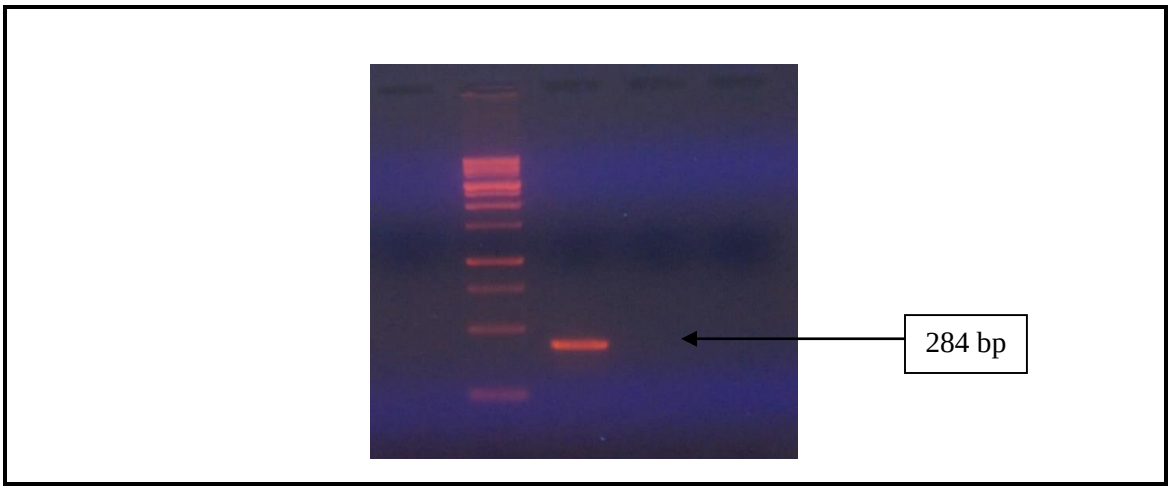


Figure-4.8 (c): Agarose gel picture of amplification of *Salmonella typhimurium*

4.11 Helminthes

Equally, the membrane based septic tank effluent used for irrigating vegetables contained 2 helminthes eggs/L. This exceeds the recommended limit of WHO of less than 1 helminthes egg/L in treated wastewater used for agricultural irrigation. WHO proposed the concentration of helminthes eggs both for restricted and unrestricted irrigation because of epidemiological concern. Results recorded that helminthes egg was absent on wheat. Mint and coriander also showed the level of helminthes less than 1 egg/L shown in Figure-9 the significant difference ($p<0.001$) prevails in helminthes egg level in treated wastewater and irrigated crops.

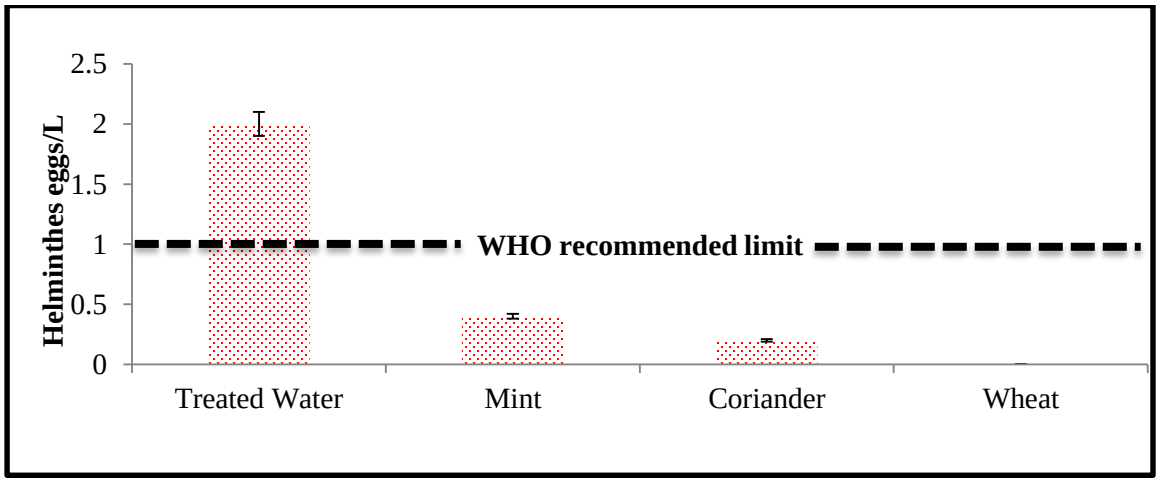


Figure- 4.9: Helminthes eggs in MBST discharge and plants

4.12. Detection of endophytes in irrigated crops

Endophytes have been found to be closely related to human pathogens or are either human or opportunistic human pathogens. This is the case of endophytic *Salmonella* strains, which have caused outbreaks and constitute a health risk for consumers of raw fruits and vegetables (Guo *et al*., 2010).

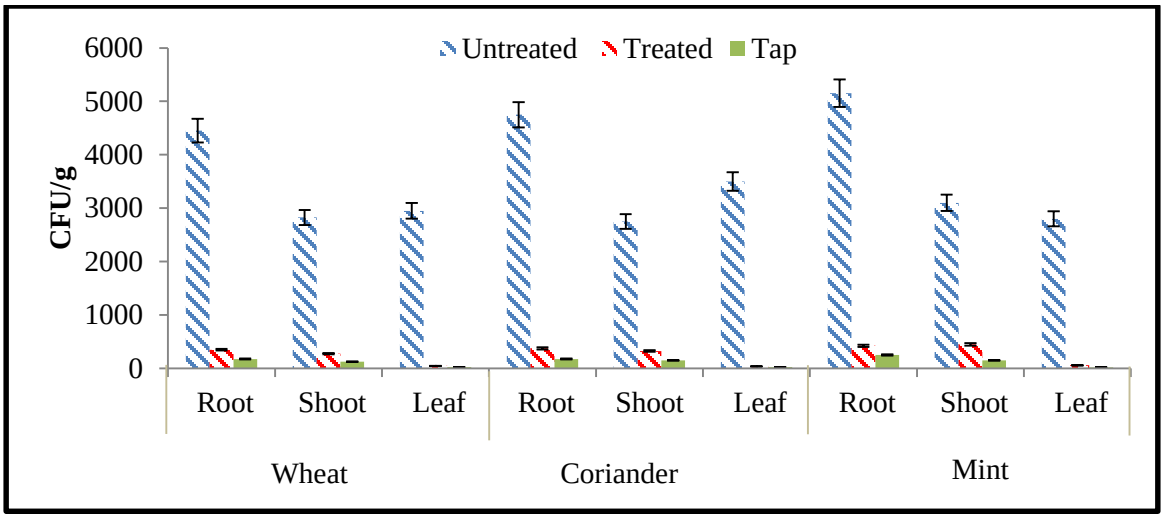


Figure-4.10: Quantification of endophytes in plants

Bars shown in Figure-4.10 clearly exibit that plants irrigated with untreated wastewater have huge accumulation of bacterial endophytes as compared to plants parts irrigated with MBST effluent. Endohpytic bacterial count shows the same trend of bacterial accumulation in plant parts.

Chapter 5

5. Conclusions and Recommendations

1. Treatment performance is better in terms of COD (73 %), turbidity (96 %), phosphorous (48 %) and bacteriological load (84.2 %) as compared to conventional septic tanks though the quality of the MBST effluent and irrigated vegetables exceed then the Internationally defined standards for the unrestricted irrigation and suitable for the restricted irrigation.
2. Plants analysed showed *faecal coliform* levels (3.4 log CFU/g), more than the $1x10^3$ per 100 g wet weight hence may be classified as undesirable for consumption according to the International Commission on Microbiological Specifications for Food (ICMSF, 1974) guidelines.
3. Statistically significant difference of 8.4 unit was observed in the plant growth when irrigated with untreated and treated water. Better growth was observed in untreated water as the root and shoot length was 15.44 and 19.07 cm respectively, higher than the treated water where the root and shoot length was 14.66 and 18.02 cm respectively. Whereas, a difference of 26 unit was observed, in plant growth, irrigated with treated water when compared with tap water, as the root and shoot length was 10.81 and 13.0 cm.

Recommendations:

On the basis of present study, it is recommend that the government enforce strict rules and legislation on adequate treatment of wastewater and effluents before discharge to the environment.

1. Proper washing and disinfection of vegetables before consumption is strongly advised.
2. UV disinfection of effluent for unrestricted irrigation by installing the UV lamp within the membrane based septic tank.
3. Membrane pore size should be less than 0.01 microns, to avoid passage of pathogenic bacteria.

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Annexure A

API results of isolated bacterial strain

EXCELLENT IDENTIFICATION

Strip	API 20 E V4.1
Profile	5 1 4 4 5 5 2
Note	

Significant taxa	**% ID**	**T**	**Tests against**			
***Escherichia coli* 1**	**99.9**	**1.0**				

Next taxon	**% ID**	**T**	**Tests against**			
Kluyvera spp	**0.1**	**0.38**	**LDC 25 %**	**SOR 25 %**	**SAC 89 %**	

GOOD IDENTIFICATION

Strip	API 20 E V4.1
Profile	4 5 0 4 5 5 2
Note	Confirm by serological tests

Significant taxa	**% ID**	**T**	**Tests against**			
Salmonella spp	**96.6**	**0.94**				

Next taxon	**% ID**	**T**	**Tests against**			
Salmonella choleraesuis	**3.0**	**0.59**	**ONPG 98 %**	**ADH 75 %**	**CIT 75 %**	

GOOD IDENTIFICATION

Strip	API 20 E V4.1
Profile	5 2 1 5 7 7 3
Note	

Significant taxa	% ID	T	Tests against			
Klebsiella pneumoniae ssp	**97.6**	**1.0**				

Next taxon	% ID	T	Tests against			
Klebsiella oxytoca	**2.1**	**0.72**	**IND 99 %**			

Complementary test(s)	5KG	METHYL RED	
Raoultella planticola	**98 %**	**100 %**	
Klebsiella pneumoniae ssp	**2 %**	**9 %**	

EXCELLENT IDENTIFICATION

Strip	API 20 E V4.1
Profile	0 7 3 6 0 0 0
Note	

Significant taxa	% ID	T	Tests against			
Proteus mirabilis	**99.9**	**1.0**				

Next taxon	% ID	T	Tests against			
***Proteus vulgaris* group**	**0.1**	**0.0**	**OD 0 %**	**CIT 12 %**	**IND 92 %**	**%**

GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	1 2 0 0 0 6 3
Note	

Significant taxa	% ID	T	Tests against			
Serratia plymuthica	**60.2**	**0.35**	**GLU 100 %**	**MAN 90 %**		
Serratia rubidaea	**20.2**	**0.18**	**GEL 82 %**	**GLU 99 %**	**MAN 99 %**	**INO 75 %**
***Pantoea spp* 1**	**8.2**	**0.17**	**CIT 13 %**	**GLU 100 %**	**MAN 99 %**	
Klebsiella pneumoniae	**5.4**	**0.2**	**CIT 18 %**	**GLU 99 %**	**MAN 96 %**	**SAC 20 %**
***Pantoea spp* 2**	**2.1**	**0.08**	**GLU 100 %**	**MAN 99 %**	**SOR 82 %**	**RHA 90 %**

Annexure B

DNA extraction method

Four major steps of DNA extraction are

1. Lysate preparation
2. Binding to column
3. Column wash
4. DNA elution

The details of the DNA extraction steps are discussed here under:

Lysate preparation

1. Wastewater sample was transferred to eppendorf tube and centrifuged at 14000 rpm. Pellet was resuspeded in lysis buffer and added to bead tube.
2. Lysis additive was added and sample was centrifuged after vortexing briefly for a min at 14000 rpm.
3. Binding solution was added to supernatant, mixed well and incubated for 5 min.
4. It was then centrifuged at 14000 rpm and supernatant was transferred to new vials. 70 % ethanol was added in equal volume and vortexed briefly.

Binding column

1. 600 μL of clear lysate was put into spin column combined with collection tube followed by centrifugation and the process was repeated depending upon lysate volume.

Column wash

1. 500 μL of wash solution-I was added in column and centrifuged and wash solution II was added.
2. It was centrifuged again followed by spinning to dry the resin.

DNA elution

1. The spin column was placed in fresh eppendorf tube and 50 μL elution buffer was added to it.
2. It was centrifuged at 2000 rpm for 2 min followed by 1 min centrifugation at 14000 rpm.
3. The eluted volume was stored at -20 °C for further use