PERFORMANCE EVALUATION AND MICROBIAL COMMUNITY DYNAMICS OF VERMIFILTRATION

Ph.D. THESIS

by

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE – 247 667 (INDIA)
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A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"PERFORMANCE EVALUATION AND MICROBIAL COMMUNITY DYNAMICS OF VERMIFILTRATION"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Civil Engineering, Indian Institute of Technology Roorkee, is an authentic record of my own work carried out during a period from December, 2011 to July, 2015 under the supervision of Dr. A. A. Kazmi, Professor, Environmental Engineering, Department of Civil Engineering, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(SUDIPTI ARORA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Date:

(**A. A. KAZMI**)
Supervisor

Dedicated to my Grandfather (Bade papa)

ABSTRACT

The present study was designed to determine the effect of earthworms and microbial community dynamics on the removal of pathogens and organic matter degradation during wastewater and solid waste treatment by vermifiltration. The study was divided in different phases. The results of a lab scale study showed that vermifiltration resulted in an effluent with biochemical oxygen demand (BOD) < 20 mg/L, chemical oxygen demand (COD) < 100 mg/L, total suspended solids (TSS) < 30 mg/L, and pathogens < 10^3 MPN/100 mL, signifying high treatment efficacy. The decay rate constant (k) for indicator organisms and pathogens was observed to be within the range of 5.99–7.96 md^{-1} and the population of total heterotrophic bacteria, total fungi and actinomycetes were reduced remarkably by 2–3 log, respectively.

The suitability of different filter media (riverbed gravel, mud balls, wooden coal and glass balls) was explored during vermifiltration and results indicated that naturally occurring riverbed material and mud balls were found to be better suited for the treatment with higher pathogen removal efficacy. The higher BOD, COD, and pathogen removal efficiency, higher microbial diversity in the filter bed, increase in earthworm's number and biomass, and no abrasions on the body walls of earthworms concluded that river bed material is a promising filter media. Overall, the observed trend of VFs in terms of treatment efficacy was observed to be riverbed material ≥ mud balls > glass balls > wooden coal.

The study further investigated the microbial community dynamics and antibacterial & enzymatic properties of microorganisms in a vermifiltration system. It included the isolation and identification of diverse microbial community from a vermifilter (VF) with earthworms and its comparison with a conventional geofilter (GF) without earthworms. The burrowing activity of earthworms promoted the aeration conditions in VF which led to the predominance of the aerobic microorganisms, accounting for complex microbial community diversity. In vitro antimicrobial assay also showed that the present microflora had strong inhibitory efficiency against pathogens *S. aureus*, *E. coli*, *P. aeruginosa* and *K. aerogenes*. The release of antimicrobial substances by earthworms and associated microflora was found to be responsible for the removal of pathogens. The enzymatic activity of microorganisms is responsible for the biodegradation and stabilization of organic matter. The kinetics evaluation showed the predominance of first order removal model during vermifiltration.

The study on the effect of seasonal temperature on the treatment efficiency and pathogen removal efficacy from wastewater was also performed. The results showed a significant effect on BOD & COD reduction, indicator organisms & pathogen removal,

earthworm population, bacterial and actinomycetes population with a variation in ambient temperature, but had no effect on TSS removal and fungi population. The study showed that higher BOD and COD removal was accomplished during the spring and autumn period when the mean temperature was 25-27°C. This temperature range is optimum for the earthworm species *Eisenia fetida* for its activity, growth and reproduction and any variation in temperature from the optimum range led to decrease in treatment efficiency and earthworm population. The pathogen removal efficacy of VF increases with the increase in temperature, as shown by linear regression analysis, which implied that temperature had a significant contribution to the pathogen removal efficiency of VF. Pearson coefficient of correlation (r) derived an important relationship between the seasonal temperature and treatment efficiency, pathogen removal efficacy and microbial population during vermifiltration.

The last phase of the study brings an insight to the performance evaluation of a pilot scale VF during the combined treatment of domestic wastewater and organic fraction of municipal solid waste (OFMSW). The study showed that VF resulted in an effluent with BOD < 20 mg/L, COD < 100 mg/L ammonia NH_4^+-N ≤ 1 mg/L, nitrate NO^-_3-N > 10 mg/L, and coliforms < 10^3 MPN/100 mL and mature vermicompost with high nutrient value (C: N ratio < 20) signifying high treatment and pathogen removal efficacy. A total of 41 bacterial colony-forming units (CFUs) were isolated, out of which 12 strains were selected, that exhibited higher antimicrobial activity against tested pathogenic bacteria and fungi. The comparative sequence analysis of 16S rRNA genes showed two phylogenetically different clusters of the characterized bacterial strains. Six strains were affiliated with Firmicutes (Family *Bacillaceae* and *Enterococcaceae),* and six with γ-Proteobacteria (Family *Enterobacteriaceae*). The identified strains contribute to enhance the disinfection efficiency during wastewater treatment.

Keywords: Earthworms, Microbial community, Pathogens, Solid waste, Vermifiltration, Wastewater

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(SUDIPTI ARORA)

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LIST OF ABBREVIATIONS

Abbreviation/Symbol	*Definition*
AOB	Ammonia oxidizing bacteria
APHA	American public health association
ASP	Activated sludge process
ATCC	American type culture collection
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
CETP	Common effluent treatment plant
CFU	Colony forming unit
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribose nucleic acid
DO	Dissolved oxygen
FC	Fecal coliforms
FS	Fecal streptococci
GE	Gel electrophoresis
GF	Geofilter
HLR	Hydraulic loading rate
HRT	Hydraulic retention time
MPN	Most probable number
MSW	Municipal solid waste
MTCC	Microbial type culture collection
NCBI	National center for biotechnology information
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
OM	Organic matter
PCR	Polymerase chain reaction
PVC	Poly vinyl chloride
RNA	Ribonucleic acid
SD	Stocking density
SEM	Scanning electron microscope
SPC	Standard plate count
SS	Suspended solids

STP	Sewage treatment plant
SVI	Sludge volume index
TC	Total coliforms
TDS	Total dissolved solids
THB	Total heterotrophic bacteria
TF	Total fungi
TSS	Total suspended solids
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorous
VF	Vermifilter
VFA	Volatile fatty acid
VSS	Volatile suspended solids
WHO	World health organization
S*ymbol*	
K	Log removal
k	Kinetic constant (m/d)
ρ	Porosity
γ	Gamma
μ	Micro

CHAPTER 1

INTRODUCTION

This chapter presents the background of the study and formulation of objectives based on the identification of problems. The scope of the work is followed by the description on the structure of thesis presented here.

1.1. Background

Owing to increasing human activities, deterioration of existing fresh water resources is largely observed in developing countries. For several decades, increasing trend in urbanization along with fast and unplanned growth is resulting in wide impact on our natural resources and environment. The growing world population, relentless urbanization, and growing scarcity of quality water resources are the compelling forces behind the accelerating trend for reuse of wastewater (World Health Organization, 2010). Water pollution and freshwater depletion are currently viewed as the important problems in Asian regions (Karn and Harada, 2001). In order to address these issues, wastewater is now being looked as a resource for water and energy, rather than a waste (Jadhav et al., 2014). The choice of appropriate wastewater treatment technology that follow sustainable development approach, presents a challenge to national and regional policy makers (Kalbar et al., 2012a). Increasingly, urban areas are moving towards multiple sites for the wastewater treatment against the practice of centralized treatment (Kalbar et al., 2012b). Numerous wastewater treatment systems exists including activated sludge process, fluidized bed reactors, trickling filter, aerated lagoons, and oxidation ponds. Irrespective of the high treatment performance efficiency, these systems suffer from plentiful drawbacks (Khan et al., 2011a). These technologies are restricted to use because at times, these are prone to failure due to high investment, operational and maintenance costs, which makes them unaffordable in developing countries (Muga and Mihelcic, 2008). The cost effectiveness of wastewater collection, transportation, pumping, treatment and disposal especially in areas with low population density and dispersed households can be optimized by decentralized technology. These decentralized treatment plants are planned all over the large urban and sub urban areas (Arceivala and Asolekar, 2006). It is necessary to seek a decentralized wastewater treatment technology that is cost effective and ecofriendly (Wu et al., 2013). Currently, a variety of decentralized treatment technologies have been employed like lagoons, media filters, sequencing batch reactor, constructed wetlands, etc.

The treatment of wastewater utilizing earthworms was first suggested over 20 years ago due to its ability to biodegrade variety of organic materials. Late Prof. Jose Toha at University

of Chile first described the utilization of earthworms for wastewater treatment, which has been termed vermifiltration, in 1992. Vermifiltration is one of the most promising technologies that have gained widespread popularity since last decade, for wastewater treatment. It involves the inoculation of earthworms in a filter bed, containing suitable filter media. The body of the earthworms acts as a biofilter, which interact with microorganisms to treat wastewater. Earthworms affect various physico-chemical and biological processes inside a vermifilter (VF). These include substrate aeration, mixing, definite grinding and microbial degradation of organic matter (Yadav et al., 2010). The major features of vermifiltration technology include cost-effectiveness, sustainability, and efficiency to meet wide range of treatment, with no problem of odor, clogging or sludge accumulation. Vermifilters (VFs) are an engineered system that encompasses different treatment modules and have been effectively used to alleviate environmental pollution by removing pollutants including organic matter (OM), suspended solids (SS), pathogens, and heavy metals from wastewater. Conventional treatment systems include separate units for each contaminants removal. After secondary treatment of wastewater, the effluent may contain pathogens and other microorganisms, due to which it is not advisable to reuse the treated effluent unless it is disinfected (Jadhav et al., 2014). One of the major barriers to water treatment and reuse is apprehension regarding the health hazard of wastewater exposure to public. Out of all the contaminants in wastewater, pathogens are of major concern because of their ability to cause diseases in humans. Human pathogens are typically present in domestic sewage and needed to be removed during wastewater treatment (Arias et al., 2003). Since raw wastewater contains a wide variety of fecal microorganisms and pathogens, the reduction of bacteriological pollution in wastewater is of high priority. In order to make effluent suitable for reuse purposes, there is a need for complete removal of pathogens from water (disinfection). Disinfection processes are crucial in water treatment facilities. Until now, most of the study related to vermifiltration deals with organic matter removal and nutrients enrichment, however the removal of pathogens has been neglected. Investigations regarding the mechanism of pathogen removal and organic matter degradation, along with optimizing suitable filter media and environmental conditions are necessary for successful implementation of vermifilter.

Urban areas in Asia generate more than 700,000 tonnes of municipal solid waste (MSW) per day. In 2025, this figure will increase to nearly 2 million tonnes waste per day (World Bank, 1999). In most cases, the final treatment involves collecting, transporting and dumping the solid waste to the nearest available open space, in an uncontrolled way (Norbu et al., 2005). These sites are still observed in developing countries and can be detrimental to the

environment (Tränkler et al., 2005). Among the many technologies available such as aerobic and anaerobic biodegradation, vermicomposting has been employed since last many decades, for treatment of municipal solid waste, as it is rapid, easily controllable, cost-effective, energy saving, and accomplishes most efficient recycling of organics and nutrients (Hait and Tare, 2011). Henceforth, adopting the same principle and utilizing earthworms, vermifiltration is employed for the combined treatment of organic fraction of municipal solid waste (OFMSW) and wastewater in this study. In this context, vermifiltration system for wastewater and solid waste treatment is developed for removal of organics, nutrients and pathogens. The objective of the present study is to discuss the concept of vermifiltration process, its ecological principle, microbial community dynamics and its role to treat waste using vermifilters.

1.2. Problem Identification

So far, the performance of vermifiltration is assessed on the basis of organic matter removal or nutrients removal efficiency. There is no evidence of the removal of pathogens in a vermifilter (VF) treating domestic wastewater. Earthworms and microroganisms (residing in a VF) interact symbiotically and synergistically to treat wastewater (Zhao et al., 2010). There may be some controlling factors that affect the performance of a VF for the removal of contaminants. One such factor is the filter media, where earthworms thrive and feed upon to perform their activity. Besides this, suitable environmental conditions including temperature, pH, and moisture content are also important for the growth and functioning of earthworms. Therefore, one of the aims of this research is to select the appropriate filter media for a VF. The other aim is to study the microbial community dynamics and underlying mechanism behind the contaminants removal (including organic matter and pathogens) for treating domestic wastewater and solid waste. The effect of different seasonal temperature on the performance of a VF is further explored. The results of the study can provide insight on the mechanism and application potential of vermifiltration for waste management.

1.3. Objectives of the Study

A new concept of vermifiltration was introduced in this research, which implies that vermifilter is not only utilized for wastewater treatment, but also used for combined treatment of wastewater and OFMSW. The mechanism of vermifiltration for removal of organic matter and pathogens is mainly explored in the study. The specific objectives of the present study are:

- To test the performance efficiency of a lab scale vermifilter for removal of organic matter and pathogens
- To assess the suitability of different filter media for the vermi-treatment of wastewater

- To test the performance efficiency of a pilot scale vermifilter (utilizing selected filter media) and to understand the microbial community dynamics and underlying mechanism for removal of organics and pathogens
- To investigate the effects of seasonal temperature on the treatment efficiency of vermifiltration and establishing correlation between different parameters.
- To test the performance efficiency and microbial community dynamics of a vermifilter for the combined treatment of OFMSW and wastewater

1.4. Scope of the Study

To achieve the above-mentioned objectives, laboratory experimental studies were conducted under different conditions and methodologies. The performance and maintenance of the VF and design of the routine of experiments were the main scope of the study. In the first part, a lab scale VF was tested for the removal of organic matter and pathogens, along with pathogen removal kinetics. In the second part, the assessment of the suitability of different filter media for a VF was evaluated. In the third part, the selected filter media (from the previous study) was tested on a pilot scale VF for evaluation of performance efficiency and understanding the microbial community dynamics and mechanism behind the removal of organic matter and pathogens. A pathogen removal model was proposed, considering its affecting factors. In the fourth part, a long term continuous VF was operated for one year to evaluate the effects of seasonal temperature on the treatment efficiency of vermifiltration. The last part covers the performance evaluation of pilot-scale VF for combined treatment of OFMSW and wastewater simultaneously. A large part of the work was collecting the samples (twice in a week) from the reactor, and maintenance of the reactors during the execution of the experiments and earthworm handling, followed by the collection and analysis of data including daily visual observations in the reactors.

1.5. Structure of the Thesis

The structure of the chapters in the thesis is presented in the flowchart (Figure 1.1)

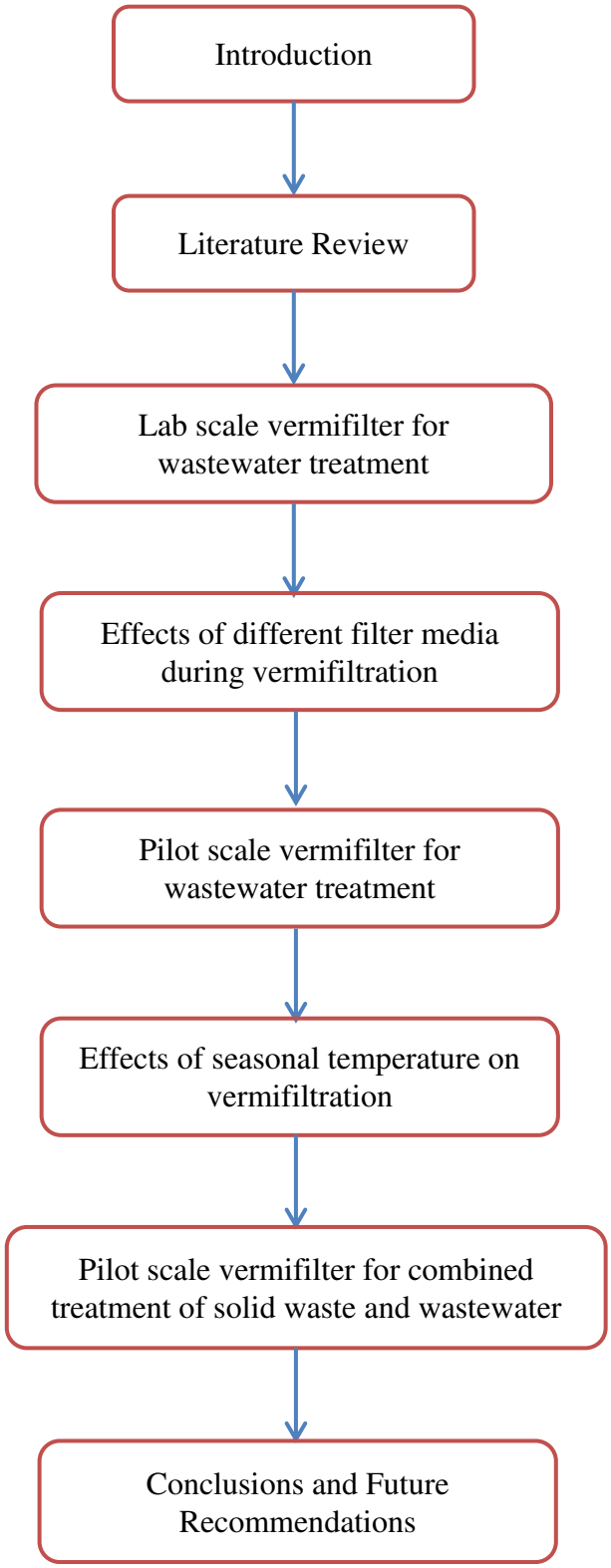


Figure 1.1: Structure of the thesis

CHAPTER 2

LITERATURE REVIEW

This chapter presents the review on the information available in different literature sources. The most related aspects of this study are presented in a summarized form.

2.1. Background Information on Vermifiltration

The last century has seen the global water demand to increase by six folds, which is more than twice of the world population growth rate. Currently 40% of the world population faces water scarcity, which is expected to rise to 60% by the year 2025 (Khan et al., 2011b). So with increasing pressure on fresh water resources, there is a need to consider wastewater for reuse. There are very few wastewater treatment facilities due to high costs of treatment process and lack of effective environmental protection law implementation. In developed countries, reduction of all pollutants (organic, inorganic contaminants and pathogens) is the primary wastewater treatment goal whereas in developing countries, the major wastewater treatment aim is to protect public health by removal of pathogens (Kivaisi, 2001). Different technologies have been employed for wastewater treatment varying from simple 2-stage processes to physico-chemical and biological processes (Pidou et al., 2008). Many centralized treatment plants are found to be unsuccessful mainly due to large cost factor associated, or unable to cope with environmental legislations. Consequently, over the last decade, a large number of decentralized wastewater treatment plants of different technology types have been installed all over the world (Singh et al., 2015). One such technology is vermifiltration, which emerged as a popular option for wastewater treatment and has been recognized as appropriate alternative to conventional treatment systems (Aira et al., 2007a). Vermifiltration is a novel technology for wastewater treatment, which adopts modern concept of ecological design and extends the existing chain of microbial metabolism by introducing earthworms. Vermifiltration using earthworms is a low-cost, ecological and sustainable technology that utilizes earthworms for domestic and industrial sewage treatment. This technology was first developed as an extension of vermicomposting for solid waste to treat anaerobically stabilized effluent from dried vine fruit industry (Athanasopoulos, 1993). In the last decade, this technology has been successfully applied for different wastewater, at a lab and pilot scale. It is more popular in China, where a vermifilter was successfully installed in a field for treating municipal wastewater, in rural village inhabiting 1000 people (Li et al., 2009a). So far, Australia and China have benefited the most from vermifiltration although some design approaches are still prevailing. In India, various researchers have proved the implementation of the technology for domestic water and

various industrial wastewater but the real accomplishment of the technology lies in understanding of the overall design criterion and understanding of mechanism behind the treatment process. Henceforth, in the present study, the mechanism of vermifiltration technology is explored for its definite use along with the design criteria and microbial community dynamics.

2.2. Design Criterion of Vermifiltration

Definition of appropriate technology for developing countries is so unclear that it may sometimes lead to the adoption of wrong technology if not rightly perceived (Tandukar et al., 2007). Grau (1996) cited failure of a low-cost technology due to lack of proper selection techniques for an appropriate technology. Furthermore, affordability is also an important factor, which determines the selection of appropriate treatment systems for a particular community. Consequently, reuse of wastewater for toilet flushing, garden irrigation or any other non-potable uses provides considerable savings in potable water and need not meet the stringent water quality requirements of potable water, however untreated wastewater can still carry risks and requires some necessary degree of treatment (Avery et al., 2007). Vermifiltration technology for wastewater treatment represents a techno-economically feasible and emerging solution for water pollution control, water conservation and reuse of water for non-potable purposes placing them in the suitable alternative for appropriate technology. Vermifiltration refers to an organic degradation process involving earthworms-microorganisms interactions. The microorganisms are responsible for the bio-chemical degradation of the organics, and earthworms further enhance the process by proliferating the growth of aerobic microflora through their burrowing activity. Earthworms can modify the microflora of a vermifilter directly and indirectly by three main mechanisms (1) comminution, burrowing and casting (2) grazing (3) dispersal. These activities may change the substrate's physico-chemical and biological status and cause drastic shifts in the density, diversity, compositions and activities of microbial communities in the VF (Zhao et al., 2014). The performance of a VF for wastewater treatment is dependent on crucial factors. There are various critical factors that affects the design of a VF which includes hydraulic loading rate (HLR), hydraulic retention time (HRT), organic loading rate (OLR), type of earthworms species used, stocking density (SD) of earthworms, filter medium (filter bed or bedding) for the earthworms and suitable environmental conditions (temperature, pH and moisture) for earthworm's growth and survival.

2.2.1. Hydraulic Loading Rate (HLR) and Hydraulic Retention Time (HRT)

Hydraulic conditions strongly influence the biogeochemical processes, biotic community composition, and the fate of pollutants in VFs (Zhang et al., 2014). Hydraulic loading rate (HLR) is defined as "the volume of wastewater that a vermifiltration system can treat in a given time (measured in area and depth of the filter bed)." HLR is mathematically calculated as the volume of wastewater applied, per unit area of VF bed per unit time as described in Equation 2.1, where HLR is hydraulic Loading Rate (m/hr), $V_{wastewater}$ is volumetric flow rate of wastewater (cum), A is area of vermifilter bed exposed (sqm) and t is time taken by the wastewater to flow through the filter bed (h) (Sinha et al., 2008).

$$HLR = {V_{wastewater}}/{(A \times t)} \qquad \text{Equation 2.1}$$

Hydraulic retention time (HRT) is "the time taken by the wastewater to flow through the VF bed in which earthworms inhabit. It is actually the time during which the pollutants are in contact with the filter bed containing earthworms. It is a controlling factor in determining the removal efficiency of contaminants. HRT depends on the flow rate of wastewater to the VF unit, volume of filter bed and quality of media used." It is mathematically expressed in Equation 2.2, where HRT is theoretical hydraulic retention time (h), V_s is volume of the VF bed, through which the wastewater flow (cum), p is porosity of the entire filter medium through which wastewater flows, $Q_{wastewater}$ is flow rate of wastewater through the VF bed (cum / h).

$$HRT = {(p \times \mathrm{Vs})}/{Q_{wastewater}} \qquad \text{Equation 2.2}$$

Various studies have been conducted in the past to evaluate the treatment performance of VF at different HLRs. Li et al. (2009a), Fang et al., (2010), and Xing et al., (2010a) investigated the effect of HLR on contaminants removal from domestic wastewater by vermifilter. The results of the studies revealed that an increase in HLR lead to decrease in treatment efficiency and adult earthworm abundance. This occurs because higher HLR leads to reduced HRT, so organic substrates are not fully degraded before being discharged from the VF. Also higher HLR leads to stronger scouring of media surface and increased humidity, which is also not beneficial for earthworm growth.

2.2.2. Organic Loading Rate (OLR)

Organic loading rate (OLR) is defined as "the amount of biochemical oxygen demand (BOD) or chemical oxygen demand (COD) applied to the VF per day per volume of filter media. It is

the influent mass rate of BOD (or COD) per unit volume that influences the performance efficacy of VF." It can be mathematically calculated as given in Equation 2.3.

$$Organic\ Loading\ Rate\ (Kg/m^3\ .day) = \frac{BOD(mg/L)\times Flow(m^3/day)\times 10^{-3}}{Filter\ Volume\ (m^3)} \qquad \text{Equation 2.3}$$

Different studies have been conducted in the past to evaluate the performance of VF under different organic loading conditions. The results highlighted that VF has a prominent ability to resist a particular level of organic load fluctuation i.e., varying inflow conditions would not disturb the effluent quality, however, it is imperative to conclude that the influent load or OLR would also affect the performance efficiency of a VF, because earthworms have the ability to consume only particular amount of food per day. Hence, OLR should be appropriately chosen before installing a VF (Li et al. 2009a; Dhadse et al., 2010).

2.2.3. Earthworms species and Stocking Density (SD)

Earthworms are burrowing animals, regarded as "soldiers of mankind" by Charles Darwin (Sinha et al., 2009a). The use of earthworms for the treatment of solid waste, known as vermicomposting, is an age-old technology, where different earthworm species are being utilized for processing and efficient recycling of municipal, animal, industrial and agricultural waste. Researchers have investigated that tiger worm (*Eisenia fetida*), Red tiger worm (*Eisenia andrei*), Indian blue worm (*Perionyx excavatus*), African night crawler (*Eudrilus euginae*) and red worm (*Lumbricus rubellus*) are best suited for vermi-treatment of solid and liquid organic wastes. Besides the type of earthworms, the number or SD of earthworms is another important design parameter during vermifiltration. As earthworms play a critical role in wastewater purification, their numbers (SD), weight (biomass), maturity and health are important factors. Domínguez and Edwards (2010) studied the effect of SD on growth and maturation of *E. andrei* in pig manure and concluded that individual worms grew more and faster at the lowest population density, whereas the total biomass production was maximum at the higher population density. At higher stocking density, worms sexually mature faster than in the lower density (Yadav et al., 2011). Monroy et al. (2006) showed that stocking density have a strong effect on earthworm's mature weight and cocoons production. In a VF, the stocking density or number of earthworms may range from several hundred to several thousands. Studies have reported that about 8-10,000 worms per sqm of the worm bed and biomass (in quantity) as 10 kg per cum of filter media are required for optimal function (Sinha et al., 2009b). Various researchers have studied the effect of different earthworm loads on the treatment efficiency of vermifiltration (Li et al., 2009b; Wang et al., 2013b). The results revealed that diversity of the

bacterial community structure were affected within the earthworm packing bed when the earthworm load reached a certain level.

2.2.4. Filter media

The selection of efficient and enduring filter media is important to provide a suitable dwelling habitat for the earthworms to perform their activity. For any system to work efficiently, it is important that the species that maintain the working of the system should be well adapted and adjusted with the habitat of system and a favorable habitat allows them to work better and more efficiently. In the previous studies, various filter media like ceramsite, quartz sand, converter slag and coal cinder were compared for the treatment efficacy of the system (Yang and Zhao, 2008; Wang et al., 2010; Xing et al., 2011). The results suggested that ceramsite is more suitable filter media, while converter slag & coal cinder could possibly enhance phosphorous removal. However, the availability of the suitable filter media is another important aspect. It would be more convincing if the locally available filter media could be utilized for vermifiltration giving promising results. Hence, suitability of locally available filter media will be quintessential for the technology to become most appropriate technology. The use and performance efficiency of locally available filter media is one of the research gaps from the existing literature, which is further explored in this study.

2.2.5. Environmental conditions

The reduction of pollutants in a VF is mainly attributed to biotic, temperature dependent activity. The influence of temperature is an important aspect when the treatment efficiency of a VF is assessed. Temperature is an important parameter for the growth and metabolic activity of microorganisms because diversity of microbial community changes with the variation of temperature (Nedwell, 1999). Earthworm is a poikilotherm, the body temperature of which is significantly associated to outside temperature, and they could die under higher or lower temperature other than optimal temperature range (Edwards, 2004). In vermifiltration, the treatment process is due to the oxidation and decomposition process of microorganisms and earthworms, therefore, the process will inevitably be affected by temperature. In a previous study on the effect of filter bed temperature on organics and nutrient removal, the results showed the optimal temperature range of 16−25°C for earthworm survival (Yin et al., 2011). Li et al. (2009a) also studied the effect of seasonal variations on treatment efficiency of vermifiltration for domestic wastewater but the study was limited for only two seasons. The studies on how temperature affects pathogen removal, earthworm growth characteristics and microbial population are limited. In addition, little is known about the effects of seasonal

temperature on the treatment efficiency (BOD, COD, TSS removal), pathogen removal efficacy, bacteria, fungi and actinomycetes population and earthworm growth and reproduction pattern.

pH and moisture content are other important factors affecting the process of vermifiltration. Hughes et al., (2007) studied the effect of pH on vermifiltration process. The likelihood of biological inhibition and disruption against different pH levels were evaluated in a VF treating wastewater and it was found that VF has an inbuilt buffering capacity for pH (Sinha et al., 2008). The results revealed that the earthworm species could survive the pH level between 6.2 and 9.7. The moisture content inside a VF is an important parameter influencing the growth of the earthworm species since the earthworm's body contains about 80% water (Gunadi and Edwards, 2003). The moisture content in VF should be in the range of 85-90% throughout, for optimum growth and activity.

2.3. Mechanism of Vermifiltration

The typical sewage treatment process, as illustrated in Figure 2.1, normally starts with preliminary screening (with mechanical grids) to exclude large material. Grit is removed to protect the pumps and ensure free movement of the water through the plant. This is followed by the primary treatment that involves the settlement and sedimentation of fine solids resulting in removal of suspended organic solid content from the water by 50%. The high concentrations of nitrogen (N) and phosphorous (P) lead to eutrophication of waterways. Therefore, to achieve the effective reduction, the effluent after the primary treatment is passed to a secondary treatment unit. This is the main biological aspect of the process and involves the two essentially linked steps of initial bioprocessing and the subsequent removal of solids due to enhanced biological activity. Oxidation and biodegradation is the fundamental basis of biological sewage treatment. Aerobic bacteria are responsible, thriving in the optimised conditions provided, leading to the significant reduction of BOD, COD, N and ammonia (NH_4^+-N) levels in the effluent. In most cases, tertiary treatment is required as an advanced final step to remove trace organics or to disinfect effluent, which adds significantly to the cost of sewage management. At the end of the process, the treated water may be suitable for reuse but there can be difficulty in finding suitable outlets for the concentrated sewage sludge produced. Sewage sludge management is another big issue, requiring a separate treatment unit (Evans and Furlong, 2003). On the contrary, vermifiltration technology encompasses all forms of treatment, i.e. primary (removal of grit, silt, etc.), secondary (biological degradation and nutrients removal) and tertiary (removal of pathogens) treatment technology into one unit. It is a compact biological wastewater treatment system as compared to other non-conventional system. The

treatment scheme of a typical wastewater treatment process and vermifiltration is illustrated in Figure 2.1.

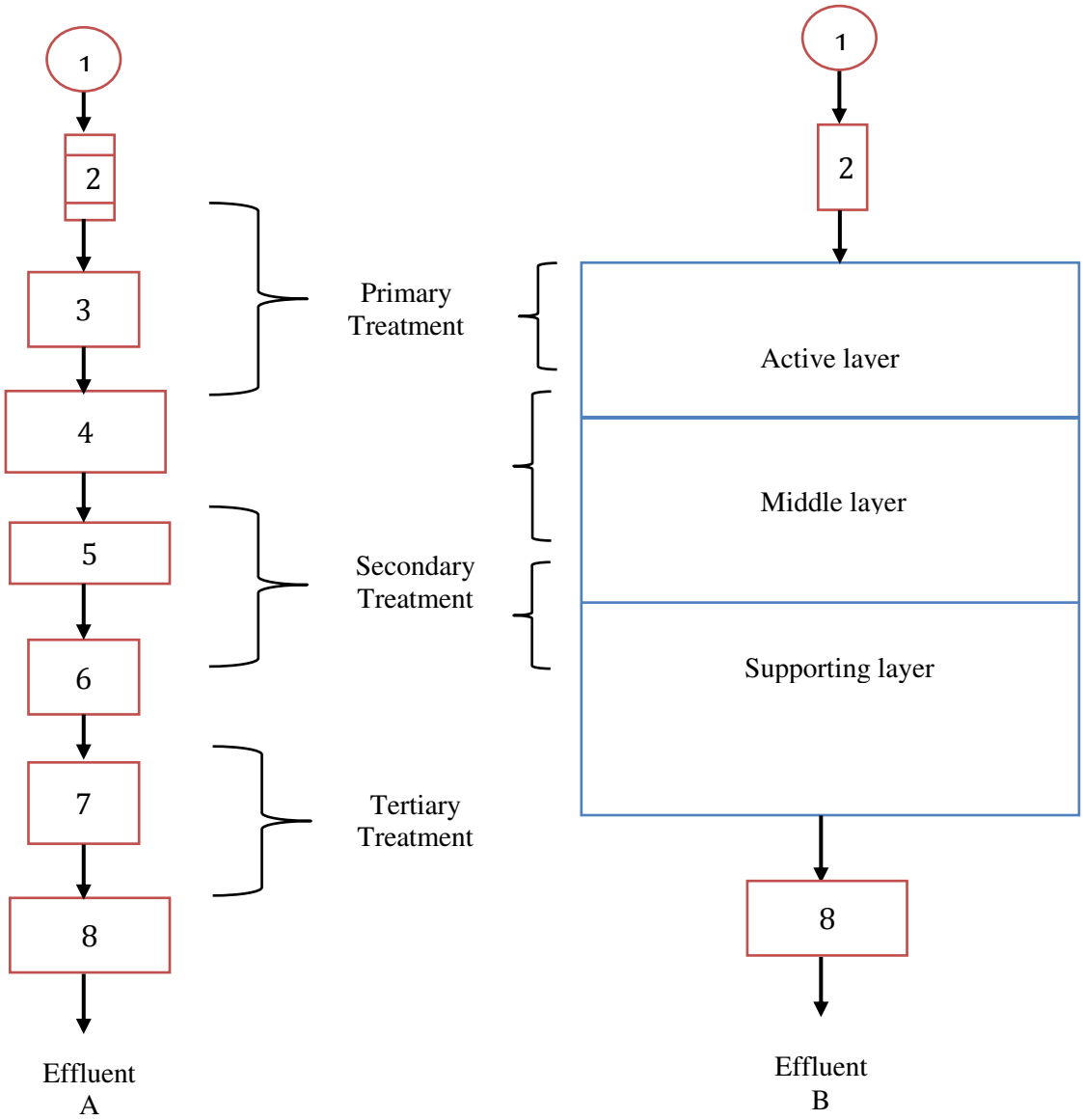


Figure 2.1: Scheme of wastewater treatment

(A) Conventional sewage treatment plant process scheme 1-Pump, 2-Screen, 3-Grit chamber, 4-Primary clarifier, 5-Aeration tank, 6-Secondary clarifier, 7-Filtration, 8- Chlorination tank

(B) Vermifiltration process scheme 1-Pump, 2-Screen (optional), Vermifilter, 8-Chlorination tank (optional)

Vermifiltration is a bio-oxidative process in which earthworms interact symbiotically and synergistically with microorganisms enhancing the degradation of organic matter by modifying its physical and biochemical properties. Earthworms are known as waste decomposers or biodegraders, proliferating other beneficial microorganisms in the VF. They perform the biodegradation of substrate by aerating, grinding, crushing and digesting the waste through their intestine. Earthworms stimulate and accelerate the microbial activity by

increasing the population of microorganisms and by improving aeration through burrowing activity. The performance of VF rely upon the relationship between earthworms and microorganisms, in which microbes perform biochemical degradation of waste material, while earthworms homogenize the material through their muscular actions and add mucus to the ingested material, thereby increasing the surface area for microorganisms. Dissolved and suspended organic and inorganic solids are trapped on the top of VF by adsorption and stabilized through complex biodegradation process. Microorganisms that populate in the filter bed along with earthworms reflect the functioning and performance of vermifiltration process (Wang et al., 2013a). Earthworms achieve greater utilization of organic matter by the enzymatic activity of microorganisms. However, the detailed knowledge on the enzymatic activity, which is responsible for organic matter degradation, is lacking. Assessment of enzymatic activity of the isolated microflora could bring an insight to the organic matter biodegradation during vermifiltration, which is explored in the present study.

It was reported by Sinha et al., (2008) that earthworms devour on the pathogens found in the wastewater. They promote the development of other microorganisms (bacteria and fungi), which are capable of producing antibiotics that kills the pathogens in wastewater resulting in sterile and disinfected effluent. However, understanding the pathogen removal efficacy of vermifiltration is still a major research gap. No attention has been paid so far to the effect of earthworms on the antimicrobial properties of the microbial community diversity and its role in VF. Thus, investigation of the microbial community dynamics can help in understanding and controlling the vermifiltration process, which is one of the objectives of this study.

The majority of nitrogen (present as organic nitrogen and NH_4^+-N) was removed mainly through rapid adsorption by the biomass in the VF. Organic nitrogen is converted to NH_4^+-N by a process known as ammonification. Nitrification, the two-step process by which ammonia is oxidized to nitrate via nitrite, plays a key role in the biological removal of nitrogen in wastewater treatment systems (Limpiyakorn et al., 2005). The adsorbed NH_4^+-N was converted to nitrate (NO_3^--N) via biological nitrification, through the formation of nitrites (NO_2^--N) as intermediates was carried out by aerobic, autotrophic bacteria using molecular oxygen as an electron acceptor (Wang et al., 2011b). Oxygen is available in abundance through the burrowing action of earthworms, which favors a microenvironment for aerobic nitrobacteria. Earthworms mediate the conversion of organic nitrogen to inorganic nitrogen thus promoting the formation of nitrate. The process involves two phylogenetically unrelated groups of obligatory chemolithotrophic bacteria: ammonia-oxidizing bacteria (AOB), which oxidize

NH_4^+-N to NO_2^--N, and subsequently nitrite-oxidizing bacteria oxidizes NO_2^--N to NO_3^--N. This results in an effluent with high concentration of NO_3^-–N, which is of significance if the water is to be reused for irrigation purposes.

In wastewater, phosphorous (P) forms occur both in soluble and insoluble phases. HPO_4^{2-}, $H_2PO_4^-$ and PO_4^{3-} ions were the main soluble-phase forms of P present in the wastewater. The effluent from the VF is mainly composed of phosphate (PO_4^{3-}) ion and is the main component of the soluble TP (99.2%). Vermifiltration is effective for removing insoluble P-forms in the wastewater into more soluble forms. The increase of soluble TP and PO_4^{3-} can be attributed mainly to the activity of earthworms (Wang et al., 2010). Activities of earthworm and associated microbes in vermi-beds promote rapid phosphate mineralization in the system causing increased concentration of PO_4^{3-} in the effluent (Hait and Tare, 2011). As PO_4^{3-} is one of the key components of water from agriculture point of view, this showed the potential of vermifiltration for wastewater reuse for irrigation purposes.

Vermifiltration technology is an earthworm driven treatment process, which can treat waste efficiently at a higher rate. The process tends to become more efficient and robust with time due to the growth of earthworms, which further proliferates the growth of aerobic microorganisms (decomposers) due to its burrowing activity. The introduction of decentralized technologies such as vermifiltration enables the recovery and reuse of resources in the form of water, nutrients and energy (Nanninga et al., 2012).

2.4. Applications of Vermifiltration

The development of a vermifilter for wastewater treatment systems is pertinent to environmental sustainability. These systems have been tested for treating different kinds of wastewater under various conditions in many countries. The Bhawalkar Earthworm Research Institute has developed a process for wastewater treatment using the species *Pheretima elongata*, where sewage was treated with HLR of 1.0 m^3/m^2d showing significant results (Athanasopoulos, 1993). Anaerobically stabilized distillery wastewater was also treated in vermifilter with OLR of 0.2 Kg COD/m^2d and showed COD and BOD removal by 90% (Athanasopoulos, 1993). Since last decade, VFs are recognized for their higher efficiency for treating municipal, domestic and industrial wastewater at a lower cost. However, most of these studies were implemented at a lab scale. The idea of vermifiltration for full-scale wastewater treatment germinated in 2005, when Xing et al. (2005) found higher performance efficiency of vermifilter. Since then, various researchers performed a pilot scale study on vermifiltration of domestic wastewater. Table 2.1 gives a comprehensive overview of the treatment performance

of various VFs for domestic wastewater treatment. Li et al., (2009a) compared a pilot scale VF installed in a village in China with activated sludge process (ASP) treatment plant. The results proved that VF has comparable treatment efficiency with ASP at a lower cost, with simpler operation and maintenance. Yang et al., (2009) and Wang et al., (2010) studied the effects of converter slag and coal cinder filter media for enhanced phosphorous removal and advanced wastewater treatment. Xing et al., (2010a) highlighted an important relationship between earthworm population dynamics and enzymatic activities with treatment efficiency of vermifilter for domestic wastewater treatment. Wang et al., (2011a) explored a novel three-tier technology involving earthworms, microorganisms and plants species *Penstemoncam panulatus* for enhancement of wastewater treatment, especially nutrient removal. The study also explored polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) for investigating the microbial community diversity in a VF. In another study, Wang et al. (2011b) investigated the dominant population of ammonia oxidizing bacteria (AOB) in a VF by 16S rRNA sequeuncing. It is known that ammonia oxidation is carried out by AOB belonging to *Beta* and *Gamma-proteobacteria* (Kasuga et al., 2010). Li et al. (2012) developed a new multi layer VF for treatment of rural domestic wastewater, which showed better efficiency. In context to India, two out of every three Indians practice open defecation which means 665 million people lack improved sanitation and thereby, India has the highest number of people who defecate in the open (Starkl et al., 2013). Therefore it is obligatory to consider various factors including economics when implementing a wastewater treatment system, as often people in India are not ready or willing to adopt these and pay for service provision. Various researchers have explored the potential of vermifiltration in India. Kharwade and Khedikar (2011) showed that a vermifilter with earthworms could significantly remove BOD, COD, and TSS as compared to a filter without worms. Tomar and Suthar (2011) integrated the technology with constructed wetland, and showed enhancement in the treatment process.

Table 2.1: *Performance Inventory of Vermifilter for Domestic Wastewater Treatment*

Vermifilter specifications Type (Material) Dimensions	Design parameters			Removal (%)							Country	Reference	Remarks
L × W × D (m) or Dia × Depth (m)	HLR ($m^3/m^2/d$)	Earthworm type/ No. ($worms/m^3$)	Filter media	BOD	COD	TSS	NH_4^+-N	TN	TP	FC			
Cuboid (concrete) 4. 0 × 2.5 × 2.0	1.0	*E. andrei* 3,000	Coarse & fine quartz sand, wood flour, chaff, turf	89.3	83.5	89.1	53.7	56.7	65.2	-	China	Li et al., 2009a	Pilot scale VF for long term operation optimizing HLR, OLR and seasonal temperature
-	-	-	Converter slag, coal cinder	86.3	71.8	88.6	58.0	24.3	16.6	-	China	Yang et al., 2009	Batch column experiments to test the suitability of filter media
Cylinder fitted with a cone (PVC) 0.6 × 0.25	4.0	*E. fetida* 1,000	Ceramic pellets (3-5 mm)	98.4	78.0	-	90.3	-	62.4	-	China	Wang et al., 2010	Evaluation of the effect of earthworms and filter media height

Cuboid Dia 1.83 m	2.4	*E. fetida* 21,000 or 30.3 Kg	Ceramsite (3-5 mm), Quartz sand (1.40-2.36 mm)	60.6	56.0	67.5	41.7	11.3	-	-	China	Xing et al., 2010a	Relationship between earthworm population, enzymatic activity & treatment efficiency
Cuboid- three stages (PVC) Stage 1 & 2 0.5 × 0.5 × 0.6 Stage 3 1.10 × 0.65 × 1.20	1.0	*E. fetida* 12.5 g/L Plant *Penstemon campanulatus*	Soil, silver sand, fine detritus, cobble stones	-	81.3		98.0	*60.0	98.4	-	China	Wang et al., 2011a	Enhancement of wastewater treatment using multistage tower VF
Cuboid (plexiglass) 0.40 × 0. 40 × 11.5	1.0	*E. fetida* 12.5 g/L	Soil, saw dust, sand, detritus, cobble stones	-	79.5	-	95.6	40.9	-	-	China	Wang et al., 2011b	Microbial earthworm ecofilter for nitrogen removal
Cuboid Depth 0.27 m	-	*E. euginae*	Gravel, sand, soil, cow dung	89.0	77.0	75.0	-	-	-	-	India	Kharwade and Khedikar, 2011	Comparison of filter with and without earthworms
Unit 1: Cylinder (plastic; 80 L) Unit 2: Rectangular	-	*Perionyx sansibaricus*	Gravels, sand, stones, sawdust, leaves, soil	-	90.0	88.6	92.7		98.3		India	Tomar and Suthar, 2011	Integration of VF with constructed wetlands

Multilayer VF 0.60 × 0.40 × 0.40	1.0	*E. andrei*	Quartz sand, wood chips, turf	85.0	75.0	91.7	72.9	-	68.8	-	China	Li et al., 2012	Treatment effects of multi layer vermifilter
Cuboid (plexiglass) 0.30 × 0.30 × 0.60	0.5	*E. fetida* 4.63 g/L (250 g)	Large gravels, ceramsite, sand, soil, wood chips	-	89.4	-	81.2	74.2	-	-	China	Zhao et al., 2012	VF with earthworms & plants at C: N ratio 6: 1 gives optimum results
Cylinder	4.2	*E. fetida* 8 g/L	Ceramsite (3-5 mm)	78.0	67.6	89.8	92.1	-	-	-	China	Liu et al., 2013	Use of gene library technology for microbial community structure
Cuboid (Plexiglass) 0.40 × 0.85	1.0	*E. fetida* 12g/L	Cobble stones, fine detritus, silver sand soil	-	82.5	-	90.0	-	-	-	China	Wang et al., 2013a	Community analysis of AOB in different seasons in microbial earthworm ecofilter
Cuboid 0.70 × 0.70 × 0.75	0.2	*E. fetida* 0, 4.5, 8.5, 12.5, 16.5 g/L	Cobble stones, fine detritus, silver sand artificial soil, rice straws		72.2		74.7	64.3	81.3			Wang et al., 2013b	Effects of earthworm loads on organic matter & nutrient removal & bacterial community diversity

Note: a) L-length, W-Width, H- Height, Dia- Diameter, BOD- Biochemical oxygen demand, COD- Chemical oxygen demand, TSS- Total suspended solids, NH4-N- Ammonia, TN- Total nitrogen, TP- Total phosphorous, FC- fecal coliforms, b) * Indicates nitrate removal

Vermifiltration can be also used as a sustainable option for individual industry or cluster of similar industries, to reduce the burden on sewage treatment plants (STPs) or common effluent treatment plants (CETPs). The technology gained its momentum when various researchers explored the technology for treating different kinds of wastewater from various industries. Table 2.2 described the studies indicating potential of earthworms for industrial wastewater treatment. Sinha et al. (2007) reported the treatment of dairy wastewater in Australia. Li et al., (2008) devised an integrated method for the development of VF to treat swine wastewater in China. In India, Ghatnekar et al., (2010) applied three-tier vermitechnology to treat gelatine industry wastewater, and Dhadse et al. (2010) reported the treatment of pharmaceutical wastewater using vermifiltration technology.

Vermifiltration technology sets the process of physical filtration, adsorption, aerobic decomposition of organics, and sludge treatment, all in one unit, which can simultaneously achieve combined treatment of wastewater and sludge stabilization. Various studies have been conducted in the past that showed the potential of VFs in combined treatment of wastewater and sludge. Table 2.3 gives a comprehensive overview of the performance inventory of various VFs for the combined treatment of wastewater and sludge stabilization. As an extension of vermicomposting for solid waste, vermifilters (VFs) were developed to treat both solid and liquid waste simultaneously (Xing et al., 2011). The capacity of VF to treat solid waste and wastewater can be attributed to the vermi-composting and vermi-filtration processes that occur within the system due to earthworm's consumption of organic matter on filter bed surface (Taylor et al., 2003). However, there exists a literature gap to assess the ability of VFs to treat organic fraction of municipal solid waste (OFMSW) and wastewater together, which is a part of this study. Investigations regarding the performance evaluation of a pilot scale vermireactor and microbial community dynamics were made to understand the vermifiltration process.

Table 2.2: Earlier Studies Indicating Potential of Earthworms in Different Industrial Wastewater Treatment

Types of wastewater	Country	Earthworm sp.	Major observations	Reference
Dairy wastewater	Australia	*E. fetida*	Significant organic matter and SS removal from organic rich dairy wastewater	Sinha et al., 2007
Swine wastewater (piggery)	China	*E. andrei*	Effective for treating wastewater from swine facility; reduces ammonia and green house gases emissions; treated effluent can be utilized to flush the manure	Li et al., 2008
Gelatin industry wastewater	India	*Lumbricus rubellus*	Application of three tier vermiculture technology to treat gelatin industry wastewater; synergistic interactions of earthworms, enzymes and microorganism (algae & fungi)	Ghatnekar et al., 2010
Herbal pharmaceutical wastewater	India	*E. eugeniae*	85-96 % BOD & COD removal, feasible option to treat toxic pharmaceutical wastewater at different hydraulic and organic loadings	Dhadse et al., 2010
Pig slurry	France	*E. fetida, E. andrei*	Earthworms reduce emissions of ammonia, nitrous oxide and methane emissions; VF contributes to Carbon & Nitrogen abatement	Robin et al., 2011
Petroleum wastewater	Australia	*E. fetida*	Effective for treating toxic wastewater; 99.9 % reduction in volatile hydrocarbons from petroleum	Sinha et al., 2012
Milk processing industry wastewater sludge	India	*E. fetida*	Potential to convert milk processing sludge into vermicompost (with high nutrients)	Suthar et al., 2012
Spent Carbon and chemical sludge from soft drink industry	India	*E. fetida*	Chemical sludge form soft drink industry can be converted into quality manure in a short span (88-100 days); good quality end products	Singh and Kaur, 2013
Paper mill wastewater sludge	India	*E. fetida*	Vermistabilization of paper mill sludge resulting in stable end product, rich in nutrients, microbial population; suitable for land applications (agronomic uses)	Negi and Suthar, 2013

Table 2.3: *Performance Inventory of Vermifilter for Combined Treatment of Wastewater and Sludge Stabilization*

Vermifilter specifications Type (material)/ dimensions L × W × H (m) or Dia × D (m)	Design parameters HLR ($m^3/m^2/d$)	Earthworm type/ No. ($worms/m^2$)	Filter media	Important findings	Country	Reference	Remarks
Cuboid (plastic)	-	Mixture of *E. fetida, P. excavatus E. euginae* (500 worms)	Gravels, sand, garden soil	98% BOD; 45% COD, 90% TSS, 98% turbidity removal efficiency; pH buffering ability, significant removal of toxic compounds, heavy metals and pathogens	Australia	Sinha et al., 2008	Mechanism of vermifiltration process
Cylinder fitted on a cone 0.25 × 0.35	4.0	*E. fetida* 25, 000	Ceramsite	74.5% BOD, 51.9% COD, 91.9% TSS, 69.3% NH_4^+-N removal efficiency; ability to buffer pH; no clogging & foul odor observed; Presence of *zoogloea, rotifer* and *vorticella* microroganisms	China	Liu et al., 2009	Ability of earthworms to decompose organics and devour solids
Cylindrical column 0.55 × 0. 30	3.0 OLR- 50 g VSS/d	*E. fetida*	Ceramic pellets (3 -5 mm)	PCR-DGGE revealed the presence of diverse microbial community in VF with higher earthworm load	China	Li et al., 2009b	Earthworm occurrence enhanced microbial diversity
Cylinder 0.30 × 0.60	3.0 OLR- 1.5 Kg VSS/m^3	*E. fetida* 32 g/L	Ceramic pellets (6-9 mm)	48.5~ 53.5% TCOD removal; 56.2~ 66.6% VSS reduction; additional 25.1% reduction in VSS due to earthworms; improved sludge settleability with a compact structure and low SVI values (33–45 mL/g)	China	Zhao et al., 2010	Earthworm–microorganism interaction for stabilization of sludge

Circular Surface area- 10.75 m^2 Dia 1.83 m	Varying HLR 2.4, 4.2, 6.0, 6.7	*E. fetida*	Quartz sand (1.9 mm)	47.26 ~ 57.55% COD, 54.78 ~59.29% BOD, 62.06 ~ 77.90% SS, and 21.01 ~ 52.58% NH_4^+-N removal efficiency; sludge reductions 38.2 ~ 44.7%; VSS/SS ratio of the effluent sludge 44.8 ~ 48.1; VFA of the effluent sludge 36.9~39.3 mg/L; Microorganisms found in the unit are *Vorticella*, *Nematode*, *Rotifer* sp.	China	Xing et al., 2010b	Vermifilter operation offers cost savings of up to 48.72%
Circular 0.25 × 3.7 m	4.8	*E. fetida* -	Ceramsite (3- 5 mm), Quartz sand (1.68- 2.05 mm)	Avg. sludge reduction 38.2~ 48.2%; VFA of the vermicast sludge range 34.1~ 39.3 mg/L, VSS/SS ratio 42.1~48.1%, SVI range 28.9–37.8 mL	China	Xing et al., 2011	Ceramsite as better filter media compared to quartz sand
Circular Vol-53.3 L, 0.30 × 0.50	OLR 2.24 Kg VSS/m^3.d	*E. fetida* 3000 worms (32g/L)	Ceramsite (10-13 mm)	Sludge reduction capability 936 ± 47 gVSS/m^3.d; VSS reduction 43.17 ± 2.0 %; Total biomass quantity 101 ± 6.6 gVSS; SVI of the treated sludge 48 ± 3.2 mL/g; Earthworm biomass increase by 41.6%; DGGE analysis showed most abundant microorganisms in VF biofilm	China	Liu et al., 2012	Comparison of VF and biofilter for sludge reduction ability, along with investigation of microbial community diversity
Cylindrical (perspex) 0.20 × 1.20	4.0 OLR 1.12 kg VSS/ m^3.d	*E. fetida* 40 g/L	Ceramsite (10-20 mm)	Fluorescence EEM spectra of humic acid like fractions indicates the maturity & stability of VF biofilm; Presence of earthworms decreases OM contents, microbial biomass, and improves microbial & enzymatic activities & community structure of VF biofilm	China	Li et al., 2013	Properties of biofilm for sewage sludge stabilization
Cylindrical 0.30 × 0.80 Vol. 56.5 L	OLR 1.36 kg VSS/m3.d	*E. fetida* 32 g/L	Ceramsite (10-13 mm)	SS reduction 34.8 ± 5.1%; VSS reduction 47.9 ± 6.2 %; Sludge reduction capability 651 ± 5 g VSS/(m3 d); Total biomass quantity in the filter 213 ± 14.4 g/VSS; 24.7% increase in earthworm biomass; greater diversity in VF biofilm; VF biofilm dominated by unclassified *Bacillus* sp., *c-proteobacteria, b-proteobacteria,* and *Actinobacteria*	China	Yang et al., 2013a	Effect of earthworm on morphology, biochemical characterization, microbial community analysis

Cylindrical 0.30 × 0.90 Vol. 49.5 L	4.0 OLR 1.38–1.51 kg VSS/m3.d	*E. fetida* 32 g/L	Ceramsite (10-13 mm)	VSS reduction 48.5 ± 3.9%; VSS: SS ratio 0.62 ± 0.03; Analysis of heavy metal chemical speciation showed that some unstable fractions were transformed into stable fractions; Zn was accumulation observed; risk analysis further supported that earthworms weakened environmental risk of heavy metals after vermifiltration of sludge	China	Yang et al., 2013b	Effect of earthworms on heavy metal stabilization of liquid state sludge
Cylindrical (perspex) 0.20 × 1.0 Vol- 31.4 L	4.0 OLR 1.10–1.28 kg-VSS/m3.d	*E. fetida* 32 g/L	Ceramsite (10-20 mm)	VSS reduction 49.9 ± 2.80 %; VSS/SS 0.63 ± 0.033; fatty acid profile analysis revealed feeding behavior and trophic relationship of earthworms and other predators	China	Xing et al., 2014	Liquid-state sludge stabilization using fatty acid profiles

Note: a) L-length, W-Width, H- height, Dia- Diameter, Vol- Volume BOD- Biochemical oxygen demand, COD- Chemical oxygen demand, TSS- Total suspended solids, VSS- Volatile suspended solids, VFA- Volatile fatty acid, NH4-N- Ammonia, TN- Total nitrogen, TP- Total phosphorous, DGGE- Denaturing gradient gel electrophoresis

2.5. Summary

The review attempts to identify and summarize the performance and evaluation of vermifiltration technology at lab and full scale. Based on the overall analysis, it is found that vermifiltration technology is not only one of the most promising technologies for rural and peri-urban sewage treatment but also for the treatment of wastewater from small agro-based industrial units. The present review evidenced the design criterion of a full scale vermifilter based on HLR, HRT, OLR, earthworm species type and number, filter bed media, and other environmental conditions that defined the treatment potential of VF. It is successfully applied and demonstrated in different countries throughout the world. The technology showed higher BOD, COD, TSS, and nutrients removal efficiency for various kinds of wastewater. The review suggests that earthworms together with microorganisms in a controlled ecosystem, termed *vermifiltration* can significantly treat wastewater. However, most of the research on vermifiltration is carried out for physico-chemical parameters. So far, it does not highlight on the removal of pathogens or the mechanism involved. Therefore, necessary studies are required to assess the pathogen removal efficiency and mechanism involved therein. Previous studies have investigated the composition of microbial community diversity in a VF by using advanced techniques like PCR-DGGE, fatty acid profiling, etc. However, other than investigating the microbial community diversity, it is more crucial to understand the dynamics and role of microbial community in a vermifilter. Therefore the present study is designed to understand the role of isolated microbial community, to enhance the understanding behind vermifiltration process and to optimize the design parameters such as filter media or seasonal temperature for vermifiltration for wastewater and solid waste treatment.

Next chapter deals with the experimental methodology.

CHAPTER 3

METHODOLOGY AND EXPERIMENTAL METHODS

3.1. Methodology and Framework

In order to accomplish the objectives, the research was carried out into different phases. The summarized form is shown in the form of flowchart, in Figure 3.1, while the detailed methodology is illustrated in Figure 3.2. The methods of sampling and analysis are described in the next section.

Phase 1.1: A lab scale study was carried out to investigate the organics and pathogen removal profile and factors affecting pathogen removal in a vermifilter, along with pathogen removal kinetics. The duration of this phase was from March 2013 to May 2013. It is discussed in Chapter 4.

Phase 1.2: Additional work was included to Phase 1 in another lab scale study to compare the effects of locally available filter media (River-bed gravels, Mud balls, Wooden coal and Glass balls) in a vermifilter for wastewater treatment. The duration of this phase was from April 2013 to June 2013. It is discussed in Chapter 5.

Phase 1.3: Based on the results of Phase 1 and Phase 2, it was clear that determining the organics and pathogen removal profile is prerequisite to explain the performance of vermifiltration process. Henceforth, a pilot scale study was carried out to investigate in details, the treatment performance and microbial community dynamics, along with antimicrobial and enzymatic property of microorganisms in a vermifilter to gain in-depth knowledge about the mechanism behind the treatment process. Attempts were also made to develop the pathogen removal model. The duration of the study was from June 2013 to September 2013. It is discussed in Chapter 6.

Phase 1.4: A long term continuous evaluation of vermifilter was carried out to investigate the effects of seasonal temperature on treatment efficiency, earthworm population and microbial diversity for one year. Attempts were made to examine the correlation between temperature and all water quality parameters during vermifiltration for wastewater treatment. The duration of this phase was from December 2012 to November 2013. It is discussed in Chapter 7.

Phase 2.1: A new concept in vermifiltration was introduced for the combined treatment of organic fraction of municipal solid waste (OFMSW) and domestic wastewater. A pilot scale

VF was evaluated for the performance efficiency for the removal of organics, nutrients and pathogens. The duration of the study was from October 2013 to December 2013.

Phase 2.2: The next sub-phase includes the investigation of the microbial community dynamics, antimicrobial activity and the phylogenetic relationship of the isolated bacterial species by 16S rRNA sequencing, was further determined during the period from January 2014 to June 2014. Phase 2.1 and 2.2 is discussed in Chapter 8.

The final conclusions of the study along with future recommendations are discussed in Chapter 9.

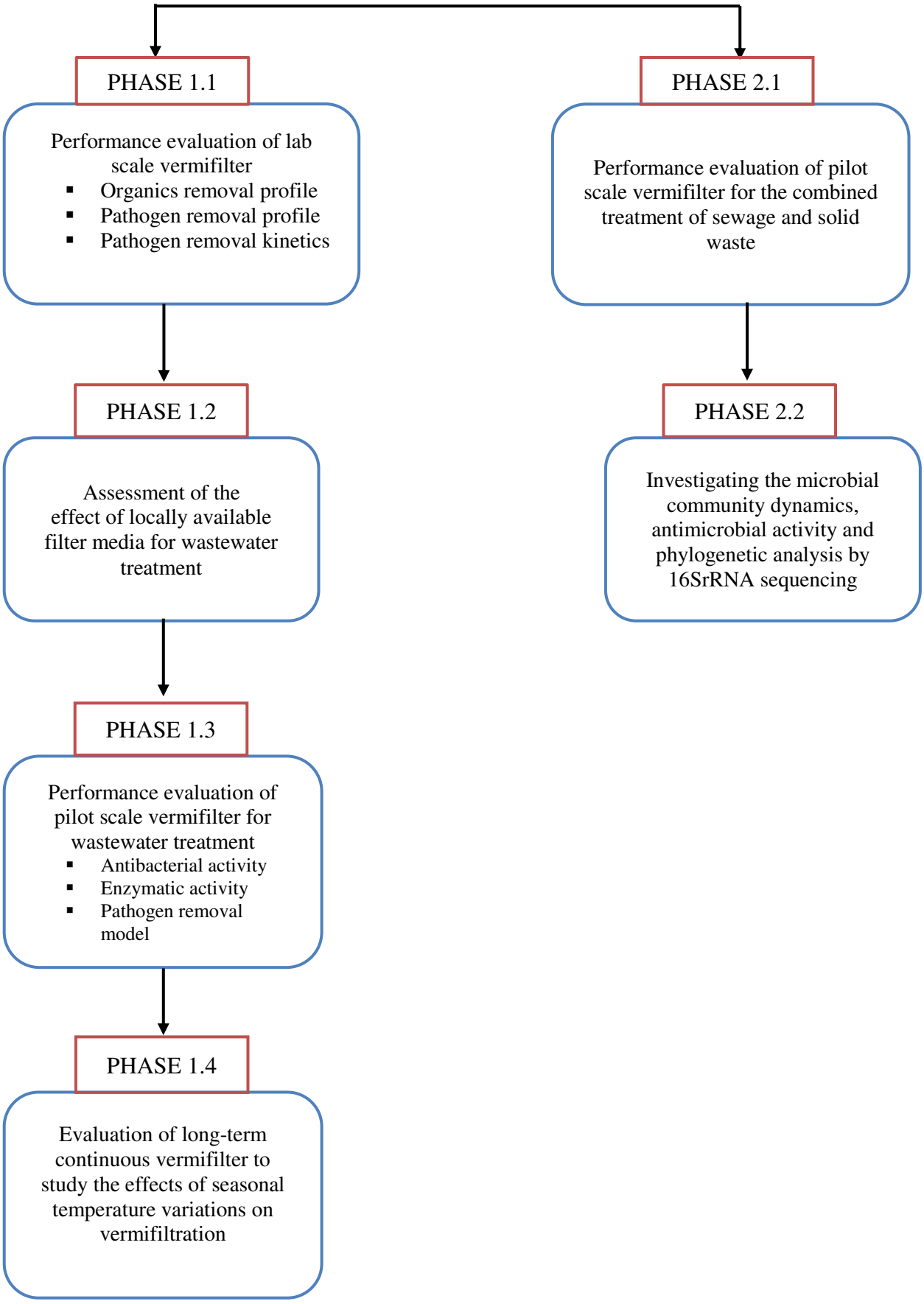


Figure 3.1: Methodology of the research (summarized form)

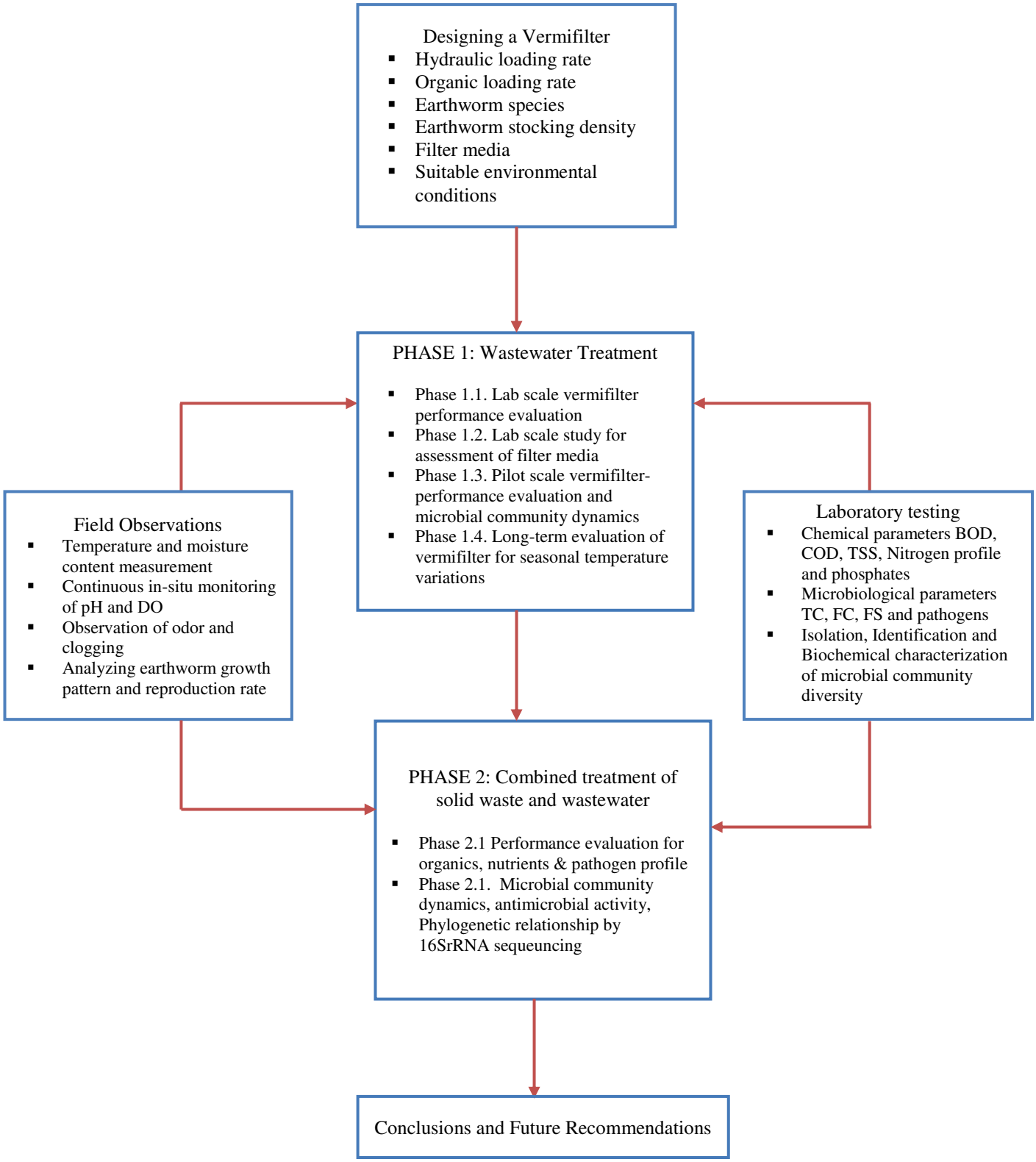


Figure 3.2: Detailed methodology of research

3.2. Experimental Methods

Different experimental approaches were used in the study to accomplish the stipulated objectives. To test the performance efficacy of VF, various physico-chemical and microbiological analyses were carried out according to the methods described below. Samples of wastewater (influent and effluent) were collected in sterile plastic bottles, twice a week and solid waste samples were collected once in 30 days, throughout the experimental period.

3.2.1. Physico-chemical parameters

pH, temperature and dissolved oxygen (DO) of samples were measured daily in situ using a Hach multi-parameter kit (Hach, USA). BOD was measured by azide modification method and COD was measured using potassium dichromate method (APHA, 2005). NH_4^+-N, NO_3^--N and TP were determined using Nessler's reagent spectrophotometric method, ultraviolet spectrophotometric method and acid digestion using stannous chloride method, respectively (APHA, 2005), as described in Table 3.1.

For solid waste samples, 10 g of sample was dissolved in 90 mL of sterile distilled water and shaked mechanically for 2 h at 100 rpm and analyzed for pH, temperature, moisture content, total nitrogen (TN), NH_4^+-N and NO_3^--N. Moisture content was determined by weight loss of sample at 105°C for 24 h. Total organic carbon (TOC) was determined by Shimadzu (TOC-VCSN) solid sample module (SSM-5000A).

The percentage reduction can be calculated by using Equation 3.1, where, C_i and C_0 represent the influent value and effluent value, respectively.

$$Percentage\ reduction\ (\%R) = \frac{(Ci-Co)}{Ci} \times 100 \qquad \text{Equation 3.1}$$

3.2.2. Microbiological parameters

Water and solid waste samples were analyzed for indicator microorganisms like total coliforms (TC), fecal coliforms (FC), fecal streptococci (FS), and pathogens such as *Escherichia coli*, and *Salmonella*. TC, FC, and FS were evaluated using Lauryl Tryptose broth, A1 broth, and Azide Dextrose broth, respectively (APHA, 2005), as shown in Table 3.2. The population of *E. coli* was enumerated by SPC on MacConkey agar media after incubation for 24–48 h at 37°C (Bhatia et al., 2012). The population of *Salmonella* was enumerated on the plates of Modified Semisolid Rappaport–Vassiliadis media after incubation for 17–24 h at 42°C (Bhatia et al., 2012). The total bacteria count was determined using Nutrient agar media after incubation for 24–48 h at 37°C. Total fungi was determined using Potato Dextrose agar, for 48-72 h at 28°C. Actinomycetes population was determined on starch casein agar media for 72 h at 28°C

(Kadam et al., 2009). All the specific media were obtained from Vikas Scientific Co. supplied from Hi-Media Laboratory Pvt. Ltd., India. The Pathogen removal efficiency is calculated using Equation 3.2, Where, C_{in} and C_{out} represents the influent pathogen concentration and effluent pathogen concentration, respectively.

$$Log\ Removal\ Value\ (K) = log_{10}\ {}^{Cin}/_{Cout} \qquad \text{Equation 3.2}$$

3.2.3. Wastewater composition

During the Phase 1 of the study, synthetic wastewater was used as the influent. The synthetic wastewater was composed of tap water, glucose, urea, $NaHCO_3$, NH_4Cl, KH_2PO_4, K_2HPO_4, $CaCl_2$, and $MgSO_4.7H_2O$ to give the ratio of COD/N/P as 300/30/1 in a way such that it simulates actual domestic wastewater of medium strength (Seetha et al., 2010). It was prepared every day and agitated manually for 5 min before being pumped into the VF. It was seeded with 5% actual domestic sewage for initializing the growth of coliforms and microbial population. The reason behind using synthetic wastewater was that, Phase 1 was continuous study that required huge amount of sewage, which was not available near the study area. Also, variations in the real wastewater (sewage) in the institute campus were found to be very high. So, in order to standardize the procedure, maintaining all other conditions constant, synthetic domestic wastewater was used that imitate the actual sewage, to maintain the constant conditions for the study. During Phase 2, actual wastewater was collected from nearby sewage pumping station and was introduced into VF through perforated PVC pipes (1.5 mm diameter). This is because, during this phase, VF was allowed to operate only for 6 h per day (80 L for 6 h) at room temperature. The availability of this amount of wastewater was not an issue during this phase of the study.

Table 3.1: *Methods of Analysis for Physico- chemical parameters*

Parameter	***Principle***	***Instrument/ Technique used***
Temperature (°C)	Metric	Thermometer
pH	Metric	Digital pH meter (calibrated)
TSS (mg/L)	Gravimetric	SS estimated as the weight of solids material retained on a pre-weighted filter after filtering a known volume of sample and drying the filter at 105^{o}C until a constant weight is reached
DO (mg/L)	Metric	HACH Multi-parameter kit
BOD (mg/L)	Volumetric	Azide modification method, Incubation for 3 days at 27°C
COD (mg/L)	Closed reflux, Colorimetric	Potassium dichromate method, Hach COD System (DR/ 4000 U Spectrophotometer) set at λ = 600 nm
NH^{+}_{4}-N (mg/L)	Colorimetric	Direct Nesslerization method using spectrophotometer, λ = 425 nm
NO^{-}_{3}-N (mg/L)	UV fluorescence	Ultraviolet spectrophotometric method, λ = 220 nm and 270 nm
TP (mg/L)	Colorimetric	Acid digestion using stannous chloride method, λ = 650 nm

Table 3.2: *Methods of Analysis for Microbiological Parameters*

Parameter	***Method***	***Media***	***Incubation temperature and time***	***Expression of results***
Total coliforms (TC)	MPN	Lauryl Tryptose Broth (pH-6.8±0.2)	35 ± 0.5°C, 24−48 h	MPN/100mL
Fecal coliforms (FC)	MPN	A-1 Broth medium (pH 6.9 ±0.2)	35 ± 0.5°C for 3 h and 44.5 °C for 21 h	MPN/100mL
Fecal streptococci (FS)	MPN	Azide Dextrose Broth (pH 7.2)	35 ± 0.5°C for 24−48h	MPN/100mL
Total heterotrophic bacteria (THB)	SPC	Nutrient Agar (pH 7.0)	37 ± 0.5°C for 24−48 h	CFU/mL
Total fungi (TF)	SPC	Potato Dextrose Agar	25^{o}C for 72 h	CFU/mL
Actinomycetes	SPC	Starch Casein Agar	27^{o}C for 72 h	CFU/mL
E. coli	SPC	MacConkey Agar	35 ± 0.5°C for 24−48h	CFU/mL
Salmonella	SPC	Modified Semisolid Rappaport-Vassiliadis	42°C for 17−24h	CFU/mL

CHAPTER 4

LAB SCALE VERMIFILTER FOR WASTEWATER TREATMENT

A lab scale vermifilter was installed and operated for the duration of 70 days from March 2013 to May 2013 to study the treatment of domestic wastewater, with special focus on the removal of organics and coliforms. The results were quantified and presented, and emphasis was placed on the pathogen removal kinetics.

4.1. Introduction

In recent years, the magnitude of the problem of sewage treatment and management has increased due to augmenting industrialization and urbanization. The main problem of pollution is excessive sewage generation and its discharge in the nearby water bodies (Belmont et al., 2004). Due attention has not been given to solve the problem of sewage pollution, which contributed to the worsening of the water quality (Khursheed et al., 2014). One of the greatest concerns about sewage pollution is occurrence of enteric pathogens, which leads to disease outbreak around the world (Paul et al., 1997; Pundsack et al., 2001). Recent outbreak of water-borne diseases has raised concerns regarding the safety of water reuse (Curriero et al., 2001). The necessity to remove pathogens from wastewater is long recognized and study on the assessment of the pathogen removal efficacy of any treatment system is essential (Zhang et al., 2007). The removal of pathogens from wastewater can be approached by either removal processes and/or inactivation (disinfection) processes. Conventionally, pre-treatment via coarse filters (e.g. gravel, sand, etc.) reduces turbidity and microbial population. Storage reservoirs can offer pathogen removal by permitting settlement and allowing time for bacterial and virus death outside the host environment. However, these simple treatment systems are infrequently sufficient in themselves to meet the pathogen removal standards (Amy et al., 2000). The disinfection of pathogens is the second important approach. Oxidation, heat or ultraviolet treatments are used. Oxidation reacts with the organic structure of the pathogens, heat kills pathogens by exceeding thermal tolerances, whilst ultraviolet radiations disrupts the genetic material of a cell through a mechanism that ultimately restricts replication, though a large cost factor is associated with all these technologies (Wolfe, 1990; Undabeytia et al., 2014).

Vermifiltration technology has the ability to remove chemical and biological contaminants in a single unit. The performance of vermifilter is controlled by an ecosystem of living organisms (earthworms and microorganisms), which is responsible for biodegradation of organic matter and biological inactivation of pathogens found in wastewater. So far, most of the studies related to vermifiltration concern the removal of organics and inorganic nutrients;

however, the pathogen removal part has been neglected. Sinha et al., 2008 reported that earthworms engulf the pathogens found in the wastewater. They promote the development of bacteria and fungi species, which are capable of producing antibiotics that might kill the pathogens in wastewater. This could be the possible reason for the removal of pathogens by vermifilter; however, there is no quantitative information available so far. There is limited literature for the removal efficacy of pathogens in a vermifilter. Therefore, investigations regarding the pathogen removal efficacy of vermifiltration are necessary for optimizing and understanding the process.

4.2. Objectives

The specific objectives of the present study are:

- To obtain the BOD, COD, coliforms (TC, FC, and FS) and pathogen removal profiles in a lab scale vermifilter
- To determine the removal efficiency of total heterotrophic bacteria (THB), total fungi (TF) and actinomycetes in a vermifilter
- To evaluate the pathogen removal kinetics in a vermifilter

4.3. Experimental Methodology

4.3.1. Description of lab scale vermifilter

A lab scale VF of dimensions $0.30 \times 0.25 \times 0.60$ m^3 made of polypropylene material was set up as shown in Figure 4.1 to treat synthetic domestic wastewater. The pictorial view of VF is shown in Figure 4.2. The VF consisted of 55 cm of filter bed packed with different materials, with an empty space of 5 cm at the top for aeration purpose. The filter bed consisted of four layers, and the composition is described in Table 4.1. At the top layer, earthworm species *E. fetida* were inoculated at stocking density of 10,000 worms per cum. These worms were allowed to acclimatize for about seven days before the experiments. After stabilization phase, VF was allowed to run for 10 weeks continuously at constant HLR of 1.3 $m^3 m^{-2} d^{-1}$. The VF consisted of a glass rod distributor with 1-2 mm perforations for its uniform distribution and effluent was collected at the bottom of the VF

Table 4.1: *Description of Filter Bed Layers*

Layer (from the top)	Filter material	Particle size	Depth
Layer 1 Active layer	Mature vermigratings	600-800 µm	15 cm
Layer 2 Second layer	Small gravels	6-8 mm	15 cm
Layer 3 Third layer	Sand	1-2 mm	10 cm
Layer 4 Supporting layer	Large gravel and stones	12-14 mm	15 cm
Empty space			5 cm
Total depth			60 cm

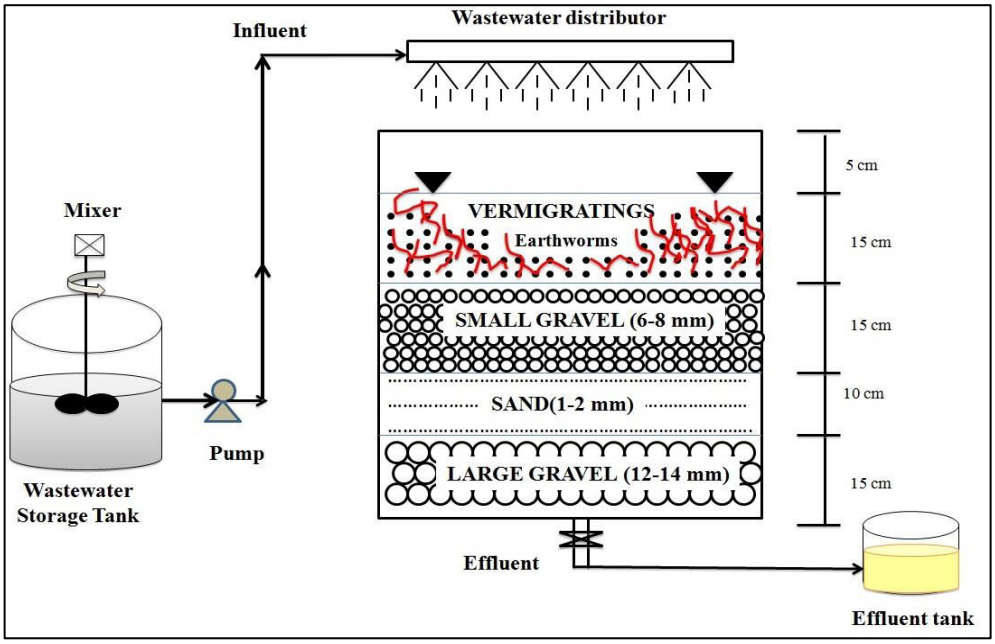


Figure 4.1: Schematic diagram of lab scale VF

Figure 4.2: Pictorial view of lab scale VF

4.3.2. Sampling and analysis

The influent and effluent samples were collected in sterile plastic bottles twice a week and analyzed immediately for physico-chemical and microbiological parameters, as explained in section 3.2. Statistical analysis was carried out for data interpretation. The results were expressed as mean ± standard deviation.

4.3.3. Pathogen removal kinetics

The average value of indicator organisms in influent and effluent permits the calculation of area-based first order bacterial decay constants (k, md^{-1}) using Equation 4.1 (Arias et al., 2003), where, C_o represents indicator organisms in the effluent (MPN/100 mL), C_i represents indicator organisms in the influent (MPN/100mL) and q represents hydraulic loading rate ($m^3m^{-2}d^{-1}$).

$$Co/Ci = \exp(-k/q) \qquad \text{Equation 4.1}$$

Isolating k in Equation 4.1 yields,

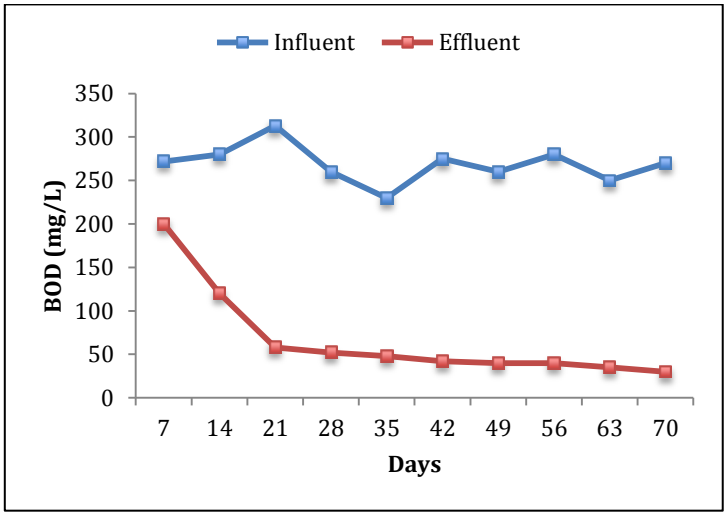
$$k = q \times \ln\frac{Ci}{Co} \qquad \text{Equation 4.2}$$

4.4. Results and Discussions

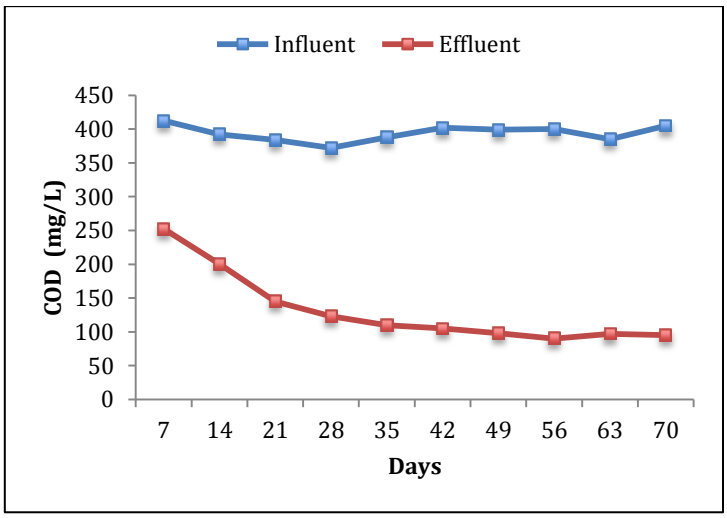
4.4.1. Physico-chemical contaminants removal

Table 4.2 shows the performance efficacy of the lab scale VF. The average pH of the influent was 7.7 ± 0.8, and effluent was found to be 7.2 ± 0.3, which is close to neutral showing the inherent buffering capacity of earthworms to neutralize pH during vermifiltration. The temperature of the influent was observed to be 25 ± 2°C and effluent was 26.5 ± 1.5°C, which was within the optimum temperature range for earthworm species *E. fetida* (Tripathi and Bhardwaj, 2004). The mean concentration of BOD and COD in influent was 269 ± 22 mg/L and 394 ± 12 mg/L, respectively. Figure 4.3 (a) and (b) showed the variation of influent and effluent concentrations of BOD and COD with time, respectively. BOD and COD concentration decreased remarkably during the study, with mean removal efficiency of 84.8% and 73.9%, respectively. The decrease in effluent concentration after 21-28 days is attributed to the fact that introducing earthworms in the VF allows accumulated organic matter to degrade at a fast rate. The burrowing activity of earthworms loosens the filter media to improve air permeability allowing BOD and COD removal. There are various enzymes present in the gut and intestines of earthworms, responsible for biodegradation of organic matter. So this clearly gives the possible reason for organics removal in VF. Total suspended solids (TSS) and total dissolved solids (TDS) were reduced during vermifiltration significantly. The maximum TDS

removal in vermifilter was observed to be 82%. This could be attributed to the ingestion of organic and inorganic solid particles in wastewater by earthworms, which excrete them as finer particles. These finer particles are further trapped in the voids of VF and causes high removal efficiency of TSS and TDS from wastewater (Sinha et al., 2008). The TSS removal for VF was observed to be 90%. Various physical, chemical and biological reactions take place in vermifiltration process including the adsorption of molecules and ions, oxidation–reduction of organic matter, the behaviour of earthworms and their synergetic effects with microorganisms (Bouché and Soto, 2004), responsible for higher treatment efficiency of vermifilter. Also, during the study, there was no problem of odor or clogging observed throughout, which signifies high performance efficiency of VF.



(a)



(b)

Figure 4.3: Variation in (a) BOD and (b) COD with time (70 days)

Table 4.2: *Influent and Effluent Quality*

Parameter	Influent	Effluent	Percentage Reduction
pH	7.7 ± 0.8	7.2 ± 0.3	-
Temperature (°C)	25 ± 2	26.5 ± 1.5	-
BOD (mg/L)	269 ± 22	41 ± 7	84.8%
COD (mg/L)	394 ± 12	103 ± 11	73.9%
TDS	400 ± 100	72 ± 12	82.0%
TSS	289 ± 106	29 ± 9	89.9%

Note: All values are arithmetic Mean ± standard deviation, n = 20

4.4.2. Indicator organism removal

The removal of BOD and COD from wastewater creates unsuitable environment for pathogens to live in a VF and thus makes septic bacteria more susceptible to die-off (Kadam et al., 2008). There is a possibility that BOD and COD removal is related to pathogen removal. Overall, the removal of indicator bacteria was 99.5−99.9% for the three groups of bacteria (TC, FC, and FS), signifying efficiency of the system to remove pathogens. *E. coli* is a common inhabitant of the intestinal tract of warm-blooded animals, and its presence indicates possible fecal contamination. The mean concentration of TC, FC, FS and *E. coli* in influent were 1.1×10^6, 1.1×10^5, 4.5×10^5, 2.4×10^4 MPN/100 mL, respectively. The mean log removal (K) as calculated using Equation 3.2, for TC, FC, FS and *E. coli* were 2.41, 2.66, 2.00 and 1.98, respectively during the study. Figure 4.4 shows the mean log removal profile of indicator organisms and the results showed that VF can reduce fecal counts (FC and *E. coli*) to less than 10^3 MPN/100 mL which is below the standards given by WHO for wastewater reuse for irrigation (World Health Organization, 1989) indicating that effluent from VF is safe and hygienic for irrigation reuse.

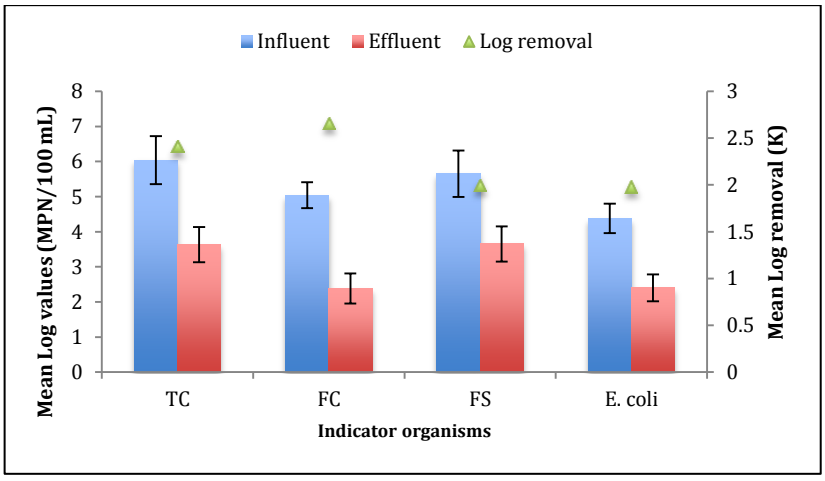


Figure 4.4: Mean values of indicator organisms in influent and effluent and log removal

4.4.3. Pathogen removal kinetics

Based on the average indicator bacteria population in influent and effluent and HLR, the area-based rate constants (k, md^{-1}) for TC, FC and FS, and *E. coli* were estimated according to Equation 4.2. The values of k are illustrated in Table 4.3. These k values are comparable with the reported values of bacterial die-off rate constants for *E. coli* and FS (Reddy et al., 1981). Different researchers have reported area based bacterial removal rate constants for the vertical flow wetland to be 3.2, 3.3, and 2.1 md^{-1} for TC, FC and FS, respectively (Arias et al., 2003; Kadlec and Wallace, 2009), 0.2-0.5 md^{-1} for constructed wetlands (Hench et al., 2003), 0.03-0.05 md^{-1} for waste stabilization ponds (García et al., 2008) and 0.1-0.2 md^{-1} for algae and macrophytic systems (García et al., 2008).

Table 4.3: *Pathogen removal rate constants (k)*

Parameter	C_i	ln (C_i)	C_o	ln (C_0)	ln (C_i/C_o)	q ($m^3.m^2.d^{-1}$)	k ($m\ d^{-1}$)
TC	1.1×10^6	13.91	4.3×10^3	8.37	5.54	1.3	7.20
FC	1.1×10^5	11.60	2.4×10^2	5.48	6.12	1.3	7.96
FS	4.5×10^5	13.02	4.5×10^3	8.41	4.61	1.3	5.99
E. coli	2.4×10^4	10.08	1.1×10^2	4.70	5.38	1.3	6.99

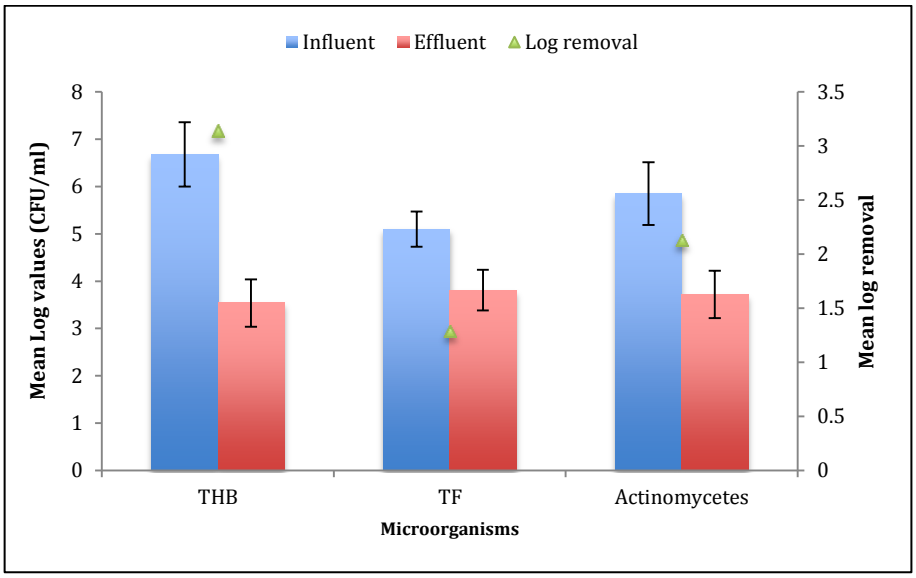
Note: Ci= influent concentration, Co= effluent concentration, ln= natural logarithmic value, q = HLR

4.4.4. Microbial population removal

The mean value of total heterotrophic bacteria (THB) and total fungi (TF) in the influent was 4.5×10^6 and 1.1×10^5 CFU/mL, respectively. There was order of difference in the level of actinomycetes in influent viz. 1.6×10^6, 4.7×10^5 and 7.5×10^4 CFU/mL but in the effluent, the levels were stabilized to (3.6-6.8) $\times 10^3$ CFU/mL. It could be due to variations in the characteristics of sewage and due to better environment in VF for their proliferation than removal. The log removal for population of bacteria, fungi and actinomycetes in VF during the study was illustrated in Figure 4.5. The population of THB and TF were reduced in effluent by log 3.14 and 1.29, respectively. The removal of microbial population can be attributed to the presence of earthworms. Earthworms are capable of reducing organic matter content, thus making the environment unsuitable for other microorganisms and pathogens. Under favorable conditions, there exists a symbiotic relationship between earthworms and microorganisms that enhances the decomposition of organic matter and causes the release of coelomic fluids from earthworm's body cavity (coelom). This fluid has anti-bacterial properties, which destroy all the pathogens from the media in which it inhabits (Valembois et al., 1992). But there is no

supporting evidence for the actual mechanism responsible for removal of pathogens in a vermifilter.

It has also been reported that earthworms engulf the pathogens present in wastewater and promote the development of other microorganisms (bacteria and fungi), which are capable of producing antibiotics that will also eventually kill the pathogens (Sinha et al., 2008). The removal of pathogens, FC and *E. coli* from wastewater was observed to be more rapid when processed by *E. fetida* (Bajsa et al., 2003). The filter media inside VF is rich in its microbial ecology and hosts environment for diverse and dense microflora, due to which the system inside VF is very complex and heterogeneous. There is a resistance by indigenous microflora to any new addition (Ellis and McCalla, 1978), which develops a competition for space and nutrients that limits the pathogen survival in the filter bed. Besides, the pathogens in the VF are subjected to various toxic and antibiotic secretions, which cause their death. These are the possible reasons for removal of pathogens. Further studies are necessary to give an in-depth knowledge about the underlying mechanism.



Note: THB- total heterotrophic bacteria, TF- total fungi, all values are mean, bars are standard deviation, n =20

Figure 4.5: Mean values of microbial population in influent and effluent and log removal

4.5. Summary

The salient features of the study are as follows:

- The present lab scale study for 70 days revealed that the population of coliforms and pathogens reduced considerably in the effluent. This clearly demonstrates the disinfection (removal of pathogens) ability of vermifiltration. This study is the first of its kind to give an idea about the pathogen removal process in a vermifilter.

- The vermifiltration process is able to reduce BOD and COD significantly by 74-85% and coliforms by 99.9% from wastewater, henceforth it can be successfully utilized as an effective technology for domestic wastewater treatment.
- The pH of the VF effluent is neutralized, demonstrating the natural intrinsic buffering ability of earthworms, with no problem of odor or clogging observed throughout the study.
- However, further research studies are essential to explore the actual mechanism and in depth understanding about the pathogen removal. More investigation is required in this aspect to give the confirmatory evidence and the features of the antimicrobial activity as a mechanism process, are further explored in the study.

Next Chapter deals with the assessment of the effects of different filter media on vermifiltration.

CHAPTER 5

EFFECTS OF DIFFERENT FILTER MEDIA DURING VERMIFILTRATION

The effect of different, locally available filter media as a vermifilter bed was compared during vermifiltration for domestic wastewater treatment. Emphasis was laid on the removal of organic matter and pathogens, quantification of microroganisms and earthworms along with the observations of the abrasions on the body of earthworms.

5.1. Introduction

Conventional sewage treatment processes involves high cost, large energy consumption, and high maintenance. As a result, implementation of these methods in developing countries for wastewater treatment has been discouraged (Sato et al., 2006). Whereas, low-cost treatment technologies such as vermifiltration have recently been implemented in various countries. Worldwide, the most common application for wastewater recycling is agricultural irrigation (Pidou et al., 2007). Therefore, it is utmost important that the wastewater be treated to the significant level, that it can be safely applied for agricultural irrigation practices.

In the past, various researchers have investigated the potential of vermifiltration for domestic and industrial wastewater treatment, which, turned out to be good, compared to conventional technologies. Vermifiltration is an organic decomposition process requiring a filter bed of suitable materials (bedding) to allow earthworms and microorganisms to interact symbiotically and synergistically. Previous studies have also reported the effect of different earthworm loads or stocking density on organic matter and nutrient removal efficiencies and the effect of different hydraulic loading rates on vermifiltration. However, attention is still not paid on the third design criterion, i.e., the filter medium of a VF, where earthworms live. Keeping this in mind, the present study is designed to evaluate the effect of different filter media as a vermifilter bed on the performance of vermifilter for domestic wastewater treatment. The experiments were conducted using four different, locally available and cost effective natural ingredients as a filter media. The aim was to make the system cost-effective along with providing the maximum treatment efficiency.

From the previous studies, it was revealed that ceramsite material was found to be relatively superior filter media, compared to quartz sand with better characteristics of sewage sludge and health of earthworms (Xing et al., 2011). Wang et al., (2010) and Yang et al., (2009) also revealed the importance of converter slag & coal cinder as suitable filter media for enhanced treatment and phosphorous removal during vermifiltration. Despite these

advancements, vermifiltration is still in its infancy and requires a better media that could bridge different issues like pollutant and pathogens removal, providing suitable dwelling habitat for earthworm's survival and functioning. Therefore, the selection of efficient and enduring filter media is the research objective of the study.

5.2. Objectives

The objectives of this study are as follows:

- To compare the effect of different filter media, i.e., riverbed materials (VFR), mud balls (VFM), glass balls (VFG) and wood coal (VFC) for vermifiltration
- To quantify the organics (BOD, COD) and pathogens (TC, FC, FS, *E. coli* and *salmonella*) removal efficiency for each filter media
- To check the growth and reproduction pattern of earthworms in each VF containing different filter media

5.3. Experimental Methodology

5.3.1. Reactor description

The study was carried out during the month of April 2013 to June 2013 for 70 days duration. Different VFs (made up of plastic material) of dimensions 0.25 × 0.20 × 0.25 m^3 were set up. Figure 5.1 showed the schematic diagram of the lab-scale VF and Figure 5.2 showed the pictorial view of the actual VFs with different filter media. The top layer or active layer (5 cm thick) consisted of thick mature vermicompost, inoculated with earthworms sp. *E. fetida* at a stocking density of 10,000 worms/m^3 of VF bed. The earthworm species were allowed to acclimatize for about one week before the study. The second layer (10 cm thick) consisted of different filter media in different VFs such as riverbed material (gravel size: 6–8 mm, VFR), mud balls (size: 4-6 mm, VFM), wooden coal (size: 2–5 mm as VFC), glass balls (size: 6 mm, VFG) respectively. The pictures of different filter media are shown in Figure 5.3. The specification and arrangement of different layers is illustrated in Table 5.1. The wastewater was applied from the top of the VFs by a 0.5 in. glass pipe with 1.5 mm perforations, using a peristaltic pump uniformly. VFs were allowed to run continuously for over a period of 70 days at HLR of 1.0 $m^3/m^2 \cdot d$ (50 litres per day). Based on the flow rate and reactor configuration, the HRT was found to be 6 h.

Table 5.1: *Description of Filter bed Layers*

Layer from top		Filter material	Particle size	Depth
Layer 1	Active layer	Mature vermigratings	600-800 µM	5 cm
Layer 2	Second layer			10 cm
	VFR	River bed Gravels	6-8 mm	
	VFM	Mud balls	4-6 mm	
	VFC	Wooden coal	2-5 mm	
	VFG	Glass balls	6 mm	
Layer 3	Third layer	Sand	1-2 mm	5 cm
Layer 4	Supporting layer	Large gravel and stones	10-12 mm	5 cm
Total depth				25 cm

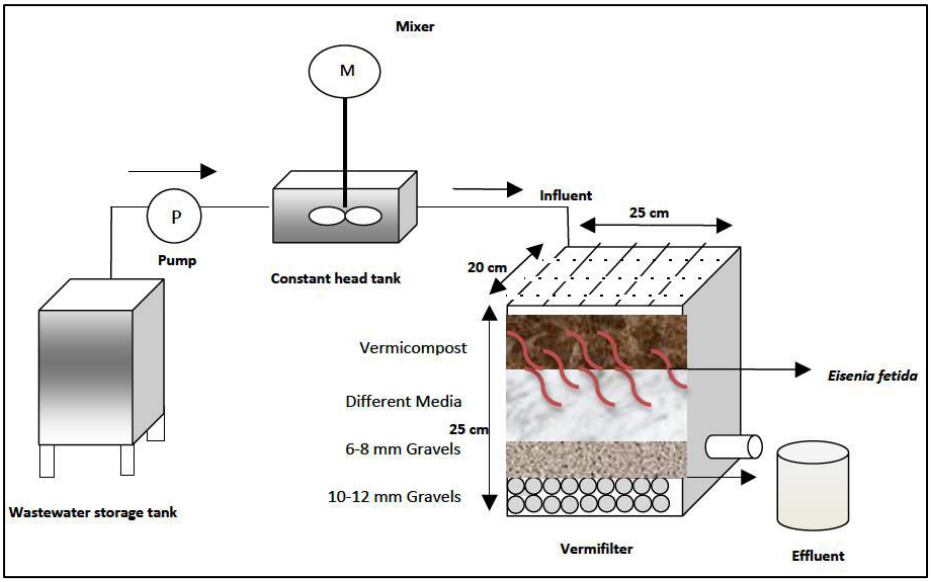


Figure 5.1: Schematic diagram of lab scale VF

Figure 5.2: Pictorial view of VFs with different filter media

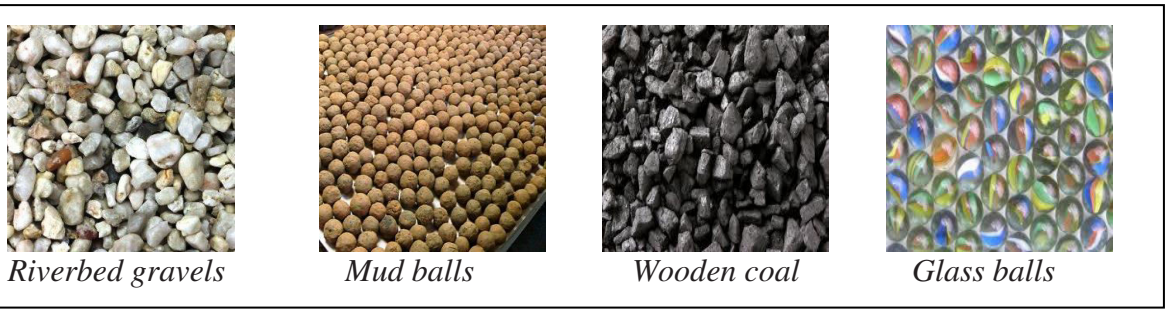


Figure 5.3: Different Filter media (Gravels, Mud balls, Wooden coal, Glass balls)

5.3.2. Sampling and analysis

The influent and effluent samples were collected in sterile plastic bottles twice a week and analyzed immediately for physico-chemical and microbiological analysis as described in Section 3.2. Earthworm number, biomass and morphology (surface structure) were also observed on the initial and final day of experiments, by isolation and hand sorting technique (Karaca, 2011). Analysis of variance (ANOVA) was conducted to analyze the data collected in all experiments using the SPSS software program, version 11.5. Mean values between different filter media were compared by using one-way ANOVA and student's *t*-test ($P \leq 0.05$).

5.4. Results and Discussions

5.4.1. Physico-chemical contaminants removal

Table 5.2 shows the performance efficiency of lab scale VFs with different filter media. The mean pH of the influent was 8.0 ± 0.3. The pH of effluent from all the VFs increased initially during the treatment, then reaching out to be in neutral range (7.6 ± 0.2) signifying the inherent capability of earthworms to act as buffering agent and neutralizing pH. Temperature is one of the key factors that affect the efficacy of wastewater treatment by vermifiltration. Throughout the study period, the average temperature of the effluent was 27.4 ± 1.5 (ranged 20-28°C). The filter media has a role in offering resistance to the adverse impact of lower and higher temperature inside a VF. Therefore the choice of filter media is important as it serves a dwelling habitat for earthworms to perform their function proficiently in optimum temperature range. DO is an important factor that signifies the environmental conditions prevailing inside the reactor. Low oxygen water is toxic to living organisms. The values of DO increased from 0.5 ± 0.5 mg/L in the influent to above 5.0 mg/L in effluent from all the VFs. This suggests that earthworms are responsible for creating aerobic conditions inside VF through their burrowing activity. The design of VF is such that oxygen could penetrate to the bottom, thus increasing the efficiency of treatment. Higher DO in effluent reduces the septic condition and brings a

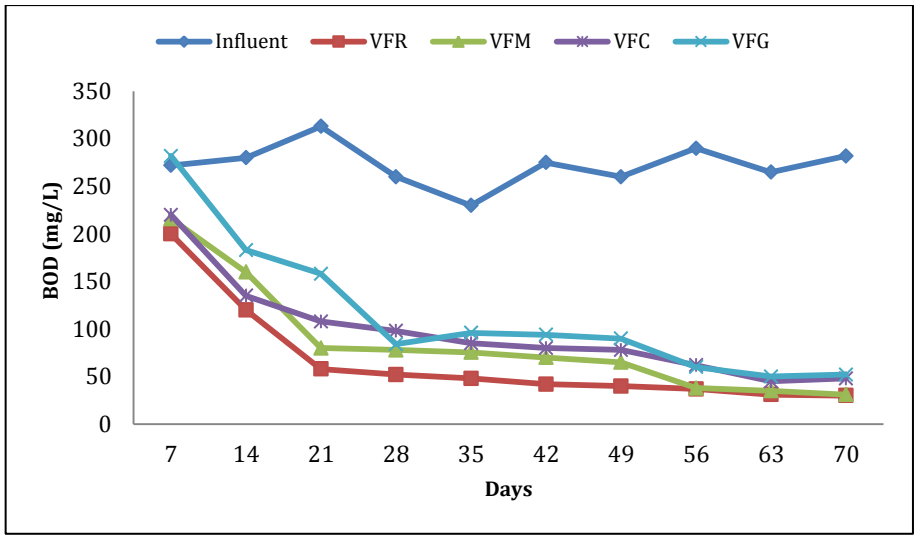
good chance of wastewater reuse for irrigation purposes (Holenda et al., 2008). It is observed that, there is no significant difference of pH, temperature and DO values between different VFs during the study.

The variations in BOD and COD removal with different filter media are illustrated in Figure 5.4 (a) and (b), respectively. It is observed that the concentrations of BOD and COD decreased remarkably during 70 days of operation. However, the maximum BOD removal efficiency was 81.3% and COD removal was 77.3% in VFR (Figure 5.5). The VF with riverbed materials (VFR) showed better efficiency as compared to other media and the possible reason behind this is the suitable endemic habitat provided by the riverbed materials (gravels) for the activity of earthworms and aerobic microorganisms to degrade the accumulated organic matter faster. In a favorable habitat the activity of earthworms and microorganisms works better. In VFG and VFC, BOD and COD removal was not significant ($P > 0.05$). This could be related to increased humidity and scouring of VF, which is not beneficial for the performance of vermifiltration process. In addition to this, physical property (specific surface area and porosity) of media also plays a significant role. Media with relatively higher surface area or lower porosity facilitates greater biomass accumulation and attains higher treatment efficiency, as evident in VFR.

Table 5.2: *Influent and Effluent Quality of Vermifilters with Different Media*

Parameter	Influent	Effluent in VFs with different filter media			
		VFR	VFM	VFG	VFC
BOD (mg/L)	300 ± 15	56 ± 15	86 ± 18	102 ± 14	111 ± 21
COD (mg/L)	418 ± 25	95 ± 10	121 ± 12	170 ± 38	185 ± 38
TDS (mg/L)	567 ± 112	258 ± 25	270 ± 30	294 ± 32	302 ± 40
TSS (mg/L)	230 ± 36	60 ± 10	88 ± 12	103 ± 18	111 ± 121
DO (mg/L)	3.2 ± 1.4	6.9 ± 1.6	6.4 ± 1.1	5.7± 1.2	5.4 ± 0.6
pH	8.0 ± 0.3	7.6 ± 0.4	7.4 ± 0.5	7.6 ± 0.5	7.3 ± 0.3

Note: All values are arithmetic mean ± standard deviation, n = 20; VFR- VF with river bed material, VFM- VF with Mud balls, VFG- VF with glass balls, VFC- VF with wooden coal



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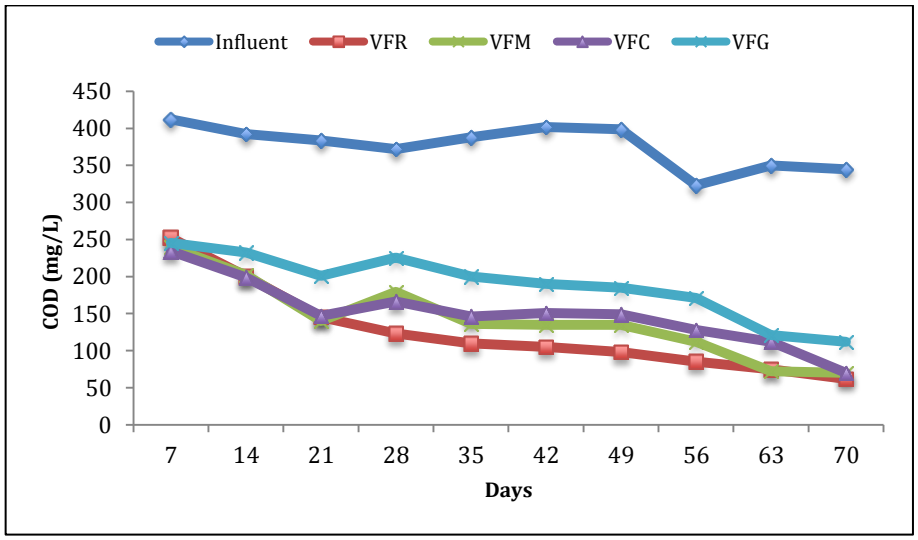


(b)

Figure 5.4: Variation in (a) BOD and (b) COD with time in different VFs

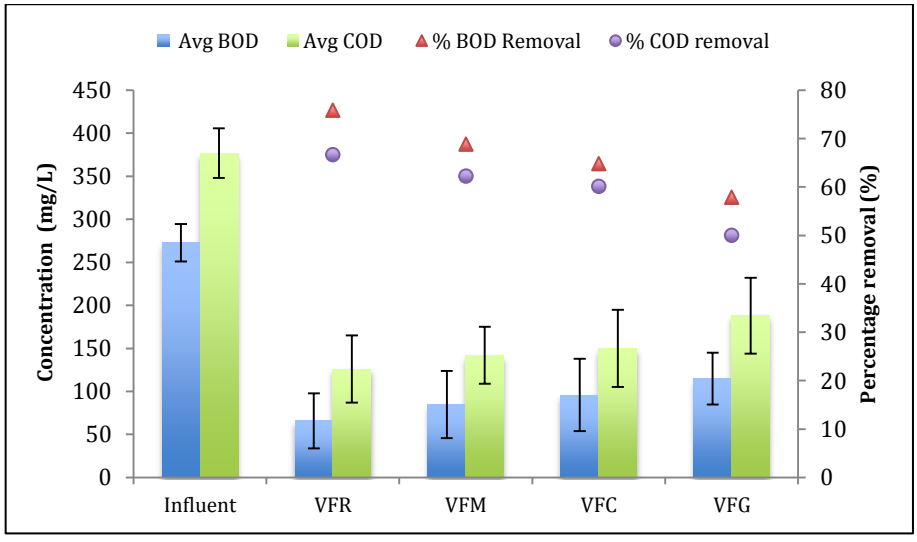
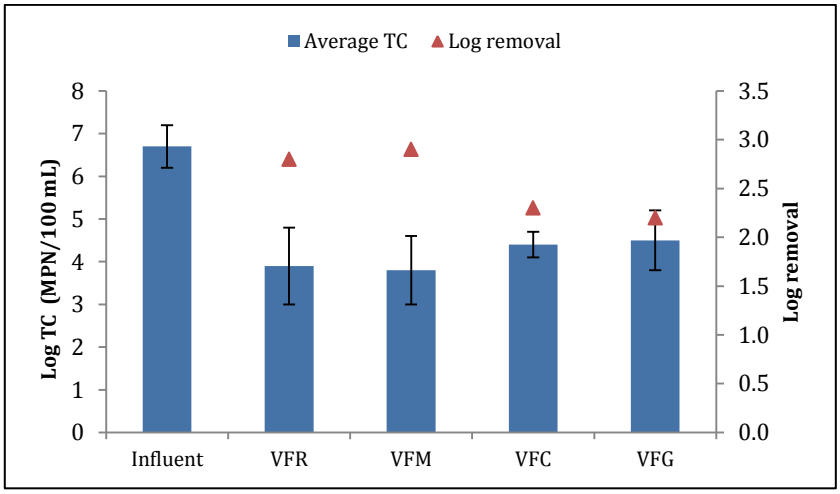


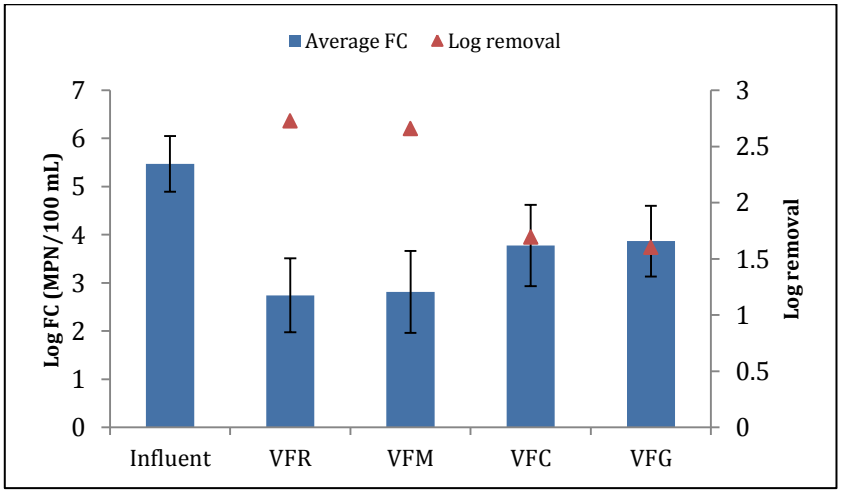
Figure 5.5: BOD and COD removal efficiency in different VFs

5.4.2. Pathogens removal

The log removal profile for TC, FC, FS, *Salmonella* and *E. coli* were comparatively higher in VFR and VFM as shown in Figure 5.6 (a), (b), (c), (d) and (e), respectively. There is no significant difference in pathogen removal efficacy between VFR and VFM (t-test, $P > 0.05$) statistically. The irregular shaped, smooth surface of gravel and spherical shaped mud balls in VFR and VFM, respectively provided a better, favorable, aerobic habitat for earthworms to thrive and interact with microorganisms, which in turn may be responsible for considerable reduction of coliforms and pathogens. Statistically, there is significant difference in pathogen removal efficacy between different vermifilters as shown by single factor ANOVA ($P < 0.05$).



(a)



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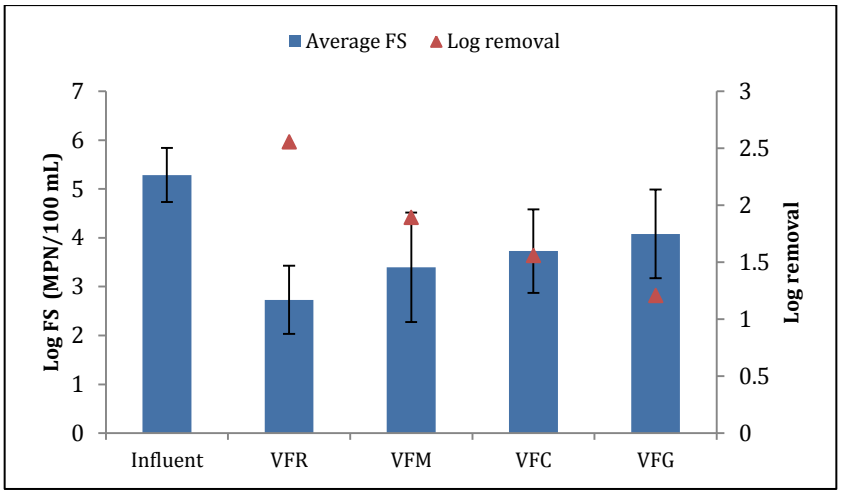

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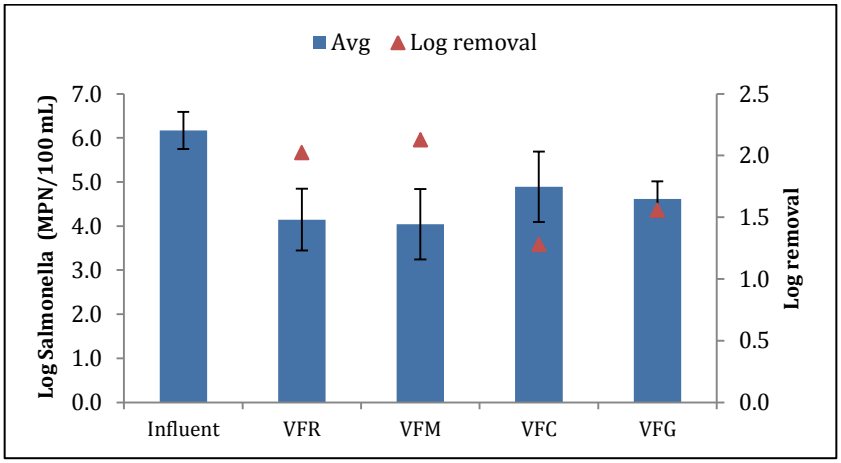

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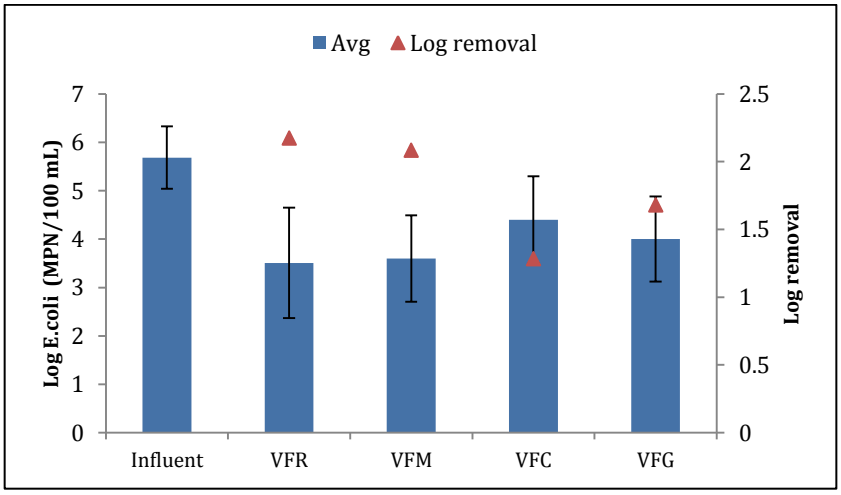


(e)

Figure 5.6: Log removal profile for (a) TC (b) FC (c) FS (d) *Salmonella* (e) *E. coli* in different VFs

5.4.3. Microbial population in different vermifilters

The standard plate count (SPC) measures the microbial population in different VFs. As shown in Table 5.3, distribution of microbial population varies with filter media. It is observed that the average population of total heterotrophic bacteria (THB) and total fungi (TF) of the active layer in VFR and VFM was found to be 99% (2 log) higher than VFC. The differences in the distribution of microbial diversity in different VFs is due to the particle size and surface structure of the media which have a greater capacity to protect microbial biomass by providing a sorptive, high surface area environment for closer microorganisms attachment (Arias et al., 2003). This suggests that the presence and activity of earthworms in its suitable habitat might result in higher microbial diversity. In vermifiltration, microorganisms (bacteria) work together with earthworms under favorable conditions to accelerate and enhance the decomposition of organic matter. Therefore in a suitable endemic environment like riverbed material, the population of bacteria will be higher to work more efficiently. For any system to work efficiently, it is important that the living species that maintain the working of the system should be well adapted and adjusted with the habitat of the system and a favorable habitat allows them to work better and more efficiently.

Table 5.3: *Microbial Community Diversity in Different VFs*

Vermifilter	Total bacteria	Total fungus	Actinomycetes
VFR (10^5 CFU/mL)	70 ± 20	27 ± 12	36 ± 10
VFM (10^5 CFU/mL)	35 ± 15	17 ± 10	25 ± 12
VFC (10^3 CFU/mL)	84 ± 53	44 ± 20	24 ± 10
VFG (10^2 CFU/mL)	60 ±10	3 ± 1.0	22 ± 12

Note: Data is represented as arithmetic mean ± standard deviation

5.4.4. Earthworm's biology

The average initial number and biomass of earthworms was 100 and 40g, respectively in all the VFs. At the end of experimental period, the percentage increase in the number and biomass of earthworms for different VFs is given in Table 5.4. The maximum percentage increase in numbers of earthworms was found in VFG by 21%. This is because, glass balls have a very smooth surface, which is an advantage for earthworms to move around and perform their functions but the development of microbial community on the surface was low (see Table 5.3). This accounts for lower treatment efficiency in VFG (Table 5.2). While in VFC, the decrease in the number of earthworms was observed. This may be due to the rough surface of coal that

resulted in the abrasions on the outer skin lining of earthworms, affecting their skin and health that resulted in the death of earthworms, accounting for lower treatment efficiency.

No clogging was observed in VFR and VFM throughout the study. There was a dynamic balance between the earthworm active mass and its number in VFR and VFM due to favorable habitat provided for earthworms. The study concluded that gravels and mud balls media showed better treatment efficiency. Riverbed gravels is a cost-effective filter media, which is easily available and can be utilized without any prior treatment and thus its use makes the system economically viable. Mud balls and glass balls are comparatively costly, whereas coal media may be completely disregarded, as it showed poor results. However, further combinations of filter media into one single unit may bring more desirable results. Overall the observed trend of performance efficiency in four VFs was VFR ≥ VFM > VFG > VFC.

Table 5.4: *Growth characteristics of earthworms*

Vermifilters	No of earthworms			Biomass of earthworms (g)		
	Initial	Final	Percentage Increase	Initial	Final	Percentage increase
VFR	100	108	7.4	40	52	23.0
VFM	100	119	15.9	40	54	22.2
VFC	100	98	- 2.0	40	46	- 6.5
VFG	100	127	21.2	40	71	42.2

5.5. Summary

- The suitability of different filter media was explored and naturally occurring riverbed material and mud balls was found to be better suited for the treatment with high pathogen removal efficacy for vermifilter.
- Overall, the observed trend of VFs in terms of treatment efficiency was observed to be VFR ≥ VFM > VFG > VFC. The possible reason is the larger particle size of filter media, good permeability, higher porosity and smooth surface.
- The higher BOD, COD, and pathogens removal efficiency, higher microbial diversity in the filter bed, higher percentage increase in earthworm's number and biomass, and no abrasions on the body wall of earthworms in VFR and VFM, concluded that river-bed and mud balls media as a promising bed material for vermifiltration.

Next Chapter deals with the performance assessment of pilot scale VF elucidating the microbial community dynamics and mechanism of vermifiltration for wastewater treatment.

CHAPTER 6

PILOT SCALE VERMIFILTER FOR WASTEWATER TREATMENT

In this chapter, the performance evaluation of a pilot scale vermifilter was compared with a geofilter (without earthworms) along with investigation of the microbial community dynamics and its functionality to understand the mechanism behind the treatment process during vermifiltration. The study involves the assessment of antimicrobial and enzymatic property of microbial community, with an in-depth understanding on the mechanism of organics and pathogen removal. Emphasis was also laid on studying the kinetics of pathogen removal.

6.1. Introduction

Wastewater generated from industrial, agricultural, and domestic activities contains high level of organics and inorganics, and numerous disease causing microorganisms that require utmost treatment before discharge into water bodies or reuse (Pradhan and Ghangrekar, 2014). Wastewater treatment plants are usually designed to efficiently remove organic pollutants and nutrients, but rarely have been planned explicitly to remove pathogens (Tyagi et al., 2011). Efficient removal of pathogenic microorganisms from wastewater is a crucial task for controlling pathogen discharge. Therefore, it is necessary to seek an onsite wastewater treatment technology that helps to remove pathogens and other contaminants from wastewater at a lower cost.

Vermifiltration is an innovative technology, which adopts modern concept of ecology by inoculating earthworms in a traditional filter. The inoculation of earthworms in conventional geofilter (GF), which is termed as vermifilter (VF), has been widely utilized to treat municipal and industrial sewage and sludge stabilization. Microorganisms (bacteria and fungi) that colonize in the VF along with earthworms reflect the performance and functioning of vermifiltration process. Earthworms decompose the organic matter by the enzymatic activity of microorganisms residing in a filter bed. Enzymes are biological catalysts giving pace and rapidity to biochemical reactions. Cellulases, amylases and proteases are a consortium of most important enzymes that causes the hydrolysis of cellulose to glucose, starch molecules to finer products such as dextrins, and proteins to amino acids (Gautam et al., 2012; Gupta et al., 2003). Organic matter is basically composed of cellulose, starch, sugars, proteins and lipids, which constitute the biodegradable portion of wastewater. These enzymes have the potential to biodegrade the substrate proficiently into simpler products. The saccharification of cellulose is a complex and poorly understood process done by cellulase enzyme (Sajana et al., 2014). Therefore, assessment of enzymatic activity of the isolated microorganisms in a vermifilter will

bring an insight to the organic matter removal during vermifiltration. Previous studies on vermifiltration focus on the treatment efficacy, however information on the microbial community dynamics is also important to maximize the treatment capacity of the system. No attention has been paid so far to the mechanism of pathogen removal and the studies on the antibacterial property of the microorganism in VF. The overall idea of the study is to gain an in-depth knowledge on the microbial community dynamics and its role in organics and pathogen removal.

6.2. Objectives

The objective of the study is to compare the treatment efficacy of a VF (with earthworms) with GF (without earthworms) as a control by continuous monitoring of physico-chemical and microbiological parameters. The specific objectives of the study are:

- To evaluate the removal rate of organics (BOD, COD), pathogens (TC, FC, FS *E. coli* and *Salmonella*) and microorganism (total bacteria, total fungi, actinomycetes) from synthetic domestic wastewater
- To isolate, identify and biochemically characterize the microbial population (bacteria, fungi, actinomycetes) from VF and GF
- To evaluate antibacterial activity of isolated microorganisms against the known bacterial pathogens (ATCC culture of Gram-Positive *Staphylococcus aureus* and Gram-Negative *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*)
- To determine the qualitative and quantitative enzymatic activity (cellulase, amylase and protease) of the isolated microorganisms

6.3. Experimental Methodology

6.3.1. Reactor description

A pilot scale reactor made up of perspex sheet, was set up and operated for 120 days. The schematic diagram of the reactor is shown in Figure 6.1 and the pictorial view of the actual reactor is shown in Figure 6.2. The reactor was divided into two chambers; one is vermifilter (VF) with earthworms and another is geofilter (GF), which is devoid of earthworms (as a control). It consisted of filter bed material (river-bed material), wastewater storage tank, wastewater distribution system and effluent collection system. Both the chambers were 80 cm long and 40 cm wide with a depth of 80 cm, and have 65 cm of packed bedding of different filter materials. An empty space or free board of around 15 cm is kept at the top for aeration purpose. The filter bed consisted of 4 layers. The description of filter bed layers is illustrated in

Table 6.1. The pump and constant head tank were installed to collect and transfer the influent (synthetic wastewater) to the reactors. Wastewater passed through different layers in sequence by gravity flow. The experiments were performed during the months of June 2013 to September 2013 (120 days), when the average extreme air temperature was 30^{o}C (ranged 25-30^{o}C), which is the optimum temperature range for earthworm species. The moisture content in VF during the study period was observed to be 85- 90% throughout, which is an optimum range for earthworm growth and activity (Gunadi and Edwards, 2003).

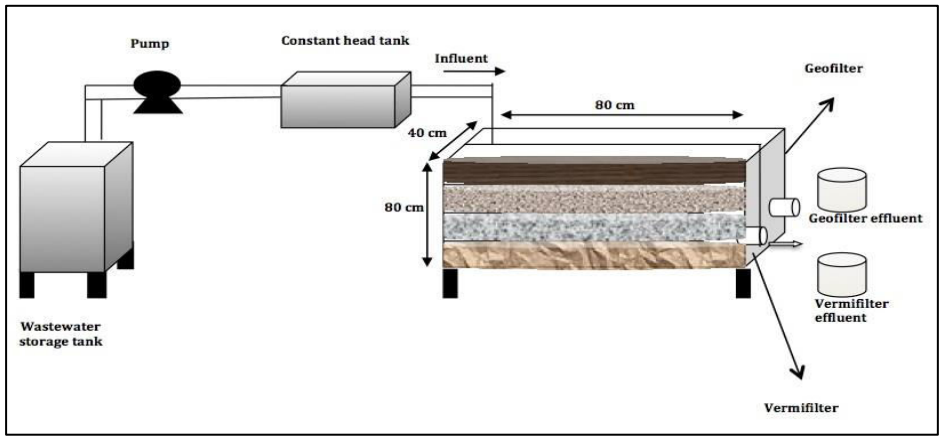


Figure 6.1: Schematic diagram of pilot scale reactor

Figure 6.2: Pictorial view of pilot scale reactor

Table 6.1: *Description of Filter Bed Layers*

Layers (from the top)		Filter material	Particle size	Depth
Layer 1	Active layer	Mature vermigratings	600-800 µm	20 cm
Layer 2	Second layer	Sand	1-2 mm	15 cm
Layer 3	Third layer	Small gravels	6-8 mm	15 cm
Layer 4	Supporting layer	Large gravel and stones	10-12 mm	15 cm
Empty space				15 cm
Total depth				80 cm

6.3.2. Design parameters for pilot scale vermi-reactor

The design parameters include HLR, HRT, stocking density of earthworms, and filter media. The HLR of the vermireactor was kept constant at 1.0 $m^3m^{-2}day^{-1}$ (320 litre per day each for VF and GF) during all experiments. Based on the flow rate and reactor configuration, the HRT was found to be 7.8 h. The VF was inoculated with earthworm species *E. fetida* at an initial earthworm stocking density of 10,000 worms/m^3. *E. fetida* was available in the institute culture laboratory, where it has been cultured from last 10 years. During experiments, the natural habitat of the earthworms was not disturbed. They were maintained in suitable environmental conditions inside VF and were not harmed or killed during the study. Continuous feeding to earthworms and the earthworm activity ensured that the microbial-earthworm ecofilter was not in a state of inundation. This reasoning holds equally well for the use of *E. fetida* in the present study.

6.3.3. Wastewater characteristics

During the study, synthetic wastewater was used as the influent as described in section 3.2.3. The influent characteristics are: pH 7.9 ± 0.1, temperature 27.4 ± 1.5°C, DO 0.35 ± 0.3 mg/L, BOD 242 ± 30 mg/L, COD 456 ± 32, coliforms 10^6-10^8 MPN/100 mL, *Salmonella* and *E. coli* 10^5-10^6 CFU/mL, THB, TF and actinomycetes in the range of 10^5-10^9 CFU/mL.

6.3.4. Start-up period

The start up of the treatment process was initiated by seeding wastewater in batch mode (160 litre for 6 h alternatively) for acclimatization of earthworms, colonization and accumulation of microorganisms in the filter bed. The initial two weeks were regarded as acclimatization period for the earthworms and microorganisms. After acclimatization, both the VF and GF were operated continuously for 120 days at room temperature.

6.3.5. Analytical procedure

Sampling frequency was twice a week. Influent and effluent samples were collected in sterile plastic bottles and analyzed within 6 h for microbiological parameters and 24 h for other chemical parameters. pH, temperature and DO were monitored at the site itself. All the parameters were monitored as described in Section 3.2. A paired sample t-test between VF and GF was performed for each parameter to analyze the differences using SPSS® statistical package. The results reported in the study have significance level (P) less than 0.05 or 0.01.

6.3.6. Pathogen removal kinetics evaluation

Four different kinetics models have been used to fit the experimental data obtained during the vermifiltration: (1) a log-linear according to the Chick' law (Equation 6.1). This model reduces disinfection to a bimolecular chemical reaction in which microorganisms are treated as molecular species. There is an extensive application of this equation in other disinfecting agents as well, like chlorine, ozone, hydrogen peroxide and chloramines. (2) A double log-linear kinetics (Equation 6.2), with a first stage of very fast ($k_1 > k_2$) inactivation and a second phase of attenuated inactivation (k_2). (3) A log-linear region followed by a 'tail' (Equation 6.3). The 'tail' shape represents the bacterial population remaining at the end of the experiment due to the presence of a population of cells resistant to the treatment. (4) An initial delay or very smooth decay at the beginning ('shoulder'), attributed to lose of cells viability during the process, followed by a log-linear decrease (Equation 6.4) (García-Fernández et al., 2015).

$$Log\left(\frac{N}{No}\right) = -k.t \qquad \text{Equation 6.1}$$

$$Log\left(\frac{N}{No}\right) = -k_1.t;\ t = [0, t_1]; Log\left(\frac{N}{No}\right) = -k_2.t;\ t = [t_1, t_2] \qquad \text{Equation 6.2}$$

$$\frac{dN}{dT} = -k_1.N \rightarrow \frac{N\text{-}N_{res}}{No\text{-}N_{res}} = e^{k_1 t} \qquad \text{Equation 6.3}$$

$$Log\left(\frac{N}{No}\right) = -k_1.t \begin{Bmatrix} N \geq N_0\,;0 \\ N < N_0; e^{-k_1\,(N-SL)} \end{Bmatrix} \qquad \text{Equation 6.4}$$

Where, N/N_0 is the microorganism concentration reductions, k_i is the disinfection kinetic rate and t is the time of treatment, N_{res} is the residual population density, and SL = Shoulder length (min^{-1}).

6.3.7. Investigation of microbial community diversity in filter media

Different filter media samples were taken randomly from four layers of VF and GF to determine the spatial microbial community diversity. Bulk samples were augured from different layers representing S1/2/3/4 for VF and S*1/2/3/4 for GF for first, second, third and

fourth layers, respectively (Table 6.1). Samples were air dried and mixed in equal proportion to obtain homogenous sample. Microbial parameters investigated were total heterotrophic bacteria (THB), total fungi (TF) and actinomycetes to assess the microbial diversity in general. Standard pour plate technique was used to develop colonies. The standard plate count (SPC) was measured as colony forming unit (CFU/g) for THB after incubation for 24−48 h with Nutrient agar media. TF population were enumerated after incubation for 4−5 days at 28°C with Potato Dextrose agar. Similarly, actinomycetes were measured as CFU/g of sample grown on Starch Casein medium for 3−5 days at 28°C (Kadam et al., 2009). After the experiments, scanning electron microscope (SEM) (Hitachi S-570, Japan) imaging was performed to obtain micrographs of the top layer from VF and GF without deformation of their surface features at 5 KX magnifications. The SEM was operated at 20 kV of acceleration voltage condition (Xing et al., 2011).

6.3.8. Isolation, identification and biochemical characterization of microbial community

Spread plate technique was used to isolate microorganisms from active layer of VF and GF. Well-isolated colonies were selected and sub cultured to a new petriplate. Cells from new colony were then picked up with an inoculating needle and transferred to an agar slant for maintenance of pure culture. Pure bacterial cultures were identified morphologically by microscopy (shape, gram staining and motility) and biochemically by API biochemical identification tests (Sugar utilization, Indole production, Citrate utilization, Methyl Red-Voges Proskauer test, Triple sugar iron utilization, Oxidase production, Catalase production, Coagulase test) according to Bergey's manual of determinative bacteriology (Holt, et al., 1994). Pure cultures of fungal isolates were identified using both macroscopic (cultural) and microscopic (morphological) features (Barnett and Hunter, 1998). For actinomycetes, cultural characteristics of cells were recorded and morphological observations were made with a microscope (Shirling and Gottlieb, 1966; Kadam et al., 2008).

6.3.9. Determination of antibacterial activity

The isolated microorganisms were tested for antibacterial activity against the known bacterial culture by agar well diffusion method as described by Parekh and Chanda (2007) and Sethi et al. (2013). Twenty-four hours fresh cultures of Gram-Positive *S. aureus* (ATCC 29213), Gram-Negative *E. coli* (ATCC 25922), *K. pneumoniae* (NCIM2719) and *P. aeruginosa* (ATCC 27853) were swabbed by sterilized cotton swab and lawns were prepared over the agar surface on the petriplate. These all strains are pathogenic in nature, procured from the Vikas scientific co., Roorkee, India. The molten Mueller-Hinton agar (Hi-media) was inoculated with 100 µl of

the inoculum (1×10^8 CFU/mL) and poured into the petriplate. Two wells were made in the inoculated plates using sterile cork borer (0.85 cm). About 80 µl cell-free supernatant was added in the first well and antibiotic streptomycin (50 mcg) was added in the second well as positive control for the experiment. Plates were then incubated at 37°C for 24 h. After 24 h, the zones of inhibition were measured in millimetre (mm). Species showing diameters between 12 to 16 mm were considered to be moderately active and with more than 16 mm were considered to be highly active. The experiment was done in triplicate and the mean values are presented.

6.3.10. Determination of enzymatic activity

Enzymatic activity was determined according to the standard methods. Protease activity was determined according to sigma's non-specific protease activity assay (Lowry et al., 1951). Cellulase activity was determined according to the method described by Ghose, (1987). Amylase activity was determined according to the Dinitro salicylate method (Miller, 1959). All the samples were analyzed in triplicate and the results were averaged. Optical density (OD) of each sample with reaction mixture was taken in a spectrophotometer (Hach DR 5000) as described by Aira et al., (2007b). Enzyme activity was expressed in units/mL.

6.3.11. Earthworm's growth rate characteristics

Isolating and hand sorting technique was used as the sampling method for observing the growth rate characteristics of earthworms (Tripathi and Bhardwaj, 2004). At the end of experiments, the biomass and number of earthworms were measured and compared with the initial biomass and number to know about the earthworm growth characteristics.

6.4. Results and Discussions

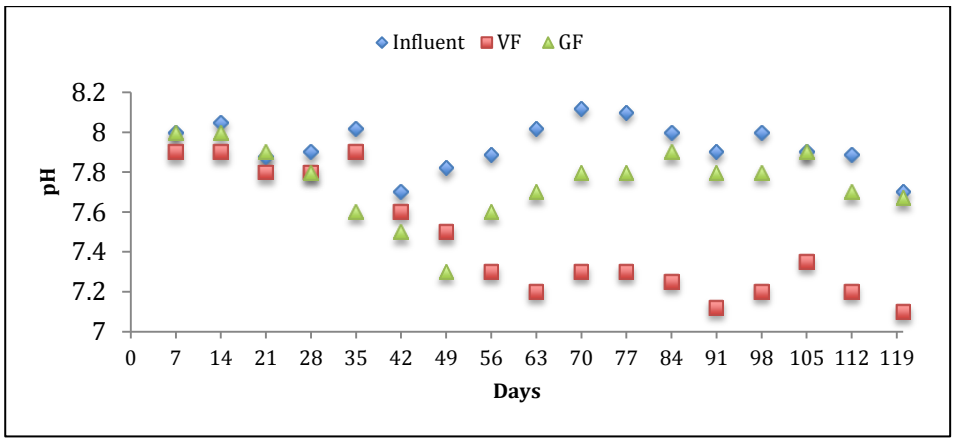
6.4.1. Physico-chemical performance

The average pH of the influent was 7.9 ± 0.1. The pH of VF effluent increased initially during the treatment, then decreasing slightly, reaching out to be in neutral range, signifying the natural ability of earthworms to neutralize pH. The pH of GF effluent also improved but it was not consistent on all days as observed in Figure 6.3(a). There was a significant variation in pH between VF and GF ($t = 6.2919$, $P < 0.001$).

Temperature is one of the key factors that affect wastewater treatment efficacy in VF. Throughout the study period, the average temperature of the influent and effluent was 27.4 ± 1.5^0 C (ranged 20-30^0 C). There was no significant difference between the VF and GF effluent temperature ($t = 0.2608$, $P > 0.05$). This is because gravel and sand filter media have a

buffering capacity to withstand the variations of temperature. It provides better resistance to the adverse impact of vermifiltration under lower and higher temperature conditions. Consequently, it proves to be a suitable dwelling habitat for earthworm *E. fetida* to thrive and perform its function proficiently in the optimum temperature range/

The average value of DO in the influent was 0.35 ± 0.3 mg/L. In VF effluent, the DO was observed to be 5.6 ± 1.1 mg/L while in GF effluent, initially DO appeared to be high as 7.06 mg/L but with time, it tends to decline and reached 1.0 mg/L as shown in Figure 6.3 (b). There is significant difference in the value of DO for VF and GF effluent ($t = 3.3119$, $P < 0.001$). This suggests that earthworms are responsible for creating aerobic conditions inside VF by their burrowing action, while the geological system in GF tends to be anaerobic after few days. The design of VF is such that oxygen could penetrate to the bottom, thus increasing the efficiency of treatment. The moisture content in VF during the study period was observed to be 85-90% throughout, which is an optimum range for earthworm growth and activity.



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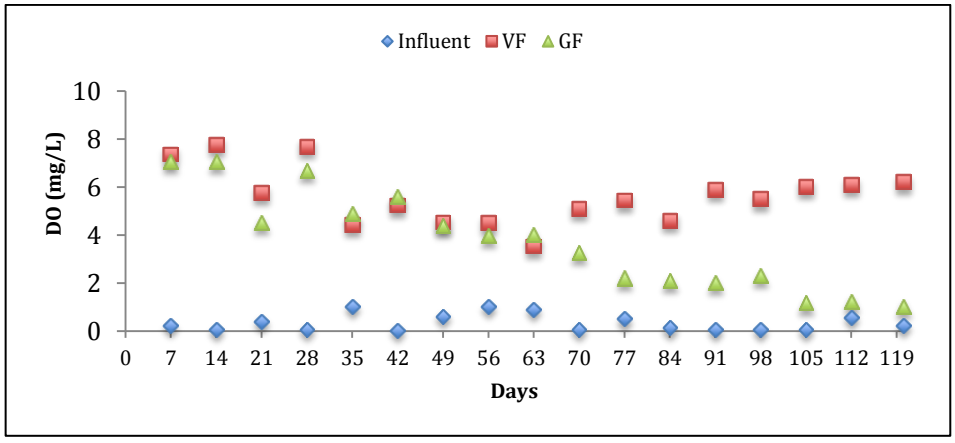
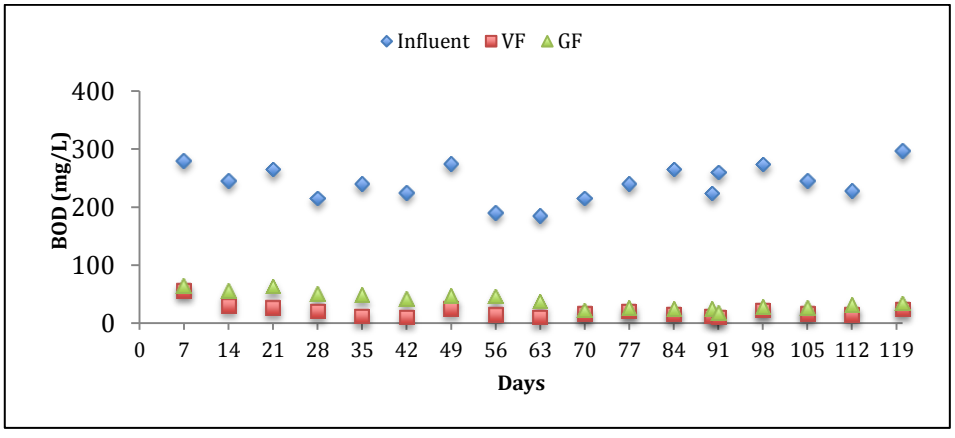


(b)

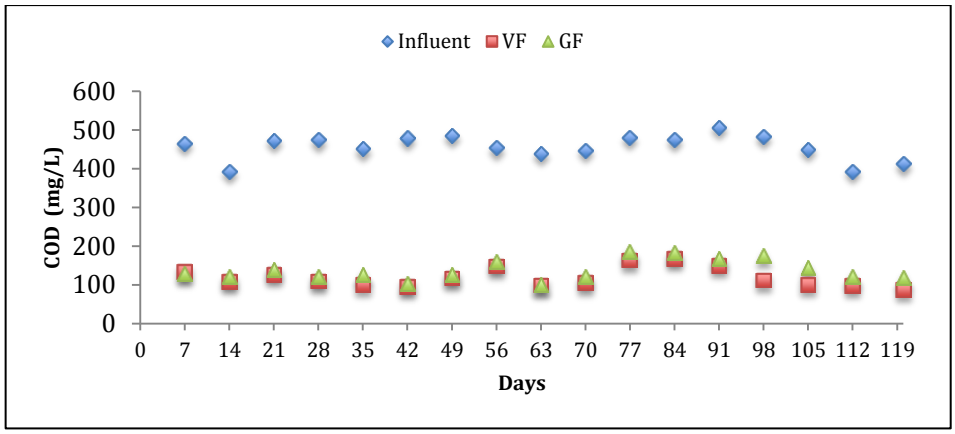
Figure 6.3: Variation in (a) pH and (b) DO during treatment period

6.4.2. Organics removal

The profile of BOD and COD for influent and effluent during the operation period is shown in Figure 6.4 (a) and (b). The organic matter measured as average BOD in the influent was 242 ± 30 mg/L. The results show that earthworms can remove BOD loads by over 92% in VF while BOD removal in GF (where only geological and microbial system works) is around 74%. The percentage BOD removal was significantly higher in VF as compared to GF ($t = 3.9258$, $P < 0.01$). The average COD removal in VF is over 74% whereas in GF is 68%. The percentage COD removal was significantly higher in VF as compared to GF ($t = 2.7214$, $P < 0.01$). Higher BOD and COD removal in VF is attributed to the activity of earthworms and its associated microorganisms that degrade the wastewater organics by its enzymatic activity (Rajpal et al., 2012). Though significant removal of BOD and COD is achieved by the microbial-geological system unaided by earthworms (in GF), however the system fails to work for longer time as it is frequently choked due to the formation of sludge and colonies of bacteria and fungi. Collectively, these results indicate that microbes are responsible for the biochemical degradation of organic matter, while earthworms are important drivers of the process, as they enhance the biodegradation through their feeding, burrowing and casting behavior. Therefore, assessment of enzymatic activity is crucial to understand the process. The results suggest the mutual relationship between earthworms and microorganisms outside earthworm gut during the first stages of organic matter decomposition. This mechanism could be similar to nutrient enrichment process as *E. fetida* modified the structure of substrate and released new nutrient pools due to its feeding and casting activities, which stimulated microbial metabolism.



(a)



(b)

Figure 6.4: Variation in (a) BOD and (b) COD during treatment period

6.4.3. Pathogens removal

The pathogen removal performance is given in Table 6.2. The average value of TC, FC and FS in the influent was 3.5×10^8, 2.0×10^6 and 3.3×10^6 MPN/100 mL, respectively. In VF, the log removal for TC, FC and FS was significantly higher as compared to GF ($t = 2.6239$, 3.5206 and 4.3058 for TC, FC and FS, respectively, $P < 0.01$). The log removal of *E. coli* and *Salmonella* was significantly higher in VF effluent as compared to GF ($t= 2.6088$ and 4.8123 respectively, $P < 0.001$). The population of THB in the influent was very high as 1.4×10^9 CFU/mL, which was reduced by 3.85 log units in VF, and 2.32 log units in GF ($t = 3.8752$, $P < 0.01$). The TF in the effluent were also reduced in VF by 3.46 log units ($t = 2.2795$, $P < 0.01$). The actinomycetes levels vary significantly in the influent as 2.3×10^6, 2.4×10^5 and 4.4×10^4 CFU/mL, but in the VF effluent, the levels were stabilized to $(2.6 - 3.8) \times 10^4$ CFU/mL. The possible reason for pathogen removal is attributed to the fact that these pathogens get subjected to various antibiotic secretions from the earthworms and associated microflora (Sinha et al., 2008). In addition, the microbes that earthworms leave behind are beneficial to the VF because they compete with pathogens for the limited nutrients. So, there is a possibility of antibacterial activity of the microorganisms that inhibits or prevents the growth of pathogens during the treatment. The concentration of pathogens (FC) was reduced considerably below 10^3 MPN/100 mL, within the WHO standards for irrigation (World Health Organization, 1989). So the investigation of the antibacterial activity of the isolated microbial species is important.

Table 6.2: *Pathogen Removal Performance of Vermifiltration*

Organisms	Influent	Effluent VF	K	Effluent GF	K
TC (MPN/100 mL)	3.5×10^8	2.5×10^5	3.15	2.0×10^6	2.24
FC (MPN/100 mL)	2.0×10^6	8.3×10^2	2.88	6.5×10^4	1.49
FS (MPN/100 mL)	3.3×10^6	1.9×10^2	3.74	3.0×10^4	2.04
THB (CFU/mL)	1.4×10^9	2.0×10^5	3.85	6.8×10^6	2.32
TF (CFU/mL)	2.2×10^6	7.5×10^2	3.46	1.6×10^4	2.14
Actinomycetes (CFU/mL)	6.5×10^5	5.2×10^4	1.09	2.5×10^5	0.4
Salmonella (CFU/mL)	1.2×10^6	1.5×10^2	3.9	1.2×10^4	2.0
E. coli (CFU/mL)	1.5×10^6	1.4×10^4	2.03	1.5×10^5	1.0

All values are average, n = 34, K = Log removal

6.4.4. Pathogen removal kinetics

All experimental data from the vermifilter were fitted to the proposed equations (Equations 6.1-6.4). Those results, which led to a minimum R^2 coefficient, were accepted as the best statistical fitting. The results of these fittings are shown in Table 6.3, including R^2 values. Kinetic parameters obtained for the different equation are comparable, as all are obtained under same experimental and reactor conditions. As expected for disinfection during vermifiltration, first-order kinetics can be considered as the most common behavior for all the results (Table 6.3). In the case of *Salmonella* and actinomycetes population, removal is represented by biphasic model (model 2, double log-linear). Total bacteria and total fungi showed less frequently observed linear behavior continued with a residual concentration (model 3, log-linear + tail). As all these equations are based on first-order kinetics (simple or modified) the kinetic constants can be directly compared to assess the best disinfection results. The 'tail' shape can be explained for the presence of remaining population of bacteria, which were not removed due to the presence of the suspended solid particles present in the wastewater which aggregates around bacterial cell, shades it and prevents the attack on its cell wall surface.

Table 6.3: *Microorganisms inactivation rates (k) during vermifiltration*

Microorganisms	k_1 (min^{-1})	R_1^2	k_2 (min^{-1})	R_2^2	SL (min)	Log (N_{res})	Model #
FC	0.033 ± 0.002	0.998	-	-	-	-	1
FS	0.013 ± 0.001	0.977	-	-	-	-	1
Salmonella	0.009 ± 0.001	0.983	0.003 ± 0.002	0.866	-	-	2
E. coli	0.024± 0.003	0.973	-	-	-	-	1
Total bacteria	0.005 ± 0.001	0.953	-	-	-	1.83	3
Total fungi	0.051 ± 0.007	0.965	-	-	-	0.30	3
Actinomycetes	0.069 ± 0.004	0.947	0.010 ± 0.001	0.970	-	-	2

Note: k = inactivation rate (linear regression of Log (concentration) versus time); R2 = regression coefficient; Model 1 = Log-lineal (k1); Model 2 = double log-lineal (k1, k2); Model 3 = Log-lineal (k1) + tail (Log (Nres)); Model 4 = shoulder (SL) + log-lineal (k1).

6.4.5. Analysis of changes in microbial community diversity

The SPC measures the population count of prevailing microbial diversity in different layers (Figure 6.5). As shown in Table 6.4, distribution of the microbial population varies with depth in VF, while in GF the microbial population is almost same in all the layers. In VF, higher bacterial diversity was observed in the top (S1) and second layer (S2). The bacterial diversity decreased gradually as the depth increased. This suggested that the presence and activity of earthworms in the first two layers might result in higher microbial diversity. Earthworms were found to dwell at a depth of 15-25 cm. Adequate oxygen and improved aerobic conditions due to burrowing action of earthworms favors the microenvironment for aerobic microorganisms. In addition, the mucus and casts produced by the earthworms are conducive to the development of diverse microbial community (Vivas et al., 2009). The difference in the distribution of microbial diversity due to depth is also due to smaller particle size of vermicompost and sand media which have a greater capacity to protect microbial biomass by providing a sorptive, high surface area environment for closer microorganisms' attachment (Arias et al., 2003). The increase in microbial population may be attributed to the mucus secreted by earthworms, which is a source of assimilable carbon and has a stimulating effect on microorganisms (Aira et al., 2007a).

Table 6.4: *Microbial Community Diversity of Filter Media of Different Layers*

Layers	THB (CFU/g)	TF (CFU/g)	Actinomycetes (CFU/g)
S1	2.8×10^9	8.3×10^5	2.4×10^7
S2	1.8×10^8	1.0×10^4	3.3×10^5
S3	1.1×10^6	1.4×10^4	1.0×10^2
S4	6.0×10^4	3.0×10^3	8.6×10^2
S*1	3.0×10^4	2.5×10^3	8.0×10^2
S*2	2.5×10^3	1.7×10^2	6.1×10^2
S*3	7.7×10^3	4.0×10^2	2.8×10^3
S*4	1.6×10^3	4.5×10^2	3.8×10^3

Note: THB- total heterotrophic bacteria, TF- total fungi, S1/2/3/4 denotes First, Second, Third and Fourth layer, respectively of vermifilter; S*1/2/3/4 denotes First, Second, Third and Fourth layer, respectively of geofilter; All values are average values

6.4.6. Identification and biochemical characterization of microbial species

Figure 6.6 demonstrates the streaking technique for isolation of bacterial species on specific media. The results of the study revealed that a total of 26 bacterial species were isolated from VF while a total of 11 bacterial species were isolated from GF (Table 6.5). Most of these species were identified, while very few species still remains unidentified. VF showed higher and diverse microbial community due to the presence of earthworms, which promote the

development of beneficial microorganisms. It is a known fact that earthworms further stimulate and accelerate microbial activity by increasing the population of microorganisms and also through improving aeration by burrowing actions. Another reason is that microbial activity in GF decreases due to adverse environmental conditions after a certain period of time. Gram staining of fresh bacterial isolates revealed three groups: Gram-Positive rods, Gram-Negative cocci and Gram-Negative rods (Figure 6.7). The bacterial species isolated from filter media includes *Bacillus, Lactobacillus, Pseudomonas, Clostridium, Klebsiella pneumoniae, K. aerogenes, Serratia marscens, Alcaligenes, Aeromonas, Neisseria, Pseudomonas aeruginosa, Micrococcus,* and *Proteus vulgaris* species. The fungal species includes *Penicillium chrysogenum, P. oxysporum* and *Fusarium oxysporum* and Actinomycetes includes *Micromonospora* and *Streptomyces*. *Bacillus* appeared to be the dominant genus in VF. These observations were consistent with the previous findings (Wang et al., 2011a; Wang et al., 2013a).

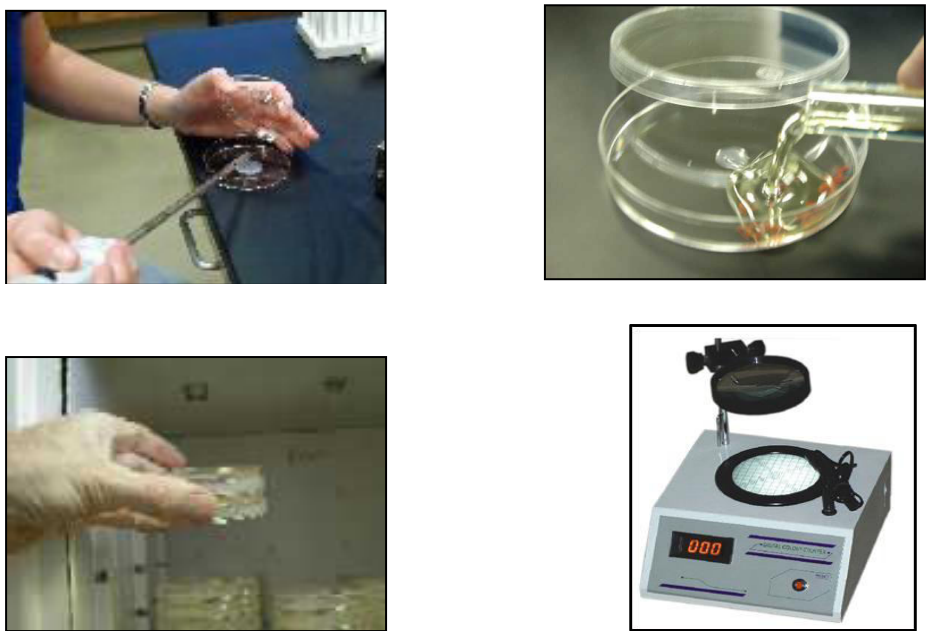
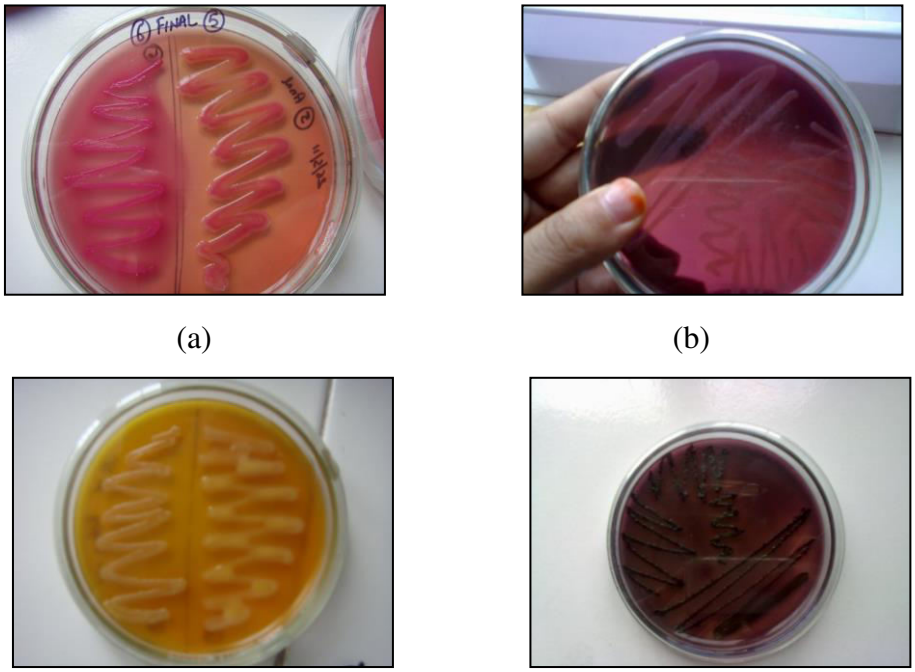


Figure 6.5: SPC technique to isolate microorganisms and colony counter

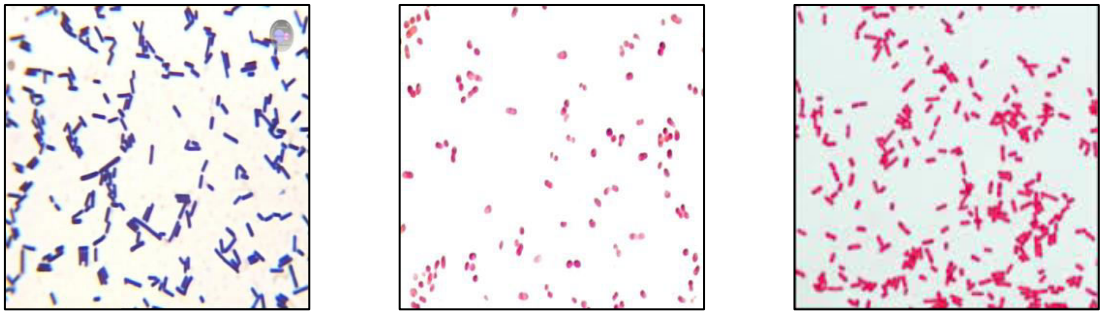


(a) (b)

(c) (d)

Figure 6.6: Streaking technique to isolate pure culture on different media

(a) Nutrient agar medium (b) Eosin Methylene Blue (EMB) agar (c) Xylose Lysine Deoxycholate agar (XLD) agar (d) Green metallic sheen colony of E. coli on EMB agar



(a) (b) (c)

Figure 6.7: Microscopic images of (a) Gram- Positive Rods (b) Gram- Negative Cocci (c) Gram- Negative Rods

Table 6.5: *Isolation, Identification and Biochemical Characterization of Bacterial Species Isolated from VF and GF*

Isolate No.	GS	Indole	MR	VP	Citrate	TSI	Catalase	Oxidase	Isolate name
Isolates from VF									
1	+	-	+	-	+	+	-	-	*Clostridium* sp.
2	+	-	-	-	-	+	+	+	*Bacillus* sp.
3	-	-	+	+	-	+	-	+	*Unidentified*
4	+	-	+	-	-	+	-	-	*Lactobacillus*
5	-	-	+	-	-	+	+	-	*E. coli*
6	+	-	+	-	-	+	-	-	*Lactobacillus*
7	+	-	+	-	-	+	-	-	*Lactobacillus*
8	-	-	+	-	+	+	-	-	*K pneumoniae*
9	-	-	-	+	+	+	-	-	*K. aerogenes*
10	-	-	-	+	+	+	-	-	*K. aerogenes*
11	-		-	-	+	+	+	+	*Alcaligenes* sp.
12	-	-	+	+	+	-	+	+	*Aeromonas* sp.
13	+	-	+	+	-	-	+	+	*Neisseria* sp.
14	+	-	-	-	-	+	+	+	*Bacillus* sp.
15	-	-	-	+	+	+	+	+	*Serratia* sp.
16	-	+	-	-	+	+	+	+	*P. aeruginosa*
17	+	-	+	-	-	+	+	+	*Clostridium* sp.
18	+	-	+	-	-	+	-	+	*Clostridium* sp.
19	-	-	+	+	-	+	-	+	*Shigella* sp.
20	+	-	-	-	-	+	+	+	*Bacillus* sp.
21	+	-	+	-	+	+	+	+	*B. cereus*
22	+	-	-	-	-	+	+	+	*Bacillus* sp.
23	+	-	+	-	-	+	-	+	*Clostridium* sp.
24	-	-	+	+	-	+	-	+	*K. pneumoniae*
25	+	-	+	-	+	+	+	+	*M. luteus*
26	-	-	+	+	+	+	+	+	*P. vulgaris*
Isolates from GF									
1	+	-	+	+	-	+	-	+	*Leuconostoc* sp.
2	-	-	-	+	+	+	-	+	*Pseudomonas* sp.
3	-	-	+	-	+	+	+	-	*Shigella* sp.
4	-	-	-	+	+	+	-	+	*Pseudomonas* sp.
5	-	-	-	+	+	+	-	+	*Pseudomonas* sp.
6	-	-	+	+	-	-	+	+	*Unidentified*
7	-	-	+	-	-	-	+	+	*Alcaligenes* sp.
8	+	-	-	-	-	+	+	+	*M. luteus*
9	+	-	+	+	+	+	+	+	*Kurthia* sp.
10	+	-	+	-	+	+	+	+	*S. aureus*
11	-	-	+	-	+	+	+	+	*Unidentified*

Note: GS= Gram staining, + is Positive test, - is Negative test

6.4.7. Antibacterial activity

The antibacterial activity of the isolated bacterial species against *S. aureus* (ATCC 29213), *E. coli (ATCC 25922), K. pneumoniae* (NCIM2719) and *P. aeruginosa* (ATCC 27853) is shown in Table 6.6. The antibacterial activity was measured in terms of zone of inhibition (mm) around the tested organism in a petriplate as shown in Figure 6.8. It was observed that *Klebsiella* showed maximum antibacterial activity with a zone of inhibition of 40 mm (which is same as control) against *K. pneumoniae. Clostridium, Bacillus, Lactobacillus* and *E. coli* showed higher antibacterial activity with a zone of inhibition of 35, 31, 31, and 30 mm, respectively against *Pseudomonas. Lactobacillus* sp. also showed zone of inhibition of 32 mm against *E. coli.* Fungal species *P. chrysogenum, P. notatum* and *F. oxysporum* showed antibacterial activity against *S. aureus* with a zone of inhibition of 29, 26 and 22 mm, respectively, and against *E. coli with* a zone of inhibition of 19, 16 and 22 mm, respectively. Fungi sp. showed very less (less than 10 mm) antibacterial activity against *K. pneumoniae* and *P. aeruginosa*. Actinomycetes species *Streptomyces, Actinomycetes* and *Micromonospora* showed antibacterial activity against *S. aureus* with an inhibition zone of 17 mm, 19 mm and 11 mm, respectively. These actinomycetes species showed very less (less than 10 mm) activity against all four tested pathogens. All these results signify that a vast diversity of microorganisms exists which exhibits the potential to inhibit or prevent the growth of other known pathogens. So there is a possibility of the mechanism that inhibit the growth of other pathogens. In an environment, which is best suited for earthworms, there exists a mutualistic interaction of earthworms and microorganisms that may further enhance the antibacterial activity and causes the death of pathogens in a VF. This important observation described the antibacterial property of the isolated microflora and a possible reason for removal of pathogens.

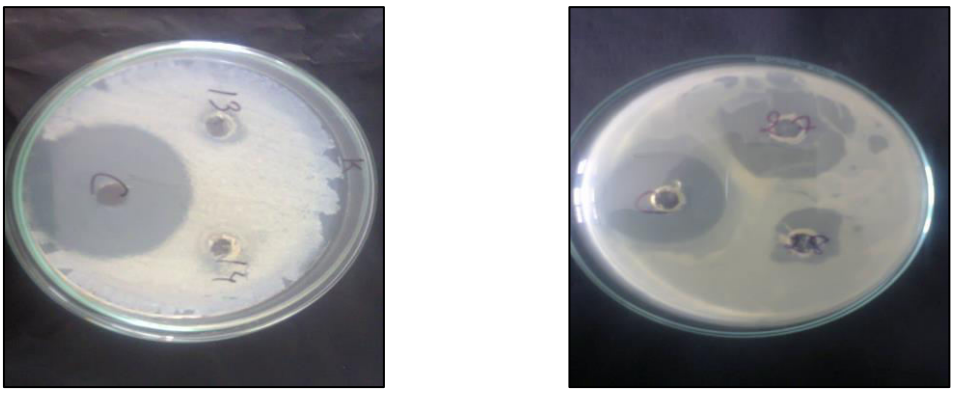
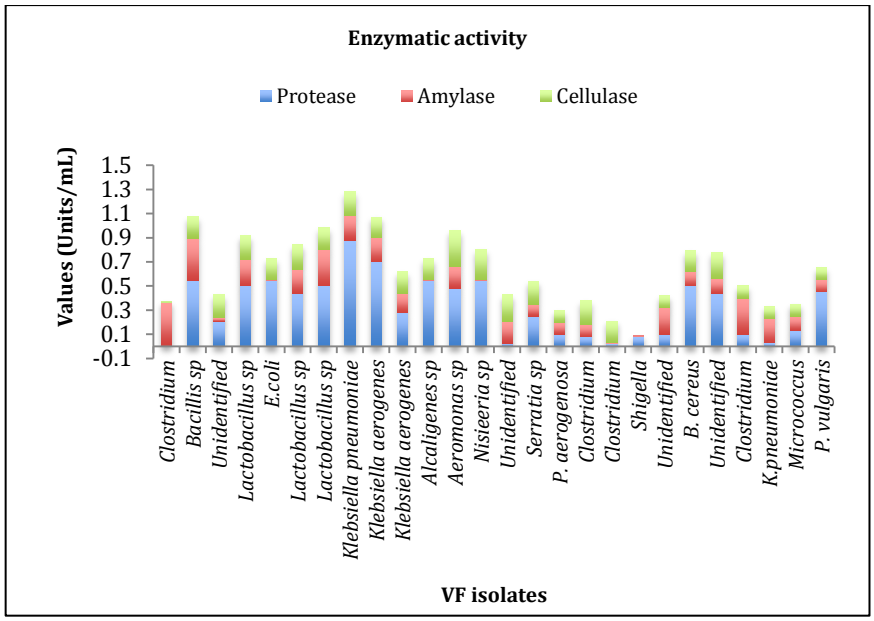


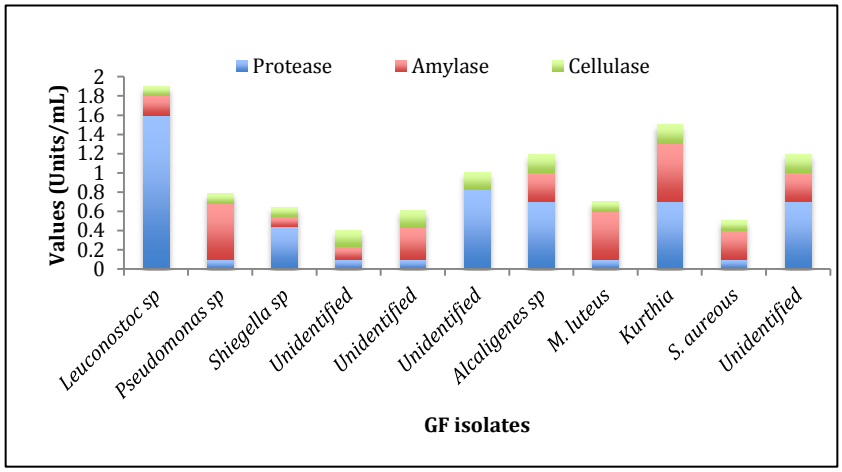
Figure 6.8: Petriplates showing zone of inhibition

6.4.8. Enzymatic activity

The production of enzymes by microorganisms which are found to degrade and stabilize the organics in wastewater is important due to their ability to decompose cellulose, proteins, starch and sugars, which guarantee the integrity of the vermifiltration system. The investigation on the enzymatic activity of the isolated bacterial species would supply vital data for understanding organic matter degradation in vermifiltration (Xing et al., 2010a). The knowledge of enzymatic activity leads to better understanding of earthworms and microorganisms interactions during organic matter decomposition. Cellulase enzymes are enzyme of the carbon cycle, because they have been associated with litter mass loss and with the turnover of carbon. Protease activity is a critical point in the nitrogen cycle as polymers are not accessible to microorganisms. Therefore, since these enzymes metabolize large organic polymers into smaller ones, analyzing them could bring information on decomposition rates of organic matter (Aira et al., (2007b). Table 6.6 showed the screening results (qualitative) of enzymatic activity and Figure 6.9 (a) and (b) showed the quantification of enzymatic activity of the bacterial isolates from VF and GF, respectively as defined in Units/mL. From the results, it was found that the enzymatic activity of the isolated microorganisms was very significant, resulting in organic matter biodegradation and the probable reason for higher BOD and COD removal.



(a)



(b)

Figure 6.9: Enzymatic activity of the isolated microorganisms from (a) VF (b) GF

Table 6.6: *Enzymatic activity and Antibacterial activity of the isolated bacterial species*

Isolate No.	Isolate	Enzymatic activity			Antibacterial activity (mm) against			
		Amylase	Cellulase	Protease	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*E. coli*
	From VF							
1.	*Clostridium* sp.	+	-	-	14	19	35	21
2.	*Bacillus* sp.	+	+	+	24	18	31	17
3.	*Unidentified*	+	+	+	16	20	23	18
4.	*Lactobacillus*	+	+	+	15	13	31	28
5.	*E. coli*	-	+	+	18	15	30	23
6.	*Lactobacillus*	+	+	+	14	18	28	17
7.	*Lactobacillus*	+	+	+	14	32	26	16
8.	*K. pneumoniae*	+	+	+	14	28	21	40
9.	*K. aerogenes*	+	+	+	10	28	12	14
10.	*K. aerogenes*	+	+	+	14	38	11	18
11.	*Alcaligenes* sp.	-	+	-	20	Nil	17	20
12.	*Aeromonas* sp.	+	+	+	18	13	16	15
13.	*Neisseria* sp.	-	+	+	14	Nil	Nil	13
14.	*Bacillus* sp.	+	+	+	13	15	Nil	23
15.	*Serratia* sp.	-	+	+	Nil	18	Nil	Nil
16.	*P. aeruginosa*	-	-	-	13	12	11	Nil
17.	*Clostridium* sp.	-	+	+	15	22	13	11
18.	*Clostridium* sp.	-	+	+	Nil	26	14	21
19.	*Shigella* sp.	-	+	+	27	14	19	16
20.	*Bacillus* sp.	+	-	-	21	Nil	15	14
21.	*B. cereus*	+	+	+	25	16	14	Nil
22.	*Bacillus* sp.	+	+	+	26	24	13	Nil
23.	*Clostridium* sp.	+	-	-	17	16	17	16
24.	*K. pneumoniae*	+	-	+	21	Nil	12	21
25.	*M. luteus*	+	-	-	25	16	16	12
26.	*P. vulgaris*	-	-	+	18	27	14	18

	From GF							
1.	*Leuconostoc* sp.	+	+	+	Nil	Nil	15	Nil
2.	*Pseudomonas* sp.	+	+	-	13	Nil	15	Nil
3.	*Shigella* sp.	+	-	+	Nil	Nil	Nil	Nil
4.	*Pseudomonas* sp.	-	+	-	14	Nil	Nil	Nil
5.	*Pseudomonas* sp.	+	+	+	11	12	16	17
6.	*Unidentified*	+	+	-	24	29	15	20
7.	*Alcaligenes* sp.	+	+	+	Nil	Nil	Nil	Nil
8.	*M. luteus*	+	-	-	18	17	12	13
9.	*Kurthia* sp.	+	+	+	28	14	15	21
10.	*S. aureus*	+	-	-	25	16	18	17
11.	*Unidentified*	+	+	+	18	26	29	Nil

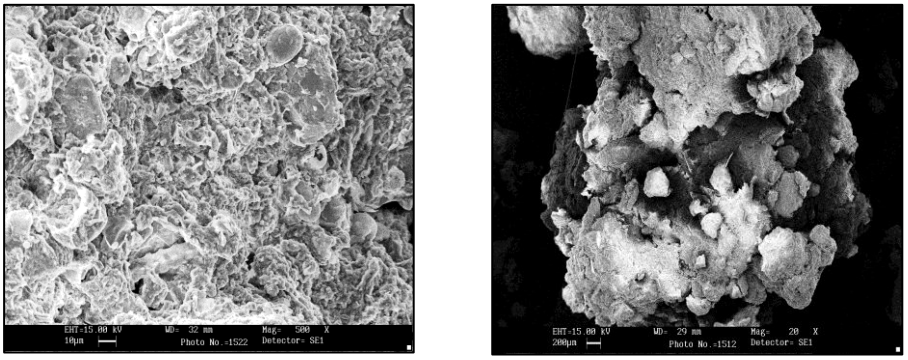
Note: GS = gram staining, + is positive test, -is negative test.

6.4.9. Earthworm's growth rate characteristics

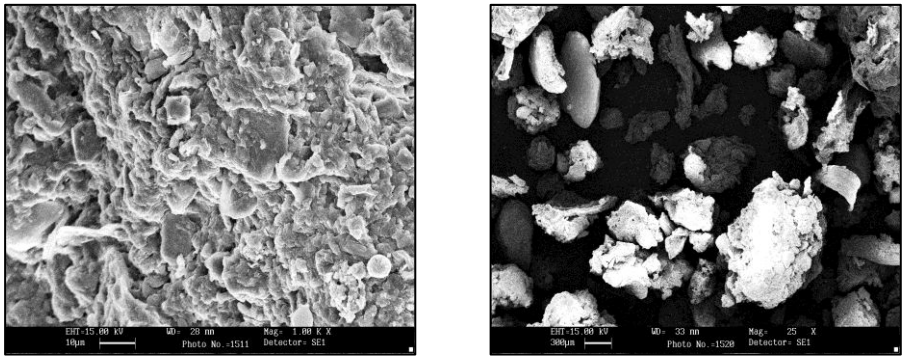
In the beginning, 800 number (weighed 450 g) of earthworm species *E. fetida* were added in the VF based on 10,000 worms/m^3 stocking density. At the end of the experiments, the number of earthworms was found to increase to 950, showing an increase in number by 16%. Earthworm biomass increased significantly by 21.7%. This could be due to the continuous substrate (organic matter) availability to the earthworms and better environmental conditions for their proliferation inside VF. This clearly indicates that *E. fetida* can be utilized for the wastewater treatment by vermifiltration.

6.4.10. Scanning electron microscopy

The typical SEM micrographs of the samples obtained after the operation period are shown in Figure 6.10. The results revealed that the vermicompost from VF had a loosely packed fluffy structure characterized by predominance of rod-shaped cells and an evident extracellular polymeric substances matrix in which the cells were embedded (Figure 6.10 (a). The GF vermicompost was characterized by a floc structure, but a remarkable reduction in rod-shaped cells was observed when compared with that of the VF vermicompost, indicating the partial disruption of extracellular polymers in the VF (Figure 6.10 (b). This signifies the maturation of VF vermicompost.



(a)



(b)

Figure 6.10. SEM images of compost from (a) VF (b) GF

6.5. Summary

- The study evaluated the performance evaluation of a pilot scale continuously operated vermifilter and explored the microbial community dynamics elucidating the mechanism of organic matter biodegradation and removal of pathogens.
- VF showed higher treatment efficiency as compared to GF, in terms of organics and pathogens removal, which is attributed to the functionality of earthworms residing in VF bed.
- It was concluded that enzymes are responsible for the biochemical degradation of organic matter, which is proven by the enzymatic activity tests.
- The antibacterial activity assay confirmed the possibility of the mechanism (antibiosis) that is responsible for removal for pathogens during the treatment.
- The work focused on selecting the potential microbial species that possess higher antibacterial and enzymatic activity that may be further investigated to enhance the treatment efficacy of vermifiltration.
- This approach can lead to deeper understanding of the role of microbial community and interactions between earthworms and microorganisms for the treatment process, during vermifiltration.

Next Chapter deals with evaluating the effects of seasonal variations of temperature on the treatment efficiency.

CHAPTER 7

EFFECT OF SEASONAL TEMPERATURE ON VERMIFILTRATION

This chapter described the effect of variation in seasonal temperature on the treatment efficiency, pathogen removal efficacy, earthworm population characteristics and microbial population during domestic wastewater treatment by vermifiltration. Attempts were made to examine the correlation between temperature and other parameters.

7.1. Introduction

Vermifilters are engineered natural systems based on the symbiotic relationship between earthworms and microroganisms to treat waste. Earlier studies on vermifiltration investigated the pollutants and nutrients removal efficiency of domestic and industrial wastewater and the stabilization effect of wastewater treatment by VFs. These studies mostly focused on the effects of filter media, hydraulic loading rate, organic loading rate, or stocking density of earthworms but rarely for the effect of seasonal temperature on the treatment performance of VF. In a previous study on the effect of filter bed temperature on organics and nutrient removal, the results showed the optimal temperature range of 16−25°C is required for earthworms survival (Yin et al., 2011). Li et al. (2009a) also studied the effect of seasonal variations on treatment efficiency of vermifiltration for domestic wastewater, but the study was limited for only two seasons (Li et al., 2009a).

Temperature is an important factor for the growth and metabolic activity of microorganisms and microbial community diversity changes with the variation of temperature. Earthworm is a poikilotherm, the body temperature of which is significantly associated to outside temperature, and they could die under higher or lower temperature other than optimal temperature range (Edwards, 2004). In vermifiltration, the organic matter and pathogen removal from the wastewater is due to the oxidation and decomposition process of microorganisms and earthworms; therefore, the process will inevitably be affected by temperature. In context to India, the study becomes more important as the weather conditions here are variable throughout the year. India experiences variations of temperature to as low as 4–8°C during winter to as high as 45–48°C during summer. The studies on how temperature affects pathogen removal, earthworm growth characteristics and microbial population are limited. In addition, little is known about the effects of seasonal temperature on the treatment efficiency (BOD, COD, TSS removal), pathogen removal efficacy, bacteria, fungi and actinomycetes population and earthworm growth and reproduction pattern.

7.2. Objectives

The specific objectives of the present study are:

- To investigate the effect of seasonal temperature on VF efficiency (BOD, COD, TSS removal)
- To determine the effect of temperature on the removal of indicator organisms (TC, FC, and FS), pathogens (*Salmonella, Escherichia coli*), earthworm population and microbial population (total heterotrophic bacteria (THB), total fungi (TF) and actinomycetes)
- To evaluate the relationship and examine the correlation between temperature and water quality parameters, pathogen removal and microbial numbers

The scope of this study is limited to only indicator organisms and fewer pathogens, for evaluating the pathogen removal performance.

7.3. Experimental Methods

7.3.1. Reactor description

A polyvinyl chloride (PVC) vermifilter having dimensions 0.25 × 0.20 × 0.30 m^3 was installed and operated for one year. The schematic diagram is shown in Figure 7.1 and pictorial view of actual VF is shown in Figure 7.2. The VF consisted of filter bed, wastewater storage tank, mixer for constant mixing, peristaltic pimp, wastewater distribution system and effluent collection system. A pipe with small holes (1.5 mm in diameter) drilled in its underside was installed to distribute wastewater uniformly. An empty space or free board of around 5 cm is kept at the top for aeration purpose. The filter bed is filled with 4 layers (from bottom to top). The fourth supporting layer of 5 cm consisted of gravels of size 10-12 mm. The third layer comprised of gravels of size of 4-6 mm of depth 5 cm. The second layer consisted of sand particles of size 1-2 mm and the first layer comprised of earthworm packing bed of 10 cm of mature vermicompost. This is the active layer where earthworm species *E. fetida* are inoculated (150 in number) based on 10,000 worms/m^3 stocking density. The HLR of the reactor was kept constant at 1.0 $m^3m^{-2}day^{-1}$ (50 litres per day) during all experiments. Based on the flow rate and reactor configuration, the HRT was found to be 7.2 h. Wastewater passed through different layers in sequence by gravity flow. The experiments started during the month of December, 2012 and continued till November 2013 for four different seasons in a year. The average influent characteristics are: pH 8.0 ± 0.5, DO 0.60 ± 0.5 mg/L, BOD 328 ± 15 mg/L, COD 448 ± 32 mg/L, TSS 130 ± 9 mg/L, TC 4.26 × 10^6 MPN/100 mL, FC 3.02 × 10^5 MPN/100 mL and FS 2.82 × 10^5 MPN/100 mL, respectively.

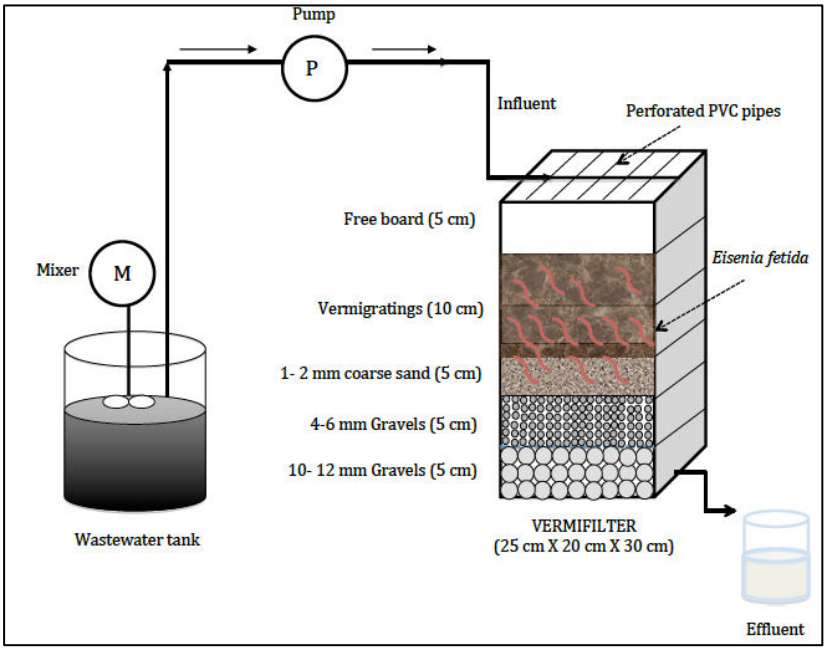


Figure 7.1: Schematic diagram of VF

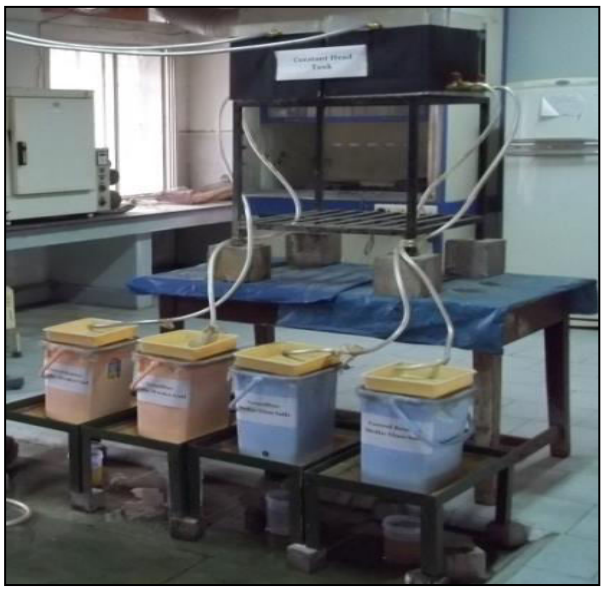


Figure 7.2: Pictorial view of VF

7.3.2. Sampling and analysis

The influent (synthetic sewage) was sampled immediately as it is prepared, and effluent was sampled after every 7 days. All samples were stored at 4°C for less than 24 h before analysis, and three replicates were assessed for every parameter in each sample. BOD, COD, TSS, DO, TC, FC, FS, *E. coli* and *Salmonella* were measured according to the standard methods as described in section 3.2.

At the beginning of every season, approximately 150 clitellated *E. fetida* with biomass measuring 100 g were introduced into VF. Growth and cocoon production were measured at the end of every period. Earthworms and cocoons produced during the experiment were

separated from the substrate material by hand sorting, after which they were counted, examined for clitellated development and weighed after washing with tap water to remove material that had adhered to their bodies (Suthar, 2009). The worms were weighed without voiding their gut content. Based on biomass change and cocoon number data, the growth rate (mg worm^{-1} day^{-1}) and reproduction rate (cocoon worm^{-1} day^{-1}) were calculated with the help of recorded data.

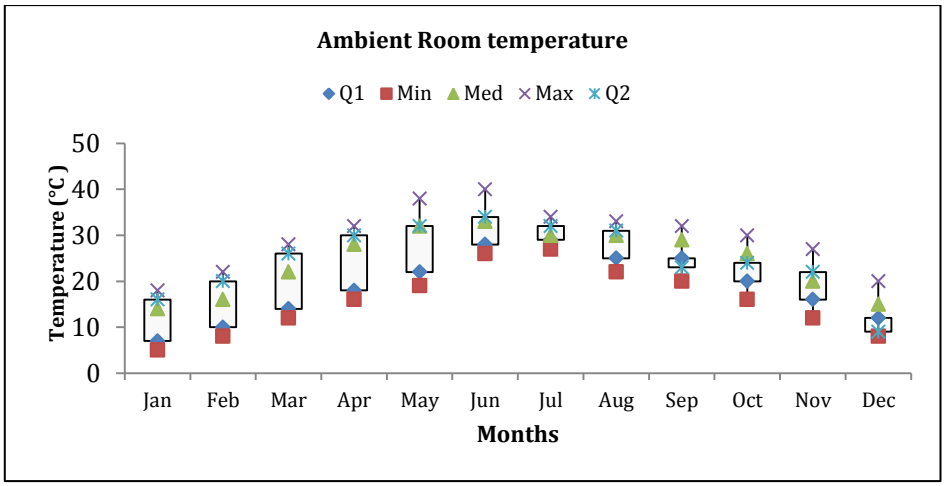
The filter media at different depths inside VF were analyzed for microbial population at the end of every season. The samples collected were mixed thoroughly to form a composite sample to avoid an accidental value at any sampling point. 1 g of sample was transferred to sterilized test tubes containing sterilized distilled water, and mixed thoroughly using a horizontal shaker for 50 min. The composite sample was then diluted using serial dilution methods and 1 mL aliquots were placed in autoclaved petriplates according to the standard methods (APHA, 2005). The pour plate method was used to enumerate bacteria, fungi and actinomycetes in nutrient agar media, Rose Bengal agar, and Kenknight's media, respectively (Singh and Suthar, 2012). The colony forming units (CFU), expressed in microbial counting, were measured. The mean values of total bacteria, total fungi and actinomycetes in the initial VF substrates were 7.55 CFU × 10^6/g, 3.16 CFU × 10^5/g and 3.29 CFU × 10^4 /g, respectively.

All the statistical analysis was done using SPSS® Version 19.0. One-way analysis of variance (ANOVA) was used to test for differences between different seasons. After the ANOVA analysis, statistically significant differences in the VF were assessed by Duncan's multiple range test. All values are reported at $P < 0.05$ levels. Karl Pearson's coefficient of correlation (r) and regression analysis ($P < 0.05$) were used to develop the relationship between temperature and various performance parameters.

7.4. Results and Discussions

7.4.1. Variation in ambient room temperature

The monthly minimum, maximum and mean ambient room temperature during the study of one year is given in Figure 7.3. Based on the differences in temperature, the experimental period is categorized into four seasons; winter (Dec–Feb, 15.6 ± 3.3°C), spring (Mar–May, 27.9 ± 5.2 °C), summer (Jun–Aug, 35.4 ± 3.8 °C) and autumn (Sept–Nov 28.5 ± 4.4 °C). The values of temperature during Jan 8–14 were significantly lower and June 22–26 was significantly higher than the means, showing extreme temperature. The moisture content in VF during the study period was observed to be 85–90% throughout, which is optimum for earthworm growth and activity.



Note: Q1 is the lower quartile; Q2 is higher quartile; Min, Max and Med are the minimum, maximum and median values.

Figure 7.3: Ambient room temperature for different months during the experimental period

7.4.2. Effect of temperature on physico-chemical characteristics

The daily mean influent and effluent temperature was recorded during the entire monitoring period and it was observed that the temperature of the effluent was almost same throughout, showing not much effect of the temperature of air on temperature of water. This may be attributed to the effect of filter media to withstand the variations of temperature. Filter media have a buffer capacity that provides better resistance to the VF from adverse impact of lower and higher temperature. The temperature of the filter media 10–15 cm below the surface was normally found in the range of 20–35.8°C throughout the experiments with few exceptional days in extreme weather. This may be attributed to the vermicompost effect that could keep the VF warm (Kale et al., 1992). Therefore, the temperature in filter material was normally higher than ambient room temperature. The difference was 1–4°C in summer and 2–6°C in winter. In January there are several days with air room temperature below 6°C. Heat preservation is important for earthworm survival so that it can adjust the filter material temperature. Consequently there was no problem of VF running even in coldest days in winter, except decrease of treatment efficiency.

The mean value of pH of the influent was 8.0 ± 0.5. The pH values of influent were higher than the effluent. The pH of VF effluent increased initially during the treatment. The initial increase in pH is due to the intense microbial activity and organic matter degradation during first few days, which led to the formation of ammonia as a consequence of ammonification of organic nitrogen. This is followed by nitrification, showed by decrease in pH, reaching out to be in neutral range i.e., 7.3 ± 0.2. This signified the natural inherent capability of earthworms to act as buffering agent and neutralizing pH. Changes in pH may be

due to the reduction in the level of alkaline material (ammonia) and incremental increase in acidic substances (nitrate) in wastewater during the vermifiltration. There was no significant difference ($P < 0.05$) between the pH of the effluent between different periods (Table 7.1) except during extreme warm days in summer. During that period, due to higher temperature, the earthworms' metabolism and respiration rate increases inside the filter bed, resulting in release of CO_2 in the wastewater. CO_2 is responsible for lowering of pH by formation of weak carbonic acid in the wastewater, due to which pH in the effluent decreases to 6.8 ± 0.2 (Sharma et al., 2013).

The mean values of DO increased from 0.6 ± 0.5 mg/L in the influent to 5.2 ± 1.0 mg/L in effluent. This suggests that earthworms are responsible for creating aerobic conditions inside VF by their burrowing action. The design of VF is such that oxygen could penetrate to the bottom, thus increasing the efficiency of treatment. No significant difference ($P < 0.05$) of DO was observed statistically between different periods (Table 7.1) except during the very hot days in summer with extreme temperature. The value of DO reached to very low state during this period. This is attributed to the increase in temperature of filter media and higher temperature in the bed increases the earthworms' metabolism and respiration rate, increasing CO_2, due to which DO in the effluent decreases (Buentello et al., 2000).

Table 7.1: *Influent and Effluent quality*[a]

Period	Influent[b]		Effluent[b]	
	pH	DO (mg/L)	pH	DO (mg/L)
Winter	7.9 ± 0.22	0.6 ± 0.48	7.5a ± 0.26	4.61a ± 0.41
Spring	8.2 ± 0.41	0.5 ± 0.68	7.3a ± 0.32	5.87a ± 1.04
Summer	7.8 ± 0.53	0.7 ± 0.24	7.2a ± 0.22	5.75a ± 1.01
Autumn	8.0 ± 0.38	0.5 ± 0.51	7.1a ± 0.19	4.57a ± 0.76

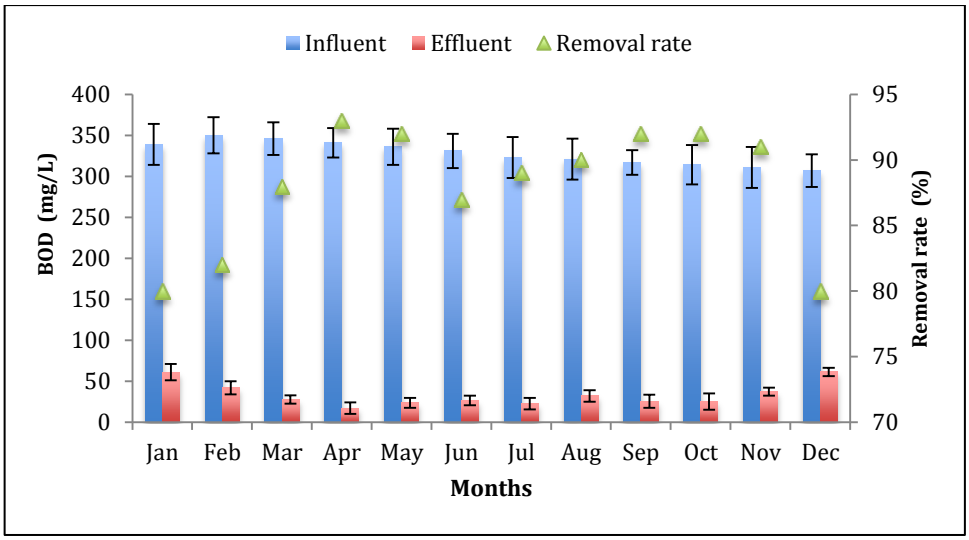
Note: a Arithmetic averages (mean ± standard deviation) of 4 seasons during the experimental period

b Values with different letters in the same column for different seasons indicate significant differences at P = 0.05 according to Duncan's multiple range test

The organic matter measured as mean BOD in the influent was 328 ± 15 mg/L. Results showed that the mean BOD removal is 90% during the entire study. Figure 7.4 (a) shows the mean concentration of BOD in the influent, effluent and percentage removal efficiency in different months. The average BOD removal efficiency was lower in the winter period (80–82%) and highest in spring (88–95%). BOD removal in VF is attributed to the activity of earthworms and its associated microorganisms that degrade the wastewater organics by its enzymatic activity. The earthworms work best in their optimum temperature range, which is

25–30°C, and beyond this temperature, the efficiency decreases. The influent mean COD concentration was 448 ± 32 mg/L. The mean COD removal in VF is 74%. Figure 7.4 (b) shows the mean concentration of COD in the influent, effluent and percentage removal in different months. This is attributed to the symbiotic activity of earthworms and its associated microflora such as denitrifiers and other heterotrophs and the enzymatic activity that helps in degradation of organic chemicals. Since sedimentation, adsorption and microbial metabolism are considered to be the primary mechanisms for BOD and COD removal; it is likely that earthworms provide a more rapid rate of degradation in its filter bed. Thus, adding earthworms to the filter bed enhances organic matter removal and VF could work best in spring and autumn period, within the optimum temperature range for earthworms. An increase or decrease in temperature other than the optimum range may lead to decrease in BOD and COD removal efficiency. Seasonal effects on BOD and COD removal efficiencies were evident, with the best and most consistent overall performance during the spring and autumn and worst during the winter months. The higher BOD and COD removal efficiency during the warm period is also attributed to the high decomposition rate and buffering characteristics of VF at optimum temperature range.

Total suspended solids (TSS) were reduced during vermifiltration significantly and the mean TSS removal in VF was observed to be 82%. This could be attributed to the ingestion of organic and inorganic solid particles in wastewater through earthworm, which excrete them as finer particles. These finer particles are further trapped in the voids of VF and causes high removal efficiency of TSS from wastewater. Figure 7.4 (c) shows the variation of TSS in the influent, effluent and mean removal efficiency in different months. TSS purification and removal rates were consistent during all periods because the main processes involved were filtration and sedimentation, which are not temperature dependent processes. Previous studies also found that seasonal variations do not affect TSS removal rates (Kadlec, 2003; Sharma et al., 2013). No significant statistical relationship between TSS removal and seasonal temperature was observed. Figure 7.5 (a), (b) and (c) shows the effect of temperature on the removal rate of BOD, COD, and TSS by regression analysis. The value of $R^2 \leq 0.6$ for BOD and COD removal rate efficiency denotes that around 60% of the variation in removal rate is influenced by the changes in temperature, rest 40% is due to some other reasons. In case of TSS removal efficiency, less significant correlation values ($R^2 = 0.02$) denotes that only 0-2% variations in TSS removal is affected by changes in temperature. This clearly indicates that temperature has no effect on TSS removal.



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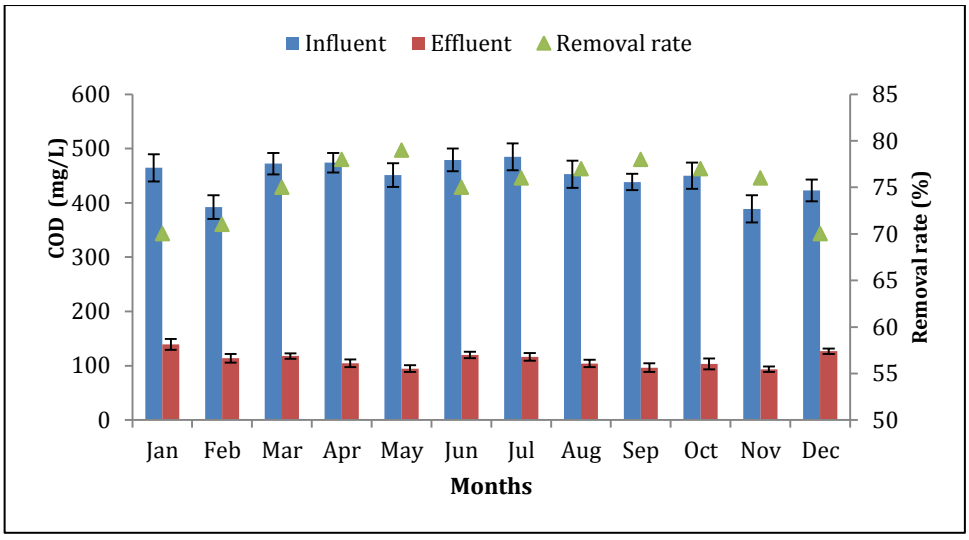

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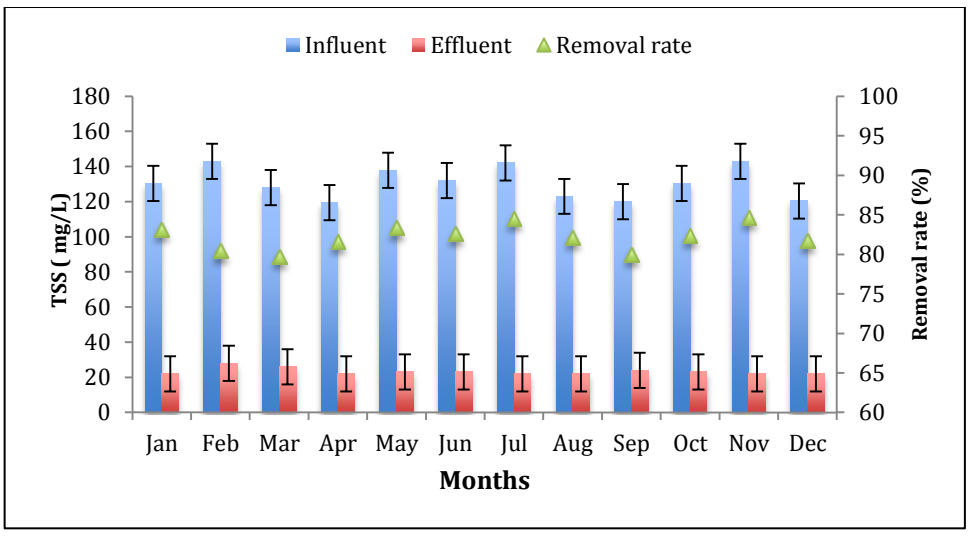
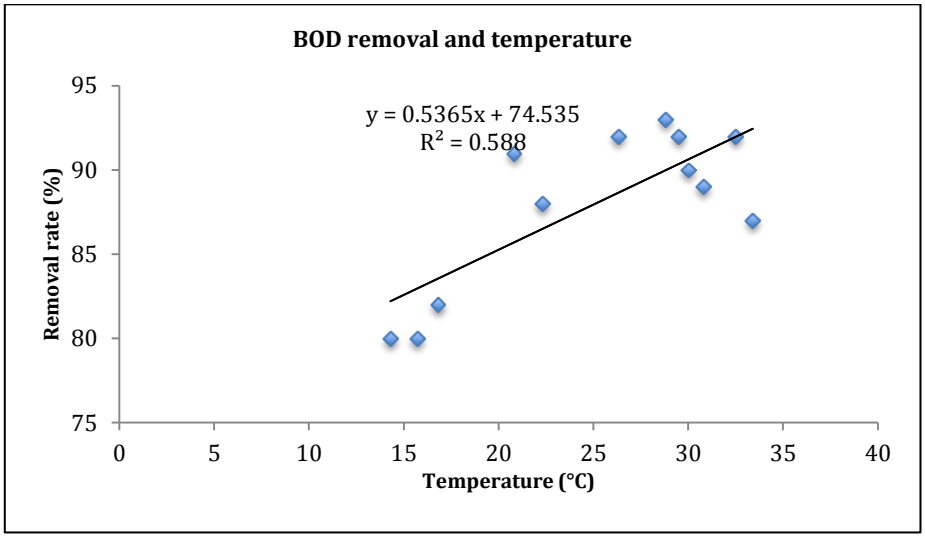


(c)

Figure 7.4: Mean concentrations of (a) BOD (b) COD (c) TSS in influent, effluent and removal efficiency in different months



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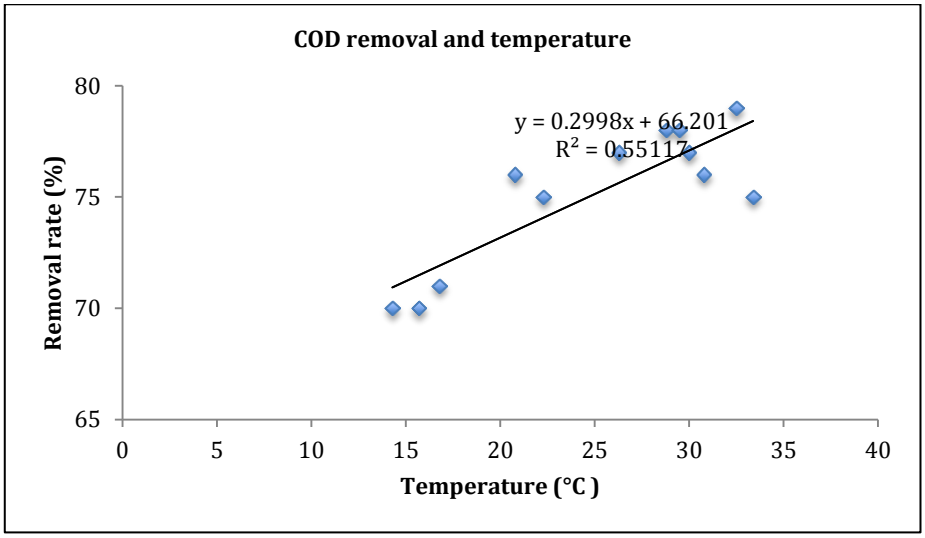

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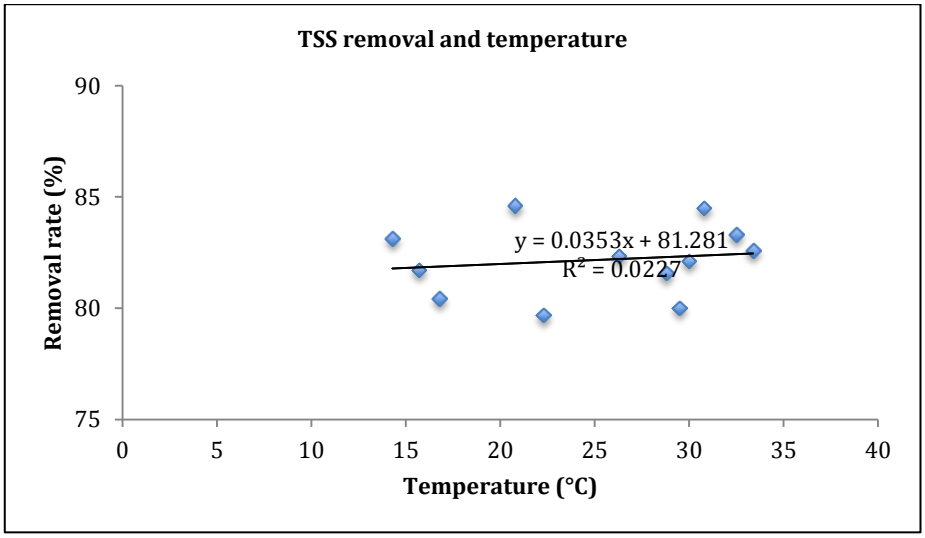


(c)

Figure 7.5: Removal rate of (a) BOD (b) COD (c) TSS plotted against seasonal temperature (°C)

7.4.3. Effect of temperature on indicator bacteria and pathogen removal

The presence of coliforms in a sample indicates its pathogenicity. The indicator organisms and pathogens removal efficiency in different periods is given in Table 7.2. There was significant difference in the TC removal efficiency in different months, ranging from 48% in winter to 99% in summer. The removal rate increased with increase in temperature and because of the high seasonal variability in the removal efficiency, these differences were statistically different ($P < 0.05$). The average FC concentration in the effluent for different seasons varied and there is a greater variability between different months, exhibiting significant seasonal difference ($P < 0.05$). The average FC concentration in the effluent reached the recommended levels of 1.0×10^3 MPN/100 mL in 8 out of 12 months. Overall, VF showed better performance during warmer months of the year. FC removal ranged from a high of 98% in summer to 53% in winter. The lower removal of FC during the winter season in VF can be attributed to the lower metabolic activity of microorganisms resulting from prolonged periods of <10°C temperatures (García-Fernández et al., 2015). In addition, decrease in the activity of earthworms during the winter season also limits the microbial activity, microbial attachment surface area and filtering capacity of the substrate. The effluent FS removal was also found to be highest in summer (99%) and lowest in winter (38%). The mean effluent FS concentration in summer (3.7×10^2 MPN/100 mL) was below WHO recommended levels of 1.0×10^3 MPN/100 mL (World Health Organization, 1989) while during winter (1.4×10^4 MPN/100 mL), the concentration was higher. The temperature range in summer provides the best and most consistent treatment for pathogen removal in VFs. The microbial growth is evident in the range of 15–47°C, with optimum temperature of 28–37°C. When temperature is far from optimal, significant changes in the metabolism of cells occur. At temperatures below 10°C, the fluidity of cell membrane is drastically reduced and the metabolism slows down. At the optimum temperature, the metabolic activity is maximal; the genetic material is unpacked and completely active, and therefore more vulnerable to stress due to increased surface of DNA (Wang et al., 2014). In our results, when temperatures rose from 15 to 35°C, we observed an increase in efficiency of the disinfection (removal of pathogens) process, which can be explained by the increased activity of the metabolism of bacteria cells. Moreover, at higher temperatures, the defense mechanisms of bacteria decrease their capability. Figure 7.6 (a), (b) and (c) shows the effect of temperature on the log removal of TC, FC and FS, respectively by linear regression analysis. The value of R^2 is in the range of 0.75-0.78, which showed that 70-75% variation in the TC, FC and FS log removal is affected by the changes in temperature, which is another significant finding for vermifiltration.

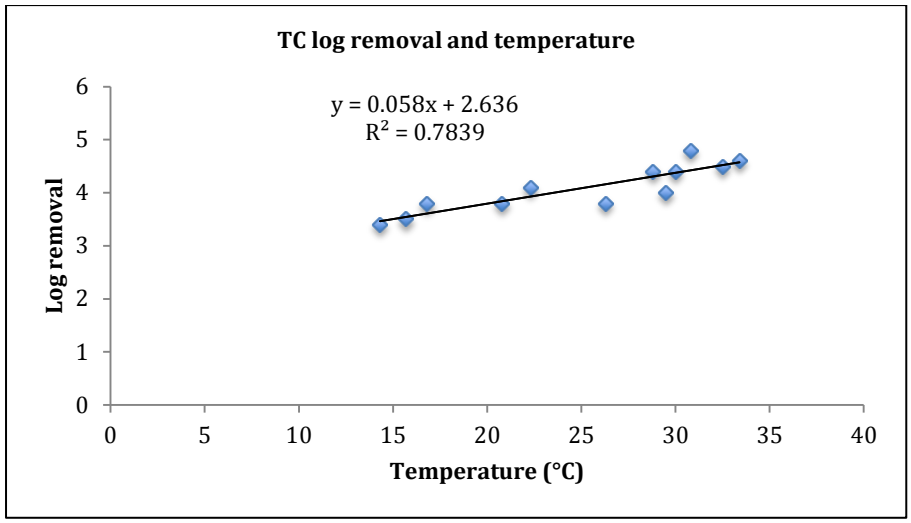
Other than *Salmonella,* all microorganisms showed mean concentration significantly higher (3 orders of magnitude) in the influent than in the effluent. The overall removal of *Salmonella* in VF is higher in summer and spring i.e., 96%, and removal of *E. coli* in summer was highest, i.e., 99.9 %. These findings showed that FS and *E. coli*, commonly used as indicator bacteria, were the most reduced microorganisms and these reductions were below the WHO guidelines of fecal bacteria limit (World Health Organization, 1989). Different processes may be involved in the removal of microorganisms in a natural wastewater treatment system. The fate, distribution and survival of microorganisms in vermifiltration are influenced by the optimum growth conditions inside a VF, including temperature of the filter bed. Filtration, sedimentation, adsorption of microorganisms to the filter bed media and associated biofilms, predation by antagonistic microorganisms, unsuitable physico-chemical conditions are thought to be the main removal mechanisms in VF (Reinoso et al., 2008). As observed, the lowest removal efficiencies were observed in winter periods. The performance of the VF in winter is poorer due to reduced DO concentration and lower microbial activity (Armstrong et al., 1982; Rivera et al., 1997). Statistical differences ($P<0.05$) in the removal of microorganisms were highly dependent on temperature and effect of temperature and seasonal variation on removal of microorganisms may vary with different species, although the scope of our study is limited to indicator organisms and few pathogens.

Table 7.2: *Influent and Effluent Average Indicator Bacteria and Pathogens Concentration (in log_{10} units) and Removal Efficiency*[a] *(in %)*

Microorganism	Influent[a]	Final effluent[a]	Removal Efficiency[b] (%)			
			Winter	Spring	Summer	Autumn
TC (MPN/100 mL)	6.63 ± 0.60	2.72 ± 1.60	47.80a	95.89b	98.78b	97.69b
FC (MPN/100 mL)	5.48 ± 0.37	2.66 ± 0.30	52.6a	97.12b	98.20b	80.21ab
FS (MPN/100 mL)	5.45 ± 0.66	2.80 ± 0.50	37.56a	95.29b	98.60b	88.89b
E. coli (CFU/mL)	4.50 ± 0.42	1.99 ± 0.10	33.93a	94.99b	99.88b	92.32ab
Salmonella(CFU/mL)	3.87 ± 0.94	1.67 ± 0.92	36.07a	96.81b	96.21b	96.51b

Note: a Values are arithmetic mean ± standard deviation, n = 48 (4 samples per month)

b Values with different letters in the same row for different seasons indicate significant differences at P = 0.05 according to Duncan's multiple range test



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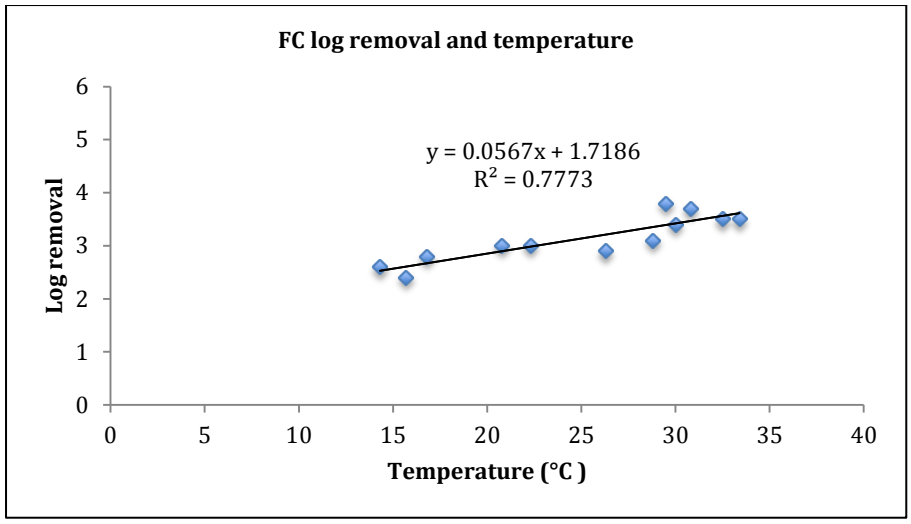

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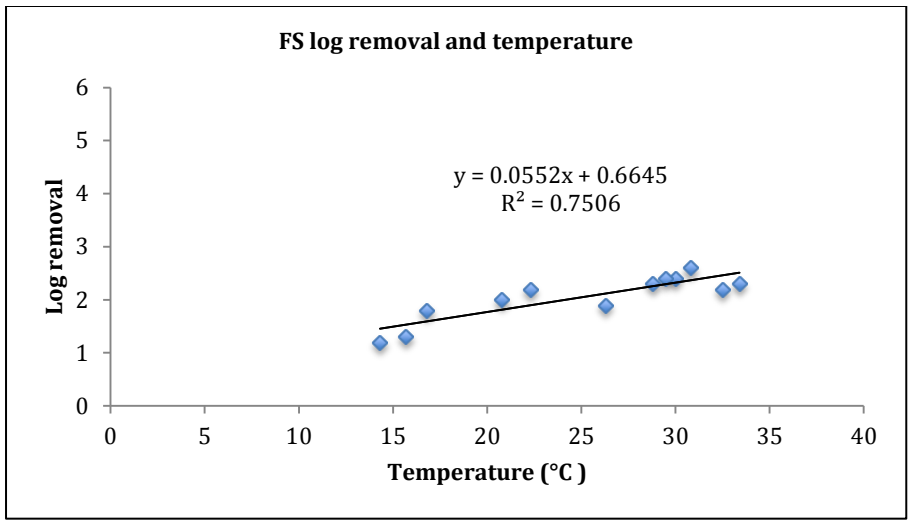


(c)

Figure 7.6: Removal rate of (a) TC (b) FC (c) FS plotted against seasonal temperature (°C)

It is a well-known fact that many biochemical reactions proceed at a faster rate as the temperature of the medium is increased. In addition, microbially mediated enzymatic reactions also behave similarly but up to a point. Each microbial process has an optimal range of temperature where maximum activity occurs. As the temperature is increased in the filter media inside VF, the growth and metabolic functions of earthworms and microorganisms will increase until an optimum temperature range is achieved. Generally, the optimal temperature of the microbial species was reported in the range of 28–37°C (Jones and Hood, 1980). It is to be noted that each microbial species and each strain has its own minimum, optimum and maximum temperature range. Earthworm species *E. fetida* or Red worms are tolerant of a wide range of temperatures from the 2°C to about 35°C, so they are utilized worldwide. According to Edwards and Fletcher (1988) the optimal breeding temperature for this species is 15-20°C, while the optimal temperature for maximum growth and waste processing ability is 25-27°C. All bacteria have their own optimum environmental surroundings and temperature in which they most thrive. Optimum temperature for maximum microbial activity may vary depending on the type of microorganisms. A mesophile is an organism that grows best in moderate temperature, neither too hot nor too cold, typically between 20 and 45 °C (Pelczar et al., 2008). Thermophiles contain enzymes that can function at high temperatures. This is the possible reason for the appreciable performance of VF in all the seasons, while the best performance is reported in spring and autumn period due to the combined symbiotic activity of earthworms and associated microflora in its optimum temperature range. Bacteria and fungi are unable to achieve temperature homeostasis and within a narrow range of temperature, their metabolic rates respond to temperature in the same manner as simple chemical reactions, increasing in rate with increasing temperature. Beyond this range, the effects of temperature become more pronounced and would inactivate or kill these microorganisms.

The relationship between temperature and biological activity is of complex nature. Temperature influences microbial community and biological activity rate. The optimum temperatures for bacterial activity in vermifiltration processes are in the range between 25°C and 30°C. When the temperature drops to about 5°C, the autotrophic nitrifying bacteria practically cease functioning and at 2°C even the chemo-heterotrophic bacteria mineralizing carbonaceous material become essentially dormant (Tchobanoglous et al., 2003). Low temperature period might cause a change in the physico-chemical properties of the VF filter bed, affecting subsequent processes. Furthermore, temperature is an important parameter in VF operation as it affects the filtration rate and has an influence on the filter bed performance along with activity of earthworms and associated microflora. Besides, temperature acts upon

the rate of bio-reactions, oxygen solubility in water, settling properties, thickness and/or porosity of filter bed layer, biodegradation of particulate organic matter and biodegradability of wastewater. These all factors resulted in the changes in the performance efficiency of a vermifilter treating domestic wastewater (Krzeminski et al., 2012).

7.4.4. Effect of temperature on microbial population

The microbial population of the VF was also analyzed at the end of every season. Relative to the initial filter, bacteria, fungi and actinomycetes numbers in the final VF filter bed were 25-1000 folds higher. Previous studies have also reported that the microbial population in ingested material increase as earthworms further stimulate and accelerate microbial activity, both by increasing the population of soil microorganisms and improving aeration by burrowing actions (Binet et al., 1998). The highest bacterial number was 90 ± 10 CFU × 10^8 /g in summer, while the lowest bacterial number was 60 ± 7 CFU × 10^6/g in winter. There was a statistically significant difference between the bacterial populations in different seasons ($P < 0.05$). During summer, when the ambient temperature was optimum and there was best living environment for microorganisms, conditions were also beneficial for rapid bacterial multiplication. Fungi populations ranged from 80 to 105 CFU × 10^5/g in VF in different seasons, and there was no significant difference between the fungi numbers. Fungi are aerobic microorganisms and can be used as an indicator of media aeration status (Fester, 2013). Filter ventilation and permeability were high because of the earthworm burrowing actions in VF. The impact of temperature on fungi population was not significant as fungi can grow over a broad range of temperature. Results for actinomycetes numbers were similar to those for bacterial numbers in the VF. The maximum final actinomycetes population was in summer (150 ± 10 CFU × 10^6/g), while the minimum actinomycetes population was in winter (50 ± 6 CFU × 10^4/g). The reason for the significant difference ($P < 0.05$) for actinomycetes population in different seasons may be attributed to earthworm activity. The higher earthworm growth and reproduction rate in summer promoted more vermicast production. Earthworms hosts millions of actinomycetes in their guts and excrete them in substrate-forming earthworm excreta or vermicast (Gopalakrishnan et al., 2011).

7.4.5. Effect of temperature on earthworm population

E. fetida showed significant growth and reproduction performance in VF in all the seasons. The changes in earthworm weight, growth rate, number of cocoons and reproduction rate in VF at the end of every season are shown in Table 7.3. After every season, the earthworm weight increased and growth rate was high. However, growth rate was significantly higher in spring

and autumn, because of the favorable environmental conditions and optimum temperature in these months. During summer, the observed difference in earthworm growth rate may be related to the higher temperature, which caused increased humidity and VF scouring that was not beneficial for earthworm growth. During the winter, when the temperature was extremely low, earthworm growth rate drastically reduced. Also the substrate moisture in VF may increase, which may have caused reduction in earthworm fecundity. Higher or lower temperature conditions may also have an adverse effect on gametes and further development of cocoons. Cocoons numbers was also statistically significant between different seasons. This is again attributed to the temperature conditions, and that the hatching success of incubated cocoons were inhibited by higher temperature.

Table 7.3: *Earthworm Growth Rate Characteristics*[a]

Seasons[b]	Earthworm weight increased (%)	Growth rate (mg day^{-1} $worm^{-1}$)	Number of cocoons	Reproduction rate (cocoons day^{-1} $worm^{-1}$)
Winter	28.3a ± 4.50	2.2a ± 0.16	52.5a ± 5.85	0.011a ± 0.0005
Spring	48.4b ± 4.33	4.5b ± 0.29	65.4a ± 6.45	0.018a ± 0.0012
Summer	38.6b ± 5.67	3.5b ± 0.18	64.9a ± 7.20	0.017a ± 0.0008
Autumn	30.8ab ± 4.59	2.9ab ± 0.19	82.0b ± 5.62	0.029b ± 0.0017

Note: a Values are means ± standard deviations

b Different letters in the same column for different seasons indicate significant differences at P = 0.05 according to Duncan's multiple range test

7.4.6. Correlation between temperature and performance parameters

Correlations (r Pearson, $P < 0.05$) were established between temperature and all the performance parameters such as pH, DO, BOD, COD, TSS removal efficiency, TC, FC and FS, *Salmonella*, and *E. coli* log removal, and microbial numbers as shown in Table 7.4. Significant positive statistical correlations ($P < 0.01$, $P < 0.05$) were reported between temperature and all other parameters except pH, DO, TSS removal and fungi number. This showed that temperature had a significant influence on the treatment efficiency (BOD and COD removal) and pathogen removal efficacy of vermifilter. This defines the significance of temperature for wastewater treatment and disinfection (removal of pathogens). This suggests that temperature should be also considered as a design parameter during vermifiltration for wastewater treatment.

Table 7.4: *Inter correlations between various parameters*

Parameters	Temp	pH	DO	BOD[a]	COD[a]	TSS[a]	TC[b]	FC[b]	FS[b]	*E. coli*[b]	*Salmonella*[b]	TB[b]	TF[b]	Actinomycetes[b]
Temp	1.00													
pH	-0.73	1.00												
DO	-0.25	-0.08	1.00											
BOD[a]	**0.83**	-0.33	-0.19	1.00										
COD[a]	**0.74**	-0.46	0.09	**0.76**	1.00									
TSS[a]	0.15	-0.31	0.00	-0.04	-0.17	1.00								
TC[b]	**0.89**	-0.72	0.11	**0.79**	**0.74**	0.26	1.00							
FC[b]	**0.88**	-0.74	0.07	**0.75**	**0.62**	0.13	**0.81**	1.00						
FS[b]	**0.87**	-0.55	-0.04	**0.89**	**0.65**	0.07	**0.88**	**0.89**	1.00					
E. coli[b]	**0.91**	-0.67	0.05	0.49	0.23	-0.12	**0.68**	**0.88**	**0.76**	1.00				
Salmonella[b]	**0.89**	-0.48	-0.11	**0.82**	0.45	0.08	**0.87**	**0.88**	**0.84**	**0.57**	1.00			
TB[b]	**0.90**	-0.54	0.03	**0.87**	**0.66**	0.00	**0.88**	**0.90**	**0.64**	**0.66**	-0.21	1.00		
TF[b]	0.28	-0.34	0.02	0.43	**0.68**	0.23	**0.90**	**0.66**	0.43	**0.65**	-0.24	**0.51**	1.00	
Actinomycetes[b]	**0.90**	-0.35	0.12	0.37	**0.69**	0.11	**0.65**	**0.69**	0.40	0.46	-0.12	0.49	**0.76**	1.00

Note: a Removal efficiency, b log removal, Bold values with underline are statistically significant at $P < 0.0001$, without underline are significant at $P < 0.05$

7.5. Summary

- Vermifiltration technology proved proficient for treating domestic wastewater during all the seasons.
- It is remarkable that during the days with extremes in temperature, the treatment still continued, however affecting the treatment efficiency of system.
- Vermifilter showed the best performance with highest BOD and COD removal efficiency during spring and autumn period when the temperature range was 25-30°C. This temperature is the optimal range for *E. fetida* growth, reproduction and activity. The pathogen removal efficacy was highest in summer.
- Overall, VF seemed to provide the consistent treatment for all wastewater parameters. However, deviation in temperature from the optimum range may lead to acceptable decrease in treatment efficiency.
- All the performance parameters were positively correlated with temperature, except TSS removal and fungi population.

Next chapter deals with the second phase of the study, for the performance evaluation of a pilot scale vermifilter for the combined treatment of sold waste and wastewater along with investigation of microbial community dynamics.

CHAPTER 8

PILOT SCALE VERMIFILTER FOR COMBINED TREATMENT OF OFMSW AND DOMESTIC WASTEWATER

This chapter is directed towards the combined treatment of organic fraction of municipal solid waste (OFMSW) and domestic wastewater in a vermifilter. Understanding different phenomena involved in the removal of pollutants, pathogens and nutrients from the waste and the microbial community dynamics can improve the performance of vermifiltration, which is explored for the first time, in this study.

8.1. Introduction

Occurrence, fate and eco-toxic effects of waste in the environment have received increasing scientific attention over the past decade, owing to its severe impact posed on human health. Fecal contamination in water sources is a major cause of various waterborne infectious diseases and has assumed global dimensions (Kumar et al., 2012). Currently, waste management represents a rising challenge and wastewater treatment plants and solid waste management facilities causes a burden on the environmental regulatory bodies because of economic, environmental and regulation factors. The costs associated with waste handling and management is very high, therefore the need for low cost, self-enhanced, self-driven, technology is long recognized (Tchobanoglous et al., 2003). Compared with other technologies used in co-treatment of wastewater and OFMSW, such as anaerobic and aerobic digestion, vermifiltration emerged as a promising and effective approach. In this context, vermifiltration technology is explored for the combined treatment of wastewater and organic fraction of municipal solid waste (OFMSW). The capacity of VF to treat OFMSW and wastewater simultaneously can be attributed to the vermi-composting and vermi-filtration process that occurs within the VF bed due to earthworm's consumption of organic matter on the surface (Taylor et al., 2003). The treatment performance of vermifiltration is dependent upon the symbiotic interrelationship between earthworms and associated microflora and organic-matter degradation and transformation due to the microorganisms present in the biofilm. In vermifiltration, microbes are responsible for biochemical degradation of organic matter and earthworms present in the VF can also remove the harmful pathogens from wastewater by engulfing them and by discharge of antibacterial coelomic fluid showing antimicrobial properties (Subramanian et al., 2010). However, information on the co-treatment of OFMSW and wastewater is limited and knowledge about the microbial community diversity, antimicrobial activity of the earthworm-associated bacteria, composition and biochemical

properties of microorganisms in vermifiltration systems is lacking. Some advanced analytical techniques, polymerase chain reaction–gel electrophoresis (PCR–GE), and scanning electron microscopy (SEM), are often used to further understand the microbial characteristics. SEM can directly reveal the microorganism profile and biofilm microstructure from magnifications of 10 times to more than 500,000 times. Thus, investigation of the microbial consortium and its functionality can help in understanding and controlling the vermifiltration process. Assessment of the antimicrobial activity of the isolated microflora and its phylogenetic relationship brings an insight to the pathogen removal mechanism during vermifiltration.

8.2. Objectives

The objectives of the study were designed keeping in mind the concern for pathogens that affect human health globally. The specific objectives of the study are:

- To evaluate the performance of vermifilter for the combined treatment of wastewater and OFMSW
- To investigate the microbial community diversity from VF by culture-dependent method
- To determine the antimicrobial (antibacterial and antifungal) activity of the isolated bacteria against known pathogens; *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and fungal culture *Candida albicans*, *Aspergillus niger* and *Aspergillus clavatus* (Standard cultures)
- To identify, characterize and establish phylogenetic relationship of the identified bacteria exhibiting higher antimicrobial activity

8.3. Experimental Methodology

8.3.1. Reactor description

A pilot scale vermifilter (VF) having dimensions 0.8 × 0.4 × 0.10 m^3 was set up as shown in Figure 8.1. It consisted of a filter bed consisting of different filter media, a wastewater storage tank for storing the influent, mixer to constantly mix the influent before it passed through the wastewater distributor to the VF, and collection system to collect the effluent. The filter bed comprised of 5 layers (total depth 80 cm) of packed filter media of different material. A free board of 20 cm is kept at the top for aeration purpose. The fourth and fifth layers (from the top) of depth 15 cm each consisted of gravels of diameter 4-6 mm and 10-12 mm, respectively. The third layer (depth 15 cm) comprised of 1-2 mm coarse sand. The second layer of depth 15 cm (known as active layer) constituted of mature vermigratings (vermicasts of earthworms and

vermicompost collected from previous laboratory experiments) acted as the earthworm-packing bed, where earthworm sp. *E. fetida* was inoculated with stocking density of 10,000 worms/m^2. At the top, 10 kg of kitchen waste (included only the organic fraction of municipal solid waste) was collected from student's mess from inside the campus and filled upto the depth of 20 cm. Wastewater was collected from nearby sewage pumping station and was introduced into the VF through perforated PVC pipes (of 1.5 mm diameter) at hydraulic loading rate (HLR) of 1.0 m^3 m^{-2} day^{-1}. The experiments were performed during the months of October 2013 to December 2013 (92 days). The initial three weeks were regarded as acclimatization period for earthworms and microorganisms to colonize and adjust with the new surrounding environment. The start up of the vermifiltration process was initiated by seeding wastewater in batch mode for 3 h per day (40 L) for acclimatization of earthworms, colonization and accumulation of microorganisms in filter bed. After acclimatization period, VF was allowed to operate for 6 h per day (80 L for 6 h) for the rest of days at room temperature. After 92 days, the biomass and number of earthworms were measured and compared with the initial biomass and number to study the earthworm growth rate characteristics by isolating and hand sorting technique.

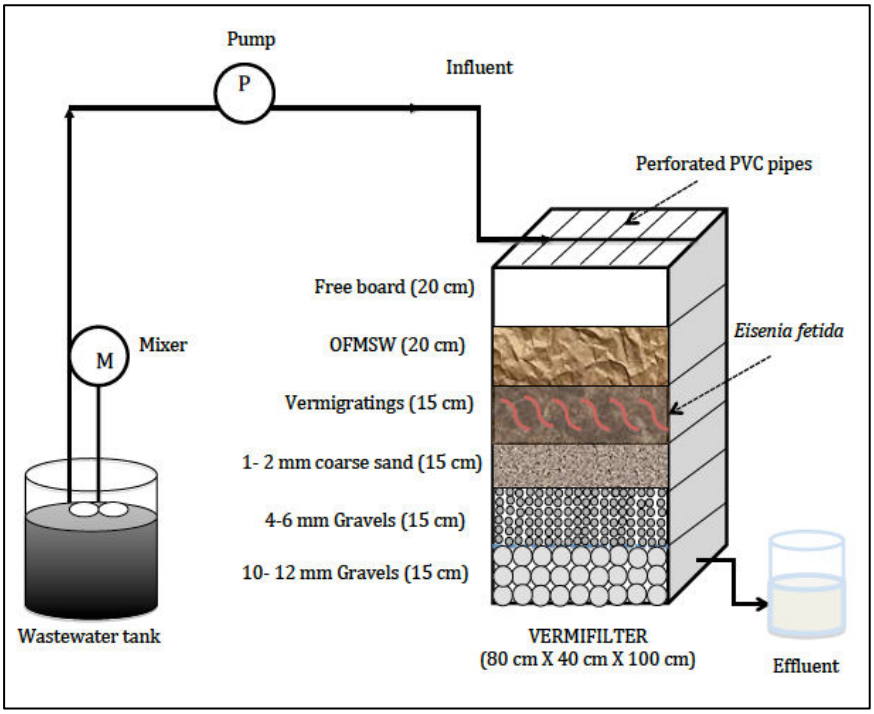


Figure 8.1: Schematic diagram of a pilot scale VF

8.3.2. Sampling and Analysis

The characteristics of initial influent and initial solid waste sample were given in Table 8.1. Treated effluent samples were collected weekly for analysis of BOD, COD, NH_4^+-N, NO_3^--N, TP, TC, FC, FS, *E. coli* and *Salmonella* as described in Section 3.2. Solid sample were collected after every 30 days and was analyzed for pH, temperature, moisture content, total

nitrogen (TN), NH_3^+-N and NO_3^- N. The total bacteria (TB), total fungi (TF) and actinomycetes count was determined as described in section 3.2.

Table 8.1: *Characteristics of Influent and Municipal Solid Waste*

Parameter	Influent (units)	Solid waste (units)
pH	8.0 ± 0.1	8.6 ± 0.2
BOD	244 ± 25 (mg/L)	-
COD	450 ± 27 (mg/L)	-
TSS	131 ± 9 (mg/L)	-
NH^+_4-N	20 ± 1(mg/L)	0.4 ± 0.2 (%)
NO^-_3-N	0.6 ± 0.2 (mg/L)	0 .3 ± 0.2 (%)
TP	5.2 ± 1.7 (mg/L)	0.96 ± 0.2 (%)
TC (log)	6.63 ± 0.6 MPN/100 mL)	6.68 ± 0.5 (MPN/g)
FC (log)	5.48 ± 0.4 (MPN/100 mL)	4.73 ± 0.6 (MPN/g)
FS (log)	5.45 ± 0.7 (MPN/100 mL)	3.56 ± 0.4 (MPN/g)

8.3.3. Isolation and identification of bacteria (Culture-dependent method)

At the end of experimental period, the filter media samples were collected from the active layer (depth 50 cm) to isolate the earthworm-associated bacterial community present in VF. All the samples were rinsed with sterile distilled water. The collected biofilm in the wash-water was centrifuged for 10 min at 8000 rpm at 4°C. Settled biofilm samples were then used to isolate microorganisms by spread plate dilution technique. Isolated colonies were selected and sub-cultured to a new petriplate for maintenance of pure culture. Pure bacterial cultures were characterized morphologically on the basis of gram staining and light microscopy, followed by biochemical identification tests according to Bergey's manual of determinative bacteriology (Holt et al., 1994). These bacterial species were assumed as the representatives of isolated microflora associated with the earthworms from the VF.

8.3.4. Determination of antimicrobial activity

The isolated microorganisms were examined for antimicrobial activity against four bacterial strains and three fungal strains by agar well diffusion method as described by Sethi et al. (2013). 24 hours fresh bacterial cultures of *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922), *K. pneumoniae* (NCIM2719) and *P. aeruginosa* (ATCC 27853) and fungal culture *C. albicans* (ATCC 10231), *A. niger* (MTCC 282*)* and *A. clavatus* (MTCC 1323) were used for the assay. These all strains are pathogenic in nature, procured from the Vikas scientific co., Roorkee, India. These strains were selected on purpose to investigate the antimicrobial activity of the isolated microflora. Positive control was kept in the experiment; streptomycin (50 mcg) for

antibacterial activity and nystatin (50 mcg) for antifungal activity as standard drugs. The zones of growth inhibition were measured after 18-24 h of incubation at 37°C for bacteria and at 28°C for 72-96 h for fungi. The sensitivity of microorganisms was determined by measuring the sizes of inhibitory zones on the agar surface and bacterial strains showing >30 mm diameter zone were considered as highly active strains. The experiment was done in triplicate and mean values are presented.

8.3.5. Culture-independent analysis (molecular method)

The identified active bacterial strains that showed higher antimicrobial activity (>30 mm zone of inhibition) are further characterized by phylogenetic analysis.

8.3.5.1. Total genomic DNA extraction from bacterial culture

The total genomic DNA was extracted from the isolated bacterial culture by method described by Bazzicalupo and Fancelli (1997) using DNA extraction Kit (Ultraclean® Microbial DNA Isolation Kit, Mo Bio laboratories Inc.) as per manufacturer's instructions. The isolated and purified DNA was used as a template for polymerase chain reaction (PCR).

8.3.5.2. PCR amplification and 16S rRNA sequencing

PCR was conducted using an EDC-810 thermal cycler. A stretch of 1465 bp was amplified from the total genomic DNA using 16S rRNA universal bacterial primers 1492r (5'-TAC CTT GTT ACG ACT T-3') and 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') (Frank et al., 2008). The PCR reaction was done using 2 μM of both primers, 50 ng of metagenomic DNA, 200 μM of each dNTPs, 1X PCR buffer, 2 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Biochem Biotech products). The PCR reaction was carried out as follows: initial denaturation (94°C for 2 min), followed by 35 cycles of denaturation (94°C for 1 min), primer annealing (48°C for 1 min) and primer extension (72°C for 1.5 min), completed with a final extension step (72°C for 10 min). The amplified products corresponding to 1465 bp were electrophoretically separated and gel purified using Gel extraction kit (Qiagen commercial kit). The purified PCR products were sent to Xcelris Genomics, Ahmedabad, India for partial sequencing of 16S rRNA gene using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rRNA gene sequence was used to carry out basic local alignment search tool (BLAST) with the nrdatabase of National Centre for Biotechnology Information (NCBI) GenBank database.

8.3.5.3. Phylogenetic tree construction

Phylogenetic data were obtained by aligning 800-850 nucleotides of different 16S rRNA sequences retrieved from BLAST algorithm available through NCBI website using CLUSTAL W program version 1.8 (Thompson et al., 1994) with standard parameters. Phylogenetic and molecular evolutionary analyses were conducted using software MEGA version 6.0 (Tamura et al., 2007). A rooted phylogram was obtained by neighbor-joining (NJ) method. An interior branch test was done (heuristic option, 1000 replications) to check the tree topology for robustness. In addition, poisson correction was applied to NJ for distance estimation and complete deletion option was used in handling gaps or missing data obtained from the alignments.

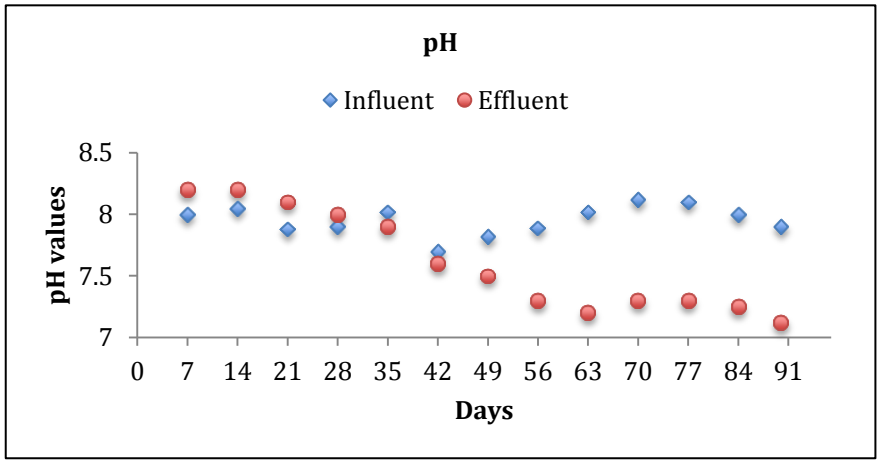
8.4. Results and Discussions

8.4.1. Physico-chemical contaminants removal

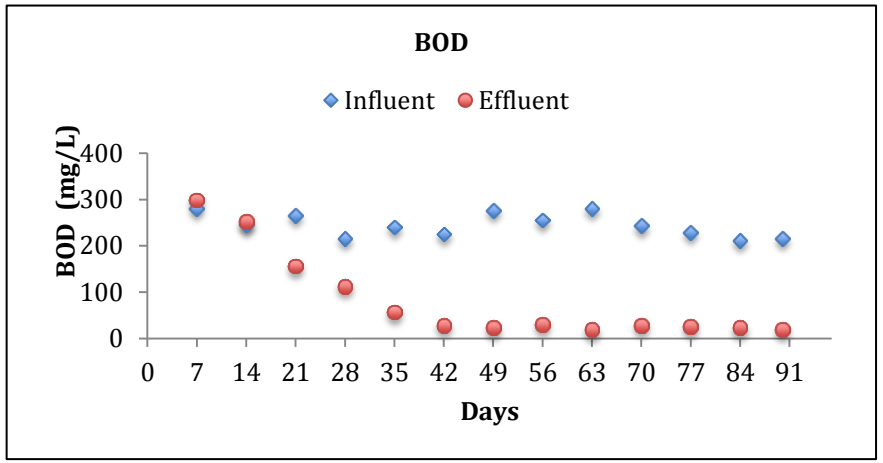
The changes in the physico-chemical characteristics of influent and effluent during the experimental period are illustrated in Figure 8.2. The changes in the characteristics of initial solid waste and final compost are shown in Table 8.2. The pH of influent remained consistent throughout, and of VF effluent increased for fewer days initially (15 days) and then decreased throughout the process, reaching 7.1 ± 0.3 (Figure 8.2 a). In case of solid waste, average pH of final compost (91st day) was 7.5 ± 1.1 from an initial value of 8.6 ± 0.2. The initial increase in pH is due to the intense microbial activity during organic matter degradation in first few days. Initially, biodegradable organic matter was digested at a higher rate, due to highly active microorganisms, which led to the formation of ammonia as a consequence of ammonification of organic nitrogen. Thus the wastewater coming from VF (leachate) showed an increase in pH value. This is followed by decrease in pH at a later stage. The decrease in pH may be due to mineralization of N and P into nitrites/nitrates and orthophosphates and bioconversion of the organic material into intermediate species of organic acids due to nitrification (Khwairakpam and Bhargava, 2009). The average DO increased from 0.8 ± 0.4 mg/L in influent to 5.1 ± 0.6 mg/L in VF effluent. This occurred as a result of passive oxygenation of dropping overflow due to the empty space and mainly due to earthworm burrowing activity. The wastewater temperature between influent and effluent did not vary greatly (data not shown). However, throughout the study period, average room temperature was 22.5°C (ranged 15–30.5°C) which falls within the optimum temperature range for *E. fetida*. The moisture content inside VF was maintained at 85–90% throughout, which is an optimum range for earthworm growth and activity (Domínguez and Edwards, 2001).

The profiles of influent and effluent BOD is shown in Figure 8.2 (b). The average BOD in influent was 244 ± 25 mg/L and BOD removal efficiency of vermifilter (with earthworms) was more than 85.5% considering first 3 weeks as acclimatization period. This acclimatization period reflected the adjustment and acclimatization of microorganisms and earthworms with each other and to the new environment. In addition, concentration of COD (Figure 8.2 (c)) declined sharply from 450 ± 27 mg/L in influent to 100 ± 14 mg/L in effluent with OM degradation occurring by 77.8%. This could be due to the availability of various enzymes that may be present in the gut of earthworms as well as due to the symbiotic and synergistic activity of earthworms and associated VF microflora. Collectively, these results indicate that microbes are responsible for the biochemical degradation of organic matter, while earthworms are important drivers of the process to enhance the biodegradation process through their burrowing and casting behavior.

During treatment process, total suspended solids (TSS) concentration in VF effluent was reduced significantly by 82.2% as shown in Figure 8.2 (d). This may be attributed to ingestion of organic and inorganic solid particles in wastewater by earthworms, which excrete them as finer particles. These finer particles are further trapped in the voids of VF and responsible for high removal efficiency of TSS from wastewater. This is the reason, why VF do not choke and work smoothly and uninterrupted.



(a)



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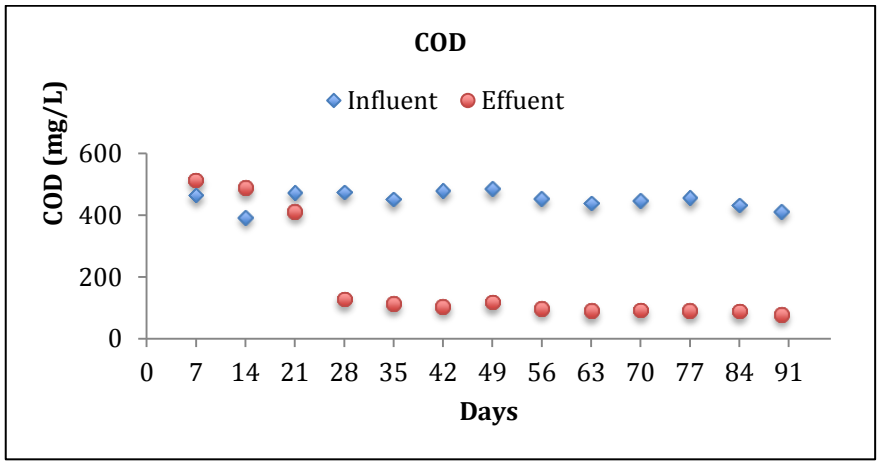
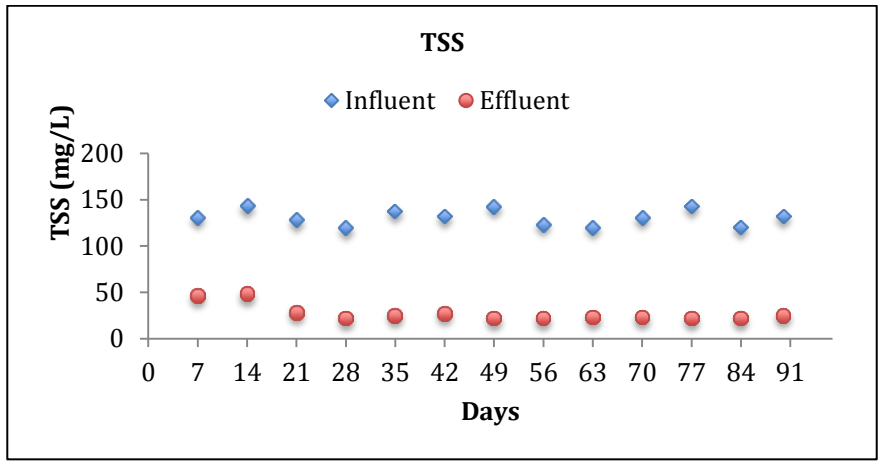


(c)

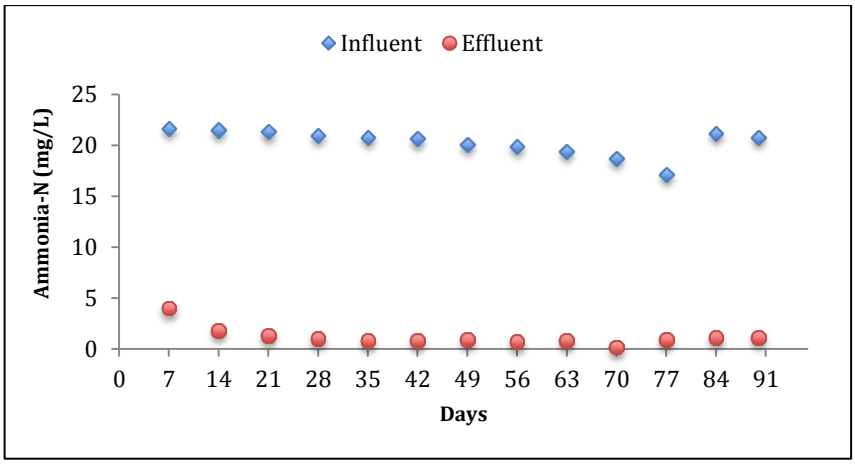


(d)

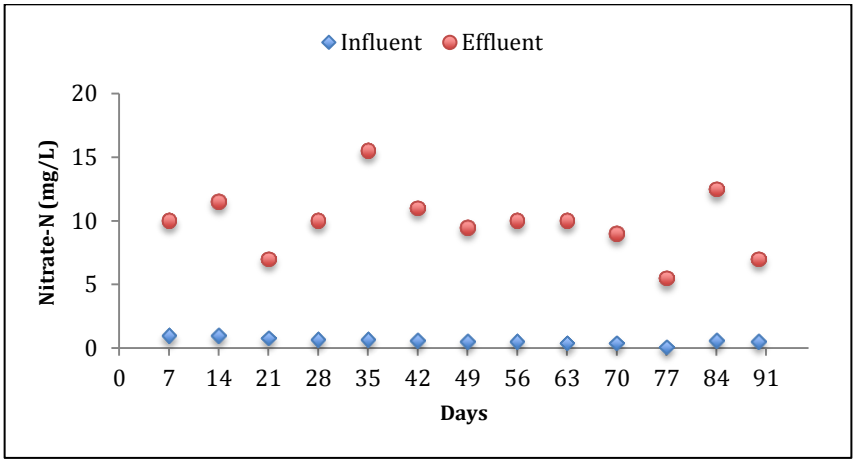
Figure 8.2: Variation in (a) pH (b) BOD (c) COD (d) TSS during vermifiltration

Nitrogen is one of the predominant contaminants in sewage and ammonia results in water pollution and eutrophication. High strength domestic wastewater discharges in certain areas of the world may cause an alarming increase in groundwater nitrate levels (Gupta and Gupta, 2001). The average concentration of NH_4^+-N in influent was 20 ± 1 mg/L. The variations in NH_4^+-N concentration in VF effluent resembled that of COD, with a steady removal efficiency reaching after 25th day, approximately 90% as shown in Figure 8.3 (a). This could be attributed to the rapid growth of ammonia oxidizing bacteria (AOB) at temperature above 15°C. Wang et al. (2011b) had investigated that majority of ammonical nitrogen (NH_4^+-N) was removed mainly in first 2 layers of VF through rapid adsorption by VF biomass and adsorbed NH_4^+-N was subsequently converted to NO_3^--N (nitrate) via biological nitrification (Figure 8.3 (b)) which was carried out by aerobic autotrophic bacteria using molecular oxygen as an electron acceptor. Meanwhile, high surface DO concentration was beneficial for aerobic microbial survival, which in turn was advantageous for nitrification. In addition, NO_2^--N (nitrite) concentrations remained low in effluent, due to the role of nitrites as intermediates in nitrification. Nitrification coupled with denitrification appears to be the major nitrogen removal process involved in VF. Conventionally, the bacterial denitrification is identified as the dissimilar, biologically catalyzed reduction of nitrate and nitrite ions to form gaseous nitrogen and/or nitrogen oxides (Gupta, 1997).

The total phosphate (TP) concentration in effluent increased significantly in VF. The change in influent and effluent TP concentration during the experimental period is shown in Figure 8.3(c). In vermifiltration process, the increased TP concentration was attributed to the activities of earthworm and associated microbes in vermifilter bed promote rapid phosphate mineralization in the system causing increased concentration of TP in the effluent. Another reason behind increased concentration of phosphorus may be attributed to the leaching of vermicast (excreta of earthworms which a strong source of nutrients (1.16% nitrogen, 1.22% phosphorus and 1.00% potassium) and mainly responsible for conversion of soil/ organic matter into vermicompost. VF process mineralize the nitrogen and phosphorous in the sewage to make it bioavailable to plants as nutrients and thus indicates the potential of vermifiltration system for treatment of wastewater to be reused for irrigation.



(a)



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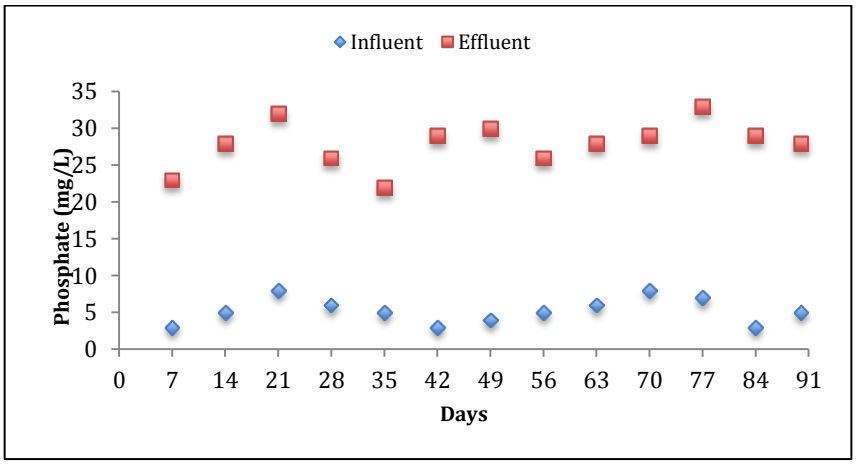

(c)

Figure 8.3: Variation in (a) Ammonia-N (b) Nitrate-N (c) Total phosphate during vermifiltration

In case of solid waste, a large fraction of total organic carbon (TOC) was lost as CO_2 and as a source of energy. TOC was used by earthworms and microorganisms for energy and constructive metabolism. The TOC content was 58.3% in initial solid waste, which was reduced to 32% in vermicompost showing 45.1% reduction (Table 8.2). The total phosphate (TP) was higher in vermicompost (1.6%) than the initial substrates (0.96%) with an increase by 66.6%. An increase in phosphates is mainly due to CO_2 emissions. This is also attributed to mineralization and mobilization of phosphorus by bacteria and phosphatase activity of earthworms (Khwairakpam and Bhargava, 2009). TN content increased in the final vermicompost by 95.9%. The reduction in organic carbon due to substrate utilization by microbes and earthworms and their metabolic activities might have led to relative increase in nitrogen. Earthworm activity enriches the nitrogen profile of vermicompost through microbial mediated nitrogen transformation, through addition of mucus and nitrogenous wastes secreted by earthworms (Bajsa et al., 2003). A decrease in NH_4^+-N occurred which corresponded with an increase in NO_3^--N at the end of the process. The end product, i.e., vermicompost obtained at the end of experimental study had lower C/N ratio (16.9), as compared to the initial value (60.1). The C/N ratio is used as an index for maturity of organic wastes, which reflects the spectra of changing carbon and nitrogen concentration of the substrate material during vermifiltration process (Edwards and Bohlen, 1996). A decline in C/N ratio to less than 20 indicates an advance degree of organic matter stabilization and reflects a satisfactory degree of maturity of organic wastes. The decrease in C/N ratio over time might also be attributed to increase in the earthworm population, which led to higher rate of substrate utilization and rapid decrease in organic carbon.

Table 8.2: *Changes in Characteristics of Solid Waste*

Parameter	0^{th} day	30^{th} day	60^{th} day	90^{th} day
Physico-chemical parameters				
pH	8.6 ± 0.2	8.5 ± 0.3	7.9 ± 0.3	7.5 ± 0.1
Ammonia- N (%)	0.4 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
Nitrate- N (%)	0.3 ± 0.2	0.9 ± 0.1	1.5 ± 0.2	1.7 ± 0.2
TOC (%)	58.3 ± 0.2	48.4 ± 0.9	39.6 ± 0.8	32.0 ± 1.1
TN (%)	0.97 ± 0.1	1.0 ± 0.2	1.5 ± 0.3	1.9 ± 0.1
C/N ratio	60.10	48.40	26.40	16.84
TP (%)	0.96 ± 0.2	1.2 ± 0.2	1.4 ± 0.1	1.6 ± 0.5
C/P ratio	60.73	40.33	28.28	20.00
Microbiological parameters				
TC (MPN/g)	6.68 ± 0.5	4.86 ± 0.3	3.72 ± 0.2	2.60 ± 0.2
FC (MPN/g)	4.73 ± 0.6	4.23 ± 0.2	3.32 ± 0.2	2.50 ± 0.3
FS (MPN/g)	3.56 ± 0.4	2.98 ± 0.5	2.2 ± 0.6	1.90 ± 0.2
Salmonella (CFU/g)	2.97 ± 0.2	2.25 ± 0.2	1.47 ± 0.3	1.25 ± 0.2
Escherichia coli (CFU/g)	2.90 ± 0.5	1.97 ± 0.2	1.50 ± 0.4	1.33 ± 0.2

8.4.2. Pathogen removal

To ensure quality of reclaimed water, biological stability needs to be managed in order to prevent microbial regrowth. Microbial regrowth in reclaimed water causes problems such as the occurrence of opportunistic pathogens and the deterioration of aesthetic quality (Thayanukul et al., 2013). Therefore, pathogens need to be removed from the wastewater with utmost care. The pathogen removal performance efficacy of VF for solid waste and wastewater is given in Table 8.2 and 8.3, respectively. The log removal of *E. coli* and *Salmonella* in VF effluent was observed to be 2.51 and 2.2, respectively. The populations of TB, TF and actinomycetes were reduced by 1.91-3.35 log. It is observed that the concentration of pathogens in VF was reduced considerably, below the WHO guidelines (World Health Organization, 1989) for water for irrigation purpose. In addition, the microbes that earthworms leave behind are beneficial for VF because they compete with pathogens for limited nutrients. In case of combined treatment of wastewater and solid waste, the possible reason for removal of pathogens may also be attributed to the antimicrobial activity. The earthworm-associated bacterial community is thought to release certain antimicrobial substance that resists the growth of pathogens, or inhibits their further growth. So, there is inevitability for determination of antimicrobial activity of bacterial community present in the active layer of VF to elucidate exact mechanism behind the disinfection in vermifiltration. Other attributed factors affecting pathogen removal may include bacterial adhesion, i.e., property of filter media to retain pathogens during filtration, unsuitable physico-chemical environment for pathogen survival and predation of pathogens to regenerate bed for further adhesion.

Table 8.3: *Changes in Microbiological Parameters of Wastewater*

Parameter	Influent	Final effluent	Log Removal
TC (MPN/100 mL)	6.63 ± 0.60	2.72 ± 1.60	3.91
FC (MPN/100 mL)	5.48 ± 0.37	2.66 ± 0.30	2.82
FS (MPN/100 mL)	5.45 ± 0.66	2.80 ± 0.50	2.65
E. coli (CFU/mL)	4.50 ± 0.42	1.99 ± 0.10	2.51
Salmonella (CFU/mL)	3.87 ± 0.94	1.67 ± 0.92	2.20
Total bacteria (CFU/mL)	6.89 ± 0.25	3.54 ± 0.14	3.35
Total fungi (CFU/mL)	4.61 ± 0.60	3.81 ± 0.72	0.80
Actinomycetes (CFU/mL)	5.63 ± 0.15	3.72 ± 0.50	1.91

8.4.3. Isolation and identification of bacterial community

58 bacterial isolates were obtained from active layer of vermifilter. These bacterial isolates were grown on nutrient agar media for sub-culturing and preserved in 10% glycerol stock

solution at -20°C. Out of 58 isolated bacteria, only 41 isolates were able to survive and remained viable after sub-culturing. Gram staining and light microscopy characterized the bacterial isolates which showed 29% of Gram-Positive Rods, 49% Gram-Negative Rods and remaining 22% Gram-Positive cocci. The isolated bacterial strains were then further identified by various biochemical tests as shown in Table 8.4 (a), (b) and (c) for Gram-Positive rods, Gram-Positive cocci and Gram-Negative rods, respectively. The isolated strains were further identified by various biochemical tests such as Citrate, Catalase, Oxidase, Methyl red-Voges Proskauer, etc. Out of 41 isolates, 37% belong to *Enterobacter* family (15 isolates), 22% belong to *Bacillus* sp. (9 isolates), 12% belong to *Enterococcus* (5 isolates), 5% belong to *Aeromonas* (2 isolates), *Clostridium* (2 isolates), *Pseudomonas* (2 isolates), *Staphylococcus* (2 isolates), and *Micrococcus* (2 isolates), and 2 % belongs to Corynebacterium (1 isolate) and 2 % were left unidentified (1 isolate) as shown in Figure 8.4. Nearly 80% of these species showed the formation of mucoid and pigmented colonies. This morphological feature is also observed in the microflora isolated from the gut of earthworm species. This finding supports the speculation that symbioses between earthworms and their associated bacteria are mutualistic, i.e. beneficial for both bacteria and earthworms. It was observed that *Clostridium* being an obligate anaerobe was also isolated, which implied that although the entire vermifiltration process is aerobic, the active layer might tend to become anaerobic by the end of experimental period, confirming that the aerobic-anoxic zone also exists in the VF.

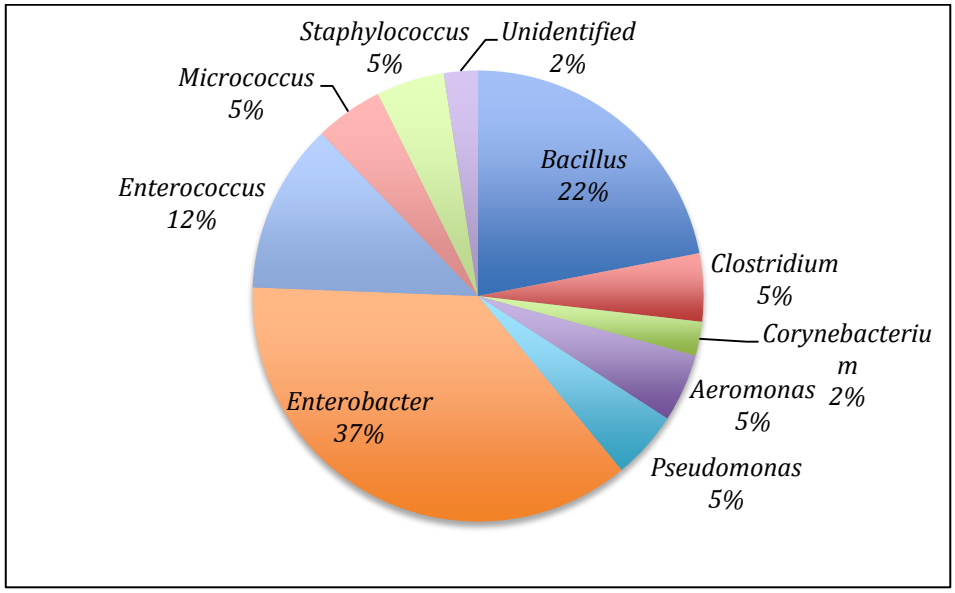

Figure 8.4: Percentage composition of bacterial isolates

Table 8.4: *Biochemical characterization and identification of isolated bacterial species (a) Gram- Positive rods (b) Gram- Positive cocci (c) Gram- Negative rods*

(a) Gram-Positive rods									
Isolate No.	Spore forming	Aerobic/ Anaerobic	Starch hydrolysis (Amylase)	Voges Proskauer	Cell diameter (width) ≥1 µm	Citrate	Acid fast staining	Catalase	Identified sp.
2	+	Aerobic	+	+	-	+	NR	NR	*Bacillus licheniformis*
8	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus thuringiensis*
14	+	Anaerobic	NR	NR	NR	NR	NR	NR	*Clostridium*
17	+	Aerobic	+	+	-	+	NR	NR	*Bacillus subtilis*
22	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus thuringiensis*
23	+	Anaerobic	NR	NR	NR	NR	NR	NR	*Clostridium*
25	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus cereus*
26	-	Aerobic	+	+	NR	NR	-	+	*Corynebacterium xerosis*
27	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus cereus*
32	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus cereus*
33	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus thuringiensis*
40	+	Aerobic	+	+	+	NR	NR	NR	*Bacillus cereus*

(b) Gram-Positive cocci

Isolate No.	Catalase	Mannitol fermentation	Hemolysis	Bile esculin	Growth w/ Tellurite	Yellow pigment (colony)	Glucose fermentation	Identified sp.
1	-	NR	γ	+	-	NR	NR	*Enterococcus faecium*
5	-	NR	γ	+	-	NR	NR	*Enterococcus faecium*
6	-	NR	γ	+	+	NR	NR	*Enterococcus faecalis*
12	+	-	NR	NR	NR	+	-	*Micrococcus luteus*
19	+	-	NR	NR	NR	+	-	*Micrococcus luteus*
21	+	+	NR	NR	NR	NR	NR	*Staphylococcus aureus*
30	+	+	NR	NR	NR	NR	NR	*Staphylococcus aureus*
34	-	NR	γ	+	-	NR	NR	*Enterococcus faecium*
35	-	NR	γ	+	-	NR	NR	*Enterococcus faecium*

(c) Gram-Negative rods

Isolate No.	Oxidase	Glucose ferment-ation	Lactose ferment-ation	Indole	Citrate	MR/VP	Hydrogen sulphide	Lysine decarboxy lase	Urease	Ornithine decarboxyla se	Motility	Identified sp.
3	+	+	NR	NR	NR	-	NR	NR	NR	NR	NR	*Aeromonas sp.*
4	-	NR	+	-	NR	+/-	+	NR	NR	NR	NR	*Citrobacter freundii*
7	-	NR	-	-	NR	NR	NR	NR	+	+	NR	*Proteus mirabilis*

9	-	NR	+	-	NR	+/-	-	NR	NR	NR	-	*Klebsiella pneumoniae*
10	-	NR	+	+	+	+	-	NR	NR	NR	NR	*Klebsiella oxytoca*
11	-	NR	+	-	NR	-/+	NR	-	NR	NR	NR	*Enterobacter cloacae*
13	-	NR	-	+	NR	NR	+	NR	+	NR	NR	*Proteus vulgaris*
15	-	NR	+	-	NR	+/+	NR	NR	NR	NR	NR	*Enterobacter intermedius*
16	+	+	NR	NR	NR	-	NR	NR	NR	NR	NR	*Aeromonas sp.*
18	-	NR	+	-	NR	-/+	NR	-	NR	NR	NR	*Enterobacter cloacae*
20	-	NR	+	-	NR	+/-	-	NR	NR	NR	+	*Serratia fonticola*
24	-	NR	+	-	NR	-/+	NR	-	NR	NR	NR	*Enterobacter cloacae*
28	-	NR	+	-	NR	-/+	NR	-	NR	NR	NR	*Enterobacter cloacae*
29	+	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	*Pseudomonas aeruginosa*
31	+	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	*Pseudomonas aeruginosa*
36	-	NR	+	+	-	NR	NR	NR	NR	NR	NR	*Escherichia coli*
37	-	NR	-	+	NR	NR	-	NR	NR	+	NR	*Providencia stuartii*
38	-	NR	-	-	NR	NR	+	NR	-	NR	+	*Salmonella enterica*
39	-	NR	-	-	NR	NR	-	NR	-	NR	+	*Serratia marcescens*
41	-	NR	-	+	NR	NR	+	NR	NR	+	NR	Unidentified

Note: + Represents Positive test; - represents Negative test; NR= not required

8.4.4. Antimicrobial activity

The antimicrobial activity of the isolated bacteria against known pathogens *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. niger*, *A. clavatus*, *C. albicans* is given in Table 8.5. The antimicrobial activity was measured in terms of zone of inhibition (mm) around the tested organism. The control standard drugs gave an inhibition zone of 40 mm. It was observed that 12 isolates, or 29% of the isolates obtained, showed higher antimicrobial activity against all pathogenic strains. These strains were regarded as active strains. All these results signify that diversity of bacterial community exists in VF that exhibits the potential to inhibit the growth of other pathogens. This important observation construed the possible mechanism for pathogen removal as previously explained. The higher activity against Gram-Positive bacteria was anticipated, since Gram-Negative bacteria are generally less susceptible to antimicrobials because of the presence of outer membrane and lipopolysaccharide layer which act as an efficient barrier (Hensyl, 1994). Sinha et al. (2008) reported that some bacteria and fungi produced by the worms also produce antibiotics, which kill the pathogens in waste. This finding excludes the need of disinfection for removal of pathogens. This also justifies that vermifiltration is a natural, self-sustaining, ecological technology that has an in-built mechanism for organic matter, nutrient and pathogen removal, all in one system.

Table 8.5: *Antimicrobial Activity of Isolated Bacterial species against Pathogens*

Isolate No.	Isolated species	Antimicrobial activity against pathogens (inhibition zone [a] in mm)						
		S. aureus	*E. coli*	*Klebsiella*	*Pseudomonas aeruginosa*	*Aspergillus niger*	*Aspergillus clavatus*	*Candida albicans*
1	*Enterococcus faecium*	18	29	22	31	24	28	33
2	*Bacillus licheniformis* [b]	35	38	32	36	34	31	29
3	*Aeromonas sp.*	16	18	22	32	14	19	22
4	*Citrobacter freundii*	23	27	10	28	12	14	29
5	*Enterococcus faecium* [b]	34	32	32	29	36	31	40
6	*Enterococcus faecalis*	26	24	13	26	24	13	0
7	*Proteus mirabilis* [b]	40	38	38	31	29	33	40
8	*Bacillus thuringiensis* [b]	40	39	33	32	36	40	39
9	*Klebsiella pneumoniae*	14	0	12	2	19	11	14
10	*Klebsiella oxytoca*	0	18	0	4	5	18	22
11	*Enterobacter cloacae*	15	22	13	11	23	30	29
12	*Micrococcus luteus*	15	22	13	11	19	18	0
13	*Proteus vulgaris*	0	12	13	11	22	13	11
14	*Clostridium perfringens*	0	0	0	7	2	0	0
15	*Enterobacter intermedius* [b]	38	37	33	34	38	32	39
16	*Aeromonas sp.*	13	12	11	0	8	20	19
17	*Bacillus subtilis*	0	8	11	18	27	14	18
18	*Enterobacter cloacae*	18	27	14	18	22	5	15
19	*Micrococcus luteus*	13	12	11	0	0	7	0
20	*Serratia fonticola*	26	24	13	0	18	0	22
21	*Staphylococcus aureus*	27	14	19	16	27	29	22
22	*Bacillus thuringiensis*	29	28	27	22	18	29	23
23	*Clostridium perfringens*	0	0	12	0	8	8	19
24	*Enterobacter cloacae* [b]	28	37	38	38	40	29	31
25	*Bacillus cereus*	17	31	26	24	13	0	12

26	*Corynebacterium xerosis*	13	12	11	0	29	28	28
27	*Bacillus cereus* [b]	36	32	39	40	40	29	39
28	*Enterobacter cloacae* [b]	39	32	33	33	35	40	31
29	*Pseudomonas aeruginosa*	0	15	22	13	11	12	22
30	*Staphylococcus aureus*	11	12	16	17	31	12	16
31	*Pseudomonas aeruginosa*	26	24	13	0	17	0	11
32	*Bacillus cereus*	11	11	12	16	17	29	28
33	*Bacillus thuringiensis*	21	14	12	0	27	32	26
34	*Enterococcus faecium* [b]	33	31	30	36	37	32	32
35	*Enterococcus durans* [b]	39	40	40	34	27	39	33
36	*Escherichia coli*	11	40	16	17	23	15	3
37	*Providencia stuartii* [b]	31	29	31	34	40	36	27
38	*Salmonella enterica*	21	0	12	21	25	16	14
39	*Serratia marcescens*	23	25	16	14	13	28	31
40	*Bacillus cereus*	18	27	14	18	34	24	0
41	*Unidentified* [b]	40	28	40	40	32	35	39

Note: 0 denotes no inhibition zone; a denotes average of three measures of the diameter of inhibition zone; b denotes species with higher antimicrobial activity

8.4.5. Phylogenetic relationship

The bacterial strains recovered from VF having higher antimicrobial activity were individually identified as different members based on comparison with those in GenBank database and their phylogenetic positions are illustrated in neighbor-joining tree as shown in Figure 8.5. Molecular identification by partial 16S rRNA gene sequencing showed that majority of bacterial isolates belong to phylum Firmicutes (50% of all strains) and Proteobacteria (rest 50%), with representatives of the class Gamma-proteobacteria. Our results are in agreement with a previous study (Wang et al., 2011a). In a previous study, the microbial community of the three-stage VF microcosm was dominated by uncultured bacterium, followed by *Bacilli* of *Firmicutes*. Less abundant bacteria were observed and classified according to phylogenetic clusters related to *Proteobacteria* and *Flavobacteria* of *Bacteroidetes* (Wang et al., 2011a). Similar findings concerning *Firmicutes* have been reported in other studies. *Bacillus* sp. (belonging to *Firmicutes*) comprised the dominant population of a VF. In contrast, in another study Zhao et al. (2010) analyzed the bacterial communities in the VF for the treatment of domestic wastewater sludge and found that species belonging to *Proteobacteria* were the dominant organisms in the VF biofilm. Vivas et al. (2009) found that *Proteobacteria* was the most abundant phylum in vermicompost made from a toxic olive-mill waste. Our results are consistent with the previous findings. In our study, the GenBank accession numbers for 12 active strains are KM246409- KM246420, respectively as given in Table 8.6. The most active strains belong to the genus *Bacillus* and *Enterobacter*. In recent years, *Bacillus* genus has attracted attention due to its high capacity to produce secondary metabolites, such as antibiotics and enzymes. *Bacillus* species are also often isolated from the gut of microflora of earthworms and comprised the dominant population of VF. These are antagonistic and functional bacteria that are crucial for the treatment efficacy of VF. Members of the genus *Bacillus*, known for production of metabolites with antimicrobial, antifungal or cytotoxic properties, were isolated from invertebrates and display high potential in the search for new antimicrobial substances.

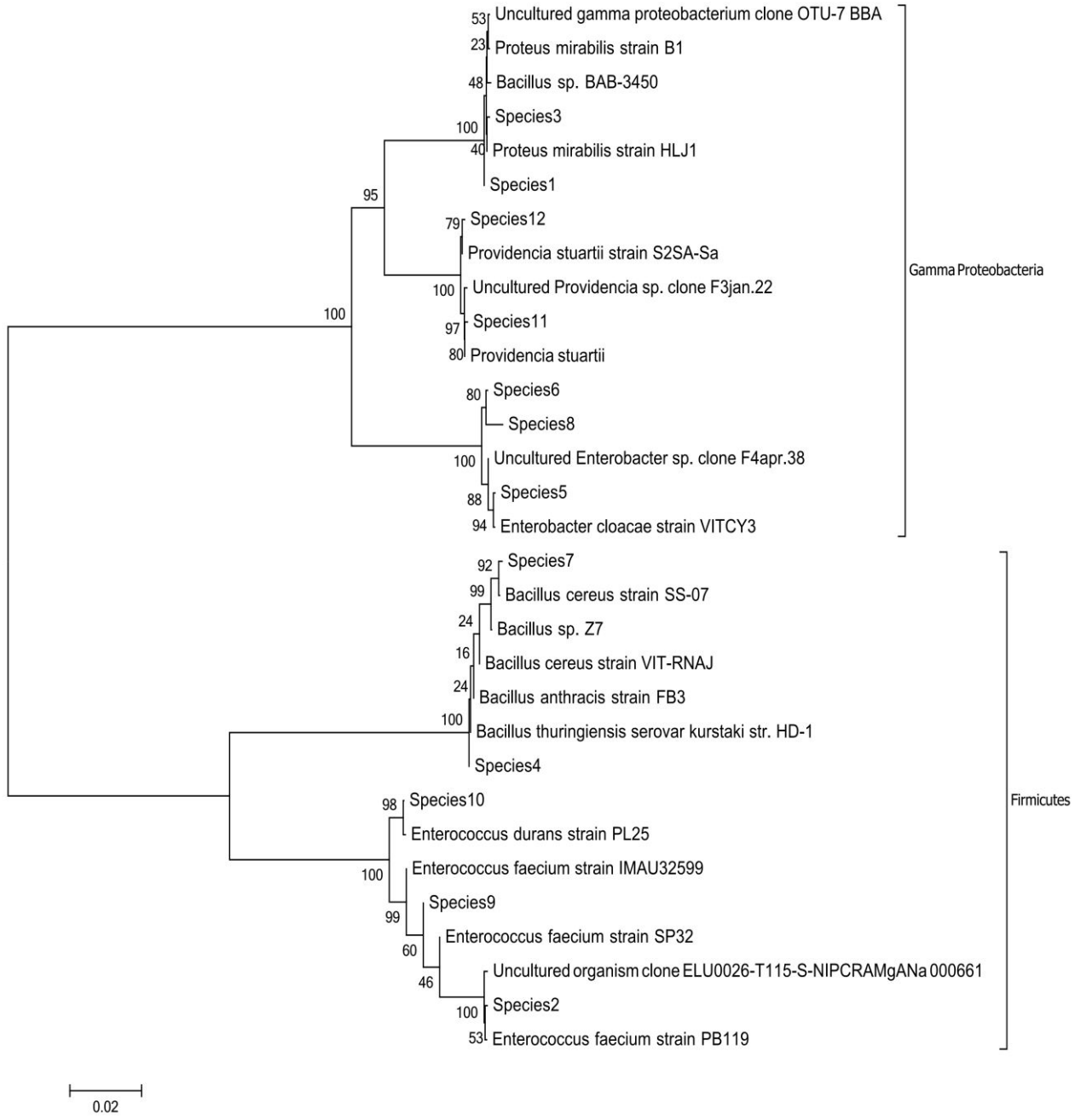

Figure 8.5: Neighbor-joining phylogenetic representation of strains and their closest NCBI (BLAST) relatives based on 16S rRNA gene sequences.

Note: Bootstrap values calculated from 1000 resamplings using neighbor joining are shown at the respective nodes when the calculated values were 50% or greater. The phyla to which the strains belong are presented on the right. The scale bar indicates the number of substitutions per nucleotide position

Table 8.6: *16S rRNA gene sequence affiliation of 12 active strains to their closest phylogenetic neighbors*

S. No.	Species Isolate No.	Gen Bank Accession No.	Closely related Sequences (AN[a])	Hom[b] (%)	Family	Phylum
1	2	KM246409	*Bacillus licheniformis* sp. BAB-3450 (KF917180.1)	99	Bacillaceae	Firmicutes
2	5	KM246410	*Enterococcus faecium* strain (JN792505.1)	100	Enterococcaceae	Firmicutes
3	7	KM246411	*Proteus mirabilis* strain HLJ1 (KF811051.1)	99	Enterobacteriaceae	ϒ Proteobacteria
4	8	KM246412	*Bacillus thuringiensis* strain LA40 (KJ534464.1)	100	Bacillaceae	Firmicutes
5	15	KM246413	*Enterobacter sp.* BSRA2 (FJ868806.1)	99	Enterobacteriaceae	ϒ Proteobacteria
6	24	KM246414	*Enterobacter cloacae* strain BAB-2824 (KF535159.1)	100	Enterobacteriaceae	ϒ Proteobacteria
7	27	KM246415	*Bacillus cereus* SS 07 (EU624445.1)	100	Bacillaceae	Firmicutes
8	28	KM246416	*Enterobacter cloacae* subsp. *dissolvens* strain 189 (KF254602.1)	100	Enterobacteriaceae	ϒ Proteobacteria
9	34	KM246417	*Enterococcus faecium* strain SP32 (JX317638.1)	100	Enterobacteriaceae	Firmicutes
10	35	KM246418	*Enterococcus durans* PL25 (JN792514.1)	99	Enterobacteriaceae	Firmicutes
11	37	KM246419	*Providencia stuartii* strain (HG427202.1)	100	Enterobacteriaceae	ϒ Proteobacteria
12	41	KM246420	Uncultured *Providencia* sp. (JGQ416767.1)	100	Enterobacteriaceae	ϒ Proteobacteria

Note: a: AN denotes Accession Number; b: Hom denotes Sequence Homology

8.4.6. Earthworm growth rate characteristics

The initial number of earthworms inoculated in VF was 800 (weighing 500 g) and changes in earthworm's number and biomass over the experimental period showed that growth of earthworms improved slowly in the VF. At the end of 91st day, the final number of fertile earthworms was increased to 1000 (750 g), showing an increase in number by 25%. In addition, several cocoons and juveniles were observed in VF. This is attributed to continuous availability of organic substrate as food to the earthworms in suitable environmental conditions inside VF. This clearly signifies the use of *E. fetida* for waste treatment

8.4.7. Scanning electron microscopy

Initially, OFMSW had relatively sparse associated microflora, although there were regions where substantial microbial numbers were seen. Eubacteria, fungi and actinomycetes represented the microorganisms, with no group being dominant (Figure 8.6). The surface of OFMSW was undamaged, showing no degradation in the initial stages. Then, as vermifiltration progressed, the microbial numbers increased noticeably. Pockets of bacteria were often found with morphologically different bacterial cells. At this stage, the breakdown of organic material was evident. At the end of 91st day, diverse microflora was seen on the surface. Microbial activity could be visualized as zones of degradation around filamentous and unicellular microorganisms. The vermicompost was dark brown (towards blackish) in color and homogeneous after 91 days of earthworm's activity. This implied that OFMSW gets converted into mature vermicompost, which seems to be completely digested (Figure 8.6).

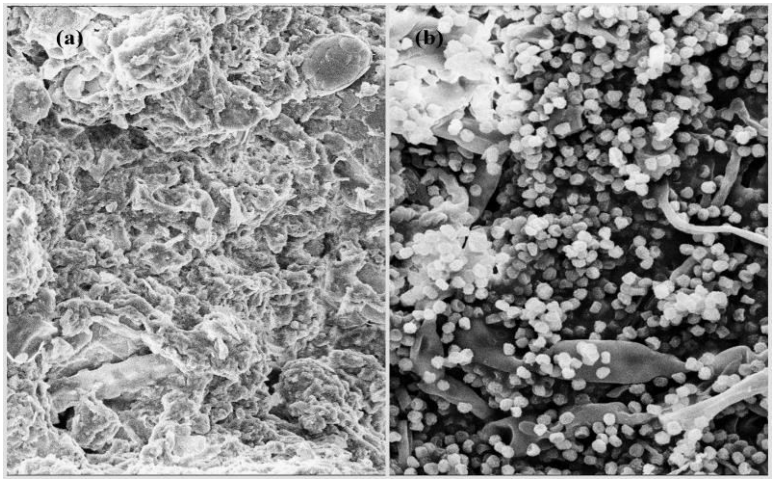

Figure 8.6: SEM images of initial OFMSW sample and vermicompost (91st day)

8.5. Summary

- Vermifiltration proved to be an efficient technology for the combined treatment of wastewater and municipal solid waste.
- The study elucidated the mechanism responsible for pathogen removal during the combined treatment of wastewater and solid waste by vermifiltration.
- The study focused on selection and identification of active bacterial strains from vermifilter that possess antimicrobial activity. These strains release a particular antimicrobial substance that inhibits the growth of pathogens that may be elucidated as the mechanism for pathogen removal.
- The study explored the phylogenetic relationship of active bacterial strains and this microbial consortium is believed to be a future source of antimicrobials adding further insight to the current knowledge of vermifiltration.
- The SEM micrographs clearly demonstrated that the final product i.e., vermicompost was exhibited by predominantly spherical cell-like structure and significantly lower number of filamentous bacteria, which implied the biodegradability of solid waste into mature vermicompost.

This study gave a completely new dimension to the field, for the treatment and management of wastewater and solid waste, using natural, ecological technology- Vermifiltration.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

This chapter deals with the conclusions drawn from the performance evaluation of lab scale and pilot scale studies for the treatment of wastewater and solid waste by vermifiltration. Furthermore, the recommendations for the future work and limitations of the study are also presented here.

9.1. Vermifiltration for Wastewater Treatment

- Vermifilter is able to remove organics (BOD and COD) and solids (dissolved and suspended) from wastewater by 75−85%. This clearly demonstrates higher treatment efficiency of vermifiltration technology for domestic wastewater.
- Vermifilter is able to reduce the pathogens (*Salmonella, E. coli*) and microroganisms (bacteria, fungi, actinomycetes) by 99.9%, thus signifying the disinfection property of the technology. This clearly signifies the applicability of the technology at a field scale, for wastewater treatment.
- Vermifiltration encompasses primary (removal of grit, sediments, SS), secondary (removal of organics) and tertiary (removal of pathogens) treatment into a single unit, in contrast to the conventional wastewater treatment plant where different treatments units are installed to treat wastewater. This excludes the need of high cost-associated separate disinfection unit such as UV or ozone to remove the pathogens.
- The use of locally available, natural filter media such as riverbed material in vermifilters, gives maximum treatment efficiency with high pathogen removal efficacy. This clearly is an advantage for vermifiltration technology from the economics point of view. Overall, the observed trend of VFs in terms of treatment efficiency was observed to be riverbed ≥ mud balls > glass balls > wood coal. This can be attributed to the larger particle size of media, good permeability, higher porosity and smooth surface.
- When a VF with earthworms, is compared to a geofilter (without earthworms), the results clearly signify the better quality of effluent from VF, with no clogging observed throughout the study, while GF tends to clog after fewer days. The burrowing activity of earthworms promoted the aeration conditions in VF which led to the predominance of the aerobic microorganisms, accounting for higher treatment efficiency.
- Earthworms residing in a VF interact with the aerobic microorganisms symbiotically and synergistically and together, they are responsible for the treatment process.

- Enzymatic activity of the microorganisms helps in biodegradation of the organic matter. Microorganism releases certain enzymes such as cellulase, amylase and protease that cause degradation of cellulose, amylase and protein components of organic matter into smaller and simpler units.
- The antimicrobial activity of the earthworm-associated microorganisms is responsible for pathogen removal. These microorganisms have the potential to release certain antimicrobial substance that may resist or prevent the growth of other pathogens. This accounts for the mechanism for removal of pathogens from the wastewater.
- The understanding and knowledge of the microbial community dynamics was developed to elucidate the mechanism for organics and pathogen removal. The work focused on selecting the potential microbial strains that possess higher antibacterial and enzymatic activity that may be further investigated to enhance the treatment efficacy of vermifiltration.
- The kinetic study revealed that the pathogen removal follows first-order kinetics during vermifiltration process.
- Vermifiltration technology proved proficient for treating domestic wastewater during all the seasons, except the days with extreme in temperature. Vermifilter showed the best performance efficiency during spring and autumn period when the temperature range was 25-30°C. This temperature is the optimal range for *E. fetida* growth, reproduction and activity.
- It is a remarkable finding that variation in temperature from the optimum range (25-27°C) led to decrease in BOD and COD removal efficiency and earthworm population, however, the pathogen removal efficacy increases with the increase in temperature, as shown by linear regression analysis.
- Pearson correlation proved that all the parameters like BOD, COD, TC, FC, FS, total bacteria, total fungi were positively correlated with temperature, except TSS removal and fungi population.
- It is a low cost technology, with approximately 80% decrease in operational cost because VF does not need aerating oxygen pumps and heavy maintenance.

9.2. Vermifiltration for Combined Treatment of Solid Waste and Wastewater

- A new aspect of vermifiltration is explored, when earthworms were utilized to treat organic fraction of municipal solid waste and wastewater together, in a pilot scale vermifilter.

- VF is known to reduce organics by 80-90% and pathogens by 99.9%, during the combined treatment of solid waste and wastewater.
- The study focused on selecting the potential bacterial strains from vermifilter that possess antimicrobial activity. These strains release a particular antimicrobial substance that inhibits the growth of pathogens that may be elucidated as the mechanism for pathogen removal.
- The study explored the phylogenetic relationship of active antibiotic-producing bacterial strains that have the ability to enhance disinfection during waste treatment.
- The mechanism of organics, nutrients and pathogen removal has been explored which is illustrated in Figure 9.1.
- Finally it is concluded that vermifiltration is a viable, efficient, and appropriate decentralized waste treatment technology as wastes gets converted into useful products; treated water for non potable use and vermicompost (rich in N, P and K) for agricultural applications.

9.3. Applications of the Technology

Vermifiltration technology, owing to its several advantages, can be successfully implemented to treat domestic wastewater and agro-based industrial wastewater, especially in developing economy. It can be installed in rural villages of India, generating nutrient rich end products, i.e., treated effluent suitable for irrigation and vermicompost as manure for fields. It is a cost effective decentralized technology, as it does not require high operation and maintenance. It is a natural, self-enhanced and self-driven technology, which is free from odor or choking problems.

9.4. Recommendations for Future Work

- Evaluation of vermifilters with actual sewage in field to test the efficacy in real time.
- Performance evaluation of vermifilter for treating different kinds of wastewater such as hospital waste or bio-medical waste.
- Investigation of the effect of inoculation of the isolated microbial consortium into the vermifilter for enhancing the vermifiltration efficacy.

9.5. Limitations of the Technology

- The technology is largely dependent on suitability and functionality of the earthworm species in suitable environmental conditions (temperature, pH, moisture)

- Sludge accumulation can be added problem, if VF is allowed to run continuously, especially in case of combined treatment of solid waste and wastewater.
- Earthworm isolation by hand sorting is a tedious task.

9.6. Limitations of the Study

- The study is limited to a lab scale and pilot scale reactor, and is yet to be tested at the field level.
- Synthetic wastewater was used, as continuous study requires large amount of sewage.
- Only fewer pathogens species were tested for antimicrobial activity.

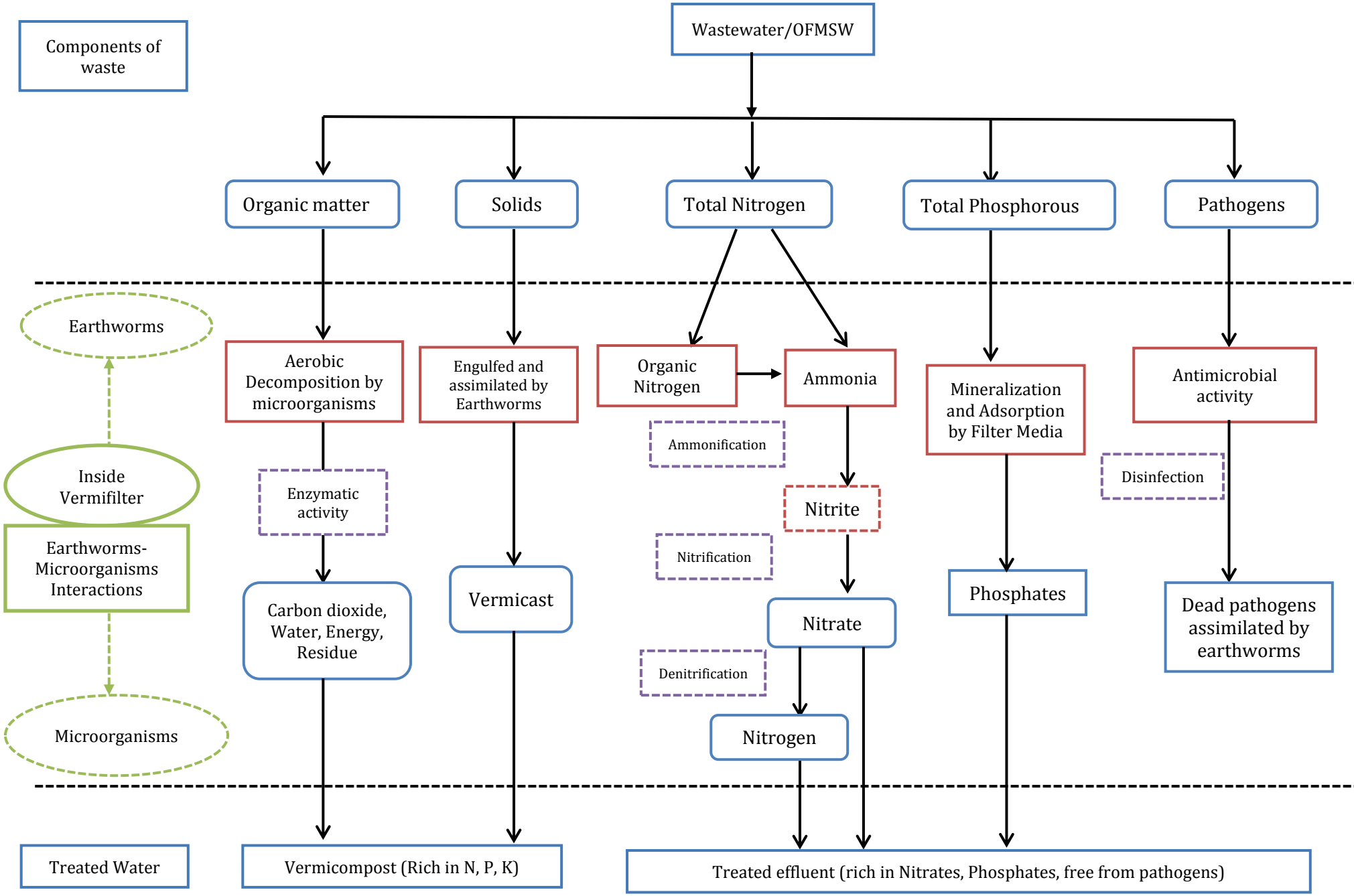

Figure 9.1: Mechanism of the vermifiltration process

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LIST OF PUBLICATIONS FROM PRESENT STUDY

JOURNALS

Published/Accepted

1. Arora, S., Rajpal, A., Bhargava, R., Pruthi, V., Bhatia, A., & Kazmi, A. A. (2014). Antibacterial and enzymatic activity of microbial community during wastewater treatment by pilot scale vermifiltration system. *Bioresource Technology*, *166*, 132–141.

2. Arora, S., Rajpal, A., Kumar, T., Bhargava, R., & Kazmi, A. A. (2014). Pathogen removal during wastewater treatment by vermifiltration. *Environmental Technology*, *35*(19), 2493–2499.

3. Arora, S., Rajpal, A., Kumar, T., Bhargava, R., & Kazmi, A. A. (2014). A comparative study for pathogen removal using different filter media during vermifiltration. *Water Science and Technology*, *70*(6), 996–1003.

4. Arora, S., & Kazmi, A. A. (2015). The effect of seasonal temperature on pathogen removal efficacy of vermifilter for wastewater treatment. *Water Research*, *74*, 88–99.

5. Rajpal, A., Arora, S., Bhatia, A., Kumar, T., Bhargava, R., Chopra, A. K., & Kazmi, A. A. (2014). Co-treatment of organic fraction of municipal solid waste (OFMSW) and sewage by vermireactor. *Ecological Engineering*, *73*, 154–161.

6. Kumar, T., Rajpal, A., Arora, S., Bhargava, R., Hari Prasad, K. S., & Kazmi, A. A. (2015). A comparative study on vermifiltration using epigeic earthworm *Eisenia fetida* and *Eudrilus eugeniae*. *Desalination and Water Treatment*, (ahead-of-print), 1–8.

Submitted

1. Arora, S., Rajpal, A., Pruthi, V., & Kazmi, A.A. Antimicrobial activity of bacterial community for removal of pathogens during vermifiltration. *Journal of Environmental Engineering (ASCE).*

2. Arora, S., & Kazmi, A.A. A Review on Vermifiltration System for Wastewater Treatment. *Water Research (Elsevier).*

3. Arora, S., & Kazmi, A.A. Reactor Performance and Pathogen Removal during the Wastewater Treatment by Vermifiltration. *Journal of Water, Sanitation and Hygiene for Development (IWA).*

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1. Arora, S., Kumar, T., Rajpal, A., & Bhargava, R. (2013, June). A low cost and sustainable alternative for wastewater treatment. *International Conference on Sustainable Innovative Techniques In Civil and Environmental Engineering (SITCEE - 2013),* Jawaharlal Nehru University, New Delhi.

2. Arora, S., Rajpal, A., Bhargava, R & Kumar, T. (2013, July). An Ecofriendly and innovative technology for wastewater treatment. *Innovative Trends in Natural/Applied Sciences and Energy Technology for Sustainable Development (ITNASETSD-2013*), Jawaharlal Nehru University, New Delhi.

3. Arora, S., Rajpal, A., & Bhargava, R. (2013, December). Performance evaluation of a vermifilter for removal of pathogens from synthetic wastewater. *8th Congress on Uttarakhand State Science and Technology congress,* Doon University, Dehradun (Young Scientist Award).

4. Arora, S., Rajpal, A., Bhargava, R., & Kazmi, A.A. (2014, October). Pathogen removal from domestic wastewater in vermifilter. *IWA conference on Global challenges: sustainable wastewater treatment and Resource recovery*, Kathmandu, Nepal (Best Poster Award).

5. Arora, S., & Kazmi, A.A. (2014, November). Microbial diversity in vermifiltration system for the combined wastewater and organic fraction of municipal solid waste (OFMWS) treatment. *11th International Symposium on Southeast Asian Water Environment (SEAWE11)*, Asian Institute of Technology (AIT), Bangkok, Thailand.

6. Arora, S., & Kazmi, A.A. (2014, December). Reactor performance and pathogen removal during the wastewater treatment by vermifiltration. *IWA 1st Specialist Conference on Municipal Water Management and Sanitation in Developing Countries*, Asian Institute of Technology (AIT), Bangkok, Thailand.