ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

PERFORMANCE OF A HORIZONTAL SUBSURFACE FLOW CONSTRUCTED WETLAND FOR THE REMOVAL OF NUTRIENTS, ORGANIC MATTER AND SELECTED PESTICIDES FROM WASTEWATER OF A FLOWER FARM, IN BISHOFTU, ETHIOPIA

BY

MEKONNEN MEAZA

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This to certify that the thesis prepared by Mekonnen Meaza entitled: **Performance of a horizontal subsurface flow constructed wetland to treat waste water discharged from flower farm, in Bishoftu Town, Ethiopia** and submitted to partial fulfillment of the requirements for the Degree Doctor of Philosophy (PhD) in Applied Microbiology, complied with the regulation of the University and meets the accepted standards with respect to the originality and quality.

Signed by the examining committee;

- Internal examiner ________________________Signature______________Date_________
- External examiner________________________Signature______________Date_________
- Supervisor______________________________Signature______________Date_________
- Chairman_______________________________Signature______________Date_________

Dedication

This thesis dedicated to my beloved family.

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Abstract

In Ethiopia, surface water pollution from streaming of wastewater of agro-industries is a major environmental and health concern that requires efficient treatment systems before they are discharged into the environment. One of the low cost, eco-friendly treatment systems is construction of different configuration of artificial wetlands to treat the waste water. The purpose of this study was to optimize treatment performance of horizontal subsurface flow constructed wetland (HSSFCW) to treat waste water laden with residual agro-chemicals including nutrients and pesticides from a flowers farm, near Bishoftu Town, Ethiopia. For this purpose, five pilot-scale (HSSFCW) units were constructed to evaluate the effect of wetland plants, hydraulic retention time, seasonal temperature, supplementing of additional substrate (bio-stimulation) and addition of effective microorganisms (EM) (bio-augmentation) and optimize the performance of the system. The CW units were single planted with Typha latifola (Tp), Cyperus papyrus (Cp) and Pennisetum purpureum (Pp) *and mixed plant with equal proportions of the three plants (Mp), operated under selected days of retention time, with addition of different volumes of molasses and EM. Water, plant tissues and sediment samples were periodically taken for analysis of physico-chemical parameters and microbial profiles following standard methods. The result showed that mean removals for* BOD_5*, COD,* NH_4^+ *and* NO_3^- *were remarkably higher (*$P < 0.05$*) in planted CW units than unplanted control. Among the single planted units,* Typha latifola *(Tp) was the most efficient. CW unit with mixed plantation (Mp) showed the best performance with mean removal of 75.9%, 75.7%, 91.6%, 72.9%, and 60.3% for* BOD_5*, COD,* NH_4^+*,* NO_3^- *and TP respectively. Extending the system operational retention time from 1 to 4 days significantly reduced (*$P < 0.05$*) the levels of* BOD_5*, COD,* NH_4^+*,* NO_3^- *and TP in out flow samples by > 42%, >33%, >48%, >36% and >29% respectively that were below the discharging limits set by*

Environmental Protection Authority (EPA) except for TP. Based on RISA profiles, higher diversity indexes of bacterial communities' were detected from Tp and Mp CW units which was positively linked with their higher treatment performance. Removal efficiency of the CWs for BOD_5, COD, NH_4^+, NO_3^- and TP showed direct dependency with intermittent water temperature where the average removal values obtained from temperature $\geq 20^0C$ were significant higher ($t > 0.177$, $p < 0.05$) compared to below it for planted CW units. Moreover, data of rDNA Illumina sequencing of the sediments bacterial community revealed a higher relative sequence abundance at all taxonomic levels (phylum, class, genus, OTUs etc') in a higher performing planted CW units compared to the unplanted control. Specifically, measures of species richness (OTUs) and Shannon diversity index (H) as well as some of the dominant bacterial taxa from the CW units showed a direct correlation ($r > 0.50$) with removal of BOD_5, COD, NH_4^+, NO_3^- and TP. Alternatively, Typha *planted CW units bio-augmented with EM at 1:1800 dilutions enhanced removal of BOD_5, COD, NH_4^+, NO_3^- and TP than its corresponding control by 7.8%, 5.7%, 8.5%, 16.1% and 15.6% respectively while both operating at 3 day of retention times. A serial increase of EM doses from 1:1800 to 1:600 also accompanied with an increase of sediment bacterial counts and remarkable reduction ($p < 0.05$) of the levels of BOD_5, COD, NO_3^- and TP in out flow samples. This corresponded to 4.4, 19.4, 0.1 and 2.5 mg/L of BOD_5, COD, NH_4^+ and NO_3^- respectively in treated effluent samples which were far below the stringent environmental tolerable standards. Moreover, the additions of sugar cane molasses (MS) and EM to the system improved the removal of 10 μg/L inflow endosulfan used as a trial pesticide by > 15 % and > 19.0% respectively with reference to the average removal efficiency of 77% obtained in untreated. Nevertheless, it was only the EM treated CW units reduced this level of endosulfan close to the permitted discharge limit of 0.1 μg/L. The high removal rate of endosulfan (≥ 77%)*

without any accumulation in plants tissue and low sediment sorption may suggest biodegradation as removal mechanism for endosulfan in the CW units. In general, this study showed HSSFCWs planted with Typha latifola or mixed plantation with equal proportion of Typha latifola, Cyperus papyrus *and* Pennisetum purpureum *effective for reducing the levels of* BOD_5, *COD*, NH_4^+, NO_3^-, *TP and endosulfan emanating from floriculture waste water below the EPA discharging limit, particularly when integrated with EM bio-augmentation. Thus, the system can be recommended for management of wastewater from point and non-point sources of floriculture industries before they are discharged into the environment.*

Key words/key phrases: Bio-augmentation, bio-stimulation, *Cyperus papyrus*, *Typha latifola*, removal efficiency, retention time

Table of contents

List of Tables

List of figures

Abbreviations and Acronyms

ANOVA	Analysis of Variance
APHA	American Public Health Association
BOD5	Five Days Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
Cp	Cyperus planted
CWs	Constructed Wetland system
CU	Constructed Wetland Unit
DO	Dissolved Oxygen
EPA	Environmental Protection Authority
EHPEA	Ethiopian Horticulture Producers and Exporters Association
EU	European Union
FWS	Free Water Surface Wetlands
FWSCWs	Free Water Surface Constructed Wetlands
HLR	Hydraulic Loading Rate
HRT	Hydraulic Retention Time

HSSF	Horizontal Subsurface Flow
HSSFCW	Horizontal Subsurface Flow Constructed Wetlands
Mg/l	Milligram per liter
Mp	Mixed planted
Pp	*Pennisetum* planted
SSF	Subsurface Flow Constructed Wetland
TN	Total Nitrogen
TP	Total Phosphorus
Tp	*Typha* planted
Up	Unplanted
VSSF	Vertical Subsurface Flow
VSSF CW	Vertical Subsurface Flow Constructed Wetland
WW	Waste water

1. Introduction

The commercialization of ornamental crops has become one of the important business sectors for job creation and earning foreign currency in more than 145 countries of the globe (Sudhagar and Phil, 2013). The ever-increasing demand for flowers in the world market has increased the trade at a rate of 10 to 15 % per year. Currently, the major markets for cut flowers are Western Europe, North America and Japan, of which the countries: the Netherlands, Germany, USA, UK, France and Switzerland covers nearly 80% of the global imports. The remaining is mainly contributed by developing countries such as Colombia, Kenya, Ecuador, Costa Rica, India, Zimbabwe, Mexico and Ethiopia.

Ethiopia has attracted several foreign investors for producing and exporting cut flowers mainly to the European markets in recent decades (Tadele Yeshiwas and Melkamu Alemayehu, 2018). The country was benefited from the industry because of low cost of production compared to other East African countries. It is one of the few emerging industries employing more than 85,000 employees and generating more than 200 million US dollars in foreign exchange for the country's economy (Mesay Adugna, 2017).

However, the sector requires nearly 60 m^3 water per day per hectare and generates a significant amount of agrochemical wasted water during the production processes. A single study conducted on flower farms established in Bishoftu indicated that 70% of the flower farms drain their wastewater to the nearby open environment without treatment (Abayneh Tilahun, 2013). Other studies also showed the potential negative impact of Ethiopian floriculture on surface waters and human health due to discharging of risky residual agro-chemicals contaminated wastewater (Malefia Tadele, 2009; Berhan Teklu *et al*., 2016a).

These studies also showed the presence of high levels (beyond maximum permissible limits) of residual agro-chemicals; nutrients and trace pesticides (spiroxamine, heptachlor, deltamethrin and endosulfan) (Jansen and Harmsen, 2011; Berhan Teklu *et al*., 2016a).in the receiving water bodies. Some of the residual pesticides are toxic and persistent in the environment that cause health hazards due to bio-accumulation and bio-magnification through the food chain. Furthermore, the levels of residual chemicals nutrients (nitrogen and phosphorus) are also detected to a level that affect receiving water quality and lead to eutrophication (Malefia Tadele, 2009; Berhan Teklu *et al*., 2016a).Therefore, this strongly necessitates the removal of these pollutants from floriculture wastewater through the using of appropriate treatment technology before discharging it into the environment.

There are various pond systems that are used for the treatment of wastewater from industries. Oxidation ponds are not that effective to treat strong wastewater from floriculture to fulfill stringent emission standards for pollutants (Lv, 2016). The employment of better activated sludge systems is not affordable for they are energy and capital intensive (Rai *et al*., 2013). These days, ecological technologies are introduced as cheap alternatives for wastewater treatment compared to the expensive ones (Wu *et al*., 2015).

Constructed wetland systems (CWs) are one of the ecological technologies used as cost effective and eco-friendly option for wastewater treatment. They are artificial wetlands planted with different types of water plants known as macrophytes that are built to emulate the functions of natural wetlands. The use of CW is aimed to take advantage of many of the processes taking place in natural wetland (Sultana *et al*., 2015). The major share of pollutant removal is contributed by biological (plant and microbial) components in the CWs (Sultana *et al*., 2015).

The CW's are attractive treatment option because they are simple to construct, operate and have low maintenance cost compared to conventional treatment methods.

There are different types of CW configurations classified on the basis of their hydrologic and water flow regimes. Broadly, they fall into two categories as free water surface CWs (FWSCW) and subsurface flow CWs (SSFCWs). The SSFCWs technology is further classified as horizontal subsurface flow CWs (HSSFCWs) and Vertical flow CWs (VSSFCWs) using water flow direction as a point of reference (Nikoli *et al*., 2010).

Although FWSCWs are simpler to design and operate, they are less effective in contaminant removal of high level of organic matter compared to SSFCWs (Wu *et al*., 2015). Moreover, VSSCWs apparently shows operational difficulty and Poor denitrification (Gorgoglione and Torretta, 2018). On the other hand, a HSSFCWs which is simple to build and easy to operate are proposed to apply for treatment of wastewater, particularly in areas of the large-scale rural farming of high malaria prevalence (Sultana *et al*., 2015).

In general, the HSSFCWs are reviewed with wide range of removal efficiencies of 28% to 99%, 20% to 70% and 30% to 60% for organic matter, total nitrogen and total phosphorus, respectively (Vymazal and Kröpfelová, 2009; Vymazal, 2017; Mesquita *et al*., 2018). These inconsistencies in removal efficiency mainly depend upon the type of plantation, types of substrate; hydrological settings and meteorological conditions (Mesquita *et al*., 2018). This implies, more studies are necessary to shed light on the feasibility of this technology in relation to those factors for treatment of wastewaters of different sources in the local context.

Since CWs are integrated with plants, they have higher removal efficiency of pollutants than those without plants (Vymazal, 2011). The CW's have also different performance efficiencies depending upon the type of plant species and different studies showed that mixed planting is more effective for pollutant removal than single species with few exceptions (Zhu *et al*., 2017). However, it is difficult to keep mixed vegetation in a long term run. Thus, the selection of plant species is one of the factors that ensure efficient removal of pollutants in CW's.

Moreover, pollutant removal performance of CWs is highly dependent on hydraulic conditions (Kotti *et al*., 2010). Optimal hydraulic retention time differs between CWs with different wetland planting (Gorgoglione and Torretta, 2018). It is speculated to be difference of removal efficacy between individual plants plus their specific interactions with wetland microbes in treatment zone of the CWs (Lv, 2016). However, there is no sufficient data revealing interactions of wetland plants, sediment microbial communities and retention time for HSSFCWs in relation to removal of organic matters, nutrients and pesticieds, particularly in remediation of wasted water of floriculture industry.

Apart from their advantage in low energy consumption and economic costs, the requirement of vast area for construction is a limiting factor in application of CWs for waste water treatment (Wu *et al*., 2015; Gorgoglione and Torretta, 2018). Linking the treatment systems with appropriate pollutant removal enhancing technology such as Bio-stimulation and bio-augmentation could minimize these limitations through shortening the required hydraulic retention time (Zhao *et al*. 2014; Meng *et al*. 2014; Huang *et al*., 2015). Unlike for soil bioremediation, there is a wide gap of information on the potential and sustainable use of these technologies for removal of pollutants from wastewater in CWs.

Bio-stimulation via nutrient supply leads to stimulating indigenous microorganisms (Garcia-Blanco *et al*., 2007) whereas bio-augmentation adds effective catabolic microbes into an indigenous microbial community (Nzila *et al*., 2016). Accordingly, sugar cane molasses primarily provide a carbon and energy source requiring for microbial growth as well as trace elements for production of enzymes involved in mineralization of pollutants. The uses of mixed effective microorganisms (EM) as bioaugments also proposed for better reduction of manifold contaminants from wastewaters (Monica *et al*., 2011). However, there are limited studies showing the feasibility of these treatment enhancing technologies for bioremediation of biocidal agro-chemicals contaminated water in HSSFCWs.

In general, wastewater management has become an important concern in floriculture industry in Ethiopia (Mesay Adugna, 2017). The industry releases significant amount of agrochemical (fertilizers and pesticides) containing wastewater to the nearby environment. The flower farms legally require feasible treatment technology to fulfill the effluent discharging limits set by regulatory agencies such as Environmental Protection Authority (EPA) (EPA, 2003). Thus, installing and optimizing sustainable treatment technology for reuse and/or safe discharge of agro-chemicals wasted water is critical to sprint an eco-friendly development of floriculture industry.

1.1. Objective of the study

1.1.1. General objective

The aim of this study was to demonstrate treatment performance of floriculture wastewater using HSSFCWs with selected plantations, hydrology and emerging technologies in a Flower Farm, nearby Bishoftu Town, Ethiopia.

1.1.2. Specific objectives

1. To examine effect of plant types on the removal of organic matter and nutrients from wastewater in HSSFCWs
2. To assess effect of hydraulic retention time the removal of organic matter and nutrients from wastewater in the CWs
3. To examine effect of seasonal temperature on the removal of organic matter and nutrients from wastewater in the CWs
4. To investigate the wetland bacterial community composition in relation to the conditions of different planting and removal performance in the CWs
5. To examine effects of EMs bio-augmentation on organic matter and nutrients removal performance of the CWs
6. To examine effects of bio-stimulation with molasses plus bio-augmentation with EMs on the removal of one of the frequently used pesticides, endosulfan in the CWs.

2. Literature Review

2.1. Overview of the Floriculture Industry

Flowers have remained an integral part of social fabric of human life. People traditionally use flowers for expressing or exhibiting their innermost feelings to God and deities, presenting to the beloved ones, praising any one or versifying any conceivable emotion. They are known to be sold throughout the year but with peak periods of Valentine's Day, Halloween, mother's day and New Year (Toumi *et al*., 2016). Flowers are also cultivated for aesthetic purposes as for their fragrance, perfumes and medicines.

Floriculture (production of flower) is a branch of horticulture concerned with the cultivation of ornamental plants with a focus on flowering plants. In general, floriculture crops are herbaceous which include bedding plants, flowering plants, foliage plants, cut cultivated greens and cut flowers. In particular, cut flowers appeared to be the most important commercial items of the floriculture industry presently (Toumi *et al*., 2016). In recent times, new techniques and technologies are introduced from production to consumption and considerably contributing for development of the floriculture sector.

Accordingly, the floriculture industry has been expanding substantially throughout the world and more than 305,105 hectare area of land was under flower production (Sudhagar and Phil, 2013). Considering the continental share; 44,444, 22,388, 215,386, 2,282 and 17,605 hectare area of land belong to Europe, North America, Asia and Pacific, Middle East and Africa and central and South Africa respectively. Among these, a total area of 46,008 hectare estimated to be used for flowers production under protected greenhouses conditions. Specifically, India has the maximum area under ornamental crops (88,600 hectare) which was followed by China (59,527 hectare).

According to Sudhagar and Phil (2013) more than 145 countries in the world cultivate ornamental crops where greater than 34% are involved in large scale production. Quite recently, many developing countries including sub-saran African countries (Cote d'Ivoire, Ethiopia, Kenya, South Africa and Uganda) are entering the market and thereby increasing the land allocated to and the market share of the industry (Mulu Gebreeyesus and Iizuka, 2010). In line with this, the total trade share of the developing countries in cut flowers has progressively and consistently showed increasing.

2.2.Ethiopian Floriculture

The history of the Ethiopian floriculture industry dates back to 1980, 39 years ago, when governmental farms started to export flowers to Europe (Daniel Hailemichael, 2013). Meskel Flowers Plc. was the first private company to engage in export oriented commercial flower farming in Ethiopia. A second private farm, Ethio-Flora, was established (in Zeway, 98kms south of Addis Ababa) soon after. Both farms were Ethiopian owned and produce summer flowers (field produced) such as *alliums, statice* and *carnations* for export to EU markets.

Within fewer than three decades, the country emerged as a global player in the cut flowers business. According to the Ethiopian Horticultural Producers and Exporters Association (EHPEA, 2015) report, there are 84 growers of which: 26 farms are locally owned, 52 are foreign owned and 6 are owned by joint venture.

Currently, the total area of land held by floriculture investors is more than 3,000 hectares in Ethiopia. The industry produces several flower species, including roses, gypsophila, Hypericum, limonium, carnations and chrysanthemum (Mesay Adugna, 2017). Importantly, the climate offers ideal conditions for cultivation of roses. As result, roses accounted about 80% of the total flower production in Ethiopia and ranked the country as the second largest exporter of roses in Africa next to Kenya and the fourth largest to the world market (Tadele Yeshiwas and Melkamu Alemayehu, 2018).

According to Mesay Adugna (2017), the country earned more than 200 million USD from floriculture that makes this sector the 4^{th} foreign currency generator of the country next to coffee, oilseeds and cereals. Moreover, the sector also provided more than 180,000 jobs opportunities of which nearly 85% is occupied by females (Tadele Yeshiwas and Melkamu Alemayehu, 2018).

2.2. 1. Prospects for growth of Ethiopian Floriculture

2.2.1.1. Climate and Topographic

Ethiopia has suitable agro-climatic conditions for the cultivation of different flowers (Rakesh Belwal and Meseret Chala, 2008; Daniel Hailemichael, 2013). The country has agricultural plan land with an altitudes between 1500 and 2500 meters around its capital city, Addis Ababa. It is with fertile soils and good water supply which are favorable for growth of various flowers. In particular, these altitude ranges characteristically known to allow the rose buds to grow larger, which is good opportunity to fetch premium prices for the growers. Moreover, many of the flowers can be produced all year round and thus, they offer opportunities to export at times when prices of importing countries are high.

2.2.1.2. Civilized population and labor force

Ethiopia is one of the top three countries in Africa with the greatest population size. The floricultural industry is labor intensive and this offers a comparative advantage to Ethiopia which has a population of more than 90 million. This provides a viable source of adequate civilized manpower for unceasing production and a potential of domestic market of flowers in the near future (Rakesh Belwal and Meseret Chala, 2008).

Ethiopia offers a relatively peaceful civic life than some African countries where investors gain lots of support. Ethiopia also has an abundant, hardworking, inexpensive, disciplined and easily trainable workforce (Daniel Hailemichael, 2013). Moreover, the cost of labor in Ethiopia is lower compared to some Asian nations and African countries such as Tunisia, Kenya that offers a comparative advantage for growers to prefer to and invest more in Ethiopia.

2.2.1.3. Governmental support

Ethiopia government also provided long-term credit through the Development Bank of Ethiopia. Investors can borrow up to 70:30 debt-equity ratios with no collateral requirement (Rakesh Belwal and Meseret Chala, 2008; Daniel Hailemichael, 2013). Interest rates are low and do not vary much. Compared to other major horticultural exporter countries in Africa, the government support in Ethiopia is clearly very attractive. For example, the fixed interest rate (around 7.5%) is very low compared to many other African countries having interest rates of around 15%.

2.2.1.4. Shift of production sites from other country

The supplementary factor contributed for development Ethiopian floriculture sector is the shift of production sites from Kenya caused by the water pollution of Naivasha Lake in Kenya (Daniel

Hailemichael, 2013). Thus, the growers in Kenya had to bear additional costs to avoid further environmental deterioration, resulting in a decline in the competitiveness of the Kenyan cut flower industry in the world markets. On the other hand, Ethiopia adopted a code of conduct for the sector in 2007 before its environmental effect becomes a serious issue.

Furthermore, the exemption of EU tariffs on flower exports from Kenya expired in January 2008 is the second reason for shift of production sites (Daniel Hailemichael, 2013), whereas Ethiopia is still exempt from the tariffs. Moreover, Kenya repeatedly experienced political violence by 2007 and in many areas including Naivasha, where many flower farms were operating (Mano *et al.*, 2011). This violence has also caused some growers in Kenya to relocate their production sites to Ethiopia.

2.2.1.5. Associations related to the sector

The Ethiopia Horticulture Producers and Exporters Association (EHPEA) established by more than 86 members of which 81 are flower producers/exporters. Then, this association has prepared the Ethiopian Horticulture Development and Marketing Strategy with support from the Dutch government and a Dutch consulate. The Strategic plan gave emphasizes for the diversification of the market and the products, i.e. not only flowers but also vegetables and fruits should be exploited as export goods.

The association has been also involved in developing informal networks with donors and organizing various forums such as international trade fairs. As a result, it has created strong connections with the donor community and secured wide support for the sector. The donors include the UK DFID, the French Development Cooperation, USAID, the Dutch government and

Different Dutch institutions such as the Dutch Center for the Promotion of Imports from Developing Countries (CBI) and Wagningen University are helping with capacity building (Daniel Hailemichael, 2013).

2.2.1.6. Strong linkage with other sectors

As the floriculture industry expands, linkage with sectors of specialists becomes more critical. Ethiopia government with the support of the Dutch government has started to consolidate higher education in horticulture. Accordingly, the Jimma University has begun to offer diplomas (BSc and MSc) in floriculture. Efforts are also underway to establish a Horticulture Practical Training Center (HPTC) within the Ethiopian-Netherlands Partnership on Horticulture.

With the expansion of the sector, complementary activities, such as propagation of planting materials, packaging, fertilizers and chemicals supplies started to emerge (Daniel Hailemichael, 2013). Beginning of companies that producing pot plants and cut flower and propagate new varieties offered good opportunity for the domestic market. This is because; the source of plant materials is slowing shifting from imports to local supply. There is also an increasing trend for imported fertilizers and chemicals to be substituted by local production though not fully realized.

2.2.1.7. Access to market

The easy way is to sell at Dutch auctions, but higher prices can be achieved through direct sales to final customers. For all Africa countries including Ethiopia, the major flower export destination is the Europe market (Rakesh Belwal and Meseret Chala, 2008; Daniel Hailemichael, 2013). Cut flowers are sold via the auction markets (mainly Dutch auctions) and/or directly to supermarkets and other retailers. Relative ease of access to the auction market means new entrants tend to begin by using this channel.

Over time, the Ethiopian floriculture has become more diversified in terms of market channels and destinations. The number of destinations has increased from 2-3 countries (all in Europe) in the early 2000s, to some 56 worldwide in 2008. There are 14 destination countries with USD 100,000 and over export value. The Europe is still the major destination accounting for around 94.5% of total export value with the Netherlands (88%) in the lead (Daniel Hailemichael, 2013).

2.2.2. Challenges of Ethiopia floriculture

Although rapid growth and development, the floriculture sector in Ethiopia is also suffer from some constraints. Particularly, it has been challenged due to lack of infrastructure, social and environmental related problems.

2. 2.2.1. Challenges in infrastructure

In general, the export oriented floriculture demands highly developed infrastructure. Although improvement has been made in the last decade, the international and domestic transportation system of Ethiopia is still not sufficient to support further development of sector in Ethiopia (Rakesh Belwal and Meseret Chala, 2008; Tadele Yeshiwas and Melkamu Alemayehu, 2018). The export of flowers to the international market is done soly from Bole International Airport in Addis Ababa. Flower farms located relatively far way for Addis Ababa. The flower growers should drive long distance using cold trucks to access the airport which will increase the transport cost, reduce the vase life and increase postharvest losses of cut flowers.

Moreover, some of the roads are not suitable and overcrowded for the transport of flowers intended for world export market using cold trucks (Tadele Yeshiwas and Melkamu Alemayehu, 2018). The refrigeration system at airport is also not sufficient enough and flowers cannot be stayed long at the airport. Thus, each flower farm has to therefore adjust the time of harvesting to the time of departure of the flights as well as the amount to be exported to the available space.

2.2.2.2. Breeders' right

Only flower breeders have the right to grow new varieties and the right is protected by an international law known as "Breeders Rights". As a result, a patent royalty should be paid when others grow the variety. Ethiopia has also launched a proclamation on Breeders Right, because, violation of "Breeders Rights" would lead to a situation out of the international market (Daniel Hailemichael, 2013). Accordingly, all of the rose and cut flower breeds come from abroad, mainly from Israel, Kenya and Netherlands. As the cut flower industry develops, the payments increases and could represent a considerable market risk for the industry.

2.2.2.3. Adoption of technology

In Ethiopia, the flower growers have not yet been able to adopt international best practice (Daniel Hailemichael, 2013). Integrating the Ethiopian floriculture production system with knowhow of the technology helps to cope up and continues with its main competitors such as Kenya and Ecuador. Thus, more investment allocating yet requested for research and technology extension services in Ethiopian floriculture sector.

2.2.2.4. Social issues

Ethiopian Floriculture frequently confronted by local communities with issues of shortage of agricultural land for its further expansion (Daniel Hailemichael, 2013). For instance, the local communities in Holeta area are found claiming as most agricultural lands and eucalyptus plantations are changed to floriculture farms. Similarly, International Labor Organization (ILO) (2006) reported the conversion of forest resources to floricultural farms as one of the side effects of floriculture expansion in the country. Moreover, depletion of water resource led to conflict of interest between the local communities and the flower farms in the country (Tadele Yeshiwas and Melkamu Alemayehu, 2018).

2.2.2.5. Management of agro-chemicals

Lack of safe management of the agro-chemicals is one of the major concerns in floriculture industry which could shadow the market image of the industry (Tadele Yeshiwas and Melkamu Alemayehu, 2018). Some devotions observed to improve the management through providing information about environmental and health effect of agro-chemicals (Malefia Tadele, 2009; Berhan Teklu *et al*., 2016a; Mesay Adugna, 2017). However, this is not enough to reduce the negative effect of the input residual agro-chemicals. There is still a gap to establish an information-exchanging system for managing the effect of agro-chemicals among research institutions, government and each flower grower.

2.3. Inputs in Floriculture Industry

The floriculture investment requests the use of higher amount of water, excess chemical fertilizers and crop protection agents compared to the other agricultural activities such as open field vegetable cultivation. A study in Ethiopia showed that the entire processes of the industry estimated to consume about 60 m^3 water per day per hectare for cultivation of roses (Mesay Adugna, 2017) and the requirement of nearly 2 liters of water per bunch of roses in the post-harvest activities (preservation and preparation) (Sahle Amare and José, 2013).

Similar to other courtiers, Ethiopian floriculture also apply high input of agro-chemicals in the form of fertilizers and crop protection agents (Mesay Adugna, 2017). The use of fertilizer is high due to the high crop demand and year-round production of cut-flowers. In Ethiopia, the amount of nitrogen and phosphorus used per hectare per cultivation is not as much as the Netherlands standards (1190 kg nitrogen per hectare and 280 kg phosphorus per hectare for rose's cultivate) (Sahle Amare and José, 2013).

According to Crop Protection Department Quarantine Office in Ethiopia (CPDQOE), the floriculture industries use more than 300 kinds of chemicals as pesticides (insecticides, fungicides and nematocides) and growth regulators (Mesay Adugna, 2017). Under the circumstances, nearly 200 kg of pesticides per hectare per year are estimated to be sprayed on flower farms. Likewise, Sahle Amare and José (2013) also reported an estimat of 1.5 gram of pesticides is applied to produce a bunch of roses. In general, the floriculture industry is apparently applying higher levels of agro-chemicals (fertilizers, pesticides) to boost quality and quantity production though potential effect of residual agro-chemicals and other wastes as output (Fig. 2.1) pose great concerns to the environment and human health.

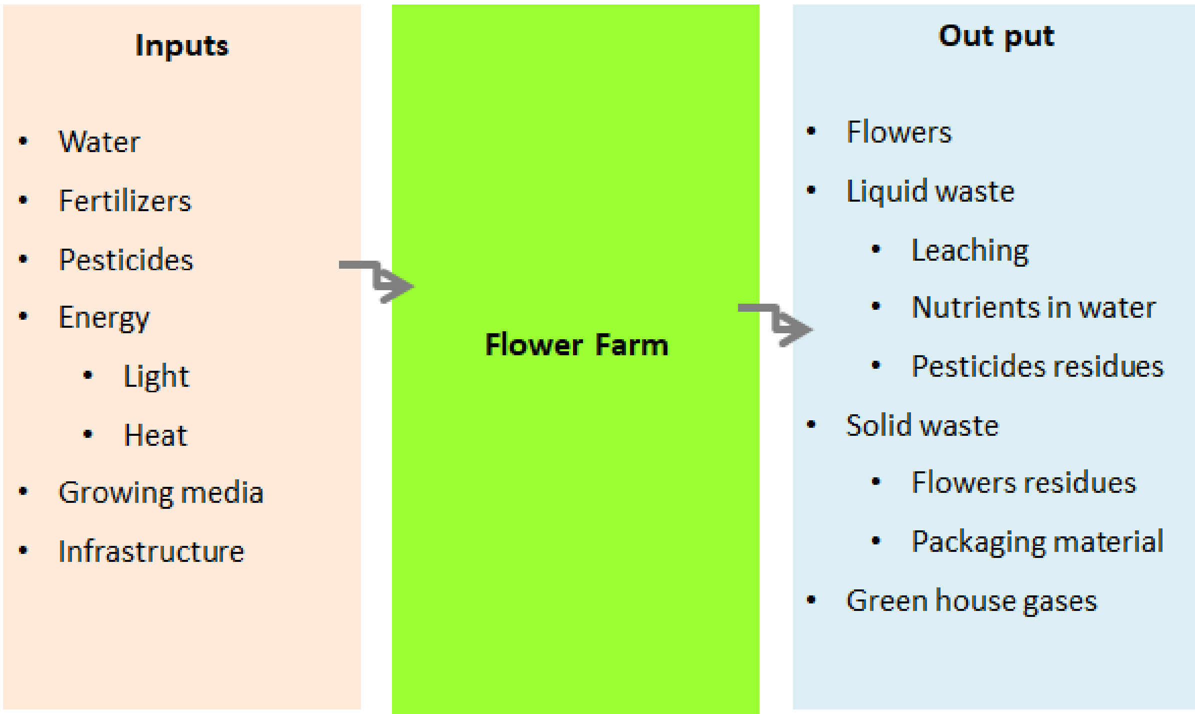


Fig. 2.1 Schematic representation of inputs and outputs in floriculture industries

Despite the significant contribution of the floriculture industry to the national economy, many issues are raised by environmentalists' mainly related to the depletion of ground water resources and pollution of surface water bodies. Different studies indicated the declining of surface water table as a consequence of intensive water use (nearly 60 m^3 per hectare per day) for flower cultivation in various parts of the globe including the savanna surrounding, Bogota (David, 2002) and Lake Naivasha of Kenya (Kinyanjui, 2009) and Lake Ziway of Ethiopia (Malefia Tadele, 2009).

Moreover, most of the flower growers are blamed for channeling their agro-chemicals contaminated water into surface water such as streams, rivers and lakes (Abayneh Tilahun, 2013). The levels of nutrients in discharging wastewater to Lake Ziway from nearby flower farm found to surpass the permissible limits of WHO, FAO and US-EPA for use as raw public water supply, irrigation, maintenance of fisheries and aquatic life respectively (Malefia Tadele, 2009). The levels of residual nutrients (nitrogen and phosphorus) are also found to strongly affect the water quality of Lake Ziway and lead to process of eutrophication. Similarly, a study conducted on the Wedecha River System which is largely influenced by discharging of the flower farms nearby Bishoftu town, Ethiopia showed high levels of organochloro pesticides such as cyhalothrin, endosulfan, profenofos and diazinon from samples of water that potentially pose health risks on both animals and humans (Berhan Teklu *et al*., 2016a).

As a matter of fact, the effect of pollution is expected to increase with the expansion of the floriculture industry, necessitates sustainable treatment facilities, particularly in developing countries which are not implemented it. Thus, flower growers particularly, in Ethiopia are looking for feasible treatment technology to assure sustainable production. Thus, installing and optimizing sustainable treatment technology for reuse or safe discharge of agrochemical-contaminated wastewater is very critical for an eco-friendly development of floriculture industry.

2. 4. Management of wastewater in Floricultures Industry

2.4.1. **Constructed wetlands for treatment of wastewater**

Constructed wetlands (CWs) are artificial wetlands that are built to emulate the functions of natural wetlands. It is aimed to take advantage of many of the processes taking place in natural wetland (Sultana *et al*., 2015). They are considered to be as one of the attractive treatment options because they are simple to construct and operate, have low maintenance cost and are sustainable compared to conventional treatment methods.

CWs have been used for treatment of various types of wastewater throughout the world since the 1950s (Sultana *et al*., 2015). They have been be tried for remediation of conventional (organic matter, nutrients, pathogens, etc.), non-conventional (heavy metals and hydrocarbons) and emerging (biocides, pharmaceuticals, steroid hormones, etc.) pollutants in a variety of wastewaters such as domestic sewage, agricultural wastewater and industrial effluents (Verlicchi and Zambello, 2014).

2.4.1.1. Types of constructed wetlands

There are different types of CWs that are described based on their hydrologic and water flow regimes. Generally, they fall into two categories as free water surface CWs (FWSCW) and subsurface flow CWs (SSFCWs). The SSFCWs technology is further classified as horizontal subsurface flow CWs (HSSFCWs) and Vertical subsurface flow CWs (VSSFCWs) based on water flow direction. Recently, different CW types have been paired into combined hybrid treatment systems as shown on Fig. 2.2.

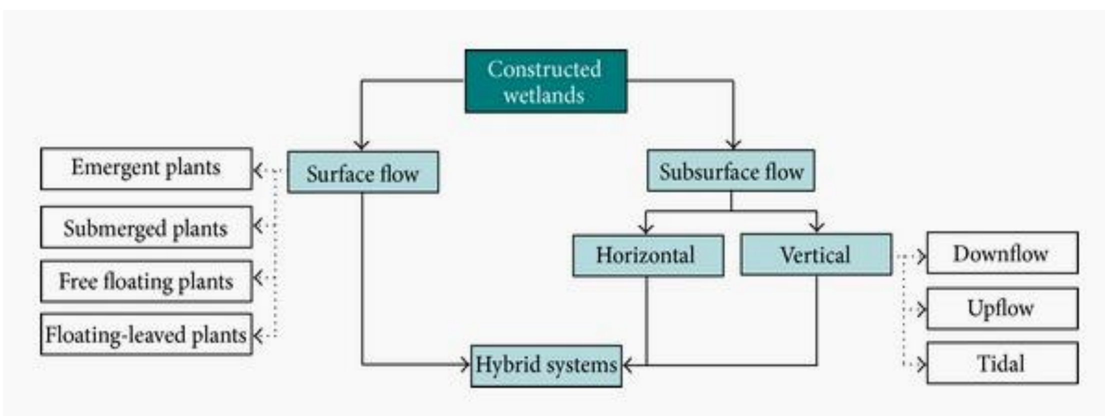


Fig. 2.2 Schematic representation the types of CWs (Adapted from Nikoli *et al*., 2010

2.4.1.1.1. Free water surface constructed wetland

A Free water surface CWs (FWSCW) consists of a shallow sealed basin, soil or other medium to support the roots of macrophytes and water control structure that maintains a shallow depth of water (5-40 cm) (Choudhary *et al*., 2011). In this system, the wastewater surface is usually above the surface of medium (Fig. 2.3). The system is aerobic with the exception of the bottom; the layer of the decomposing plant litter may provide some anoxic and anaerobic pocketets suitable, for example, processes such as denitrification. They are used for treating various kinds of waste waters including; domestic, agricultural and storm wastewaters.

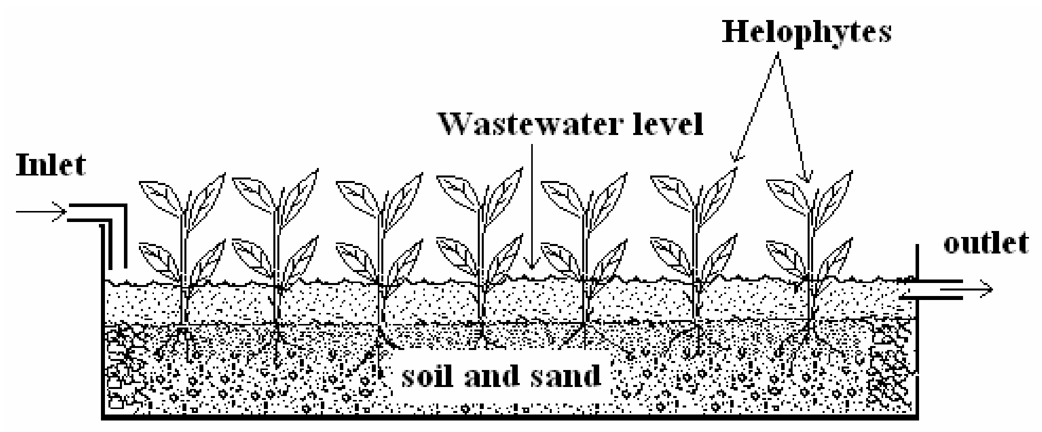


Fig. 2.3 Schematic a free water surface CWs (Adapted from Choudhary *et al*., 2011)

2.4.1.1.2. Subsurface Flow Constructed Wetlands

A subsurface flow CWs (SSFCWs) is consisted of a sealed basin with a porous medium. The bed is always sealed by an impermeable substance to avoid down leakage of the wastewater. The medium is typically composed of crushed gravel of 10–15mm diameter. In SSFCWs, the treatment is accomplished below the surface as shown in Fig. 2.4 and 2.5. According to the flow direction of water, SSFCWs are further sub-classified into Vertical and Horizontal SSFCWs.

A vertical SSF CWs (VSSFCWs) was originally introduced to oxygenate anaerobic septic tank effluents. Depending the direction of water flow (Down or Upward), the inflow water percolates down or upwards through the medium. The new batch is fed only after all the water is percolated and the bed is free of water (Fig. 2.4). This enables diffusion of oxygen from the air into the bed. Unlink upward VSSFCWs, VSSFCWs with down flow system is far more aerobic than horizontal SSFCWs (HSSFCWs) and provide suitable conditions for aerobic processes. However, VSSFCWs were not spread as quickly as HSSFCWs. This is because of the higher operation and maintenance requirement that needs to pump the wastewater intermittently on the treatment zone of the wetland.

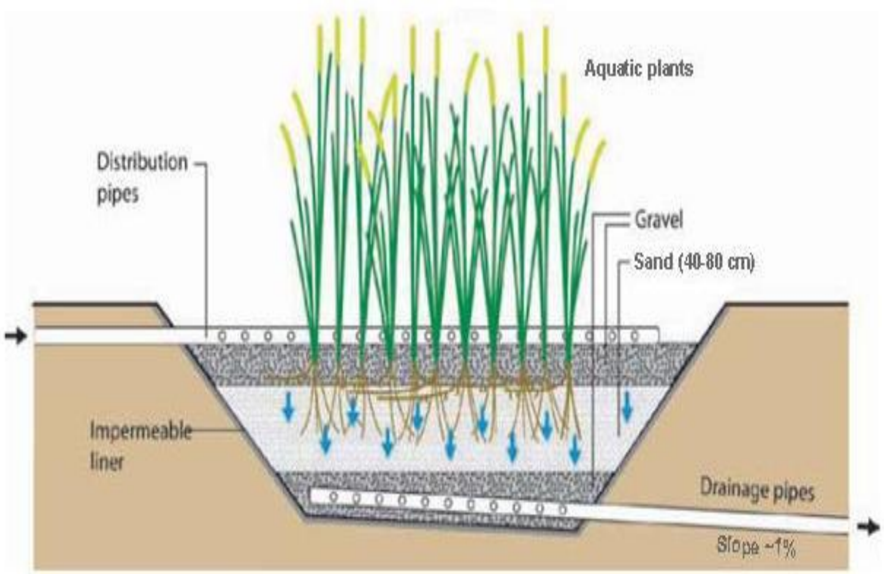


Fig. 2.4 Schematic representations of a typical down ward VSSFCWs (Adapteded from Vymazal, 2001).

HSSFCWs consist of media usually gravel or soil sealed by impermeable layer and planted with wetland vegetation (Fig. 2.5). Wastewater is fed at inlet and flows through treatment zone under the surface of media in a more or less horizontal path until it reaches outlet zone. Soil media was extensively used during the initial development of the HSSFCWs which was marketed as root zone treatment (Vasudevan *et al*. 2011). Soil was subsequently replaced by washed gravel, grain size ranges from 5 – 20mm due to problems of clogging, Any pollution-tolerant deep rooted emergent macrophyte can be potentially used, though the wide spread use of common reed (*Phragmites australis*) has led to HSSFCWs being called “Reed beds”.

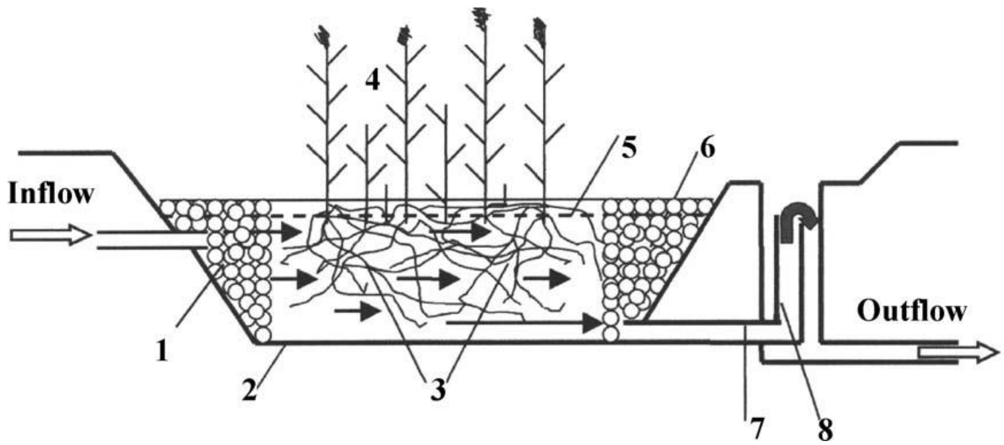


Fig. 2.5, Schematic representation of a typical HSSFCW: 1, distribution zone filled with large stones; 2, impermeable liner; 3, filtration medium (gravel, crushed rock, soil); 4, vegetation; 5, water level in the bed; 6, collection zone filled with large stones; 7, collection drainage pipe; 8, outlet structure for maintaining of water level in the bed. The arrows indicate only a general flow pattern (Adapted from Vymazal, 2001).

FWSCWs are simpler to design, operate and less expensive to construct. However, it usually achieves slower rates of pollutants removal compared to SSFCWs (Vymazal *et al*., 2007). On average, the removal efficiencies were low and ranged from 40 to 60% when low hydraulic retention times (4 to 15 days) were applied. It requires more land and extended retention time (>15 days) to achieve a nearly required level of treatment (Wu *et al*., 2015). According to Dunne *et al*. (2005) high BOD waste water are difficult to treat through the use of FWSCWs. Moreover, odor, insect’s problem and scarcity of land for construction make FWSCWs Less viable for treatment of wastewaters than SSFCWs.

Because of operational difficulty and low pollutants removal efficiency of VSSCWs, except for BOD_5 and NH_4^+ (Dunne *et al*., 2005), the use of HSSFCWs is wide spread. CWs with a HSSF configuration are successfully used for the treatment of various kinds of wastewater for more than four decades (Sultana *et al*., 2015). This includes domestic, municipal, industrial, agricultural, landfill leachate and runoff waters as shown in Table 2.1. The survey of more than 400 HSSCWs from 36 different countries around the world revealed that the highest removal efficiencies for pollutants were achieved for municipal wastewater while the least removal goes for landfill leachate.

Table 2.1 Reviewed organic matter removal by HSSFCWs (Vymazal and Kröpfelov, 2009)

Types of wastewater	BOD_5	COD
Agriculture	68.2	63.0
Industry	60.1	63.1
Landfill leachate	32.8	24.9
Municipal tertiary	60.7	-
Municipal secondary	80.7	63.2

In agriculture sector, HSSFCWs has been widely applied for treatment of wastewater of different sources i.e. dairy farms, winery, sugarcane and trout farms (Sultana *et al*., 2015). They have been used at different scales from laboratory experiments to full-scale applications with surfaces areas ranging from 0.25 to 7600 m^2 (Sultana *et al*., 2015). The mean removal efficiencies in most of the studied HSSFCWs was in the range of 28% to 99%, 20% to 70% and 30% to 60% for organic matter, total nitrogen and total phosphorus, respectively (Vymazal and Kröpfelová, 2009; Vymazal, 2017; Mesquita *et al*., 2018). This inconsistent removal efficiency mainly account for difference in the type of plantation, substrate types; composition of the wastewater, hydrological settings and other factors (Gorgoglione and Torretta, 2018). The highly variable removal efficiency usually makes it difficult to judge directly CWs is an efficient technology to reduce the levels of agro-chemicals from wastewater and consequently, contribute to reducing pollution of surface water bodies. Rather, it indicates the need of optimization of agro-chemicals removal mechanisms in newly established HSSFCWs for floriculture industry.

2.4.1.2. Mechanisms of pollutant removal in HSSFCWs

In general, the pollutant removal efficiency of treatment wetlands is dependent on complex combination of physical, chemical and biological processes (Sultana *et al*., 2015; Truu *et al*., 2015; Lv, 2016). It includes one or more of the processes of precipitation, sedimentation, adsorption, photolysis, hydrolysis and volatilization, plant uptake and microbial degradation. In particular, the process is mostly driven by critical treatment components (substrate, plants and microbes) of the CWs and the relative contribution of each process is largely determined by the nature of the pollutant type.

2.4.1.2.1. Removal of organic matter

The level of organic matter in wastewater commonly estimated through the use of BOD_5 and COD. The biodegradability of organic matter is also usually explained by the BOD_5/COD ratio in the wastewater (Saceed and Sun, 2012). The value of BOD_5 is always less than COD value. This is because; COD in principle includes the compounds which are not biodegradable. A BOD_5/COD ratio of ≥ 0.5 indicates that the organics in the wastewater are easily biodegradable while the ratio ≤ 0.3 indicates that the available organics in the waste water are difficult to degrade by microorganisms (Saceed and Sun, 2012). This ratio is commonly used as indicator to monitor the levels and nature of organic matter in wastewater.

Filtration and sedimentation are major physical processes for retention of suspended and settleable organic matter from the wastewater. The chemical processes of organic matter removal primarily attained by media of adsorption (Bruland and Richardson, 2006). Sorption of organic pollutants onto the surface of substrate involves different mechanisms, mainly achieved by hydrophobic processes. However, sorption by gravel media is less efficient in removal of organic matter though it marketed as a non-clogging material for CWs presently (Bruland and Richardson, 2006).

The major share of removal of dissolved organic pollutants is noted to be contributed by biological (plant and microbial) mechanisms in the CWs (Sultana *et al*., 2015). Wetland plants play important role in removal of various kinds of pollutants from the wastewater following various mechanisms as shown in Fig. 2.6. Plant roots do not have specific transporters for the xenobiotic organic compounds to move into the plants tissues and thus, uptake and translocation of organic pollutants within plants are driven by means of diffusion. After being taken up, the organic pollutants degraded, accumulated and volatilized as illustrated in Fig. 2.6. The metabolism might also include a series of biochemical reactions such as transformation of parent organic pollutants, conjugation of metabolites with macromolecules and incorporation of the conjugated products into plant cell walls and vacuoles (Fig. 2.6)

Though detailed mechanism is not well known, Dordio *et al*., (2011) detected a metabolite (10, 11-dihydro-10, 11-epoxycarbamazepine) of carbamazepine in the leaf tissues of *Typha spp*. indicating the occurrence of carbamazepine metabolism inside the plant tissues. Liu *et al*. (2013)

also found the conversion of ciprofloxacin HCl and oxytetracycline HCl to their epimers such as tetracycline, chlortetracycline, enrofloxacin and ofloxacin within plants tissues. Moreover, plants in CWs are well-known to stimulate the development and activities of microbial populations by means of rhizodeposition (exudates, mucigels, dead cell material, etc.). They, therefore, enhance microbial processes of organic matter removal in the rhizosphere as shown in Fig. 2.6, though the plant driven processes was not known for majority agro-chemicals.

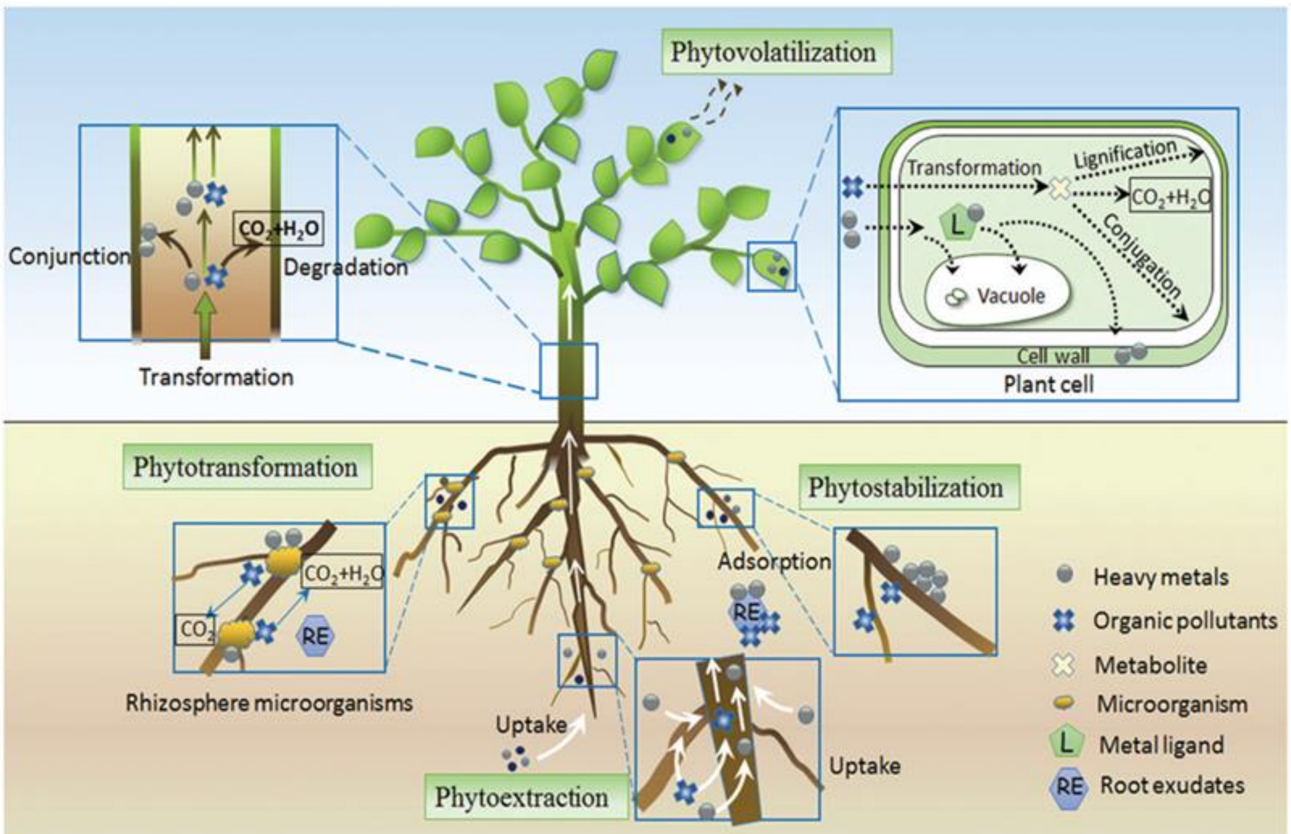


Fig. 2.6 Plant mediate-remediation of pollutants in CWs (Adapted from Truu *et al*., 2015)

Moreover, wetland microbial communities attached to surfaces of substrate and plant roots assumed to play crucial roles in the reduction of degradable organic pollutants (Guan *et al*., 2015). A few studies also found a positive correlation between numbers of various sediment microbes and removal of organic matter (Guan *et al*., 2015). In general, degradation steps are often initiated by exo-enzymes excreted by microbes that cleave functional groups or specific bonds of large molecules as shown in Fig. 2.7. This step allows internalization and metabolic utilization of the degradation products by microbes (Weber *et al.*, 2013). The degradation noted to occur through both aerobic and anaerobic mechanisms that are driven by activities of various groups of the wetland microbial consortia.

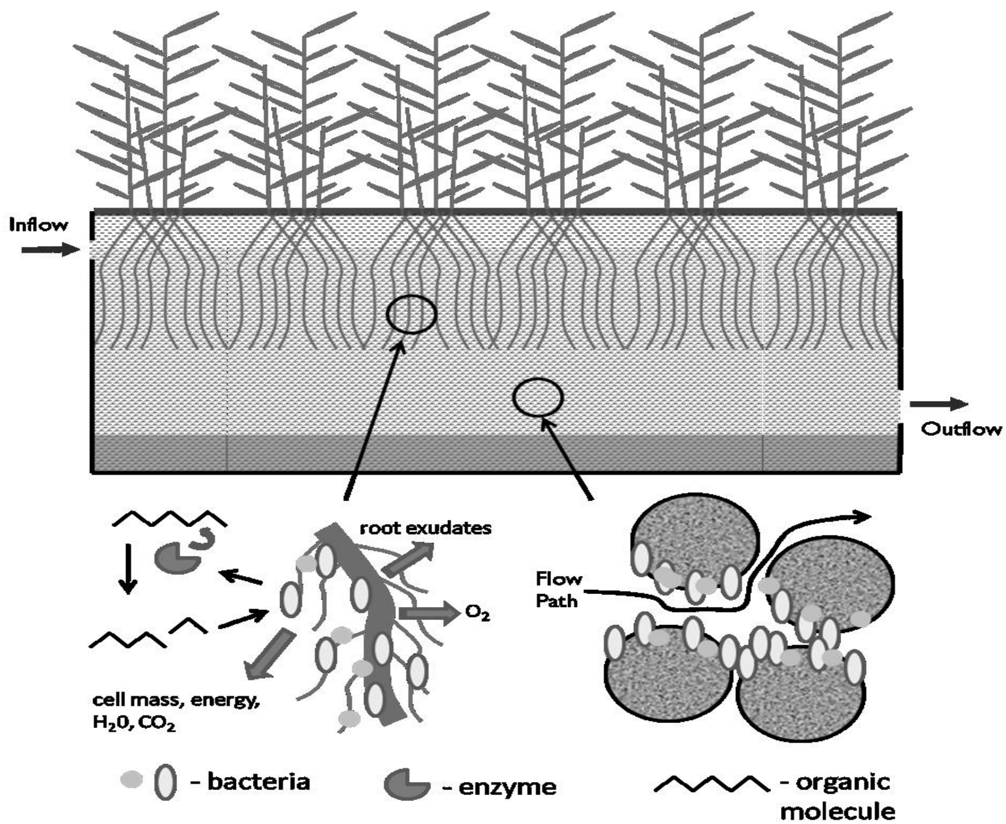


Fig. 2.7 Illustration of organic matter removal in CWs (Adapted from Weber *et al*., 2013)

Along with the aerobic degradation, anaerobic removal of organics is assumed to precede at the bottom of a SSFCWs inside the media pores lacking oxygen (Saceed and Sun, 2012). This degradation path way follows the conventional two-step process performed by anaerobic heterotrophic bacteria. In the first step, acid forming bacteria convert organic matter into new cells, organic acids and alcohols by fermentation. The second group, known as methane forming bacteria continues the oxidation (methanogenesis), converting organic compounds to new bacterial cells, methane and carbon dioxide. Both fermentation and methanogenesis assumed to occur in anaerobic zones of the CWs and their pathways are highly diverse, involving the transformations of various compounds such as iron, sulfate and nitrate.

In general, the microbial assemblage in the CWs is highly sensitive to operational and environmental conditions and thus, its structure is a good indicator for the position of CWs (Chang *et al*., 2015). Thus, studying the wetland microbial community structures in relation to the operational and environmental conditions is essential for understanding and optimization of the performance of the CWs.

2.4.1.2.2. Nutrients removal

Nitrogen and phosphorus are the most ubiquitous nutrients in relation to water quality degradation (Zhu *et al*., 2017). The removal of nitrogen and phosphorus from wastewater is one of the top priority concerns. Of the nitrogen species, ammonium and nitrate are the most targeted while phosphate and orthophosphate are the most focused upon. The removals of various forms of nitrogen and phosphorus in CWs have different pathways. For instance, ammonia removal requires aerobic conditions by chemeoautotroph bacteria while the removal of its product, nitrate under goes in anaerobic condition with availability of sufficient carbon sources. Thus, control of the transformations is described as a challenge in wetland engineering.

Nitrogen removal in CWs involves a microbial proteolysis and deamination of organic nitrogen to ammonia. Then, the level of ammonium can be reduced by processes including substrate adsorption, plant uptake, volatilization and microbial oxidation. Though plant uptake was significant at low loading rate, the conventional microbial nitrification of ammonia in CWs assumed to be dominant in ammonia removal processes (Weber *et al*., 2013). However, the heterotrophic organic matter removing microbes compete with the autotrophic nitrifying bacteria for oxygen in HSSFCWs. The natural aeration in these types of CW might not meet the oxygen demand required for both types of microorganisms. Thus, variable levels of nitrogen in HSSFCW effluents can be produced.

Unlike ammonia, nitrate is not likely to immobilize by media components and mainly remains in the water column of the CWs (Garcia-Rodríguez, 2014). It is removed through assimilation by incorporating into biomass or bio-transformed to nitrogen gas by heterotrophic bacteria through the process of denitrification. Currently, there is much attention towards biological nitrogen removal and whilst the denitrification process is generally time consuming, especially for wastewaters containing much nitrate (Foglar *et al*., 2005).

Similarly, several studies have shown that phosphorus in CWs is removed mainly by absorption of plants, accumulation of microorganisms and adsorption of substrates (Kotti *et al*., 2010). The inorganic phosphorus is immobilized by wetland plants through incorporating to organic phosphorus compounds such as ATP, DNA and RNA and then removed from the system while the plants harvested. Phosphorus is also necessary to microbes and even some of them have ability of converting poorly soluble phosphorus to dissolved inorganic phosphorus which makes ready for plant absorption.

Moreover, adsorption of media or ion exchange reported to be very important mechanism for phosphorus removal in CWs (Kotti *et al*., 2010). The iron, aluminum, calcium and magnesium compounds assumed to affect the adsorption processes. Phosphorus is known to be removed through precipitation by iron and aluminum under acidic conditions ($pH < 6$) while by calcium and magnesium under alkaline conditions ($pH > 8$). A media with significant clay content containing iron, aluminum and calcium enhance phosphorus removal. However, the replacing of clay media by the gravel with low level of these elements suggested limiting the phosphorus removal efficiency in HSSFCWs (Mesquita *et al*., 2018).

Even though there have been several studies on the processes involved in the removal of nutrients (nitrogen and phosphorus) in CWs, the results obtained in terms of removal efficiency are limited and highly variables (Mesquita *et al*., 2018). Thus, it suggests the importance of further studies for optimization of this technology to meet the increasingly strict discharge standards for treated effluents as well as their safe reuse.

2.4.1.2.3. Pesticides removal

The overall pesticides removal pathways in CWs are summarized in Fig.2.7. However, the wholes pathways may not always occur in all types of CWs simultaneously. For instance, the contribution of photodegradation can be neglected in SSFCWs. The hydrolysis is noted to be dependent on the chemical characteristics of individual pesticides (Lv, 2016). Volatilization is considered as the main mechanism for the removal of some volatile pesticides (Risco *et al*., 2015). However, due to the current increased use of less thermally labile pesticides (carbamayes, neonicotinoids, benzoyl ureas, etc.), volatilization becomes less relevant.

Pesticides adsorption processes are highly dependent on the types of media in CWs. However, gravel (instead of soil) is the more commonly used materials for CWs and provides homogenized water flow. Consequently, substrate sorption of pesticides is relatively low in CWs (Bruland and Richardson, 2006). Thus, the biological (wetland microbial and plants) are assumed to play a very important role for the removal of pesticides though the uptake, translocation and degradation processes of pesticides are still not adequately understood.

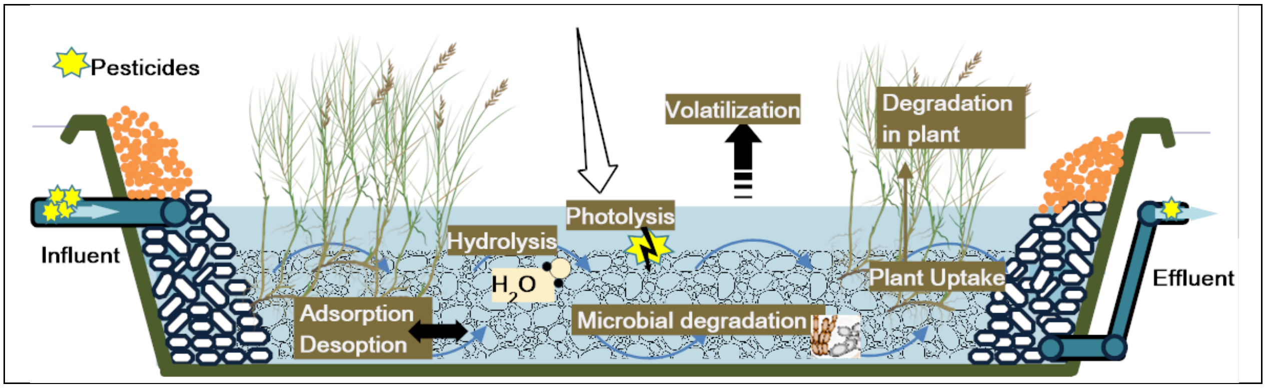


Fig. 2.8 Pesticides removal pathways in CWs (adapted from Lv, 2016).

2.4.1.3. Factors affecting treatment performance of HSSFCWs

The performance of different CW's in the removal of different types of waste water is affected by different factors. It is generally accepted that variations in the design, internal and external conditions of the CWs account for the wide differences in their removal performance (Chang *et al*., 2015; Zhang *et al*., 2016). Accordingly, factors to be taken in to consideration include; CW type, wetland plants, hydraulic conditions, level of dissolved oxygen (DO) and seasons.

2.4.1.3.1. Wetland plants

The effect of plants on the removal of pollutants from wastewater in CWs is still a controversial (Zhu *et al*., 2017). Majority of them showed better treatment performance in planted CWs compared to the unplanted corresponding while some indicated no difference between planted and unplanted CWs (Table 2.2). For instance, Vymazal, (2011) reviewed 22 articles that compared treatment efficiency of planted versus unplanted CWs and 90% them showed a positive effect of plants at least for some parameters of water quality.

Table 2.2 Comparison of treatment performances of planted and unplanted CWs (Vymazal, 2011)

Location	Vegetation	Parameters	Effect	Reference
Canada	*Phragmites australis* *Typha latifolia*	BOD_5, COD, TKN, NH_4-N	Positive effect	Naylor *et al.* (2003)
		TSS, TP, PO_4-P, NO_3-N	No effect	
Canada	*Phragmites australis* *Typha angustifolia*	TN	Positive effect	Maltais-Landry *et al.* (2009)
Greece	*Typha latifolia*	TKN, TP	Positive effect	Akratos and Tsihrintzis (2007)
		COD	No effect	
Mexico	*Phragmites australis* *Typha sp.*	FC	Positive effect	Rivera *et al.* (1995)
Morocco	*Arundo donax*	COD, TSS, NH_4-N, TP	Positive effect	El Hafiane and El Hamouri (2004)
New Zealand	*Scirpus validus*	BOD_5, TSS, FC	No effect	Tanner *et al.* (1995)
Spain	*Typha latifolia*	BOD_5, NH_4-N, FC	Positive effect	Ciria *et al.* (2005)
		TSS, COD	No effect	

A comparison of treatment performance between wetland plants also indicated difference among plant species (Table, 2.3). In general, the magnitudes of pollutant removal differences in plants species are reported to range from slight up to doubling. The differences were more often noted for nitrogen removal (especially nitrate) compared to organic matter (Akratos and Tsihrintzis, 2007; Bojcevska and Tonderski, 2007).

Moreover, the results showed that mixed vegetation is more effective for pollutant removal than single-species vegetation with few exceptions (Fraser *et al*., 2004; Zhu *et al*., 2017). For example, Zhu *et al*. (2017) reported that to mix different plants can enhance the efficiency of CWs. Whereas Fraser *et al*. (2004) claimed that the mixed plant had no significant improvement on the performance of CWs. In many cases, the specific driving forces leading to conclusion of each study is scarce. Therefore, further studies are necessary to explore whether combined planted wetland is a good strategy for enhancing organic matter and nutrient treatment.

The observed differences in plants performance are also suggested to be interpreted with careful consideration of various aspects of the plants used and external conditions. This is because, many studies showed problems of plants growth in CWs, either due to wastewater toxicity and effect of the pH of the matrix, herbivory or other unspecified reasons (Calheiros *et al*., 2007). Moreover, the health of the plant, the time taken for maturity could also be an important factor that may influence removal performance of the plant. For example, it is reported that *Phragmites australis* may take up to 3 years before reaching maturity (Vymazal and Krőpfelová, 2005) and the use of younger stage may underestimate the real efficiency of this species.

Table 2.3 Comparing pollutants removal performance between wetland plants

Water quality parameters and Species differences	Studies
SS: *Typha domingensis* > *Cyperus immensus* = *Cyperus papyrus* = *Phragmites mauritianus* COD: depend on HRT and type of wastewater	Abira *et al*. (2003)
TKN, NH_4^+, TP, PO_4: *Typha latifolia* > *Phragmites australis* BOD_5, COD: no difference	Akratos and Tsihrintzis (2007)
NO_3^-: *Typha sp.* > *Scirpus sp.*	Bachand and Horne (2000)
NH_4: *Cyperus papyrus* > *Echinochloa pyramidalis* SS, TP, PO_4: no difference	Bojcevska and Tonderski (2007)
BOD_5: *Typha latifolia* > *Juncus effuses* = *Phragmites vallatoria* TKN, NO_3: *Typha latifolia* > *Juncus effuses* > *Phragmites vallatoria* SS: no difference	Vymazal (2011)
TN: *Schoenoplectus validus* > *Typha latifolia* = *Carex lacustris* > *Phragmites australis*,	Fraser *et al*. (2004)

On the other hand, it is expected that plant species with different rhizosphere structure, root exudate release, compound uptake ability and associated different microbial community might also influence removal of pollutants differently in CWs. Some studies have shown that microbial density, diversity and activity are enhanced in the plant rhizosphere of SSFCWs compared to unplanted control (Calheiros *et al*., 2010), suggesting that plants enhance the establishment of microorganisms. While others have demonstrated no effects due to plant presence (Baptista *et al*., 2008). Thus, further research is needed in order to understand the plant-microbe interactions during removal of pollutants in CWs

2.4.1.3.2. Hydrology

Hydrology is one of the primary factors affecting wetland functions. Sufficient water supply is crucial for establishing well-functioning CWs. The flow and storage volume determine the time water spends in the wetland system as well as the degree of mixing. Thus, it influences the interactions between pollutants and the wetland critical removal components (wetlands microbial community, plants and media). Hydrological factors in wetland mainly belong to the hydraulic loading rate and hydraulic residence time.

2.4.1.3.2.1. Hydraulic loading Rate

The hydraulic loading rate (HLR) refers to the loading of a water volume per unit area over a specified time interval. It is calculated by using the equation shown below and expressed in m/d or cm/d. Organic loading rate (OLR) also indicates how many grams of organic solids are loaded per m^3 of wetland volume and unit of time. The pollutant loading rate at the inlet (LRi) is calculated as HLR multiplied by inlet concentration and expressed as kg/ m^2d or g/m^2d.

$$HLR = \frac{Qav}{As}\ ,\ LRi = HLR\ x\ CI$$

Where, HLR = hydraulic loading rate (m/d), Qav = average flow rate (m^3/d), As = surface area of wetland (m^2), LRi = inlet pollutant loading rate (g/m^2d), Ci = inlet concentration (mg/L).

Hydraulic loading rate is one of the important factors affecting treatment performance of the CWs (Saeed and Sun, 2012). Greater hydraulic loading rate increases the amount of pollutants passing through the system. Thus, pollutants accessibility to critical removal components (media, microbes and plants) of the CW increases the high absolute mass removal per unit time**.** However, at the same time the contact time between waste water pollutants and critical removal components is shortened compared to a lower hydraulic loading rate resulting in a lower pollutant removal of incoming concentration (Trang *et al*., 2010).

To date, various ranges of hydraulic loading rate have been employed to examine the performance stability of HSSFCWs (Trang *et al*., 2010; Çakir *et al*., 2015; Sultana *et al* 2015). Reviewing HSSFCWs used in treatment of agro-industrial wastewater showed the hydraulic loading rate ranges from 0.17 to 376 gm/m^2 day for organic matter, 0.007 to 2.7 gm/m^2 day for TKN and 0.004 to 4.7 gm/m^2 day for phosphorus (Sultana *et al*., 2015). Most of the studies revealed an increase in hydraulic loading rate gives higher area-specific mass removal rates in the CWs at the expense of higher outflow concentrations. According to Trang *et al*. (2010) decline of removal of organics (84% to 16%) and nitrogen (84% to 63%) observed when hydraulic loading was shifted from 0.031 to 0.146m^3/m^2/day in tropical HSSFCWs. When hydraulic rates exceed the CWs assimilating capacity, threat of pollutant release may occur (Gorgoglione and Torretta, 2018). This, therefore, suggests the needs of searching for optimum hydraulic loading rate to address the maximum pollutant removal for specific application of CWs.

2.4.1.3.2.2. Hydraulic residence time

Hydraulic residence time is the average residence time during which the wastewater remains within the CWs. Like hydraulic loading time, hydraulic residence time is also a key design parameter which affects the removal of contaminants in CWs (Conn and Fiedler, 2006). It is determined by the mean working volume of the wetland system divided by the influent flow rate as shown in the equation below. Hydraulic over loading occurs when the flow exceeds the design capacity, thus reducing the actual hydraulic residence time.

$$HRT = \frac{LWND}{Qav} \quad \text{or} \quad HRT = \frac{Vn}{Qav}$$

Where, HRT= hydraulic retention time, L = length of wetland (m), W = width of wetland (m), N = media porosity, D = water depth (m), Vn = Working Volume of the wetland and Qav = Average flow rate (m^3/d)

Several studies have been conducted to evaluate the effect of hydraulic retention time in relation to pollutants removal in CWs (Akratos and Tsihrintzis, 2007; Zhao *et al*., 2014; Çakir *et al*., 2015; Merino-Solís *et al*., 2015; Sultana *et al*., 2015). They indicated that pollutants (organic matter, nutrients and pesticides) removal was generally enhanced by extending the hydraulic retention time in CWs. Akratos and Tsihrintzis (2007) found higher removal of organic matter, total nitrogen and phosphorus due to increase of hydraulic retention time from 6 to 20 days in pilot-scale HSSFCWs. An optimal hydraulic retention time from 2 to 7 days for organic matter and 1 to 4 days for nitrogen and phosphorus removal reviewed in Sultana *et al*. (2015). Extended retention without clogging and partial clogging may increases treatment efficiency. However, too long hydraulic residence time may leads to clogging of filter material of the CWs. Thus, to obtain the required optimal retention time, it is necessary to test the organic matter and nutrients removal under different hydraulic retention time.

2.4.1.3.3. Seasonal temperature

Environmental conditions should be considered when constructing wetlands (Almuktar *et al*., 2018). Temperature modifies the rates of several key biological processes, affect water quality parameter such as level of dissolved oxygen and determinant of evapotranspirative water loss processes in CWs (Almuktar *et al*., 2018). Some of the previous studies indicated low seasonal temperature to suppress removal rates of organic matter and nutrient in CWs (Akratos and Tsihrintzis, 2007; El-Refaie, 2010; Kotti *et al*., 2010; Trang *et al*., 2010; Almuktar *et al*., 2018). For example, Trang *et al*. (2010) studied the wetland behavior in tropical conditions. They found out that there is a significant ($p < 0.05$) impact of higher operation temperature on improving the treatment process in less time, mainly associated with the rate of organic matter degradation, nitrification and denitrification processes. In the contrast, a high temperature may positively affect the system performance due to the high biological activities which resulting in over all improved wastewater treatmen efficiency. However, available studies on CWs with specific design and operational conditions under different seasonal temperature are rare in tropical conditions.

2.4.1.4. Strategies for enhancing performance of HSSFCWs

The treatment performances of CWs were often dependent on the optimization of various operational parameters and environmental conditions. Along with, different strategies are also currently available to enhance treatment performance of CWs to comply with the increasingly strict water quality standards for wastewater discharging (Wu *et al*., 2015). Bio-stimulation and bio-augmentation are among the strategies that are widely applied for bio-remediation of contaminated soil but less common from water purification by the CWs.

2.4.1.4.1. Bio-stimulation

It is established that the microbial pollutant removal mechanism in wetlands are influenced by availability of supporting nutrients. The additions of external nutrient sources stimulate the indigenous microorganisms in CWs to increase their pollutants removal efficiency (Wu *et al*., 2015b; Meng *et al*., 2014). For instance addition of adequate electron donor such as organic carbon is instrumental in the removal of nitrate through the processes of denitrification in cases of deficiency of organics in wastewater (Wu *et al*., 2015b). Apart from the removal of nitrogen pollutants, the addition of utilizable organic carbon and organic substrates hypothesized to improve removal of emergent organic pollutants such as pesticides (Meng *et al*., 2014; Zhao et al., 2014). It assumed to stimulate wetland microbial consortia for increasingly consuming the organic contaminants directly as a carbon source or indirectly via co-metabolism (Kumar *et al*., 2006).

Lin *et al*. (2002) reported that addition of fructose significantly (> 90%) improved removal efficiency of treating groundwater in CWs. Rustige and Nolde (2007) also employed acetic acid as a carbon source and attained a 75% denitrification rate of treatment of landfill leachate in CW. Furthermore, Lu *et al*. (2009) studied the effect of glucose on denitrification in CWs and found that the nitrate removal was improved by 60%. Since the addition of sugars such as glucose and fructose is costly in CW systems, the search for low-cost carbon source such as sugar cane molasses would be a best alternative. Cane molasses serves as a source of carbon and energy as well as provides trace minerals for growth of the microbes that mineralize the pollutants in CWs.

2.4.1.4.2. Bio-augmentation

Bio-augmentation is the application of a pre-adapted pure bacterial strain or consortium and genetically engineered bacteria into treatment systems (Wu *et al*., 2015). It suggested that bio-augmentation is a suitable way for accelerating biodegradation rate in CWs treating various wastewaters even containing some toxic contaminants (Zhao *et al*., 2014).

The effect of the addition of *Bacillus subtilis FY99-01 strains* efficiently enhanced denitrification with a maximal nitrate removal by 36 % in a riparian wetland (Pei *et al*., 2010). Similarly, influence of adding a consortium of six denitrifying bacteria for treating polluted river water enhanced the removal efficiencies to 75 %, 96%, 96 % and 90 % for COD, TN, NH_4^+ and TP respectively (Shao *et al*., 2014). In addition, Zhao *et al*. (2014) demonstrated that bio-augmentation to increase the removal efficiency of the pesticide endosulfan up to 97% in CW's.

In general, bio-augmentation is assumed to reinforce microbial populations in biological waste treatment systems so that they can effectively reduce the pollutants load by transforming it into less dangerous compounds. However, the effectiveness of bio-augmentation for contaminant removal is not always a success story (Wu *et al*., 2015). The introduced microorganism (s) may fail to become active in the treatment plant due to influence by indigenous microbiota, presence of predators (bacteriophages, protozoa) or unable to adapt to the new environment. Though some researches demonstrated better efficacy through bio-augmentation, finding cost effective, eco-friendly and competent bio-augmenters is yet to be desired for the full-scale application (Zhao *et al*., 2014).

In conclusion, the removal of pollutants in CWs relies on a diverse range of physical, chemical and biological factors. The treatment success is also influenced by different parameters including design, plant types, season, retention time, etc. Thus, it is essential to appraise the effect of the key influencing factors to optimize the treatment performance of newly established CWs. Enhancing technology like bio-stimulation and bio-augmentation are coming into the forefront for improving treatment performance of CWs.

References

Abayneh Tilahun (2013). Environmental impacts of floriculture industry in debrezeit town: a need for strategic environmental assessment, MSc. Thesis, Addis Ababa University

Abira, M. A., Ngirigacha, M. H. W. and van Bruggen, J. J. A (2003). Preliminary investigation of the potential of four tropical emergent macrophytes for treatment of pre-treated pulp and papermill wastewater in Kenya. *Water Science and Technology* **48(5):**223-3.

Akratos, C.S. and Tsihrintzis, V. A. (2007). Effect of temperature, HRT, vegetation and porousmedia on removal efficiency of pilot-scale horizontal subsurface flow constructed wetlands. *Ecology Engineering* **29**:173–91.

Bachand, P.A.M. and Horne, A. J. (2000). Denitrification in constructed free-water surface wetlands: II. Effects of vegetation and temperature. *Ecology and Engineering* **14:**17–32.

Baptista, J. D. C., Davenport R. J., Donnelly, T., Curtis, T. P. and Rayne, D. (2008). The microbial diversity of laboratory scale wetlands appears to be randomly assembled. *Water Research* **42(12):**3182–3190.

Berhan Teklu, Amare Hailu, Wiegant, D.A., Scholten, B.S. and Van den Brink, P.J. (2016a). Impacts of nutrients and pesticides from small and large scale agriculture on the water quality of Lake Ziway, Ethiopia. *Environmental Science Pollution and Research http://dx.doi.org/10.1007/s11356-016-6714-1*.

Bojcevska, H. and Tonderski, K. (2007). Impact of loads, season and plant species on the performance of a tropical constructed wetland polishing effluent from sugar factory stabilization ponds. *Ecological Engineering* **29:** 66–76.

Bruland, G.L. and Richardson, C.J. (2006). Comparison of soil organic matter in created, restored and paired natural wetlands in North Carolina. *Wetlands Ecology and Management,* **14(3):**245-251.

Çakir, R., Gidirislioglu, A. and Çebi, U. (2015). A study on the effects of different hydraulic loading rates (HLR) on pollutant removal efficiency of subsurface horizontal-flow constructed wetlands used for treatment of domestic wastewaters. *Journal of Environmental Management* **164**:121-128.

Calheiros, C.S.C., Rangel, A.O.S.S. and Castro, P.M.L. (2007).Constructed wetland systems vegetated with different plants applied to the treatment of tannery wastewater. *Water Research* **41(8):**1790-1798.

Calheiros, C.S.C., Teixeira, A., Pires, C., Franco, A.R., Duque, A.F., Crispim, L.F. C., Moura, S.C., and Castro, P. M. L. (2010). Bacterial community dynamics in horizontal flow constructed wetlands with different plants for high salinity industrial wastewater polishing. *Water research* **44:**5032-5038.

Chang, J. J., Wu, S. Q., Liang, K., Wu, Z. and Liang, W. (2015). Comparative study of microbial community structure in integrated vertical-flow constructed wetlands for treatment of domestic and nitrified wastewaters. *Environmental Science and Pollution Research* **22(5):**3518–3527.

Chazarenc, F., Brisson, J. and Comeau, Y. (2007). Slag columns for upgrading phosphorus removal from constructed wetland effluents. *Water Science Technology* **56(3):**109-115.

Choudhary, A.K., Kumar, S. and Sharma, C. (2011). Removal of Chemical Oxygen Demand and Color from Pulp and Paper Mill Wastewater Using Horizontal Subsurface Flow Constructed Wetland. *Proceeding International Conference on Emerging Technologies for Sustainable Environment.*

Ciria, M.P., Solano, M.L. and Soriano, P. (2005). Role of Macrophyte *Typha latifolia* in a Constructed Wetland for Wastewater Treatment and Assessment of Its Potential as a Biomass Fuel. *Biosystems Engineering* **92(4):**535–544.

Conn, R.M. and Fiedler, F.R., (2006). Increasing hydraulic residence time in constructed stormwater treatment wetlands with designed bottom topography. *Water Environment Research* **78:**2514–2523.

Daniel Hailemichael, H. (2013). Evaluation and development of floriculture supply chain in Ethiopia, to attenuate environmental impact and logistics cost, M.Sc Thesis, Addis Ababa University

David T. (2002). The Bloom on the Rose, Looking into the Floriculture Industry. *Environmental Health perspectives* **110(5)**:240-247.

Dordio, A., Ferro, R., Teixeira, D., Palace, A.J., Pinto, A.P., Dias, C. M. B. (2011). Study on the use of *Typha spp.* for the phytotreatment of water contaminated with carbamazepine. *Inernational Journal of Environmontal Analetical Chememistry* **91:**654–667.

Dunne, E.J, Culleton, N., Donovan, G. O, Harrington, R. and Olsen, A.E. (2005). An integrated constructed wetland to treat contaminants and nutrients from dairy farmyard dirt water. *Ecological Engineering* **24:**221–234.

EPA (2003). Environment Protection (Standards for effluent discharge) Regulations 2003.

Ethiopian Horticulture Producer Exporters Association (EHPEA) (2015). EHPEA resource centre. *http://www.ehpea.org.*

Foglar, L., Briski, F., Sipos, L. and Vukovic M., (2005). High nitrate removal from synthetic wastewater with the mixed bacterial culture, *BioresourcesTechnology* **96:**879–88.

Fraser, L. H., Carty, S. M. and Steer, D. (2004). A test of four plant species to reduce total nitrogen and total phosphorus from soil leachate in subsurface wetland microcosms. *Bioresource Technology* **94:**185–192.

Garcia-Blanco, S., Venosa, A. D., Suidan, M. T., Lee, K., Cobanli, S., Haines, J. R., (2007) Bio-stimulation for the treatment of an oil-contaminated coastal salt marsh. *Biodegradation* **18:**1–15.

Garcia-Rodríguez, A., Matamoros, V., Fontàs, C. and Salvadó, V. (2014). The ability of biologically based wastewater treatment systems to remove emerging organic contaminants: *a review. Environment and Science Pollution Resources* **21**:11708–11728.

Gorgoglione, A. and Torretta, V. (2018). Sustainable Management and Successful Application of Constructed Wetlands: A Critical. Review **10**: 2-19.

Guan, Y., Zhang, Y., Zhong, C. N., Huang, X. F., Fu J. and Zhao, D. (2015). Effect of operating factors on the contaminants removal of a soil filter: multi-soil-layering system. *Environmental Earth Science* **74:**2679–2686.

Huang, X., Liu, C., Li, K., Su, J., Zhu, G. and Liu, L. (2015). Performance of vertical up-flow constructed wetlands on swine wastewater containing tetracyclines and tetgenes. *Water Research* **70:**109–117.

Jansen HC, Harmsen J (2011) Pesticide monitoring in the Central Rift Valley 2009-2010. Report 2083. Alterra, Wageningen, *http://library. wur.nl/WebQuery/clc/1966024.*

Kinyanjui, J. W. (2009). Assessment of challenges encountered by small scale cut-flower sector in central kenya in complying with environmental standards, *MSc. thesis Jomo Kenyatta University of Agriculture and Technology*

Kotti, I. P., Gikas, G. D. and Tsihrintzis, V. A. (2010). Effect of operational and design parameters on removal efficiency of pilot-scale FWS constructed wetlands and comparison with HSF systems. *Ecological Engineering* **36:**862-875.

Kumar, M., and Philip, L. (2006). Bioremediation of endosulfan contaminated soil and water–Optimization of operating conditions in laboratory scale reactors. Journal of Hazardous Materials 36(2):354–364.

Lin, Y. F., Jing, S. R., Wang, T. W. and Lee, D, Y. (2002). Effects of macrophytes and external carbon sources on nitrate removal from groundwater in constructed wetlands. *Environment Pollution* **119**:413–420.

Liu, F., Xiao, R. L., Wang, Y., Li, Y., Zhang, S. L., Luo, Q., Wu, J. S. (2013). Effect of a novel constructed drainage ditch on the phosphorus sorption capacity of ditch soils in an agricultural headwater catchment in subtropical central China. *Ecological Engineering* **58:**69-76.

Lu, S, Hu, H., Sun, Y. and Yang, J. (2009). Effect of carbon source on the denitrification in constructed wetlands. *Journal of Environmental Science* **21**:1036–1043.

Lv, T., Zhang, Y., Zhang, L., Carvalho, P. N., Arias, C. A., Brix, H. (2016). Removal of the pesticides imazalil and tebuconazole in saturated constructed wetland mesocosms. *Water research* **91**:126-136.

Ly (2016). Removal of emerging organic pollutants in constructed wetlands: imazalil and tebuconazole as model pesticides. PhD Dissertation Aquatic Biology Department of Bioscience Aarhus University.

Malefia Tadele (2009). Environmental impacts of floriculture industry on Lake ziway with particular reference to water quality, A Thesis Report Submitted to Addis Ababa University School of Graduate Studies as a Partial Fulfillment of MSc. Degree in Environmental Science, Addis Ababa University.

Maltais-Landry, G., Chazarenc, F., Comeau, Y., Troesch, S. and Brisson, J. (2009). Effects of artificial aeration, macrophyte species and loading rate on removal efficiency in constructed wetland mesocosms treating fish farm wastewater. Journal of Environmental *Engineering and Science* **6:**409–414.

Mano, Y., Yamano, T., Suzuki, A. and Nov, T. M. (2010), Local and Personal Networks in Employment and the Development of Labor Markets: Evidence from the Cut Flower Industry in Ethiopia, *GRIPS Discussion grorup Paper 10-29.*

Mano, Y. and Suzuki, A. (2011). Agglomeration Economies for Industrial Development: The Case of the Ethiopian Cut flower Industry.

Meng, P., Pei, H., Hu, W., Shao, Y.and Li, Z. (2014). How to increase microbial degradation in constructed wetlands: influencing factors and improvement measures. *Bioresources Technology* **157**:316–326.

Merino-Solís, M. L., Villegas, E., Anda, J. d. and López-López, A. (2015). The Effect of the Hydraulic Retention Time on the Performance of an Ecological Wastewater Treatment System: An Anaerobic Filter with a Constructed Wetland. *Water* **7:**1149-1163.

Mesay Adugna (2017). Review on environmental effects of Ethiopian floriculture industry. *Asian Research Journal of Agriculture* **4(2)**:1-13.

Mesquita, C., Albuquerque, A., Amaral, L. and Nogueira, R. (2018). Effectiveness and Temporal Variation of a Full-Scale Horizontal Constructed Wetland in Reducing Nitrogen and Phosphorus from Domestic Wastewater. *Chemical Engineering* **2(3)**:1-24.

Monica, S., Karthik, L., Mythili, S. and Athiavelu, A. (2011). Formulation of Effective Microbial Consortia and its Application or Sewage Treatment. *Journal of Microbial and Biochemical Technology* **3(3)**:051-055.

Mulu Gebreeyesus and Lizuka, M. (2010). Discovery of the flower industry in Ethiopia: experimentation and coordination. *URL: http://www.merit.unu.edu*

Naylor S. J., Brisson, M. A., Labelle, A. Drizo and Cameau, Y. (2003). Treatment of feshwater fish farm effluent using constructed wetland: the role of plants and substrate. *Water Science and Technology* **48(5):**215-222.

Nikolic V. (2010). "Constructed Wetland Application in Wastewater Treatment: Processes in Serbia." *BALWOIS 2010-Ohrid, Republic of Macedonia -25, 29.*

Nzila, A., Razzak, S. A., and Zhu, J. (2016). Bio-augmentation: An Emerging Strategy of Industrial Wastewater Treatment for Reuse and Discharge. *International Journal Environmental Research and Public Health* **13(846):**1-20.

Pei, Y. S., Yang, Z. F. and Tian, B. H. (2010). Nitrate removal by microbial enhancement in a riparian wetland. *Bioresource Technology* **101**:5712–5718.

Rai, U.N., Tripathi.R.D. Singh, N.K., Upadhyay, A.K., Dwivedi, S., Shukla, M.K., Mallick, S., Singh, S.N. and Nautiyal, C.S. (2013). Constructed wetland as an eco-technological tool for pollution treatment for conservation of Ganga River. *Bioresources and Technology* **148**:535–541.

Rakesh Belwal and Meseret Chala (2008). Catalysts and barriers to cut flower export, A case study of Ethiopian floriculture industry. *International Journal of Emerging Markets* **3(2*):*** 216-235.

Risco, C., Rodrigo, S., López-Vizcaíno, R., Yustres, A., Sáez, C., Cañizares, P., Navarro, V. and Rodrigo, M. (2015). Electrochemically assisted fences for the electroremediation of soils polluted with 2, 4-D: a case study in a pilot plant. *Separation and Purification Technology* **156:**234-241.

Rivera, F., Warren, A., Ramirez, E., Decamp, O., Bonilla, P., Gallegos, E., Calderón, A. and Sánchez. (1995). Removal of pathogens from wastewaters by the root zone method (RZM). *Water Science Technology* **32:**211–218.

Rustige, H. and Nolde, E. (2007). Nitrogen elimination from landfill leachates using an extra carbon source in subsurface flow constructed wetlands. *Water Science and Technology* **56:**125–133.

Saeed, T. and Sun, G. (2012) A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. *Journal of Environment Management* **112:**429–448.

Sahle Amare and José, P. (2013). Environmental life cycle assessment of Ethiopian rose cultivation. *Science of the Total Environment* **443**:163–172.

Shao, Y.Y., Pei, H.Y., Hu, W.R., Chanway, C.P., Meng, P.P., J, Y. and Li, Z. (2014). Bio-augmentation in lab- scale constructed wetland microcosms for treating polluted river water and domestic wastewater in northern China. *International Biodeterioration Biodegradation* **95:**151–159.

Sudhagar, M. and Com, M. Phil. (2013). Production and Marketing of Cut flower (Rose and Gerbera) in Hosur Taluk. *International Journal of Business and Management Invention* **2(5):**15-25.

Sultana, M.Y., Mourti, C., Tatoulis, T., Akratos, C.S., Tekerlekopoulou, A.G. and Vayenas, D.V. (2015). Effect of hydraulic retention time, temperature and organic load on a horizontal subsurface flow constructed wetland treating cheese whey wastewater. *Journal Chemical Technology and Biotechnology* **91**:726–732.

Tanner, C. C., Clayton, J. S. and Upsdell, M. P. (1995). Effect of loading rate and planting on treatment of dairy farm wastewaters in constructed wetlandsdII. Removal of nitrogen and phosphorus. *Water Research* **29**:27-34.

Toumi, K., Vleminckx, C., Loco, J. V. and SChiffers, B. (2016). A survy of pesticides residues in cut flowers from various countries, *Communications in Agricultural and Applied Biological Sciences* **81(3**):493-502.

Trang, N. T. D., Konnerup, D., Schierup, H. H. and Chiem, N. H. (2010). Kinetics of pollutan removal from domestic wastewater in a tropical horizontal subsurface flow constructed wetland system: effects of hydraulic loading rate. *Ecological Engineering* **36:**527-535.

Truua, J., Truu, M., Espenberg, M., Nõlvak, H. and Juhanson, J. (2015). Phytoremediation and plant-assisted bioremediation in soil and treatment wetlands: A Review. *Journal of the Open Biotechnology* **9**:1-16.

Vasudevan, P., Griffin, P., Warren, A., Thapliyal, A. and Tandon, M. (2011). Localized domestic wastewater treatment: part I – constructed wetlands (an overview) *Journal of Scientific and Industrial Reasearch* **70:**583-594.

Verlicchi, P. and Zambello, E. (2014). How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? *Review Science of the Total Environment* **470**:1281–1306.

Vymazal, J. (2001). Types of Constructed Wetlands for Wastewater Treatment: Their Potential for Nutrient Removal. In Transformations of Nutrients in Natural and Constructed Wetlands; Vymazal, J., Ed.; Backhuys Publishers: Leiden, the Netherlands, pp. 1-93.

Vymazal, J. (2011). Constructed wetlands for wastewater treatment: five decades of experience. *Environmental Science Technology* **45(1):**61-69.

Vymazal, J. (2017). The use constructed wetlands for nitrogen removal from agricultural drainage: A Review. *Scientia agriculturae bohemica* **2**:82–91.

Vymazal, J. and Kr€opfelova, L. (2005). Growth of *Phragmites australis* and *Phalaris arundinacea* in constructed wetlands for wastewater treatment in the Czech Republic. *Ecological Engineering* **2:**606–621.

Vymazal, J. and Kröpfelová, L. (2009). Removal of organics in constructed wetlands with horizontal sub-surface flow: A review of the field experience. *Science of the Total Environment* **407**:3911-3922.

Vymazal, J., (2007). Removal of nutrients in various types of constructed wetlands. *Science of the total Environment* **380:**48-65.

Weber, K.P. and Gagnon, V. (2013). Microbiology in Treatment Wetlands. *Sustainable Sanitation and Practical* **18**: 25–30.

Wu, H., Zhang, J., Ngo, H.H., Guo, W., Hu, Z., Liang, S., Fan, J. and Liu, H. (2015). A review on the sustainability of constructed wetlands for wastewater treatment: design and operation. *Bioresources and Technology* **175:**594–601.

Yeshiwas and Alemayehu (2018). Social, economical and environmental issues of floriculture sector development in Ethiopia, *Review of Plant Studies* **5(1):**1-10.

Zhang, D., Luo, J., Lee, Z. M. P., Grasberg, M. R., Liu, Y., Tan, K. S. and Ng, J. W. (2016). Characterization of microbial communities in wetland mesocosms receiving caffeine-enriched wastewater. *Environmental Science Pollution Research* **23**:14526–14539.

Zhao, C., Xie, H., Mu, Y., Xu, X., Zhang, J., Liu, C., Liang, S., Ngo, H.H., Guo, W., Xu, J. and Wang, Q. (2014). Bioremediation of endosulfan in laboratory-scale constructed wetlands: effect of bio-augmentation and Bio-stimulation. *Environmental Science Pollution Research* **21**:12827–12835.

Zhu, H., Zhou, Q. W., Yan, B. X., Liang, Y. X., Yu, X. F., Gerchman, Y., and Cheng, X. W. (2017). Influences' of vegetation type and temperature on the performance of constructed wetlands for nutrient removal. *Water Science and Technology in press*.

3. Effect of Plant Types, Hydraulic Retention Time and Seasonal Temperature on Performance of Horizontal Subsurface Flow Constructed Wetland Treating Floriculture Wastewater in Bishoftu Town, Ethiopia

Abstract

The ever-increasing flower farms are the source of income generation and international trade to Ethiopia. However, the farms generate a significant amount of wastewater containing different agro-chemicals and discharge it to the environment without treatment. This necessitates the search for sustainable and low-cost treatment system. To this end, a horizontal subsurface constructed wetland (HSSFCW) was configured in a Flower Farm in Bishoftu Town, Central Ethiopia. The treatment performance of this system was evaluated on the basis of plant types, hydraulic retention time and seasonal temperature. Five pilot-scale horizontal subsurface flow CWs (HSSFCW) units were established. Three of the units were single planted with Typha latifola *(Tp),* Cyperus papyrus *(Cp) and* Pennisetum purpureum *(Pp), the fourth unit was mixed planted with the three plants (Mp) and the fifth unit was unplanted (Up). The influence of 4 retention times (1, 2, 3 and 4 days) and seasonal temperature (cold, < 20 ^{0}C and warm, ≥ 20 ^{0}C) were examined. The physico-chemical and microbial parameters of water and sediment samples were analyzed following standard methods. The result showed that the removal efficiency of the treatment system for BOD_5, COD, NH_4^+ and NO_3^- was significantly higher ($p < 0.05$) in planted CW units than unplanted unit. Among the single planted units, those planted with* Typha latifola *was more efficient. However, the CW unit with mixed plantation showed the best performance with mean removal efficiency of* 75.9%, 75.7%, 91.6%, 72.9%, and 60.3% *for BOD_5, COD, NH_4^+, NO_3^- and TP respectively. The removal of BOD_5, COD, NH_4^+ and NO_3^- with reference to discharging standards was reached with 4 day hydraulic retention time. Based on RISA profiles, the higher diversity indexes of bacterial communities' were detected from Tp and Mp CW units* *and showed a positive relation with their treatment efficiencies. Removal efficiency of the CWs for BOD_5, COD, NH_4^+, NO_3^- and TP showed direct relationships with periodic seasonal temperature where average removal were significant higher ($t > 0.177$ and $p < 0.05$) for warm season* ($\geq 20^0$C *) compared to below. All taken together, HSSFCW planted with* Typha (Tp) *or equal proportion of the* Typha latifola, Cyperus papyrus *and* Pennisetum purpureum *(Mp), working with 4 days retention time during the warm season can reduce organic matter and nutrients from flower wastewater to the standard before they discharged to the receiving environment.*

Keywords: Horizontal subsurface flow CWs (HSSFCWs), plant species, removal efficiency, seasonal temperature, water retention time,

3.1. Introduction

For the last three decades, the flower and other horticultural farms have been expanding in Ethiopia. The sector produces several flower species, including *Rosa, Gypsophila*, *Hypericum, Limonium*, *Carnations* and *Chrysanthemum* (EHPEA, 2015). In fact, Ethiopia is ranked as the second largest exporter of roses in Africa next to Kenya and the fourth largest supplier of flowers to the world market. Currently, there are 84 foreign and local companies from which the country earned more than 200 million USD and the sector becomes one of the top five foreign exchange earners to the country in 2016 (Mesay Adugna, 2017). Moreover, the sector provided more than 85,000 jobs opportunities of which around 85% is occupied by females in the country (Tadele Yeshiwas and Melkamu Alemayehu, 2018).

The floriculture industry requires higher amount of water, chemical fertilizers and crop protection agents compared to the other agricultural activities such as open field vegetable cultivation. Mesay Adugna (2017) noted that the sector requirements nearly 60 m^3 water per day per hectare for rose cultivation and 2 liters of water per bunch of roses in the post-harvest activities, preservation and preparation in Ethiopia. As a consequence of these, flowers cultivation processes accompanied with discharging of excessive wastewater contaminated with residual agro-chemicals (fertilizers, pesticides) to the environment.

Discharging of agro-chemicals wasted water from floriculture could strongly affect the quality of recieving water bodies. It is reviewed in Mesay Adugna (2017) to contribute for eutrophication of surface water bodies that suppresses activities of other aquatic organisms including higher plants and animals. Therefore, the removal of residual agro-chemicals from wastewater is one of the top priority concerns for flower industries before they discharge them to the environment.

The introduction of stringent laws and regulations in Ethiopia necessitate construction of low cost, easily applicable and environment friendly treatment technologies. It could be alternatives to conventional wastewater treatments facilities that require significant capital investments and running costs. To this end, ecological technologies such as construction of artificial wetlands (CWs) are drawing the attention to adopt the system to treat wastewater emanating for different sources (Wu *et al*., 2015).

Different configurations of CW are available for treatment of wastewater of which a horizontal subsurface flow CWs (HSSFCWs) is often recommended. This is because; it is simple to build easy to operate and offers better alternative treatment oxidation zones (Sultana *et al*., 2015; Gorgoglione and Torretta, 2018). However, there was inconsistencies in their performance due to different system related factors (bed depth, size of media and plant selections), application related factors (influent concentrations and hydraulics conditions) and environmental variables such as temperature (Kotti *et al*., 2010; Wu *et al*., 2015; Mesquita *et al*., 2018). Therefore, specific study for understanding how those factors influence the successful application of the system under the local prevailing conditions is remained important.

Apart from the configuration of the CWs, types of wetland plants also influence treatment performance of the system (Zhu *et al*., 2017; Gorgoglione and Torretta, 2018). It has been reported that cattails (*Typha latifola*), papyrus (*Cyperus papyrus*) and elephant grass (*Pennisetum purpureum*) are widely planted in CW for remediation of organic pollutants with high nutrient removal efficiency to improve the quality of wastewater for discharge (Akratos and Tsihrintzis, 2007; Brisson and Chazarenc, 2009). There are also conflicting reports on the efficiency of either single planting or mixed planting for better removal of pollutants from wastewaters (Zhu *et al*., 2017).

Fraser *et al*. (2004) claimed that the mixed planting had no significant improvement on the performance of CWs. On the hand, Mustapha *et al*. (2015) showed that mixing different plants in CWs can enhance the efficiency of CWs. Thus, selection of best performing planting condition is a concern in order to ensure efficient removal of pollutants. In this study, the locally adapted *Typha latifola*, *Cyperus papyrus* and *Pennisetum purpureum* may serve as efficient plant species in CW for treatment of wastewater from floriculture industry.

The removal of organic matter and nutrients in CWs is mainly attributed to the metabolic and enzymatic activities of microbes and plants (Sultana *et al*., 2015; Zhang *et al*., 2016). Greater hydraulic loading rate promotes quicker passage of wastewater through the wetland media. This may reduce the optimum pollutants contact time with the CWs removal components (microbes, plants and media). Previous studies showed wide operative hydraulic retention time for pollutant removals ranging from 2 to 30 days (Wu *et al*., 2015; Almuktar *et al*., 2018) where the optimal removal mainly depended on the specific planting design (e.g., single and mixed planting) and climatic conditions (Gorgoglione and Torretta, 2018; Mesquita *et al*., 2018). Thus, studying how performance of wetland plants and associated microbial community respond to water retention time in the CWs is still a concern.

This study was, therefore, initiated to understand the effect of single planting (*Typha latifola; Cyperus papyrus* and *Pennisetum purpureum)* and mixed planting (*Typha latifola + Cyperus papyrus + Pennisetum purpureum*) on removal of organic matter and nutrients from flower wastewater compared to unplanted control under different hydraulic and seasonal conditions in the HSSFCWs. The study could provide useful information for enhancing removal performance of CWs as well as future design and operation management under tropical climatic conditions.

3.2. Materials and Methods

3.2.1. Description of the treatment system

Pilot-scale experimental HSSFCWs were established in a privately owned flower farm nearby Bishoftu Town, 55Km south of Addis Ababa, Ethiopia. The CW consisted of five horizontal subsurface flow CW (HSSFCW) units. Fig. 3.1 indicates a schematic representation of the experimental CW units. The surface area of each CW unit was 5.2 m^2 (length: 4.0 m and width: 1.3 m) that is estimated base on the first-order design equation proposed by Kickuth for sizing of HSSFCWs (Vymazal, 2008). The treatment zone of each CW unit was filled with gravels of a particle size of 2 – 4 mm to a highest of 0.7m. Then, each CW unit considered to have a subsurface working volume of 3.64 m^3 (4.0 m length * 1.3 m width * 0.6 m depth * 0.4 poresity) which provides a continuous inflow rate of 0.312 m^3 wastewater per day while operating at 4 days of hydraulic retention time.

Three of the CW units were single planted with cattails *(Typha latifola),* papyrus (*Cyperus papyrus)* and Elephant grass (*Pennisetum purpureum)* and are designated as Tp, Cp and Pp respectively. The fourth unit is mixed planted with equal proportion of the three plants and labeled as Mp and the fifth unit was unplanted and symbolized Up as shown in Fig. 3.1. The plants were selected based on their abundance along water courses, receiving the wastewater in the vicinity of the study area and literature recommendation for wastewater treatment (Akratos and Tsihrintzis, 2007; Golda *et al*., 2014).

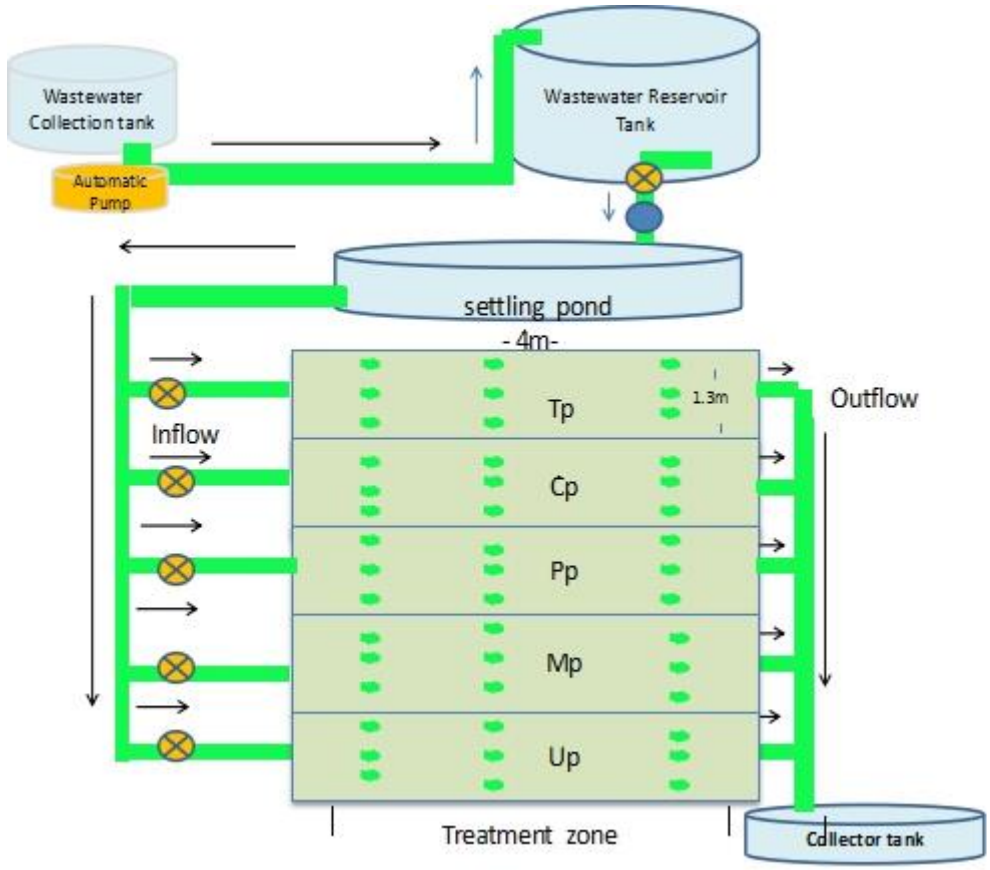


Fig. 3.1 Schematic view of the CW units, flow control valves, electromagnetic flow meter, - Sampling site, ⟶ Flow direction

3.2.2. Experimental procedures

The wastewater from the production area was collected in to a collection tank where it was pumped automatically into a reservoir tank. Then, it was flowing at fixed flow rate to a settling pond where primary polishing of the wastewater takes place. The wastewater was allowed to pass through screens installed within the redirect channels along its way to reduce portion of the suspended solids before entrance of each CW units. The arrangement of the inflow and outflow pipes within the CW units shown in Fig. 3.1. The composition of the inflow wastewater to each CW unit was with the average influent parameters of pH: 7.57 ± 0.2, temperature: 21.5±3.5^{0}C, DO: 2.3±0.9, BOD_5: 87.3±5.6 mg/L, COD: 152.1±7.5 mg/L, NH_4^+: 2.23±3.0 mg/L, NO_3^-: 12.2±2.2 mg/L and TP: 5.45±4.8mg/L.

The CWs with this configuration was acclimatized for 3 months and then operated and monitored for 22 months. During the operation period, potential effects of plant species, hydraulic retention time and seasonal temperature on organic matter and nutrients reduction performance of the CWs were evaluated using: (1) three planting conditions (single planted, mixed planted and unplanted), (2) four different hydraulic retention times (1, 2, 3 and 4 days) and (3) two seasonal temperature (warm and cold) as well as (4) the planting and hydraulic conditions in relation to RISA profiles of sediment microbial communities. The flow rate was adjusted by a flow controlled valves and electromagnetic flow meter that was supplied by Sensotronic System, Ahmedabad, Gujarat, India.

3.2.3. Sample collection

Inflow and outflow water samples (1L) were collected per week using polyethylene bottles from each CW unit except at time of shifting from one operation condition to another. Accordingly, a total of 12 samples were collected while monitoring the effect of plants for 3 months, 12 samples (3 samples per each retention time) in conducting test of retention time for 4 months and 30 samples (15 from each cold and warm) were considered for monitoring effect of temperature while operating the CWs in 15 months.

Along monitoring of retention time, sediments samples (1gm) were also collected using sterile 50 ml plastic tubes from treatment zones of each CW unit for analysis of microbial community. The sediments (fine gravels and accumulated organic matter) were collected at a depth of 25-35 cm from a distance of 1, 2 and 3 m intervals (in longitudinal direction of the 4 m l**e**ngth of CWs) and at a distance of 0.3, 0.6, and 0.9 m (crosswise direction of the 1.3 m wide CWs) (Fig. 3.1). Thereafter, equal proportion of the 9 sub-samples were mixed and homogenized in order to generate one composite representative sample. Collected water and sediment samples were kept in a cooler and immediately transported to the laboratory for analysis.

3.2.4. Sample analyses `

Temperature and pH were monitored using portable meters (Portable pH/EC/TDS/^{o}C Meters, HI 9811-5, Hanna instrument). Dissolved oxygen (DO) was measured following the procedure APHA 4500-O G. using Membrane Electrode DO meter, ExStik* DO600, Extech, Botson. Tempereture, pH and DO measuring on site were carried out along with sampling of water and sediments for analysis of other water quality and microbial parmeters.

Biochemical oxygen demand after 5 days (BOD_5) was determined following APHA 5210 B. procedures for 5-Day BOD Test and chemical oxygen demand (COD) was measured by APHA 5220 B. Open Reflux Method (APHA, 2012). Total phosphorus (TP) was also measured following the protocol APHA 4500-P C colorimetric Method. The level of ammonium (NH_4^+) was determined using APHA 4500-NH_3 C. Titrimetric Method while nitrate (NO_3^{--}) was following procedure in APHA-4500- NO_3^- with spectrophotometer method (APHA, 2012).

Total genomic DNA of each sediment sample was extracted using Power soil™ DNA isolation kit (Mo bio Laboratories) according to the manufacturer's instructions in microbial ecology loboratory facitliies in Leibniz-Institute of Freshwater Ecology and Inland Fisheries at IGB, Germany. Three replicate DNA extractions of individual sediment samples were carried out and pooled together in equal proportions in order to obtain representative samples. DNA quality was verified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). The extracted DNA was stored at –20^{0}C until the succeeding analyses were conducted.

A ribosomal intergenic spacer analysis (RISA) was used to assess the bacteria communities' profiles in the CW units. It is based on examining variation of the intergenic spacer regions (ITS) between 16S rRNA and 23S rRNA as ITS region varies greatly in length and nucleotide sequences among bacteria. Universal primers ITSF (5‘-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') were used as they are more sensitive to detect OTUs in RISA fingerprint analysis following the procedure used in Cardinale *et al*. (2004).

Table 3.1 PCR reaction mixture

Reagents	Amount (µl)	Reference
PCR buffer(1xTEB + dNTPs + $MgCl_2$)	10.0	
Taq pol (0.05U)	0.5	
Primer-f-(20 Pico mole)	1.0	
Primer-r (20 Pico mole)	1.0	Ramette *et al*., 2009
Sample DNA(1ng-1µg)	5.0	
PCR-H_2O	32.5	
Total	**50.0**	

The RISA-PCR mixture (50 µl) was used 10µl PCR buffer (Promega, Madison, WI); 2.5 mM $MgCl_2$ (Promega); 0.25mM deoxynucleoside triphosphate (mix dNTPs) (Promega); bovine serum albumin (3 µg/µl); 40 Pico mole universal primer ITSF and ITSReub, 0.05U GoTaq poly and 1ng-1µg of extracted DNA as shown in Table 3.1, The PCR mix was similar to the PCR-mix applied in Ramette (2009) with some modification.

PCR reaction was carried out in MasterCycler® X50 (Eppendorf, Hamburg, Germany) which enables extreme rapid changes in temperature. Amplification was done according to the procedure used in Ramette *et al*. (2009) as follows: with an initial denaturation at 94 ^{0}C for 3 min, followed by 30 cycles of 94 ^{0}C for 45 s, 55^{0}C for 45 s, 72 ^{0}C for 90 s, with a final extension at 72°C for 5 min. Following PCR amplification, the amplicons were separated in a 5% polyacrylamide gel matrix and images were recorded using an image analyzer (Li-Cor Inc., Lincoln, NE, USA).

3.2.5. Data analysis

The removal percentage was determined as shown below: where R% is removal percentage, C is the concentration of BOD_5, COD, NH_4^+, NO_3^- and TP in mg/L, subscript i and o represent inflow and outflow samples respectively.

$$R\% = \frac{\mathrm{Ci} - \mathrm{C,o} * 100}{\mathrm{Ci}}$$

All experimental data except the molecular data were expressed at least means of triplicates with standard deviation. Graphical analyses were performed using Microsoft excel. The matrix of RISA profiles were manually performed using the presence–absence bands. Bands were encoded by a number, 0 (absence) and 1 (presence). Bray-Curtis similarity between the RISA profiles of each CW unit were computed and subject*e*d to cluster analysis to produce dendrogram. Statistical significance differences between means of removal efficiencies were analyzed through the use of one-way analysis of variance (ANOVA) and t-test using SPSS (IBM) Version 20.0.

The sedimens bacterial phylogenic analysis was carried out using Past software, version 4.01. Differences in performance among the treatments were considered to be statistically significant if $P \leq 0.05$.

3.3. Results and Discussion

3.3.1. Effect of wetland plants on removal of pollutants

Table 3.2 represents the average removal efficiencies of organic matter (BOD_5 and COD) and nutrients (NH_4^+, NO_3^- and TP) in each CW unit. The highest removal for BOD_5 (75.9%) and COD (75.7%) was achieved in mixed planted unit (Mp), which followed by *Typha* planted (Tp) (BOD_5:73.6% and COD: 73.8%), *Cyperus* (Cp) (BOD_5: 71.2% and COD: 70.7%), *Pennisetum* (Pp) (BOD_5: 63.6% and COD: 63.5%) and the lowest removal corresponded to unplanted unit (Up) (BOD_5: 57.8% and COD: 60.3%)(Table 3.2). Enhanced removal for NH_4^+ and NO_3^- was also attained in Mp (NH_4^+: 91.6% and NO_3^-: 72.9%) and in Tp (NH_4^+: 90.4% and NO_3^-: 72.1%) compared to the rest planted units (Cp and Pp) and unplanted control (Up) (Table 3.2). However, removal for total phosphorus (TP) is limited and ranging from 61% with *Typha* (Tp) to 41% with unplanted (Up) which may related with expected low levels of iron, calcium, magnesium and aluminum hydrous oxides in gravel media for improved adsorption (Mesquita *et al*., 2018).

In general, this removal performance was in the range of the mean removal efficiencies reported in most of the reviewed HSSFCWs from treatment of agricultural and flower wastewaters which was 28% to 99% for BOD_5 and COD in Vymazal (2009), Vymazal and Kröpfelová (2009), Kimani *et al*. (2012) and Sultana *et al*. (2015). It was also in the range of the reported removal percentages from 20% to 80% and 30% to 60% for nitrogen (NH_4^+ and NO_3^-) and total phosphorus (TP) repectively in (Zhou *et al*., 2004; Vymazal, 2007; Vymazal, 2009; Kimani *et* *al*., 2012; Gorgoglione and Torretta, 2018 and Mesquita *et al*., 2018). The exhibited inconsistence in performance of removal often depend on inflow concentrations, type of media used, plants types, operated hydraulic loading rate and hydraulic retention time (Vymazal, 2017; Mesquita *et al*., 2018).

Table 3.2 Pollutant removal statistics of differently planted CW units operating at 4 days of retention time. Note: $p < 0.05$ between a vs. b, c vs. d and e vs. f among columns, outflow (mg/L), RE = Removal efficiency (%)

Parameter	Inflow(mg/L)	Tp		Cp		Pp		Mp		Up	
		Outflow	RE (%)	Outflow	RE (%)	Outflow	RE (%)	Outflow	RE (%)	Outflow	RE (%)
BOD_5	103.3±4.3	27.3±4.0	73.6±3.8ac	29.7±4.9	71.2±2.9a	37.6±6.0	63.6±2.8ad	24.8±4.5	75.9±4.4ac	43.4±7.1	57.8±3.1b
COD	193±4.5	50.6±6.9	73.8±3.2ac	56.6±7.9	70.7±3.6a	70.6±20.1	63.5±5.1ad	46.9±5.4	75.7±2.4ac	76.8±9.4	60.3±2.8b
NH_4^+	7.2±1.3	0.9±0.5	90.4±5.2a	1.2±0.4	83.9±4.4a	1.1±0.6	87.7±4.0a	0.6±0.4	91.6±4.0a	1.6±0.6	77.4±6.1b
NO_3^-	14.4±3.2	3.9±2.0	72.1±2.5a	4.6±2.3	68.5±1.7a	4.6±2.5	67.8±5.1a	4.0±1.9	72.9±3.2a	5.6±2.7	57.7±7.1b
TP	5. 3±2.5	2.0±1.0	61.0±4.0a	2.1±1.0	60.3±1.5a	3.1±+1.5	41.7±0.6	2.1±.9	60.3±4.5a	3.1±1.5	41.0±3.5b

In most cases, the removal findings > 40% in both planted and unplanted CW units might suggest involvement of others removal processes such as media adsorption-priciptation and microbial degradation (Table 3.2). However, a significantly enhanced ($p < 0.05$) reduction of organic matter (BOD_5 and COD) and nutrients (NH_4^+, NO_3^- and TP) in planted CW units than unplanted (Up) except TP in Pp indicates a substantial contribution of the plant species on performance of the CWs. This is consistent with the findings of previous study (Zhang *et al*., 2016) which reported higher removal for water quality parameters (COD, NH_4^+, NO_3^- and PO_4^{3-} by 6.4%, 15.5%, 30.6% and 12.1% respectively) in *Typha* planted HSSFCWs receiving caffeine-enriched wastewater compared to the unplanted control in tropical environment. The higher removal efficiency in planted units could be plant assisted microbial processes in rhizosphere (Bastviken *et al*., 2005; Vymazal, 2011) and plant uptaking (Chan *et al*., 2008; Zhu *et al*., 2017) where their nutrients mass balance analysis showed a plant up taking accounting for 10–15% of the nutrients removal in CWs.

Among the single planted CW units, mean removal were in the order of Tp > Cp > Pp (Table 3.2) where both BOD_5 and COD removal were significantly higher ($p < 0.05$) in Tp CW unit compared to Pp. The enhanced removal for *Typha* may associate with its vigorous root systems to provide oxygen and exudates for enhanced rhizoshphere microbial removal processes in the CWs. The vigorous growth of the plant also reported to sequester a high amount of nutrients in the above ground biomass which estimated to be between 400–600 kg nitrogen per hectar (Vyzmal, 2017). A similar plant performance difference for removal of organic matter and nutrients were also observed in the work of Akratos and Tsihrintzis (2007) on which higher removal efficiency was observed in *Typha* planted HSSFCW units compared to *Phragmites australis*.

Moreover, an important enhanced removal of organic matters and nutrients observed in CW units with mixed planting (Mp) (Table 3.2). This could be attributed to functional complementarity among *Typha latifola*, *Cyperus papyrus* and *Pennisetum purpureum.* The complementarity reported to be attributed to the temporal and spatial compensations in plant activity as well a root affinity for different microorganisms (Peng *et al*., 2014; Rodriguez and Brisson, 2016; Zhu *et al*., 2017). The overall result of this study demonstrates that plant presence, plant types and plant combination in CW are important factors affecting removal of organic matter and nutrients from floriculture wastewater in HSSFCWs. Accordingly, the combination of the selected species of *Typha*, *Cyperus and Pennisetum* (Mp) and *Typha* (Tp) from single planting could offer a good approach in flower industry for enhancing treatment performance of HSSFCWs as it observed from removal of organic matter and nutrients in this study.

3.3.2. Effect of hydraulic retention time on removal of pollutants

In this study, effect of four different (1, 2, 3 and 4 days) retention times on removal of organic matter and nutrients were assessed in differently planted CWs (Fig. 3.2). The effluent concentrations at 1 day of retention time were beyond the acceptable discharging limit i.e. > 30 and > 120 mg/L for BOD_5 and COD respectively considering all the CW units. On the other hand, a higher removal of BOD_5 (> 69%) and COD (> 64%) was obtained from higher retention time of 4 days compared to removal of BOD_5 (< 35%) and COD (< 45%) that obtained from 1 day retention time. At retention time of 4 days, the CW units demonstrated to be effective in removing the inflow organic matter to acceptable limits for wastewater discharging set by Ethiopia EPA (2003). The difference in the removal rates for the two retention times could be duration of contact between dissolved organic matter and critical removal components (media, plants and microorganisms) of the CW units.

Moreover, the removal of NH_4^+ statistically increased as a result of extending the retention time from 1 day to 2 day as well as from 2 day to 3 day in CW units. However, more statistically enhanced NH_4^+ removal efficiency (by >17% to 54% considering all of the CW units) was obtained at 4 day of retention time which was by far below EPA permissible limit of 1mg/L for NH_4^+ in treated effluents (EPA, 2003). This result was in contrast to Akratos and Tsihrintzis (2007) which showed a retention time of 8 days was not adequate for permissible removal of NH_4^+ in HSSFCW units treating domestic wastewater. Higher inflow NH_4^+ (38.4 mg/l) in their inflow wastewater compared to lower inflow (7.2 mg/l) in this study may account for higher removal (> 90%) of NH_4^+ and attaining below discharging standard at a shorter retention time of 4 days.

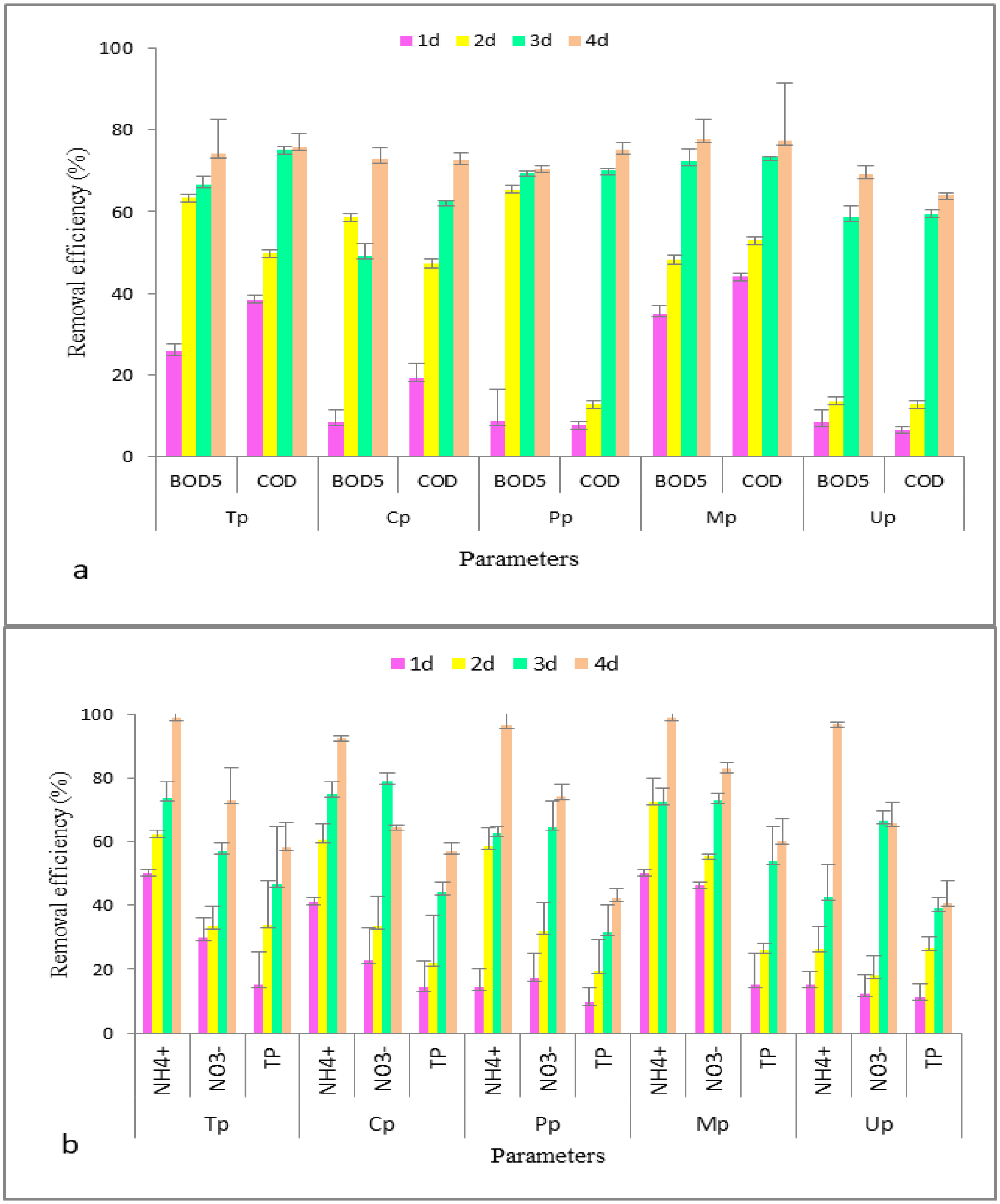


Fig. 3.2 Removal efficacy (a) BOD_5 and COD (b) NH_4^+, NO_3^- and TP at various retention times

Further increase of NO_3^- removal (by > 16.0%, 13.0%, 9.0%, 9.0% and 1.0%, in Tp, CP, Pp, Mp and Up respectively) obtained at 4 day of retention time compared to the 3 days. A maximum NO_3^- removal efficiency of 73% was obtained at a retention time of 4 day in a *Typha* planted HSSFCW. The removal efficiency of NO_3^- was significantly lower compared to the removal of NO_3^- (97%) obtained from *Typha angustata* planted subsurface flow constructed wetland unit for same 4 day of retention time in Ghosh and Gopal, (2010). This noticeable removal difference could be emanated from difference in CW configuration, operational parameters, composition of waste water and other external factors. With reference to the EPA maximium permissible limit of 10 mg/L for NO_3^-, the result obtained in this study showed a residence time of 4 day can be applied for passable removal of NO_3^- from floriculture wastewater in HSSFCWs.

Compared to 3 days of hydraulic retention times, there was a significant increase ($P < 0.05$) in phosphorus (TP) removal from all CW units, except in Up unit upon 4 days of retention times. However, it is insufficient to meet the required EPA phosphorus discharging standard (1mg/L) in all CW units which necessitating hydraulic retention times of higher than 4 day. Akratos and Tsihrintzis (2007) suggested extending hydraulic retention time to increase the contact time between removal components of the CWs and reduce TP to a permissible level than the other pollutant parameters (BOD_5, COD, NH_4^+ and NO_3^-) in HSSFCW units treating secondary waste water. Thus, extended retention time (> 4 days) and/or others phosphorus removal enhancing approachs need to be considered for addressing the TP removal limitation.

3.3.3. Effects of plant species and retention time on microbial community

The relationship of bacterial community diversity with plant species and water retention time was analyzed using ribosomal intergenic spacer analysis (RISA) profiles of samples from each

CW units (Fig. 3.3a). The cluster analysis of the RISA profiles showed related fingerprints per CW unit regardless of the retention time (Fig. 3.3b) except Tp1, Cp4, Pp3 and Pp4.

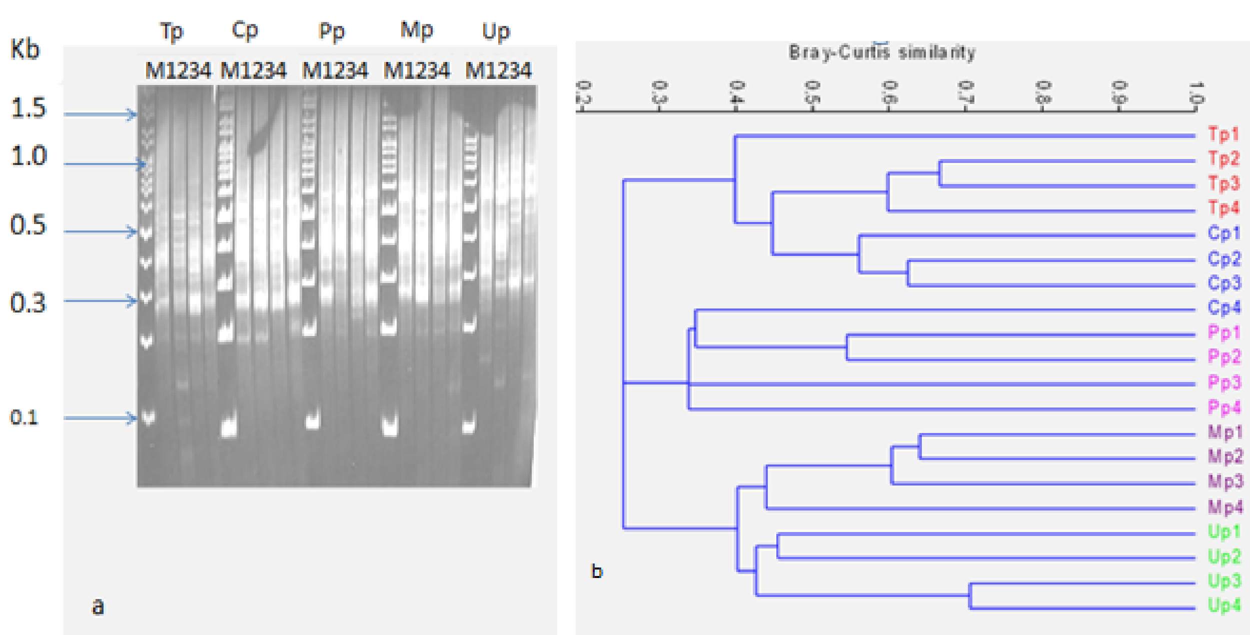


Fig. 3.3 (a) RISA profiles from sediments of CW units resolved on 5% polyacrylamide and (b) dendrogram revealing relatedness of RISA fingerprints, M – molecular size marker, 1, 2, 3 and 4 retention time in days.

Previous studies in Calheiros *et al*. (2010a) and Zhang *et al*. (2016) also showed plant species influence the structure of microbial communities in CW units due to difference in plant activity and root affinity for microbial colonization. In this study, the samples clustered in most cases with similarities > 50% per CW units (Fig. 3.3b), demonstrating certain bacteria populations groups are specific per CW unit.

Moreover, clustering of the RISA fingerprint per change of retention time per CW unit seems have a shift of bacteria community profiles (Fig. 3.3b). It implies the influence of the shift of retention time on sediment bacterial community compositions. To the overall, the RISA fingerprint results indicating both wetland planting conditions and shift of retention time to have a greater influence on modulating of sediment bacterial community.

Table 3.3 represents the diversity indices of the bacteria community based on RISA profiles. It showed apparently different values per CW units as well as the tested retention times (Table 3.3). The higher Shannon's diversity indexes (2.39) in both Tp and Mp CW units linked with their higher treatment performance for organic matter and nutrients compared to the other CW units. The overall diversity per retention time (Table 3.3) also positively correlated with the overall removal of BOD_5, COD, NH_4^+, NO_3^- and TP per retention time (Fig. 3.2) i.e the higher overall diversity index (2.28) corresponded to the higher all over removal efficiency at the extended retention time of 4 days. Corroborating the present study, Calheiros *et al*. (2010a) reported Shannon's diversity index > 1.0 for HSSFCWs with high efficiency of organic removal from wastewater. In general, the results obtained from analysis of the bacterial diversity reinforce the difference revealed by cluster analysis per plant types and retentions time in the CW units.

Table 3.3 Shannon diversity (H) and the Simpson's indexes (D) of RISA profiles

CW Units	Shannon-Wiener (H)					Simpson's (D)				
	R1	R2	R3	R4	**avg.**	R1	R2	R3	R4	**avg.**
Tp	2.08	2.30	2.49	2.71	**2.39**	0.88	0.90	0.92	0.93	**0.91**
Cp	2.20	2.08	2.20	2.37	**2.16**	0.89	0.78	0.89	0.92	**0.89**
Pp	1.95	2.20	2.08	2.20	**2.10**	0.86	0.86	0.88	0.89	**0.88**
Mp	2.57	2.30	2.57	2.49	**2.39**	0.89	0.89	0.92	0.92	**0.91**
Up	1.79	1.79	1.95	1.61	**1.78**	0.83	0.83	0.86	0.80	**0.83**
Overall	**2.12**	**2.13**	**2.26**	**2.28**	**2.16**	**0.87**	**0.85**	**0.89**	**0.89**	**0.88**

3.3.4. Effect of seasonal temperature

Fig. 3.4a presents the trend of seasonal water temperature that was monitored for 15 months in the CW units along with the collection of water and sediment samples. The difference of the daily water temperature among the CW units ranged between 0.1 and 3.3 ^{0}C (Fig. 3.4a). One-way ANOVA showed the difference among the units was not significantly ($P > 0.05$) and could not make an important performance difference between the CW units (El-Refaie, 2010)

On the other hand, the mean seasonal water temperature ranged between a minimum of 13.5 ^{0}C in cold season to maximum of 25.5 ^{0}C in warm (Fig. 3.4a). With reference to the temperature value (20 ^{0}C) used to calculate temperature dependent removal constant (K) in treatment wetland (EI-Refaie, 2010), a higher water temperature ($\geq 20^{0}$C; 22.3±1.2^{0}C) mostly detected from end of February to end of June while the lower water temperature ($< 20^{0}$C; 16.9±1.3^{0}C) from October to February (Fig. 3.4a). Based on the t- test results, the difference between overall average values of the temperature in cold season and warm season was significant ($t = -14.2$ and $p < 0.001$) in all CW units. Such wide seasonal water temperature variation in CWs was also detected in previous studies on this issue in Akratos and Tsihrintzis (2007) and EI-Refaie (2010) and found to affect the treatment performance of engineered wetland systems.

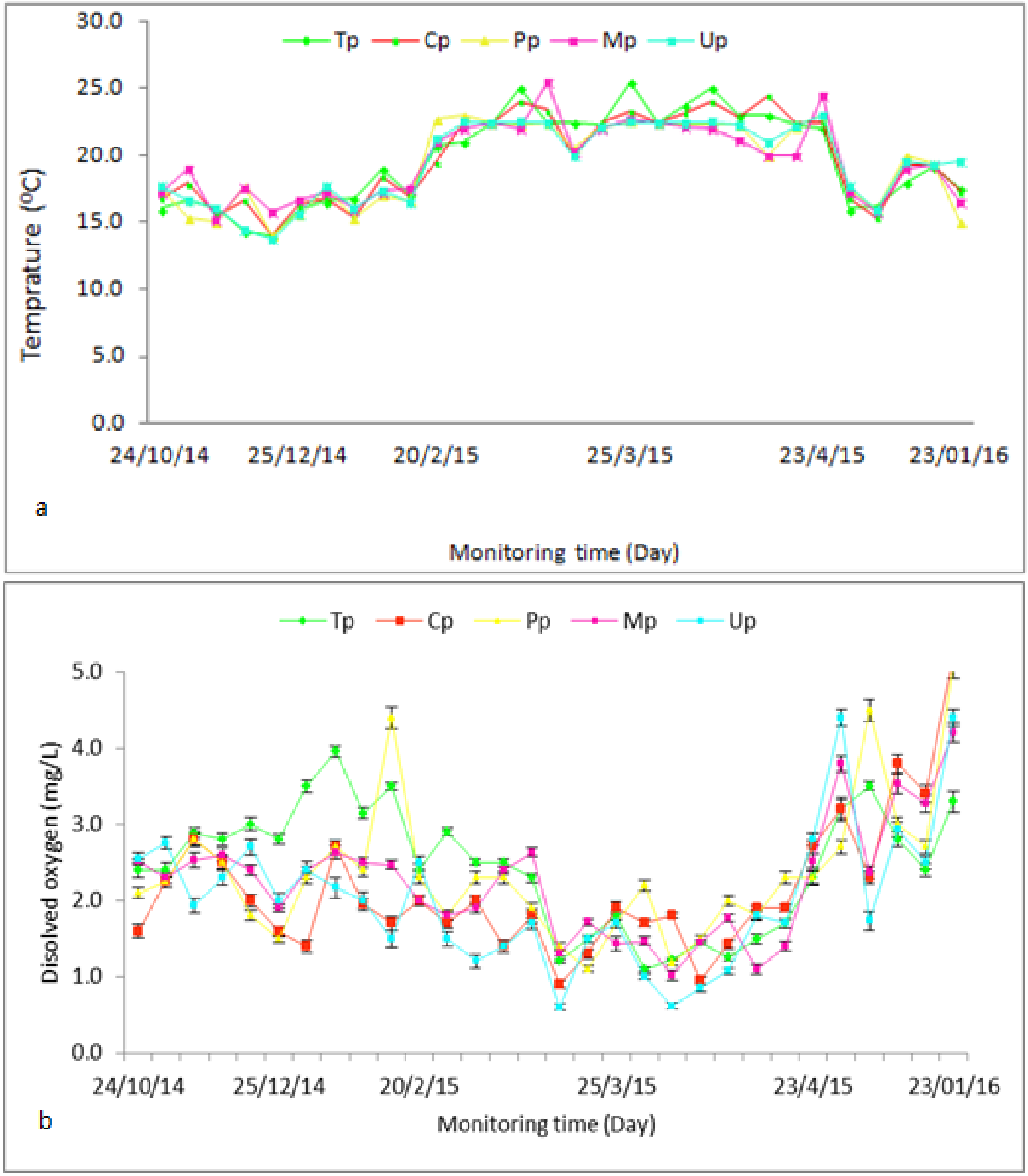


Fig. 3.4 Changes of (a) water temperature and (b) DO in CW units along the operation period

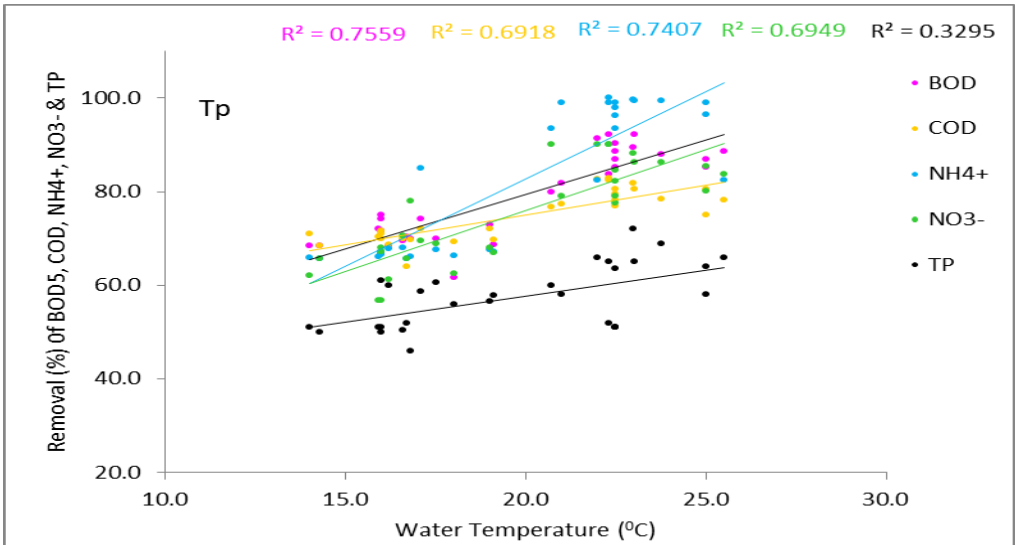
Fig. 3.4b denotes the trend of dissolved oxygen (DO) in the CW units. The level of DO showed strong inverse relation (r^2 = 0.58; P = 0.0001) with the seasonal water temperature i.e. highest levels of DO > 4mg/L was detected in cold season and 2mg/L in warm season (Fig. 3.4a). This results were comparable to other findings where DO showed negative relation with water temperature in CWs (Akratos and Tsihrintzis, 2007; Ketti *et al*., 2010). In general, the solublity of oxygen decreases with increasing of water temperature. The high rate of aerobic dependent metabolisms in the CW units such as organic matter degradation and nitrification may also contribute for low level detection of oxygen in the warm climatic conditions (Vymazala and Kröpfelova, 2009; Kotti *et al*., 2010).

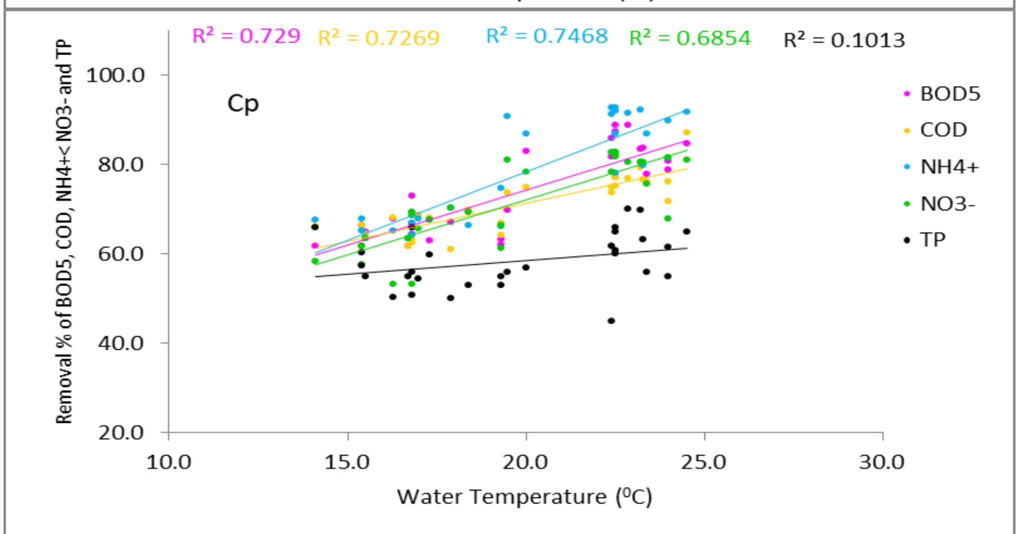
Fig. 3.5 represents correlation chart of pollutants removal with seasonal water temperature in the CW units. The significant scatter suggests involvements of several other factors in addition to temperature which affected the removal processes (Kotti *et al*., 2010). However, the direct relationship ($r^2 > 0$) of removal efficiencies of BOD_5, COD, NH_4^+, NO_3^- and TP with the temperature, specifically indicating a seasonal temperature dependency of the performance of the CW units for reduction of organic matter and nutrient from the wastewater. This result was in contrast to the findings of Akratos and Tsihrintzis (2007) and El-Refaie (2010) from temperate and tropical conditions respectively that showed insignificant and negative correlations for removal of organic matter (BOD_5, COD) vs. seasonal wastewater temperature in HSSFCWs.

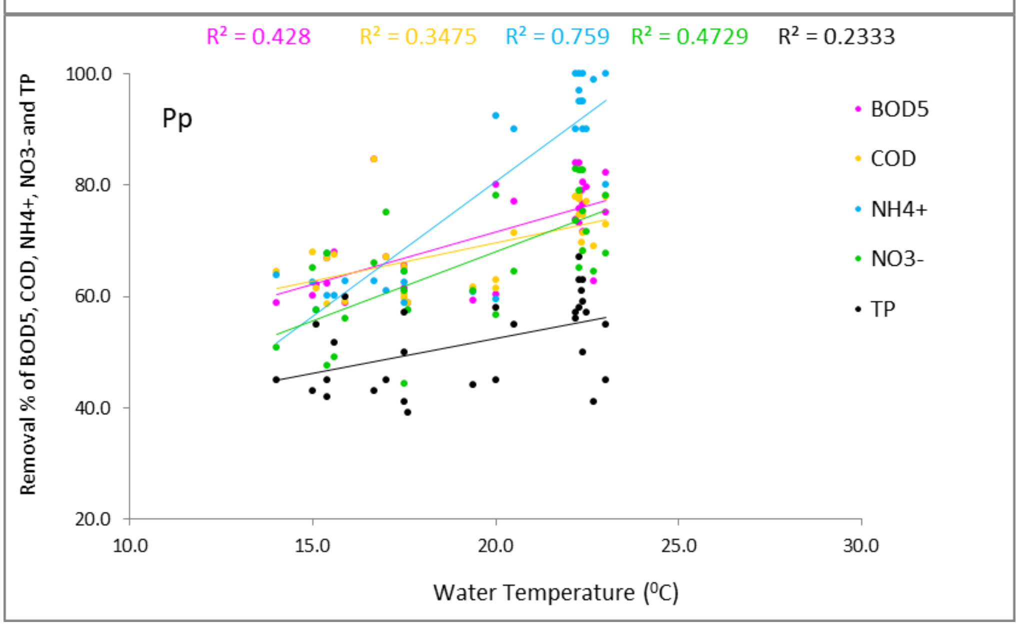
Moreover, some of the parameters in this study are also strongly correlated with changes of the temperature in the CW units. For instance, the NH_4^+ removal rate was strongly dependent ($r^2 > 0.70$, $p < 0.001$ except in Up unit) on the temperature (Fig. 3.5). It could be related to the high sensitivity of NH_4^+ removal processes to change of temperature, such as NH_4^+ volatilization, microbial and plant enzymatic activities (Almukta *et al*., 2018). The removal of BOD_5 also showed high temperature dependence than that of COD, especially in units Tp, Cp and Up (Fig.

3.5). This is in line with the BOD_5 removal mechanism, where microbial degradation in the attached sediment plays the dominant role (Kotti *et al*., 2010).

On the other hand, the relative weak correlation ($r^2 < 0.20$, $p > 0.05$, in Cp and Up) between removal of TP (total phosphorus) and seasonal temperature (Fig. 3.5) implies its overall removal was less dependence on temperature. The major processes for phosphorus retention namely; adsorption and precipitations are mostly temperature independent. Whereas the temperatur dependence biological processes such as microbial and plant uptake, contribute marginally to the over all phosphorus removal (Vymazal, 2011). Previous results of wetland phosphorus removal data from cold to warm climates also showed minimal effects of temperature, ($r^2 < 0.05$) for the range of -3^{0}C to 30 ^{0}C (Kadlec *et al*., 2005; El-Refaie, 2010). As temperature increases, there will be also enhanced plant litter breakdown in the CWs which releases phosphorous to the water and thus, may account for the weak correlation of phosphorus removal with temperature. A similar conclusion was reached for an increase of phosphorous level as response to high temperature from the tropical wetland in Almukta *et al*. (2018).


R² = 0.7559
R² = 0.6918
R² = 0.7407
R² = 0.6949
R² = 0.3295
Tp
Removal (%) of BOD5, COD, NH4+, NO3- & TP
Water Temperature (⁰C)
BOD
COD
NH4+
NO3-
TP


R² = 0.729
R² = 0.7269
R² = 0.7468
R² = 0.6854
R² = 0.1013
Cp
Removal % of BOD5, COD, NH4+< NO3-and TP
Water Temperature (⁰C)
BOD5
COD
NH4+
NO3-
TP


R² = 0.428
R² = 0.3475
R² = 0.759
R² = 0.4729
R² = 0.2333
Pp
Removal % of BOD5, COD, NH4+, NO3-and TP
Water Temperature (⁰C)
BOD5
COD
NH4+
NO3-
TP

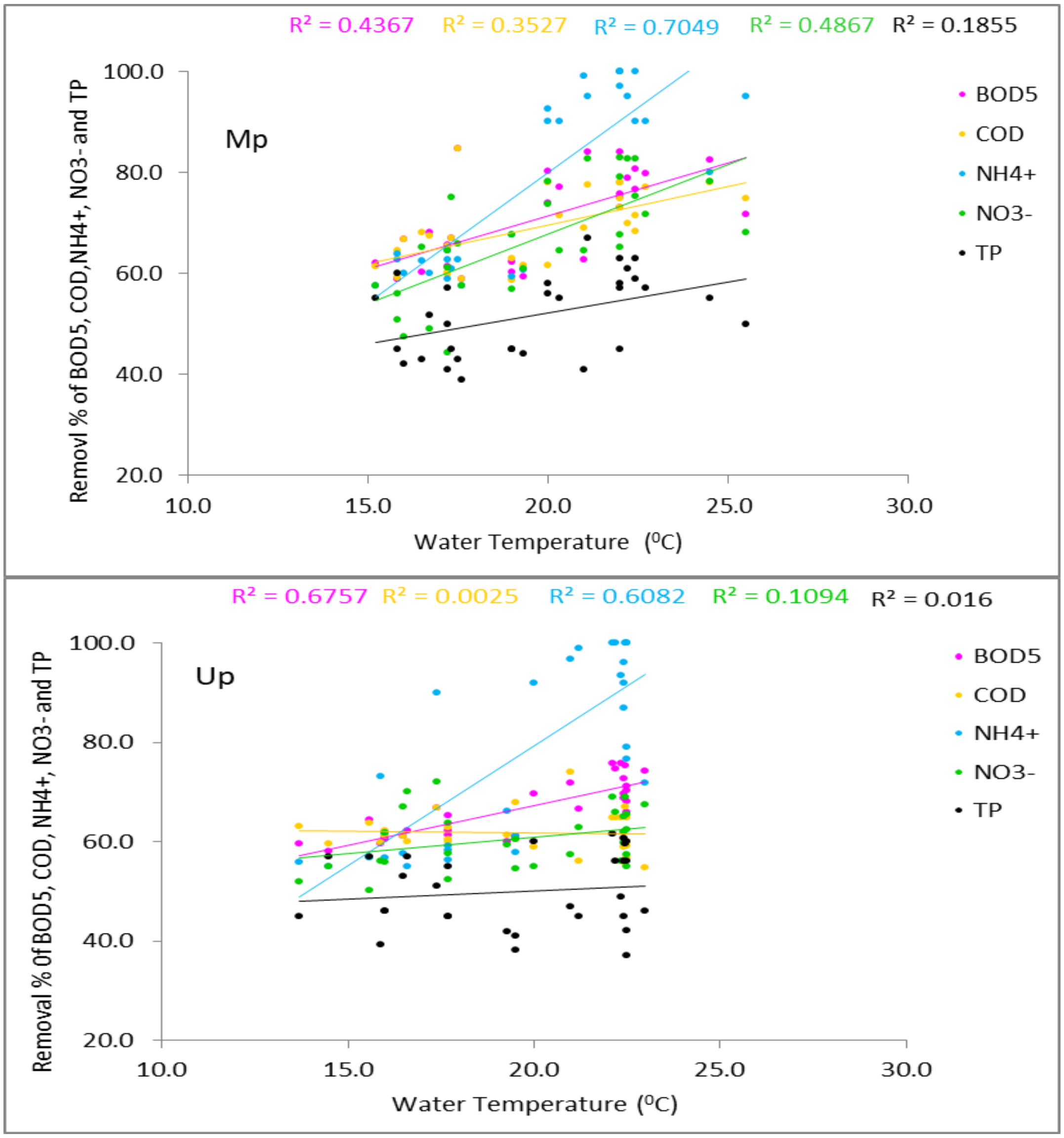


Fig. 3.5 Correlation charts in CW units (Tp, Cp, Pp, Mp and Up): removal Vs. temperature

Table 3.4 also represents summary of the removal statistics for cold and warm seasons. On the base of t-test analysis, the overall removal performance difference was significant ($t = 14.424$ and $p < 0.01$) between the two seasons. The removal performance difference between the two temperature range were also significant ($t > 0.177$ and $p < 0.05$) in each CW units except for COD, NO_3^- and TP in Up CW units and NO_3^- and TP in Mp CW unit. This result also reinforces the correlation analysis in Fig. 3.5 and previous findings in Kotti *et al*. (2010); Khisa *et al*. (2014) and Mesquita *et al*. (2018) which showed organic matter and nutrients removal tend to be higher in warm seasons than those obtained for cold periods in both temperate and tropical conditions.

Table 3.4 Summary of mean removal percentage statistics for temperature < and ≥ 20 ^{0}C, n = 15, * = p > 0.05.

Parameters	Cold season (mean: 16.9±1.3^{0}C & range: 13.5 ^{0}C -19.7^{0}C)					Warm season (mean: 22.3±1.2^{0}C & range: 20 -25.5 ^{0}C)				
	Tp	Cp	Pp	Mp	Up	Tp	Cp	Pp	Mp	Up
BOD_5	69.5±2.6	64.5±2.8	63.4±6.7	75.1±4.0	61.5±1.9	87.4±3.7	82.0±5.1	77.0±5.5	89.1±2.9	71.4±3.3
COD	69.9±1.4	65.3±2.6	64.2±6.4	76.1±3.8	61.8±1.9*	79.5±2.4	76.6±3.5	73.4±4.8	82.4±3.1	61.5±5.2*
NH_4^+	68.0±4.8	67.0±2.9	61.4±2.8	76.7±8.2	61.4±9.3	95.8±5.8	88.4±4.9	93.4±6.6	96.9±4.6	92.2±9.4
NO_3^-	65.9±5.4	63.3±5.7	58.7±8.4	75.6±5.4*	59.2±6.6*	84.9±4.5	79.9±3.9	74.5±7.1	78.2±5.1*	62.0±5.0*
TP	54.2±4.8	56.2±5.0	47.1±6.2	56.7±4.5*	47.8±6.6*	60.8±7.0	60.8±6.4	56.3±6.8	61.2±7.5*	52.1±8.0*

In general, the overall analysis of this study highlighted the potential effect of seasonal temperature on performance of CWs for organic matter and nutrients from floriculture wastewater under tropical condition. The treatment performances decreased due to low temperature of the cold season in continuously operating CWs and thus, may fail to meet the discharging limits. Hence, the effect of the changes in pollutants concentration by seasonal temperature should be considered when designing and operating CWs to make sure of effective purification.

3.4. Conclusion

In this study, the planted CW units showed a higher removal efficiency of BOD_5, COD, NH_4^+, NO_3^- and TP compared to unplanted corresponding and supported positive effect of plants in CW. Among the single planted CW units, the *Typha* planted (Tp) showed better on overall removal performance for BOD_5, COD, NH_4^+, NO_3^- and TP than *Cyperus* planted (Cp) and *Pennisetum* planted (Pp). However, the three plants in combination from Mp unit indicated a substantial enhanced treatment performance than all single planted planted (Tp, Cp and Pp units). A retention time of 4 days was found adequate for acceptable removal of BOD_5, COD, NH_4^+ and NO_3^- from floriculture wastewater in HSSFCW. The clustering analysis of RISA fingerprints highlighted that the type of wetland plant and water retention time seems to have a major effect on the established sediment bacterial communities where their diversity positively correlated with pollutant removal efficiency in the CWs. Moreover, the results obtained from removal efficiencies of BOD_5, COD, NH_4^+, NO_3^- and TP showed a seasonal dependency, with higher values during the warmer period. All the above results demonstrated that *Typha* and mixed planting CWs operating with 4 days of retention time could be a good strategy to enhance the purification of nutrients under most temperature regimes.

Reference

Akator and Tsihrintzis, V. A. (2007). Effect of temperature, HRT, vegetation and porousmedia on removal efficiency of pilot-scale horizontal subsurface flow constructed wetlands. *Ecology Engineering* **29**:173–191.

Almuktar, S. A. A. A. N., Abed1, S. N. and Scholz1, M. (2018). Wetlands for wastewater treatment and subsequent recycling of treated effluent: a review. *Environmental Science and Pollution* **25:**23595–23623.

APHA (2012). Standard Methods for the Examination of Water and Wastewater, 22nd ed. Washington DC, USA.

Bastviken, S. K., Eriksson, P. G., Premrov, A. & Tonderski, K. (2005). Potential denitrification in wetland sediments with different plant species detritus. *Ecological Engineering* **25**: 183–190.

Brisson, J. and Chazarenc, F. (2009). Maximizing pollutant removal in constructed wetlands: Should we pay more attention to macrophytes. *Science and Total Environment* **407**:3923–3930.

Calheiros, C.S.C., Teixeira, A., Pires, C., Franco, A.R., Duque, A.F., Crispim, L.F. C., Moura, S.C., and Castro, P. M. L. (2010a). Bacterial community dynamics in horizontal flow constructed wetlands with different plants for high salinity industrial wastewater polishing. *Water research* **44:**5032-5038.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E. Sorlini, C. Corselli, C. and Daffonchio, D. (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Applied Environmental Microbioogy* **70:**6147– 6156.

Chan, S. Y., Tsang, Y. F. & Chua, H. (2008). Domestic wastewater treatment using tidal-flow cinder bed with Cyperus alternifolius. *Aquatic Ecosystem Health and Management* 11:206–211.

El-Refaie, G. (2010). Temperature impact on operation and performance of Lake Manzala Engineered Wetland, Egypt. *Journal of Ain Shams engineering* **1**:1-9.

EPA (2003). Environment Protection (Standards for effluent discharge).

Ethiopian Horticulture Producer Exporters Association (EPEA) (2015). EHPEA resource centre. http://www.ehpea.org..

Fraser, L. H., Carty, S. M. and Steer, D. (2004). A test of four plant species to reduce total nitrogen and total phosphorus from oil leachate in subsurface wetland microcosms. *Bioresource Technology* **94:**185–192.

Ghosh, D. and Gopal, B. (2010). Effect of hydraulic retention time on the treatment of secondary effluent in a subsurface flow constructed wetland. *Ecological Engineering* **36:**1044-1051.

Golda, A. E., Poyyamoli, G. and Nandhivarman, M. (2014). Efficacy of phytoremediation potential of aquatic macrophytes for its applicability in treatment wetlands: A review of developments and research. *International Journal of Water Research Environmental and Engineering* **6(10):**267-278.

Gorgoglione, A. and Torretta, V. (2018). Sustainable Management and Successful Application of Constructed Wetlands: *A Critical Review* **10:**2-19.

Kadlec, R.H., Tanner, C.C., Hally, V.M. and Gibbs, M.M. (2005). Nitrogen spiraling in subsurface-flow constructed wetlands: Implications for treatment response. *Ecological Engineering* **25:**365–381.

Kimani1, R. W., Mwangi, B. M. and Gichuki, C. M. (2012). Treatment of flower farm wastewater effluents using constructed wetlands in lake Naivasha, Kenya. *Indian Journal of Science and Technology* **5(1**):1870–1878

Khisa, K. (2014). The Efficacy of a Tropical Constructed Wetland for Treating Wastewater during the Wet Season: The Kenyan Experience. *Journal of Environment and Earth Science* **4(15):**2224-3216.

Kotti, I.P., Gikas, G.D. and Tsihrintzis, V.A. (2010). Effect of operational and design parameters on removal efficiency of pilot-scale FWS constructed wetlands and comparison with HSF systems. *Ecological Engineering* **36:**862-875.

Mesay Adugna (2017). Review on environmental effects of Ethiopian floriculture industry. *Asian Research Journal of Agriculture* **4(2):**1-13.

Mesquita, C., Albuquerque, A., Amaral, L. and Nogueira, R. (2018). Effectiveness and Temporal Variation of a Full-Scale Horizontal Constructed Wetland in Reducing Nitrogen and Phosphorus from Domestic Wastewater. *Chemical Engineering* **2(3)**:1-24.

Mustapha, H.I., Bruggena, J.J.A. and Lens P.N.L. (2015). Vertical subsurface flow constructed wetlands for polishing secondaryKaduna refinery wastewater. *Ecological Engineering* **84**:588–595.

Peng, L., Hua, Y., Cai, J., Zhao, J., Zhou, W. and Zhu, D. (2014). Effects of plants and temperature on nitrogen removal and microbiology in a pilot-scale integrated vertical-flow wetland treating primary domestic wastewater. *Ecological Engineering* **64**:285–290.

Ramette, A. (2009). Quantitative Community Fingerprinting Methods for Estimating the Abundance of Operational Taxonomic Units in Natural Microbial Communities. *Applied Environmental Microbiology* **75(8**):2495–2505.

Rodriguez, M. and Brisson, J. (2016). Does the combination of two plant species improve removal efficiency in treatment wetlands? *Ecological Engineering* **91**:302–309.

Sultana, M.Y., Mourti, C., Tatoulis, T., Akratos, C.S., Tekerlekopoulou, A.G. and Vayenas, D.V. (2015). Effect of hydraulic retention time, temperature and organic load on a horizontal subsurface flow constructed wetland treating cheese whey wastewater. *Journal Chemical Technology and Biotechnology* **91**:726–732.

Tadele Yeshiwas and Melkamu Alemayehu (2018). Social, economical and environmental issues of floriculture sector development in Ethiopia. *Review of Plant Studies* **5(1)**:1-10.

Vymazal, J. (2007). Removal of nutrients in various types of constructed wetlands: a review. *Science of the Total Environment* **380**:48–65.

Vymazal, J. (2008). Constructed wetlands for wastewater treatment. *Wate*r **2**:530–549.

Vymazal, J. (2009). The use constructed wetlands with horizontal sub-surface flow for various types of wastewater: a review. *Ecological Engineering* **3(5):**1–17.

Vymazal, J. (2011). Plants used in constructed wetlands with horizontal subsurface flow: a review, *Hydrobiologia* **674**:133–156.

Vymazal, J. (2017). The use constructed wetlands for nitrogen removal from agricultural drainage: a review. *Scientia Agriculturae Bohemica* **2:**82–91.

Vymazal, J. and Kröpfelová, L. (2009). Removal of organics in constructed wetlands with horizontal subsurface flow: a review of the field experience. *Science of the Total Environment* **407**:3911-3922.

Wu, H., Zhang, J., Ngo, H.H., Guo, W., Hu, Z., Liang, S., Fan, J. and Liu, H. (2015). A review on the sustainability of constructed wetlands for wastewater treatment: design and operation. *Bioresources and Technology* **175:**594–601.

Zhang, D., Luo, J., Lee, Z.M.P., Grasberg, M.R., Liu, Y., Tan, K. S. and Ng, J.W. (2016). Characterization of microbial communities in wetland mesocosms receiving caffeine-enriched wastewater. *Environmental Science and Polluiont Research* **23**:14526–14539.

Zhou, Q., Zhang, R., Shi, Y., Li, Y., Paing, J. and Picot, B. (2004). Nitrogen and phosphorus removal in subsurface constructed wetland treating agriculture stormwater runoff. *In Proceedings of the 9th International Conference Wetland Systems for Water Pollution Control, Lyon, France 26–30*.

Zhu, H., Zhou, Q. W., Yan, B.X., Liang, Y.X., Yu, X.F., Gerchman, Y., and Cheng, X.W. (2017). Influences' of vegetation type and temperature on the performance of constructed wetlands for nutrient removal. *Water Science hand Technology in press.*

4. Bacterial Community Profile and Their Removal Efficiency in Differently Planted Horizontal Subsurface Flow Constructed Wetland Treating Floriculture Wastewater in flower farm, Bishoftu Town, Ethiopia

Abstract

Bacterial communities associated with wetland plants are major partners in the removal of pollutants from constructed wetland (CWs) treatment plants. However, there is still a knowledge gap on the link among bacterial community structure of wetland, plant composition and treatment efficiency under tropical conditions. The objective of this study was to characterize the bacterial community structure of a differently planted horizontal subsface flow constructed wetland (HSSFCW) in relation to their organic matter and nutrients removal efficiency. Sediment samples were analyzed from treatment zones of single planted (Cp) mixed planted (Mp) and unplanted (Up) CW units. The bacterial diversity was analyzed using high throughput sequencing of the 16S rDNA gene. A total of 2793, 2554 and 2282 operational taxonomic units (OTUs) with 26, 28 and 22 phyla wee identified from samples of Cp, Mp and Up CW units respectively. The firmicutes *were dominant in planted CW units, contributing to 40.9% of the bacterial flora in single planted (Cp) CW units whereas* Proteobacteria *contributing to 56.4% of the flora in the unplanted (Up) units.* Trichococcus, Chlorobium *and* Thermomonas *were the dominant genera representing a share of 18.1%, 13.5%, and 14.2% of the microflora in the Cp, Mp and Up units respectively. This analysis showed that higher relative sequence abundance was recorded at all taxonomic levels (phylum, class, genus, etc) in planted CW units than the unplanted control unit. These higher relative sequence abundances also corresponded to the higher removal efficiencies of BOD_5, COD, NH_4^+, NO_3^- and TP obtained in the planted CW units. Species richness (OTUs and Chao) and Shannon diversity index (H) as well some of the specific bacterial taxa were positively correlated (r > 0.50) with removal of BOD_5, COD, NH_4^+,*

NO_3^- and TP. In general, our data showed the sediment bacterial communities are influenced by CW planting condition which in turn affects the pollutant removal efficiency of the CWs. The results from this study will contribute to our understanding how wetland planting could affect sediment bacterial community profiles and consequently the pollutants removal from the wastewater.

Keywords: Bacterial community, firmicute, pollutants removal, Proteobacteria, waste water treatment

4.1. Introduction

The use of excess agro-chemicals in agricultural and horticultural activities contributes to point and non-point source pollution of soil and aquatic environments which are implicated with health and environmental hazard (Berhan Teklu *et al.*, 2016a). Conventional stabilization pond wastewater treatment technologies are considered less efficient in the treatment of most of the agro-chemicals (Miège *et al.*, 2009). Thus, the residual chemicals that discharge into rivers, streams and other surface waters may not attain-tolerable limits after such treatment.

Constructed wetlands (CWs) have gained attention for remediation of agricultural wastewater (Vymazal, 2011). They have been successfully used to reduce a wide range of pollutants from various types of wastewaters through combined effects of wetland plants and associated microbial communities (Verlicchi and Zambello, 2014). Although active research on the importance of artificial CWs with different plant systems has begun since the 1950's, the recognition of the rhizosphere microbes as a "hotspot" for the degradation of pollutants is quite recent. Thus, a few studies showed the relationship amongst diversity of wetland bacterial communities, wetland plants and types of waste water and efficiency of nutrient removal of the CWs (Zhang *et al.*, 2016).

Bacterial communities are distributed mainly on the surface of the roots (rhizosphere) of the macrophytes, biofilms surrounding the general media (sediment) and water column of the treatment system (Weber, 2016). These microbial communities offer suitable ecological niches to support a diversity of metabolic pathways to enhance rapid degradation of waste constituents in CWs. It is also established that sediment microbial communities dominate transformation of pollutants rather than the free bacteria in the water column (Weber and Gagnon, 2014).

The wetland microbial taxonomic and functional diversity is influenced by design and operational conditions of the CWs (Button *et al*., 2015; Wang *et al*., 2016; Weber, 2016). Wetland plants (macrophytes) in the constructed wetlands accommodate more microbial communities (Yan *et al*., 2017). The plant species had a remarkable modulating effect on the structure of sediment microbial community though microbial affinity of different planting approaches was largly unknown.

In Ethiopia, Adey Desta *et al*. (2014) reported the microbial structure of a constructed wetland integrated with anaerobic-aerobic reactors treating tannery wastewater in Modjo town, Ethiopia. The authors showed diverse bacterial phyla and implicated with the removal of various carbon containing pollutants. However, there is still a need to show the relationship between microbial community structures of different CWs treating different wastewaters. The current study shows the composition, structure and diversity of the bacterial communities in a horizontal subsurface flow constructed wastewater (HSSFCW) system treating wastewater from a flower farm in Bishoftu Town, Ethiopia.

4.2. Materials and methods

4.2.1. Components of the constructed wetland system

In this study, a horizontal subsurface flow constructed wetland system (HSSFCW) was configured according to Fig. 4.1. The CW had three units; single planted (Cp), mixed planted (Mp) and unplanted (Up) units (Fig. 4.1). The Cp unit was planted with *Cyperus papyrus;* whereas the Mp contained equal proportions of *Cyperus papyrus, Pennisetum purpureum* and *Typha latifola*. The third unit (Up) did not contain any plant and used as a control. All the units were monitored at a hydraulic retention time of 4 days. These CWs units were selected based on wide difference in treatment performce compared to the other units in our previous study.

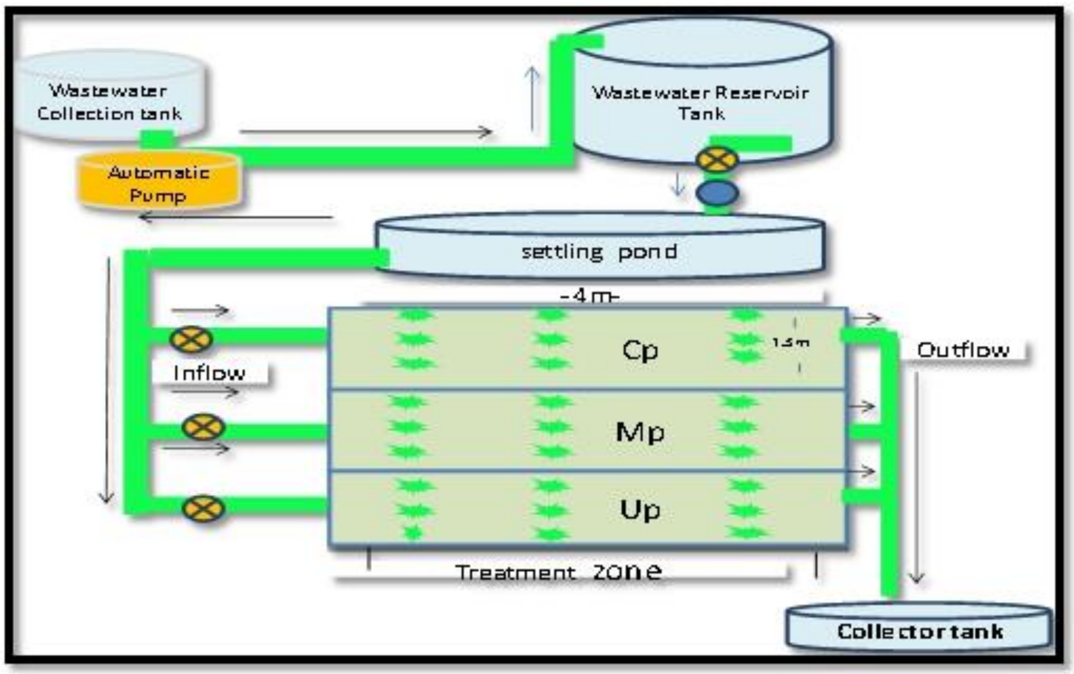


Fig. 4.1 Schematic view of the CW units: flow control valves, electromagnetic flow meter, Sampling site and ⟶ Flow direction.

4.2.2. Sample collection and analysis

Inflow and outflow water samples (1L) were collected per week for five times from each CW units. Sediment samples were also parallely collected from the treatment zones of each CW unit with a depth range of 25-35 cm. The sediments were taken from a distance of 1, 2 and 3 m intervals in longitudinal direction of the 4 m long CW and at a distance of 0.3, 0.6, and 0.9 m crosswise direction of the 1.3 m wide of the CW unit where plant roots, biological activity and nutrient levels assumed primarily to occur. The sub-samples were composited and collected using sterile 50 ml plastic tubes which then immediately transported to the laboratory for further analysis.

The water samples were analyzed for selected physico-chemical parameters (Table 4.1) using standard methods in APHA (2012). Accordingly, biochemical oxygen demand after 5 days (BOD_5) was determined using the procedure of APHA 5210 B. for 5-Day BOD Test. Chemical oxygen demand (COD) was measured following the procedure APHA 5220 B. Open Reflux Method. Ammonium (NH_4^+) was determined following procedure in APHA 4500-NH_3 C. Titrimetric Method and nitrate (NO_3^-) following the procedure in APHA-4500- NO_3^- with spectrophotometer method. Total phosphorus (TP) was measured using the protocol APHA 4500-P C Vanadomolybdophosphoric Acid Colorimetric Method. The percentage removal of pollutants (removal efficiency) was determined as follows.

$$R\% = \frac{\mathrm{Ci} - \mathrm{Co} * 100}{Ci}$$

where R% is pollutants removal percentage, C is the concentration of pollutants in mg/L, subscript i and o represent inflow and outflow samples, respectively.

Table 4.1 Sampling sites and parameters

Samples	Parameters determined
Inflow H_2O	inflow BOD_5 , COD, NH_4^+, NO_3^- ,TP
Outflow H_2O	outflow BOD_5 , COD, NH_4^+, NO_3^-,TP
Sediments from treatment zones of CWs	Bacterial community structure

The sediment samples were used for bacteriological analysis. Thus, total genomic DNA of each sediment sample was extracted using Power soil^{TM} DNA extraction kit (Mo bio Laboratories) according to the manufacturer’s instructions in microbial ecology loboratory facitliies of Leibniz-Institute of Freshwater Ecology and Inland Fisheries at IGB, Germany. Three replicate

DNA sample extraction were carried out from individual sediment samples and pooled together in equal proportions to obtain representative samples. Then the DNA was stored at –20 ^{0}C for subsequent Illumina Amplicon HiSeq sequencing machine through the use of primers 27F and 519R (Lane, 1991). Library construction and sequencing were performed at MrDNA Molecular Research, Shallowater, Texas.

4.2.3. Data analysis

Statistical analyses were carried out using PAST software (Hammer *et al*., 2001). Richness estimators (number of OTUs and Chao I) and diversity indices (Shannon-Wiener - H) were used for computing richness and diversity of the sediments bacterial communities. Non-metric multi-dimensional scaling (NMDS) based on Bray-Curtis similarity and Simpson's dendrogram were used to represent the positions of the individual samples of the three differently planted CW units. Biplot principal component (PC) analysis was performed to identify the bacterial taxa mainly responsible for dissimilarity between sediment bacterial assemblages in the three CW units.

Data were analyzed by one-way ANOVA to detect the statistical significance ($P \leq 0.05$) of differences between means values of parameters. Pearson's correlation analysis was used to determine the links between sediment bacterial community composition and over all pollutant removal efficiency of the CW units.

4.3. Result and Discussion

4.3.1. Bacterial taxonomic richness and diversity

In this study, the OTU and Chao1 data indicated higher species richness in planted CW units i.e. in Cp (single planting) and Mp (mixed planting) compared to unplanted units suggesting that the plant rhizosphere offered supporting habitats and niches for the microbes (Table 4.2). The result was comparable to species richness of higher OTUs of 2232 and Chao of 2564 detected in *Cyperus* planted SSFCW units treating a secondary effluent compared to OTUs of 1941 and Chao of 2380 recorded from unplanted control units in Yan *et al*. (2017).

The Shannon diversity index (H) was higher in planted CW units (4.93, 5.22) than unplanted ones (4.7) (Table 4.2) which was similar to the one recorded (4.9-5.3) from a constructed wetland treating tannery wastewater (Adey Desta *et al*., 2014). The result was much higher than the expected high Shannon (H) index of 2.0 recorded from CWs and other aquatic systems (Garrido *et al*., 2014).

Table 4.2 Bacterial communities' richness and diversity indices

CW units	Count	OUT	Chao1	Evenness	Shannon(H)
Cp	99039	2793	3423	0.04	4.93
Mp	63982	2554	3106	0.07	5.22
Up	76392	2228	2718	0.06	4.72

4.3.2. Bacterial community compositions

The diversity of the bacterial communities existing in the differently planted CW units was cataloged at the phylum, class and genus levels. The relative sequence abundance of the bacterial communities at a phylum level is represented in Fig. 4.2. A total of 28 bacterial phyla were obtained from the CW units which were 26, 28 and 22 phyla in Cp, Mp and Up units respectively. The number of phyla obtained was slightly lower than the 31 phyla recorded from a constructed wetland planted with *Phragmites australis (Cav.)* established to treat tannery wastewater nearby the study area (Adey Desta *et al*., 2014).

The dominant phyla were *Bacteroidetes, Firmicutes* and *Proteobacteria* contributing to 67.2 % - 86.7 % of the total phyla recorded from the CW units (Fig. 4.3a). The phyla *Proteobacteria* and *Bacteridoites* were more dominant in the unplanted CW unit than the planted units; whereas the phylum *Firmicutes* was much higher in the planted Mp (32.1%) and Cp unit (40.9%), compared to the unplanted units (4.0%). Thus, there is a remarkable difference in relative abundance of the dominant phyla amongst the CW units (Fig. 3a).

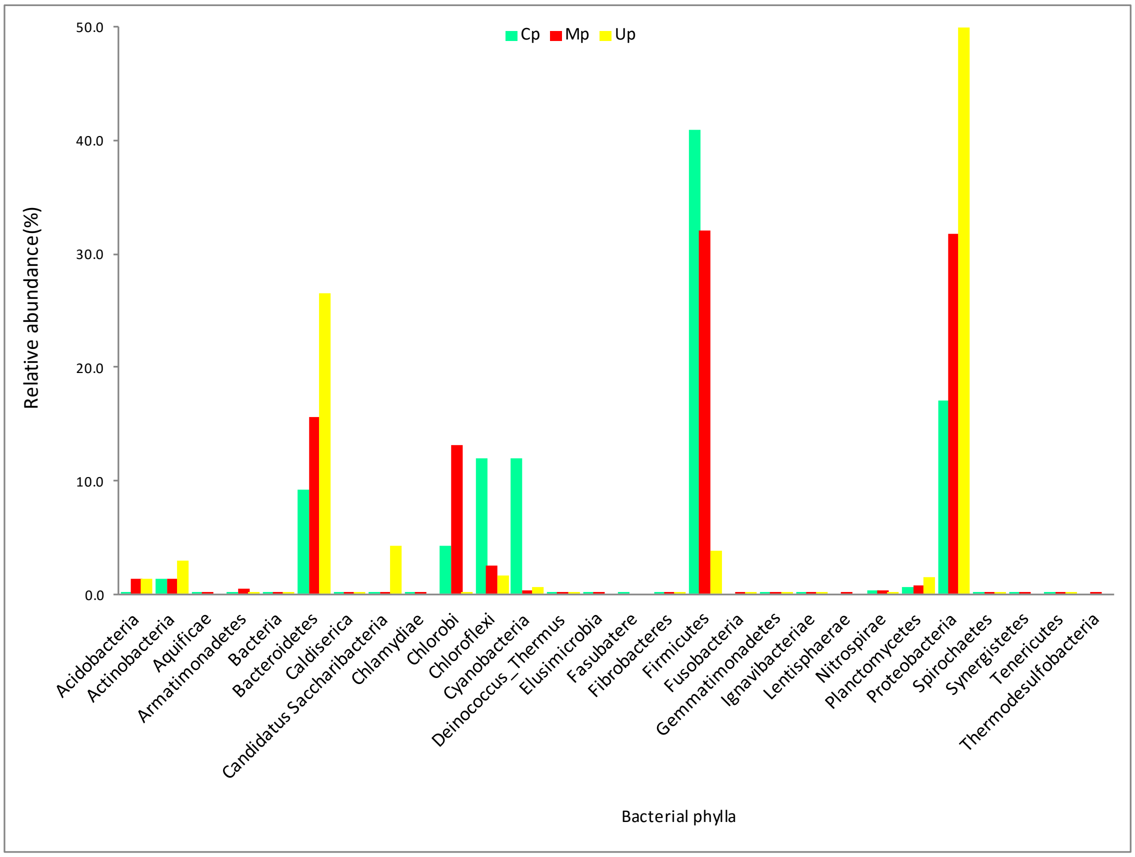


Fig. 4.2 Bacterial community composition at the phylum level in the three CWs units

Although the number of phyla (28 phyla) recorded in this study was closer to the number of phyla (31 phyla) recorded from a CWs treating tannery waste water in (Adey Desta *et al*., 2014), the overall distribution pattern of the dominant bacterial phyla was different. The authors reported that the *Firmicutes* contained 40% of all the sequences analyzed, followed by *Proteobacteria* and *Bacteroidetes*, representing 30% and 15% of the sequences, respectively. The difference may be due to varying operational factors of CWs (e.g., design, type of plant species, wastewater characteristics and sequencing methods employed within the bio-informatics pipeline (Deng *et al*., 2014).

The bacterial profile also corresponded to a number of 50, 50 and 46 classes in Cp, Mp and Up units, respectively (Fig. 4.3b) which was not significantly different from the 52, 42 and 50 number of classes observed from three different types of HSSFCWs treating secondary sewage effluent in Wang *et al*. (2016). The most dominant classes in the CW units were *Flavobacteria* (>3.8) (*Bacteroidetes*), *Alphaproteobacteria* (> 5.1) *and Gammaproteobacteria* (> 5.8) (*Protobacteria*), *Clostridia* (> 1.0) and *Bacilli* (> 2.9) (*Firmicutes*) and *Chlorobia* (> 1.0) *(Chlorobi*) of which *Flavobacteria* existed in Cp (37.4%) and *Clostridia* in Mp (20.9%) respectively as the most dominant classes. The result was considerably different from Adey Desta *et al.* (2014) who reported the class *iacteroidia* and *Betaproteobacteria* were the second most dominant group in the constructed wetland sites. Some of the members of the majority dominat classes observed in this study reported to play important roles in the removal of orgamic and nutrient pollutants (Srivastava *et al*., 2017).

Likewise, a total of 544, 503 and 456 bacterial genera were identified in the Cp, Mp and Up units respectively. Among them, 37 genera were the most frequently detected ones with a relative abundance of greater than 0.01 (Table 4.3). This result was nearly 2-times higher than the total number of bacterial genera (260) identified from sediment samples of *Typha* planted HSSFCWs receiving caffeine-enriched wastewater in Zhang *et al*. (2016). Wang *et al* (2016) revealed that such difference could attributed to the compsition of wastewater, operational parameters, environmental conditions and type of methods used to estimate diversity.

The genus *Thermomonas* in the class *Gammaproteobacteria* (14.2%) and *Flavobacterium* from the class *Flavobacteriia* (13%) were dominant in the Up sample; whereas the genera *Chlorobium* of the class *Chlorobia* (13.5%) and *Oscillatoria* in the class *Cyanobacteria* (11.5%) were abundant in Mp and Cp units. In addition, the genus *Trichococcus* in the class *Bacilli* was abundant in both planted units; CP (18%) and MP (9.0%). The bacterial composition observed in this study which was dominated by distnict genera in different treatment units was a pattern similar to those found in (Adey Desta *et al*., 2014; Wang *et al*., 2016). All taken together, the bacterial composition analysis showed that the three CW units (Cp, Mp and Up) did not share 28.6% of the phyla, 12.0% of the classes and 32.0% of the genera which suggest compositional difference among the units and certain bacteria populations groups are specific per CW unit.

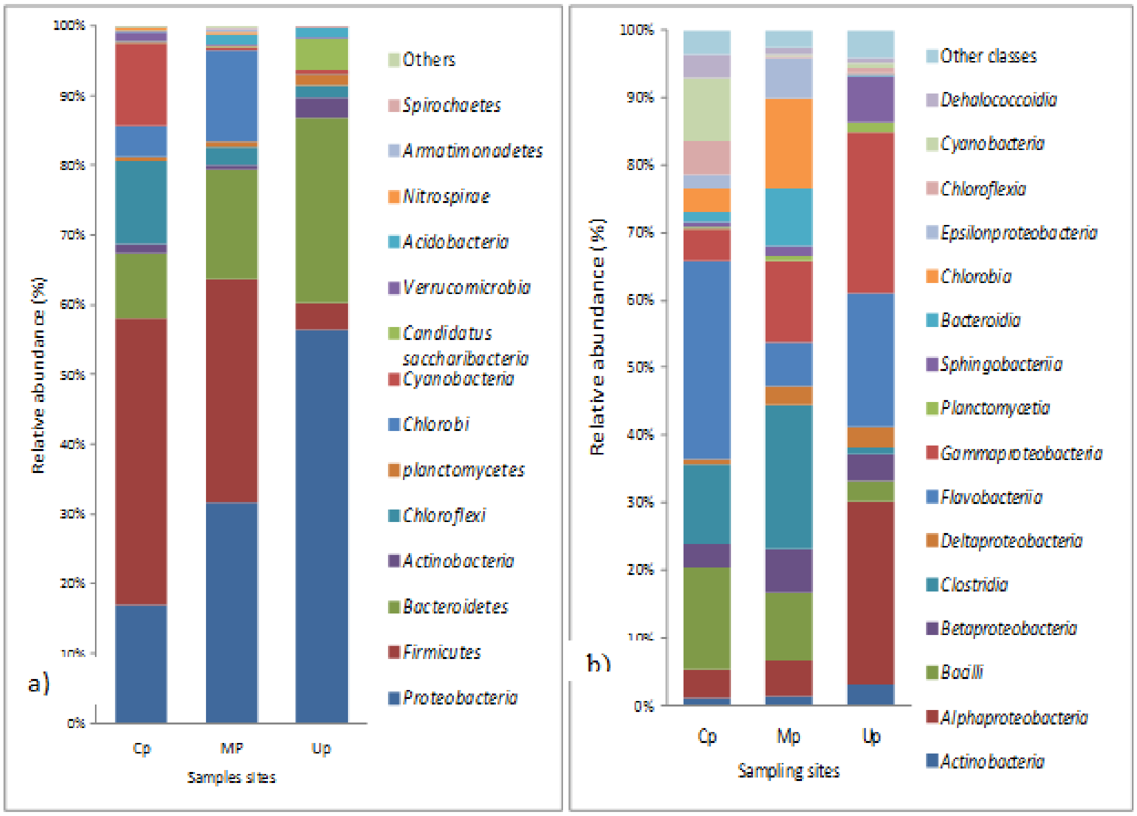


Fig. 4.3 Comparison of quantitative contribution of sequences affiliated with different phyla (a) and classes (b) from total sequences in samples of the CW units, 'Others' indicate all rare taxa combined.

Table 4.3 Relative abundance (RA) of the bacterial general at a cut off > 0.01

	Bacterial taxa		RA in CW units		
	Class	*Genus*	Cp	Mp	Up
1	*Alphaproteobacteria*	*Erythromicrobium*	0.309	0.019	0.976
2	*Alphaproteobacteria*	*Novosphingobium*	0.282	0.272	1.782
3	*Alphaproteobacteria*	*Rhizobium*	0.222	0.321	3.045
4	*Alphaproteobacteria*	*Rhodobacter*	0.399	0.298	1.681

5	*Alphaproteobacteria*	*Saccharibacter*	0.182	0.469	2.815
6	*Alphaproteobacteria*	*Sphingomonas*	0.724	0.623	2.841
7	*Alphaproteobacteria*	*Sphingopyxis*	0.077	0.199	3.406
8	*Bacilli*	*Exiguobacterium*	0.687	0.473	1.364
9	*Bacilli*	*Trichococcus*	18.065	9.345	0.092
10	*Bacteroidia*	*Bacteroides*	1.173	2.018	0.037
11	*Bacteroidia*	*Macellibacteroides*	1.446	2.430	0.012
12	*Bacteroidia*	*Paludibacter*	0.938	2.826	0.037
13	*Betaproteobacteria*	*Rhodocyclus*	0.646	2.106	0.021
14	*CandidatusSaccharibacteria*	*CandidatusSaccharimonas*	0.093	0.159	0.289
15	*Chlorobia*	*Chlorobium*	4.248	13.470	0.046
16	*Chloroflexia*	*Chloroflexus*	6.085	0.352	0.587
17	*Clostridia*	*Acetobacterium*	1.344	1.522	0.041
18	*Clostridia*	*Anaerovorax*	3.016	4.749	0.029
19	*Clostridia*	*Clostridium*	6.570	7.576	0.325
20	*Clostridia*	*Fusibacter*	0.073	3.126	0.135
21	*Clostridia*	*Ruminococcus*	0.840	1.021	0.009
22	*Cyanobacteria*	*Oscillatoria*	11.541	0.109	0.038
23	*Dehalococcoidia*	*Dehalococcoides*	4.414	0.877	0.041
24	*Deltaproteobacteria*	*Desulfocurvus*	0.009	0.019	1.820
25	*Deltaproteobacteria*	*Geobacter*	0.319	2.010	0.043
26	*Epsilonproteobacteria*	*Sulfurospirillum*	0.408	1.119	0.039

27	*Epsilonproteobacteria*	*Wolinella*	2.087	1.195	0.017
28	*Erysipelotrichia*	*Erysipelothrix*	6.301	0.793	0.018
29	*Flavobacteriia*	*Cloacibacterium*	2.132	4.643	13.068
30	*Flavobacteriia*	*Flavobacterium*	1.611	1.253	4.626
31	*Gammaproteobacteria*	*Acinetobacter*	0.475	7.065	2.351
32	*Gammaproteobacteria*	*Pseudomonas*	0.078	0.475	1.829
33	*Gammaproteobacteria*	*Pseudoxanthomonas*	0.046	1.414	3.556
34	*Gammaproteobacteria*	*Thermomonas*	1.150	0.653	14.164
35	*Gammaproteobacteria*	*Thiocapsa*	2.062	0.199	0.051
36	*Sphingobacteriia*	*Flavihumibacter*	0.215	0.563	2.913
37	*Sphingobacteriia*	*Parasegetibacter*	0.016	0.015	1.776

4.3.3. Similarity of bacterial compositions

Based on Non-metric Multidimensional scaling (NMDS) of OTU data, the two planted CWs were plotted together in the same ordination plot (Fig. 4.4a) indicating higher similar bacterial communities compared to the unplanted unit. Hierarchical clustering of OTUs also indicated a remarkable difference (> 45%) between the planted vs. unplanted CW units which was < 30% within planted CW units (Fig. 4.4b) which suggesting plants had an influence on bacterial composition. The dissimilarities between CWs were mainly associated with rhizosphere effects, where plant roots modify their surrounding environment by releasing a variety of root exudates and providing surface area for attachment (Adey Desta *et al.*, 2014: Yan *et al*, 2017; Ma *et al.*, 2018).

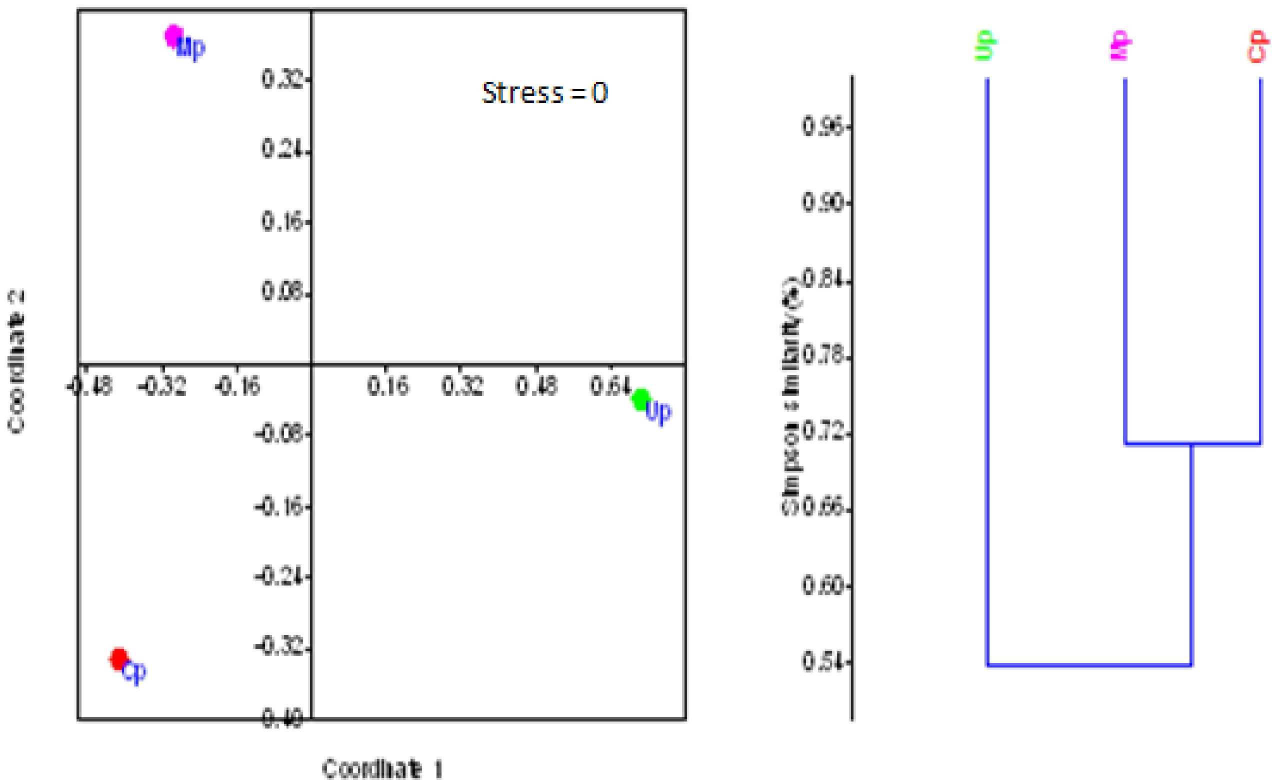


Fig. 4.4: NMDS (a) and Cluster (b) analysis of the relative abundance of bacterial OTUs

Moreover, the biplot principal component analysis (PCA) of the OTU data illustrated the degree of the bacterial richness towards planted CW units (Fig. 4.5). PC1 showed 74.8% variation between the planted CW units (Cp and Mp) and unplanted units (Up). Zhang *et al.* (2015) showed strong correlation of sediment microbial community with plant diversity using this analysis. The difference obtained in this study was higher than the PC1 values of 59.4% reported between rhizosphere and sediment bacterial community structure in pilot-scale CWs in Ma *et al.* (2018) indicating that the unplanted samples have higher dispersion from the planted (Cp and Mp) samples in bacterial compositions.

The PCA identified seven taxa (OTU-9, OTU-10, OTU-11, OTU-12, OTU-22, OTU-37 and OTU-278) as important factors in relation to the ordination of the three CW units (Fig. 4.5). The taxa (OTU-10, OTU-11, OTU-12, OTU-22 and OTU-37) with high positive PCI loading were more abundant in planted CW units whereas taxa with high negative PCI loading (OTU-9 and OTU-278) were more abundant in unplanted CW units (Fig. 4.5).

Taxa with positive loadings on PC1 (OTU-10, OTU-11, OTU-12, OTU-22 and OTU-37) belonged to *Chlorobium* (*Chlorobi*), *Oscillatoria* (*Cyanobacteria*), *Trichococcus* (*Firmicutes*), *Chloroflexus* (*Chloroflexi)* and *Erysipelothrix* (*Firmicutes)* respectively. Taxa with negative loading on PC1 (OTU-9 and OTU-278) belonged to the genera *Cloacibacterium* (*Bacteroidetes)* and *Thermomonas* (*Proteobacteria)* respectively. The length of the lines indicates the proportion of variation in species abundance where long lines correspond to species contributing more to the data set variation. Acordingly, long lines correspond to the 7 bacterial genera implies their relative abundance mainly accounted for microbial composition variation among the CW units in Fig. 4.5. This PCA result based on OTUs data highlighted presence of variations in bacterial richness among the CW units, which may be contributing for removal performance difference among the CW units (Zhang *et al*., 2015).

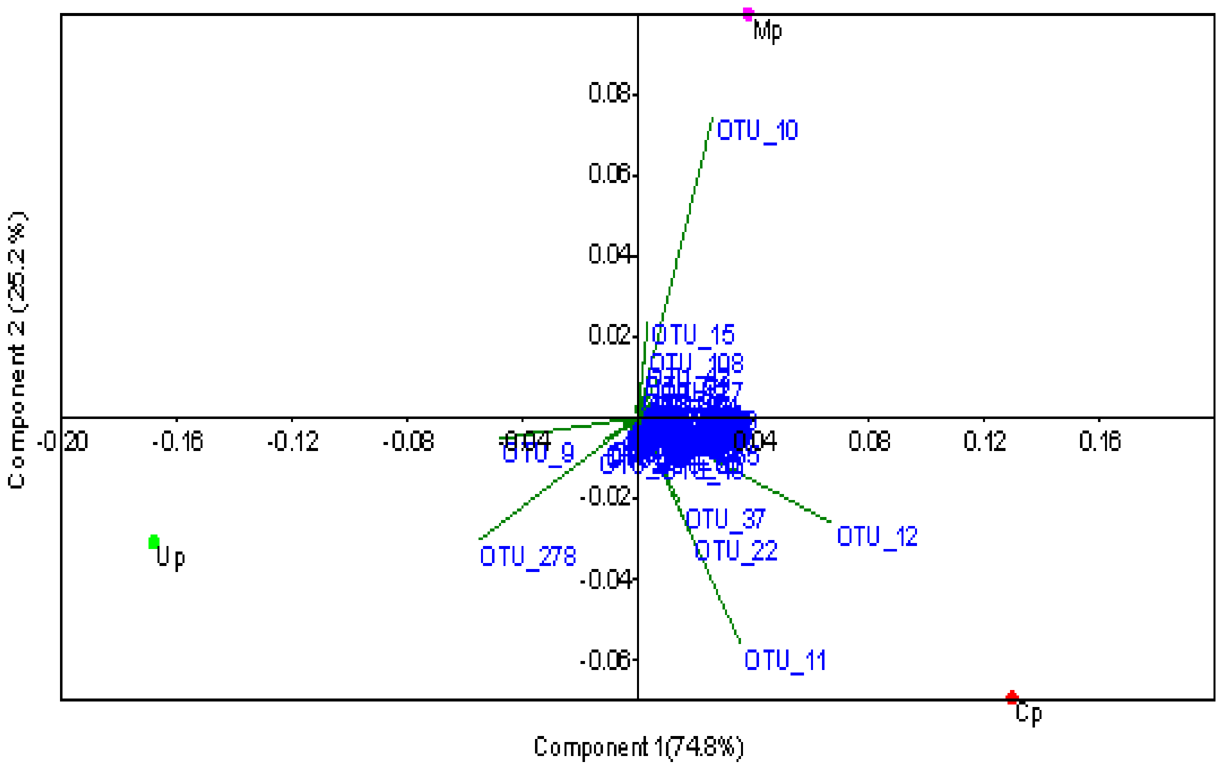


Fig. 4.5 Principal Component (PC) analysis of the OTU abundance between CW units

4.3.4. Bacterial taxa and pollutant removal efficiency

In most cases, the removal efficiencies of BOD_5, COD, NO_3^- and TP were significantly higher ($P < 0.05$) in planted CW units compared to unplanted control (Table 4.4). In all cases, the Mp showed better removal (11-21%) in all parameters. It followed by Cp unit except NH_4^+ compared to the Up unit.

Table 4.4 Removal efficiencies of BOD_5, COD, NH_4^+, NO_3^- and TP in the CW units, n = 3, $p < 0.05$ between a vs. b and a vs. c

Parameters	Removal % in		
	Cp	Mp	Up
BOD_5	$75.9{\pm}3.0^{a}$	$77.8{\pm}1.9^{a}$	$69.2{\pm}3.0^{b}$
COD	72.5±3.6a	$77.3{\pm}0.9^{a}$	64.1±0.7b
NH_4^+	92.3±1.2a	99.0±1.0a	97.0±4.4a
NO_3^-	64.5±4.5b	$82.7{\pm}1.2^{a}$	65.8±5.8b
TP	$57.0{\pm}8.5^{a}$	$60.3{\pm}9.5^{a}$	$40.7{\pm}4.0^{b}$

In general, bacterial community richness (OTUs and Chao1) as well as diversity (Shannon-H) index indicated a positive correlation with mean removal efficiencies of BOD_5, COD, NH_4^+, NO_3^- and TP (Table 4.5). These results were similar to the study of Zhang *et al*. (2015), where microbial biomass carbon (MBC) and Shannon diversity index (H) were respectively positively correlated with the removal of BOD_5 (r = 0.74; r = 0.92), COD (r = 0.65; r = 0.41), NH_4-N (r = 0.71; r = 0.83) and NO_3-N (r = 0.71; r = 0.80) in different CW configurations.

Oopkaup *et al*. (2016) also showed strong positive correlations between species richness (number of OTUs) and removal efficiency of NH_4^+ (r = 0.61) and NO_3^- (r = 0.83) as well as between bacterial diversity and removal efficiency of NH_4^+ (r = 0.82), NO_3^- (r = 0.52) and BOD_5 (r = 0.65) in HSSFCW mecosomes treating municipal wastewater. The positive correlation often considered as predictor of removal efficiencies that are mainly driven by bacterial processes (Button *et al*., 2015).

Table 4.5 Pearson's correlation analysis of bacterial community variables and pollutants removal efficiency of the CW units

Taxa	BOD_5	COD	NH_4^+	NO_3^-	TP
Number of OUT	0.870	0.810	0.640	0.830	0.850
Shannon (H)	0.856	0.909	0.985	0.893	0.880
Bacteroidetes	0.938	0.971	0.996	0.961	0.953
Firmicutes	0.954	0.915	0.782	0.929	0.939
Chloroflexi	0.500	0.400	0.155	0.433	0.458
Cyanobacteria	0.409	0.304	0.053	0.338	0.364
Bacilli	0.890	0.830	0.660	0.850	0.870
Chlorobia	0.796	0.859	0.961	0.840	0.824
Clostridia	0.910	0.950	0.940	0.940	0.930
Flavobacteria	0.025	-0.087	-0.337	-0.051	-0.023
Gammaproteobacteria	-0.914	-0.863	-0.707	-0.881	-0.894
Chlorobium	0.790	0.850	0.960	0.830	0.820
Chloroflexus	0.736	0.807	0.971	0.785	0.767
Oscillatoria	0.710	0.781	0.944	0.758	0.741
Erysipelothrix	0.641	0.712	0.875	0.690	0.672
Trichococcus	0.369	0.440	0.603	0.417	0.400

In this study, majority of the dominant bacterial taxa also showed positive correlation ($r > 0.50$) with removal of BOD_5, COD, NH_4^+, NO_3^- and TP. Specifically, relative abundance of *Bacteroidetes* and *Firmicutes* showed strong positive correlation ($r \geq 0.97$) with the removal efficiencies of nutrients (Table 4.5) which related to degradation of organic matter and nutrients in different treatment plants (Zhong *et al.*, 2015). The positive relation between the phyla *Chloroflexi* and *Cyanobacteria* (Table 4.5) and the removal of nutrients from waste water was previously reported for different CW systems (Dubey *et al*., 2011).

The correlation analysis for the dominant class also showed a positive relation ($r \geq 0.65$) between relative abundance of *Bacilli*, *Chlorobia* and *Clostridia* and removal efficiency of the CW system (Table 4.5). Other studies also revealed the association of these bacterial genera with the removal efficiency of CW integrated with other wastewater treatment components (Adey Desta *et al*., 2014; Zhao *et al*., 2017).

Moreover, majority of the important bacterial genera in relation to ordination of the CW units (Fig. 4.4) including *Chlorobium; Chloroflexus; Oscillatoria, Trichoccus and Erysipelothrix* had a strong positive correlation with treatment performance of the CWs (Table 4.5). It implies the relative abundance was closely linked to mineralization of organic matters in the CW units. The strong correlation of *Chlorobium* with removal of NH_4^+ ($r = 0.96$), was comparable to the results in Edberg *et al*. (2012) and gave an insight of the role of this genus in the transformation of NH_4^+ in the CW units. Vijayakumar (2012) and Dai *et al*. (2018) also showed strong positive correlation of *Oscillatoria* and *Trichococcus* with the removal efficiency of the nutrients from biological treatment systems and suggested a potential application of these groups in bio-augmentation and bioremediation of wastewater in biological treatment systems.

4.4.Conclusion

In this study, 16S rDNA Illumina Miseq sequencing analysis revealed the bacterial community compositions of HSSFCW treating floriculture wastewater planted with different macrophyte species. The wetland plants maintained higher bacterial and cyanobacterial community composition compared to unplanted units. It was shown that species richness, diversity as well as relative abundance of some groups have a positive influence on removal efficiency of organic matter from the CW units. This research provides information on bacterial communities among HSSFCWs established with different planting, suggesting the importance of multiple planting of HSSFCW to best support diverse groups of wetland microbial communities for better remediation of pollutants**.**

References

Adey Desta, A. F., Assefa, F., Leta, S., Francesca, S., Mark, W., Moses, N. and Djikeng, A. (2014). Microbial Community Structure and Diversity in an Integrated System of Anaerobic-Aerobic Reactors and a Constructed Wetland for the Treatment of Tannery Wastewater in Modjo, Ethiopia. *DOI:10.1371/journal.pone.0115576.*

APHA (2012). Standard Methods for the Examination of Water and Wastewater, 22nded. Washington DC, USA.

Berhan Teklu, Amare Hailu, Wiegant, D.A., Scholten, B.S. and Van den Brink, P.J. (2016a). Impacts of nutrients and pesticides from small and large scale agriculture on the water quality of Lake Ziway, Ethiopia. *Enviromental Science Pollution Reserch* http://dx.doi.org/10.1007/s11356-016-6714-1

Button, M., Nivala, J., Weber, K.P., Aubron, T. and Müller R. A. (2015). Microbial community metabolic function in subsurface flow constructed wetlands of different designs. *Ecological Engineering* **80:**162–171

Dai, Y.M., Zhang, L.L., Yan Li, Y., Li, Y.Q., Deng, X.H., Wang, T.T., Yang, F., Tian, Y.Q., Li, N., Zhou, X. M., Liu1, X.F, and Li., J. (2018). Characterization of *Trichococcus paludicola sp. nov*. and *Trichococcus alkaliphilus sp. nov.,* isolated from a high-elevation wetland, by phenotypic and genomic analyses. *International Journal of Systematic Evolution and Microbiology* **68**:99–105.

Deng, Y., Cui, X., Herna´ndez, M. and Dumont, M.G. (2014). Microbial Diversity in Hummock and Hollow Soils of Three Wetlands on the Qinghai-Tibetan Plateau Revealed by 16S rRNA Pyrosequencing. *PLoS ONE 9(7): e103115. doi:10.1371/journal.pone.0103115.*

Dubey, K.S., Dubey, J., Mehra, S., Tiwari, P. and Bishwas A. J. (2011). Potential use of *cyanobacterial* species in bioremediation of industrial effluent. *African Journal of Biotechnology* **10(7):**1125-1132.

Edberg, F., Andersson, A. F. and Holmstrom, S. (2012). Bacterialcommunity composition in the water column of a lake formed by a formeruranium open pit mine. *Microbial Ecology* **64**:870–880.

Garrido, L., Sánchez, O., Ferrera, I., Tomàs, N. and Mas, J. (2014). Dynamics of microbial diversity profiles in waters of different qualities: Approximation to an ecological quality indicator. *Science of Total Environmental* **468**:1154–1161.

Lane, D. J. (1991). *16S/23S rRNA sequencing*, p 115–175, Wiley, New York, NY.

Ma, X., Song, X., Li, X., Fu,S., Li, M. and Liu, Y. (2018). Characterization of Microbial Communities in Pilot-Scale Constructed Wetlands with *Salicornia* for Treatment of Marine Aquaculture Effluents. *Hindawi https://doi.org/10.1155/2018/7819840.*

Miege, C., Choubert, J.M., Ribeiro, L., Eusebe, M., Coquery, M. (2009). Fate of pharmaceuticals and personal care products ii wastewater treatment plants.,Conception of a database and first results. *Environmental Pollution* **157**:1721-1726.

Oopkaup, K., Truu, M., Nõlvak, H., Ligi, T., Preem, J., Mander, O. and Truu, J. (2016). Dynamics of Bacterial Community Abundance and Structure in Horizontal

Subsurface Flow Wetland Mesocosms Treating Municipal Wastewater. *Water* **8(457):**1-25.

Srivastava1, J. K. Chandra, H., Kalra, S. J. S., Mishra, P., Hena Khan, H. and Poonam Yadav, P. (2017). Plant–microbe interaction in aquatic system and their role in the management of water quality: a review. *Applied Water Science* **7**:1079–1090.

Verlicchi, P. and Zambello, E. (2014). How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? *A review. Science of Total Environmental* **470:**1281-1306.

Vijayakumar, S. (2012). Potential Applications of *Cyanobacteria* in Industrial Effluents-A Review. *Journal of Bioremedical Biodegradation* **3(6):**1-6.

Vymazal, J. (2011). Long-term performance of co nstructed wetlands with horizontal sub-surface flow: Ten case studies from the Czech Republic. *Ecological Engineering* **37**:54–63.

Wang, P., Zhang, H., Zuo, J., Zhao, D., Zou, X., Zhu, Z., Jeelani, N., Leng, X. and An, S. (2016). A hardy plant facilitates nitrogen removal via microbial communities in subsurface flow constructed wetlands in winter. *Scientific Reports | 6:33600 | DOI: 10.1038/srep33600.*

Weber, K.P. (2016). Microbial Community Assessment in Wetlands for Water Pollution Control: Past, Present, and Future Outlook. *Review Wat*er **8:**503.

Weber, K.P.; Gagnon, V. (2014). Microbiology in Treatment Wetlands. *Sustainable Sanitary Practice* **18**: 25–30.

Yan, Q., Min, J., Yu, Y., Zhu, Z., Feng, G. (2017). Microbial community response during the treatment of pharmaceutically active compounds (PhACs) in constructed wetland mesocosms. *Chemosphere* **186**:823-831.

Zhang, D., Luo, J., Lee, Z.M.P., Grasberg, M.R., Liu, Y., Tan, K. S. and Ng, J.W. (2016). Characterization of microbial communities in wetland mesocosms receiving caffeine-enriched wastewater. *Environmental Science Pollution Research* **23:**14526–14539.

Zhang, Y., Pedro, N., Carvalho, Lv, T., Arias, C., Brix, H. and Chen, Z. (2015). Microbial density and diversity in constructed wetland systems and the relation to pollutant. *Water Science and Technology in press*.

Zhao, K., Xua, R., Zhanga, Y., Tanga, H., Zhoub, C., Caob, A., Zhaoa, G. and Guoa, H. (2017). Development of a novel compound microbial agent for degradation of kitchen waste. *Brazlian Journal of Microbiology* **48**:442–450.

Zhong, F., Wu, J., Dai, Y., Yang, L., Zhang, Z., Cheng, S., and Zhang, Q. (2015). Bacterial community analysis PCR-DGGE and 454-pyrosequencing of horizontal subsurface flow constructed wetlands with front aeration. *Applied Microbiology and Biotechnology* **99:**1499–1512.

5. Bio-augmentation of Effective Microorganisms in Horizontal Subsurface Flow Constructed Wetlands for Enhanced Removal of Organic Matter and Nutrients from Floriculture Wastewater in Flower Farm, Bishoftu Town, Ethiopia

Abstract

Low cost waste water treatment of agricultural and industrial wastes is one of the formidable tasks of waste management. Constructed wetlands (CWs) are becoming popular as low cost waste treatment systems for they involve both plants and microorganisms to enhance the process. Apart from the naturally occurring microorganisms, additions of commercially available effective microorganisms (EM) are supposed to augment the treatment systems. The aim of this study was to examine the efficacy of bio-augmentation of effective microorganisms (EM) in removal of organic matter and nutrients from wastewater of the ever-expanding floriculture industry in Ethiopia. Typha planted horizontal subsurface flow constructed wetland (HSSFCW) units were used for simultaneous operation of EM-treated and control (no EM) units. Changes in physicochemical and microbiological parameters were measured to monitor the bio-remediation processes. Our results showed that the CW units bio-augmented with EM at 1:1800 dilutions remarkably reduced NO_3^- *and TP by 16.1% and 15.6%, respectively compared to the control. The shift in EM dose found to have a positively correlation to the total count of sediment bacterial. Increasing EM doses from (1:1800) to (1:600) also statistically (*$p < 0.05$*) reduced the levels of* BOD_5*, COD,* NO_3^- *and TP by > 19% > 11 % > 16% and > 16% respectively. Furthermore, the 1:600 EM-dosed CW unit operating with a retention time of 3 days showed higher* BOD_5 *(24.3%), COD (11.6%),* NO_3^- *(22.8%) and TP (15.4%) removal performance compared to a shorter retention time of solely 1 day. This corresponded to 4.4, 19.4, 0.1, 2.5 and 1.7mg/L of* BOD_5*, COD,* NH_4^+*,* NO_3^- *and TP respectively in outflow samples which were far below the environmental maximum tolerable standards of EPA. These results indicate EM*

bioaugment in HSSFCW could be a good prospective for bio-remediation of organic matter and nutrients from floriculture wastewater so as to produce better outflow quality having less pollution effect on the receiving environment.

Keywords: Bio-augmentation, Floriculture wastewater, water retention time, removal efficiency

5.1. Introduction

Protection of aquatic environment from point sources of wastewater pollutions depends largely on the development of efficient wastewater treatment technologies (Zhou *et al*., 2018). Recently, more attention has been given to bioprocesses as promising technology for the advantages of cost saving and less secondary pollutions (Zhang *et al*., 2017). Accordingly, constructed wetlands system (CWs) represent one of the low-cost biological treatment technologies that depends on mainly both marshy plants and associated microorganisms for removal of wastewater contaminants (Vymazal, 2011; Wu *et al*., 2015).

In principle, poor performance of bioprocesses is commonly associated with the lack of a sufficient number of specific microbes possessing a key metabolic route to transform targeted contaminants (Herrero and Stuckey, 2015). Therefore, designing and operating efficient wastewater treatment configuration necessitate the development of proper conditions where most desirable microorganisms can be selected for and maintained in their physiological conditions. Taken together, it is great significance of investigating the efficiency and sustainability of biological wastewater treatment technologies from prospective of the ratio of microbial density to nature of contaminants are being treated.

The operation of biological processes often face challenges of system instability and performance deterioration due to stresses conditions, including temporary shock of toxic chemicals in wastewater, temperature fluctuation and other factors (Zhang *et al*., 2017). This occasionally leads to wetland microbial community under sizing and insufficient to eliminate pollutants from the wastewater, particularly in field conditions. To this ending, an approach of bio-augmentation has been suggested into CWs to improve the treatment performance through providing effective microbial consortia for targeted removal processes and cooperating in a synergistic ways (Meng *et al*., 2014).

Bio-augmentation involves the addition of highly concentrated and specialized microbial cultures i.e pre-adapted pure bacterial culture or a consortium of natural or genetically engineered microbes to detoxify pollutants and their harmful metabolites for bio-remediation (Meng *et al*., 2014; Zhao *et al*., 2014). The bio-augments are assumed to reinforce the microbial populations' residing in CWs with mixture of microbial consortia having the key metabolic pathways so that they can effectively reduce the contaminant load by transforming it into less dangerous compound.

Effective microorganisms (EM) constitute one of the commercially available multi-purpose mixed cultures based on the original formula developed by Higa and Parr (1994) and are known as EM1 It contains a combination of about 80 selected species of beneficial microorganisms, consisting of actinomycetes, lactic acid bacteria, phototrophic bacteria and yeasts (Monica *et al*., 2011; Lananan *et al.,* 2014). They are supposed to enhance degradation of complex solid and liquid wastes into simpler ones through secretion of organic acids, enzymes, antioxidants and metallic chelates (Mandalaywala *et al*., 2017).

Several studies showed EM improved pollutants removal from different wastewater treatment in lab-scale reactors and pond systems (Monica *et al*., 2011; Namsivayam *et al*., 2011; Karamany *et al*., 2013; Maalim *et al*., 2013; Lananan *et al*., 2014; Rashed and Massoud, 2014; Priya *et al*., 2015; Victoria and Uma Maheswari, 2016; Mandalaywala *et al*., 2017). However, Karamany *et al*. (2013) reported EM improved rate of reduction of BOD_5 and COD in activated sludge reactor, necessitating further studies on the relation between EM applications on CWs, under field conditions.

The rapid development of large-scale floriculture industries in different areas of Ethiopia is part of the current major agriculture transformation in Africa (Tadele Yeshiwas and Melkamu Alemayehu. 2018). Floricultures use various pesticides and chemical fertilizers, which may affect water quality of receiving water bodies due to discharge of residual agro-chemicals. The potential risks of the chemicals on environment and human health were also implicated in Berhan Teklu *et al*. (2016a) and Malefia Tadele (2009) which necessitates remediation measures before discharging to the environment. Thus, the major aim of the present study was to evaluate the efficacy of low-priced commercially available EM1 bioaugments for removal of residual organic matter and nutrients from the wastewater in an intermediate field-scale horizontal subsurface flow constructed wetland (HSSFCWs) in flower farm, Beshoftu town.

5.2. Materials and Methods

5.2.1. Description of the treatment system

Fig. 5.1 represents a schematic sketch of five pilot-scale *Typha* planted horizontal subsurface flow constructed wetlands (HSSFCWs) units (CU1, CU2, CU3, CU4 and CU5) used for this specific experiment. The surface area of each CW unit was 5.2 m^2 (length: 4.0 m and width: 1.3

m) and working volume of 3.64 m^3 (4.0 m length * 1.3 m width * 0.6 m depth * 0.4 poersity) that is estimated base on the first-order design equation first proposed by Kickuth for sizing of HSSFCWs (Vymazal, 2008). The treatment zone of each CW unit was filled with gravels of a particle size of 2 – 4 mm. to a height of 0.7 m. The CW units were dosed with different EM concentrations with same mix of wastewater.

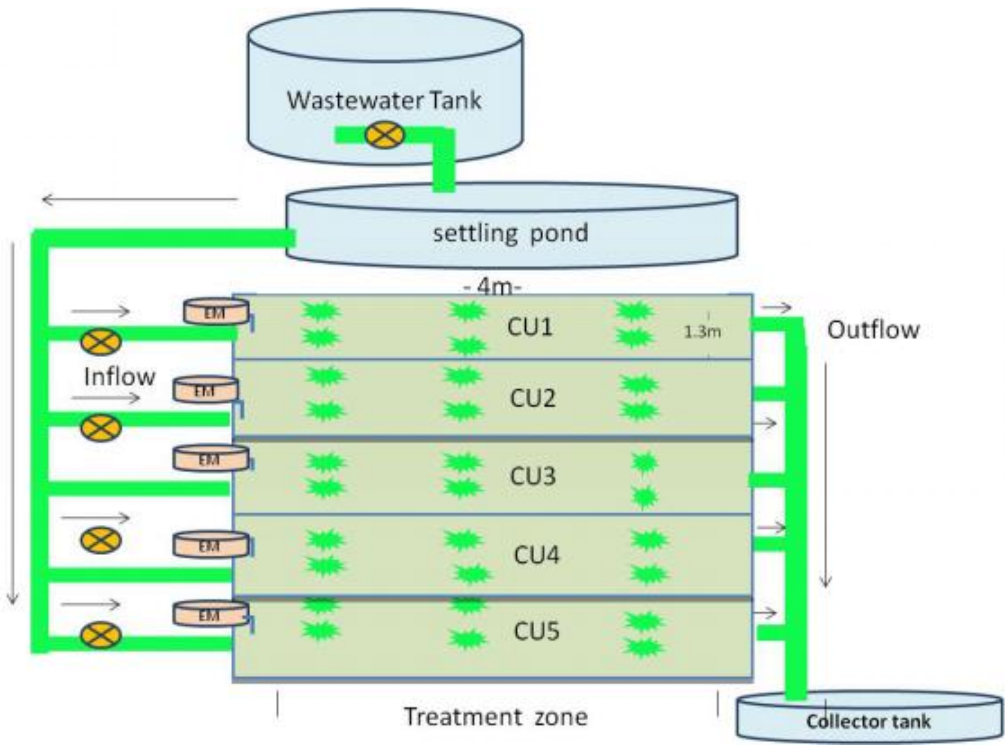


Fig.5.1 Schematic of the experimental CWs: - CU1-5 = constructed wetland unit, ⊗ = flow control valves, ✚ = Sampling site and ⟶ = Flow direction.

5.2.2. Experimental procedures

The treatments were adjusted to five different (1:1800, 1:1200, 1900. 1:720 and 1:600) EM: wastewater dilutions after trials of EM augmenting (CE) with reference to control group (CC). These dilutions were chosen in accordance of EM-dilutions (1:1000) that shows good potential of organic matter biodegradation in bioreactors (Karamany *et al*., 2013). The product was based on the original formulation developed by Higa and Parr (1994) known as EM-1. The activated EM were purchased from WOLJEEJII private EM supplies in Bishoftu town, Ethiopia, which was prepared by mixing 1 volume of EM1 dormant stock solution to 1 volume of molasses solution and 18 volumes of chlorine-free water following the manufacturer's protocol (Higa, 1996).

The added EM1, molasses and water were well mixed in a clean airtight plastic container and incubated at room temperature for around 15 days. The pH of the product shifting from approximately initially neutral (6.8) to acidic (3.7) indicated the completion of the activation process (Monica *et al.*, 2011). The total bacterial density was monitored by viable cell counts, which approximately yielded 8.67 log CFU/ ml at the end of the activation.

Finally, the prepared EM solution was added to the EM treatment CW units in a specific proportion of EM solution to wastewater. During inoculation, the mean microbial counts of EM solutions mixed with wastewater in log of CFU/ml were, 4.72, 4.87, 4.18, 5.54 and 6.32 for 1:1800, 1:1200, 1:900, 1:720 and 1:600, respectively. The 1:1800, 1:1200, 1:900. 1:720 and 1:600 EM-wastewater mixtures were introduced the treatment zones of the replicated CW units (CU1, CU2, CU3, CU4 and CU5) operating in parallel (Fig. 5.1).

The efficiency to remove organic matter and nutrients was also tested within three different wastewater retention times in the CW units (1, 2 and 3 days which was less than 4 days where optimal removal obtained with no EM addition in our previous study) using EM dilution of 1: 600. A CW unit without addition of EM was included as a control group. The retention time for the treatment system was adjusted to selected days of retention time by a flow control valve and an electromagnetic flow meter that was supplied by Sensotronic System, Ahmedabad, Gujarat, India.

5.2.3. Sampling processes

Inflow and outflow water samples were collected weekly for four months at the inlet and outlet of the experimental CWs for physicochemical analysis. Wetland sediments samples (fine gravels and accumulated organic matter) were collected to examine the dynamic of wetland bacterial counts in response to EM bio-augmentation at a depth of 25-35 cm from a distance of 1, 2 and 3 m intervals (in longitudinal direction of the 4 m long CW) at a distance of 0.3, 0.6, and 0.9 m (crosswise direction of the 1.3 m wide CW) (Fig. 5.1). Thereafter, the 9 sub-samples were mixed and homogenized in order to generate one composite representative sample. Collected water and sediment samples were kept in a cooler and immediately transported to the laboratory for further analysis. The samples were all run at least three times for one experimental testing.

5.2.4. Water quality and bacterial density monitoring

The following parameters were measured from inflow and outflow samples to evaluate the removal efficiency of the system following Standard Methods (APHA, 2012). These include; Biochemical oxygen demand after 5 days (BOD_5), chemical oxygen demand (COD), ammonium (NH_4^+), nitrate (NO_3^-) and total phosphorus (TP).

Sediment samples were prepared to appropriate dilutions (to a range of 10^{-5}-10^{-12}) in normal saline solutions (0.85%) from which 0.1 ml was inoculated on nutrient agar plates to count colony forming units (CFU) following the protocol of Gerhardt *et al*. (1994). The cultures were incubated at 37°C for 48 hours. Plates with identifiable and countable colonies were selected and counted. All results obtained were expressed on a log basis per gram of the sediments.

5.2.5. Data analysis and statistics

The removal efficiency was determined as shown below: where R% is removal percentage, C is the concentration in mg L^{-1} of the tested parameter, subscript i and o represent inflow and outflow samples respectively.

$$R\% = \frac{\mathrm{Ci} - \mathrm{Co} * 100}{\mathrm{Ci}}$$

Graphical analyses were performed by using Microsoft office excel and Past software, version 4.01. When applicable, the data were analyzed through t-test and one-way analysis of variance (ANOVA).

5.3. Result and Discussion

5.3.1. Effect of EM addition on pollutants removal

The addition of EM (bio-augmentation) in the CW units (CE) working under 3 days of retention times showed variations in the removal of several components of the organic matter in reference to the control group (CC) (Fig. 5.2). Compared to the control, the CWs dosed with EM to wastewater at ratio of 1:1800 markedly enhanced the removal efficiencies of NO_3^- and TP by 16.1% and 15.6%, though the removal of BOD_5, COD, and NH_4^+ was limited to 7.8%, 5.7% and 8.5% respectively. The EM bio-augment may reforce the removal of organic matter and nutrients through secretion of enzymes and antioxidants (Mandalaywala *et al*., 2017) and degradation of organic matter.

This result was in contrast to the report of Karamany *et al.* (2013) that showed remarkable differences in BOD_5 (30.0%) and COD (17.5%) between EM treated and control groups while treating domestic sewage in activated sludge process reactors using 1:7500 EM to wastewater ratio. Monica *et al*. (2011) showed that treatment of sewage waste water with EM (1:300) reduced the level of BOD_5 and COD by 51% and 54% respectively compared to the control in a bioreactor. Although many of the hitherto studies were focused on activated and other pond systems, data of this experiment showed the application of EM can also enhance the removal of some important pollutant constituents of wastewater under the more complex CW systems at field conditions.

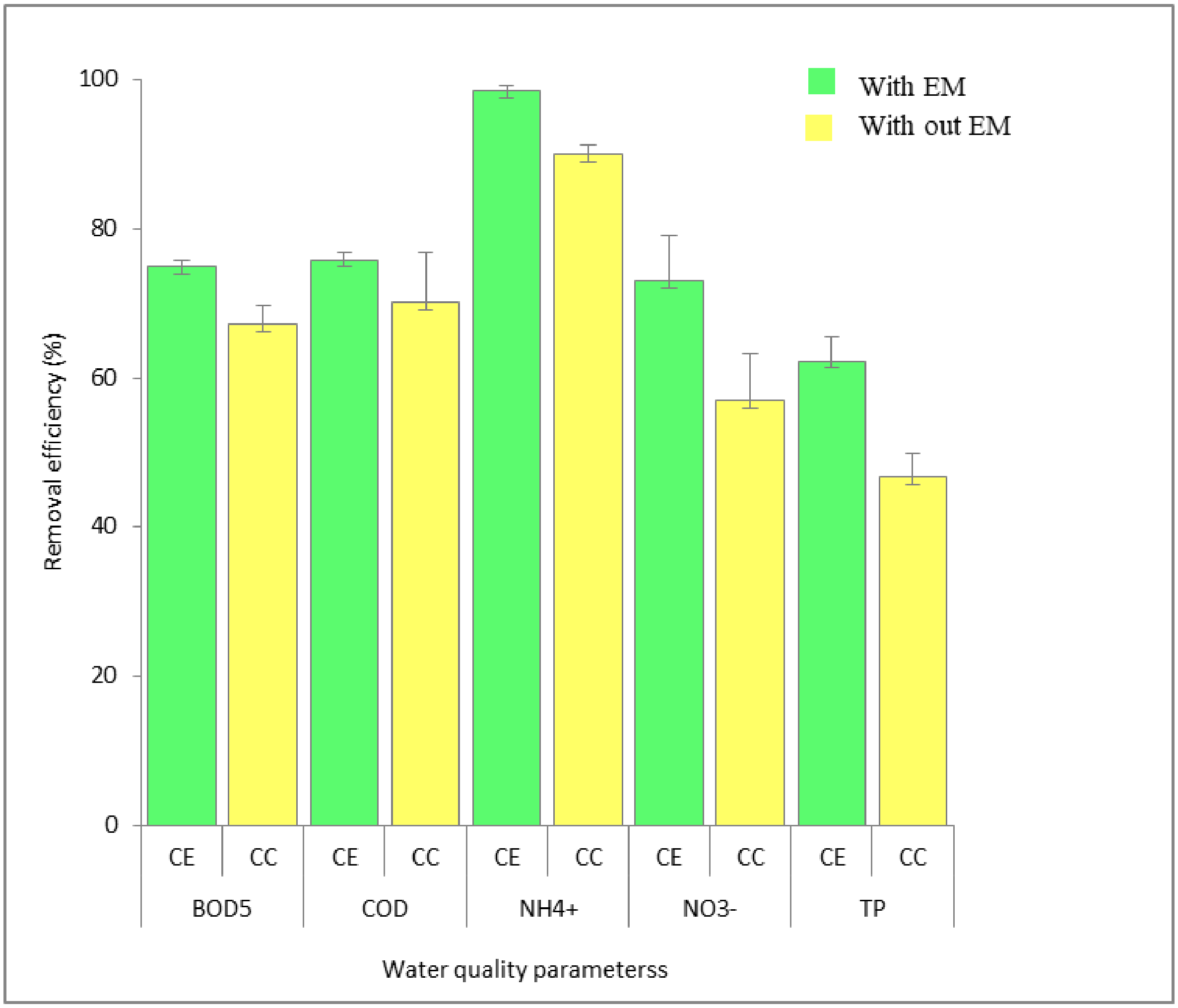


Fig. 5.2 Removal statistics between EM and with no EM for Biological oxygen demand after 5 days (BOD_5) and chemical oxygen demand (COD), ammonium (NH_4^+), nitrate (NO_3^-) and total phosphorus (TP), CE - CW treated with EM and CC – CW with no EM treated

5.3.2. Concentration effect of EM on pollutants removal

In this study, administration of five different EM to wastewater dilutions (1:1800, 1:1200, 1:900, 1:720 and 1:600) also indicated important differences on treatment performance of the CW units (Table 5.1). It was found that the removal efficiency were 75.0%, 75.9%, 91.8%, 73.0% and 60.5% for BOD_5, COD, NH_4^+, NO_3^- and TP respectively when the EM dose was lower (1:1800). While increasing the EM dilution to 1:1200 in the CW units, the removal efficiency was elevated to 78%, 77.6%, 92.7%, 76.0% and 63.6% for the parameters respectively.

In general, the levels of BOD_5, COD, NH_4^+, NO_3^- and TP importantly decreased by 19.0%, 11.2%, 4.5%, 15.1% and 14.5% respectively in CW units, due to increasing of EM dilution from 1:1800 to 1:600 which corresponded to an overall reduction by > 10% as shown in Table 5.1. In consistent with the removal results (Table 5.1) in this study, Karamany *et al.* (2013) found a reduction of the levels of both BOD_5 and COD by 20.5% from primary treated domestic wastewater as response of a step-wise increasing of EM dose from 1:7500 to 1:1000 in bioreactor. The slight performance differences could be differences in treatment configuration as CWs is complex system and highly influenced by fluctuation of several environmental variables (Wu *et al.*, 2015). The composition of wastewater could be also different with reference to presence of removal processes inhibitory chemicals that could affect the growth and activity of degrading microorganisms or the added EM bioaugments.

Moreover, one–way ANOVA results demonstrated that the mean reductions of BOD_5, COD, NO_3^- and TP in out flow samples were statistically ($p < 0.05$) higher at a dilution of 1:720 and 1:600 compared to that of 1:1800. However, the mean removal difference between applying 1:720 and 1:600 was relatively small, i.e. 0.5%, 1.4%, 0.7%, 3.1% and 3.4% for BOD_5, COD,

NH_4^+, NO_3^- and TP respectively that implying a sort of steady state, except for NO_3^- and TP. Thus, it can be put forwarded that a concentration of EM with a dilution around 1:600 could be applied for enhanced wastewater treatment performances of HSSFCWs, particularly in floriculture industry.

Table 5.1 The dose-dependent EM: wastewater (WW) removal efficiency (%) of the HSSFCW upon 3 days of retention time

EM: WW	BOD_5	COD	NH_4^+	NO_3^-	TP
1:1800	75.0±2.9	75.9±2.4	91.8±1.7	73.0±1.9	60.5±2.9
1:1200	78.0±4.2	77.6±2.9	92.7±1.1	76.0±1.2	63.6±2.2
1:900	83.0±2.5	80.7±1.7	94.0±2.2	80.7±2.2	68.0±2.3
1:720	93.5±3.1	85.7±1.9	95.6±2.1	86.4±1.5	73.2±1.5
1:600	94.0±2.1	87.1±2.5	96.3±1.5	89.5±1.2	76.6±2.4

5.3.3. Effect of EM doses on wetland bacterial counts

Microbiological analysis also showed that bacterial counts in treatment zones of the CW units changed as response of EM-augmentation (Fig. 5.3a). In general, the mean bacterial cell density increased as function of increasing of EM concentration in the CWs. The counts increased variably by a log values (CFU/gm) of 0.33, 0.36, 0.61 and 0.70 due to the change of EM dilution from 1:1800 to 1:1200, 1:1200 to 1:900, 1:900 to 1:720 and 1:720 to 1:600 respectively. Overall, the bacterial counts showed a total difference by log of 2.00 between the highest (1:600) dilution and the lowest (1:1800) (Fig. 5.3a), may suggest positive influence of EM addition on the density of sediment bacterial communities of the CW units. As a result, it can be applied for reducing limitations of treatment performance of the CWs due to the undersized or lack of orientation-degrading microbial population.

Some previous studies also found a similar microbial trend in bioreactors, i.e. an increase of microbial density by log values of 0.40 CFU/ ml due to increasing of EM dilution from 1:2500 to 1:1000 in Karamany *et al*. (2013). And an increased of total bacterial count by log of 1.21 to 1.31 respectively due to addition of EM at a dilution of 1:10,000 with interval of 5 days in Namsivayam *et al*. (2011) and Victoria and Uma Maheswari (2016). Some studies noted microbial counts decreased (Lananan *et al*., 2014) or totally failed growing after bio-augmentation to new sites, including CWs due to stresses by abiotic and biotic factors (Gentry *et al*., 2004). In contrast this, the wetland bacterial count increased in our CW units, which was positively related to the amounts of EM dosed (Fig. 5.3a) and could suggest their continued and co-existence in the system.

The increase in bacterial count showed a direct relationship with removal performance (Fig.5.3b). The change in log CFU of wetland bacterial count per dilution of EM in Fig. 5.3a positively correlated to change in removal efficiency of BOD_5, COD, NH_4^+, NO_3^- and TP in Table 5.3) though the correlation is weak, i.e. $r < 0.25$ except for BOD_5 as presented in Fig.5.3b. Thus, these results could also support an establishment of a surviving and functioning bacterial population in the treatment zone of the experimental CW units due to EM bio-augmentation.

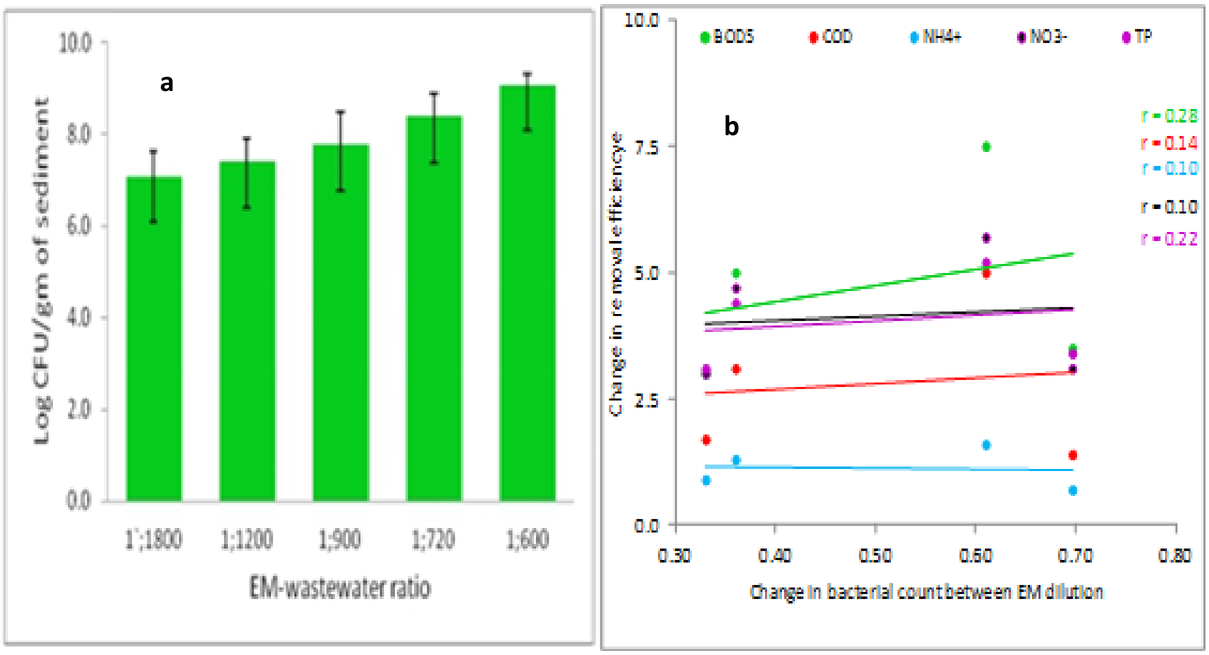


Fig. 5.3 (a) Density of sediment bacteria in treatment zones of the CW units in dependence of EM dilution (b) correlation between change bacterial density per EM dilution and removal efficiency.

5.3.3. Effect of retention time on treatment performance

Fig. 5.4 represents influences of three different hydraulic retention times (1, 2 and 3 days) on removal of organic matter and nutrients in 1:600 EM dosed (CE i.e. C1, C3) and non-dosed (CC i.e. C2, C4) CW units. Comparing data obtained between 1 and 3 day, the mean removal markedly improved for BOD_5, COD, NH_4^+, NO_3^- and TP by 24.3%, 11.6%, 3.2%, 22.8% and 15.4% in CE and 34.7%, 25.0%, 20.2%, 21.6% and 10.2% in CC respectively for 3 day of retention time. A similar trend was also found in Victoria and Uma Maheswari (2016) where an increased removal of BOD_5, COD, NO_3^- and TP by 35.2%, 7.4%, 11.9% and 15.6% respectively due to shifting of the incubation time from 5 to 10 days during treatment of wastewater in EM dosed Laboratory bioreactor. This denotes retention time was an important factor on treatment performance of the CW units regardless of EM bio-augmentation.

On the other hand, higher removals (by > 27.0%, > 5.0%, > 21.0%, > 31% and > 10% for BOD_5, COD, NH_4^+, NO_3^- and TP respectively) were obtained in 1:600 EM dosed (CE) CW units than the corresponding control (CC) while considering all tested retention time (1, 2 and 3 days). T-test analysis of the data obtained at each retention time between CE and CC showed as the removal difference were significant ($P < 0.05$). An influence of EM dosing also observed in Monica *et al*., (2011) where 3ml/L (1:333) EM treatment remarkably reduced the BOD_5 and COD by > 50% than the corresponding control at 3 days of retention. Though the difference between previous and our study could attribute to difference in operational parameters and other external factors where the experiments conducted, the results obtained demonstrates the potential of EM-augments for enhancing treatment performance regardless of the wastewater retention time.

All taking together, the 1:600 EM-dosed (CE) CW units operating at retention time of 3 days statistically reduced the level of BOD_5, COD, NO_3^- and TP by > 11% compared to solely 1 day (Fig. 5.4). This is corresponded to 4.4, 19.4, 0.1, 2.5 and 1.7 mg/L for the parameters respectively in outflow samples which were by far below the environmental tolerable limits of EPA. This tends to suggest an improvement in the degradation and assimilation of organic matter and nutrients leading to a better efficacy of CWs as response of EM addition. Therefore, the use of EM bioaugment at a dilution of 1:600 with retention time of 3 days seems optimal conditions for improved removal of organic matter and nutrients from floriculture wastewater in our HSSFCWs.

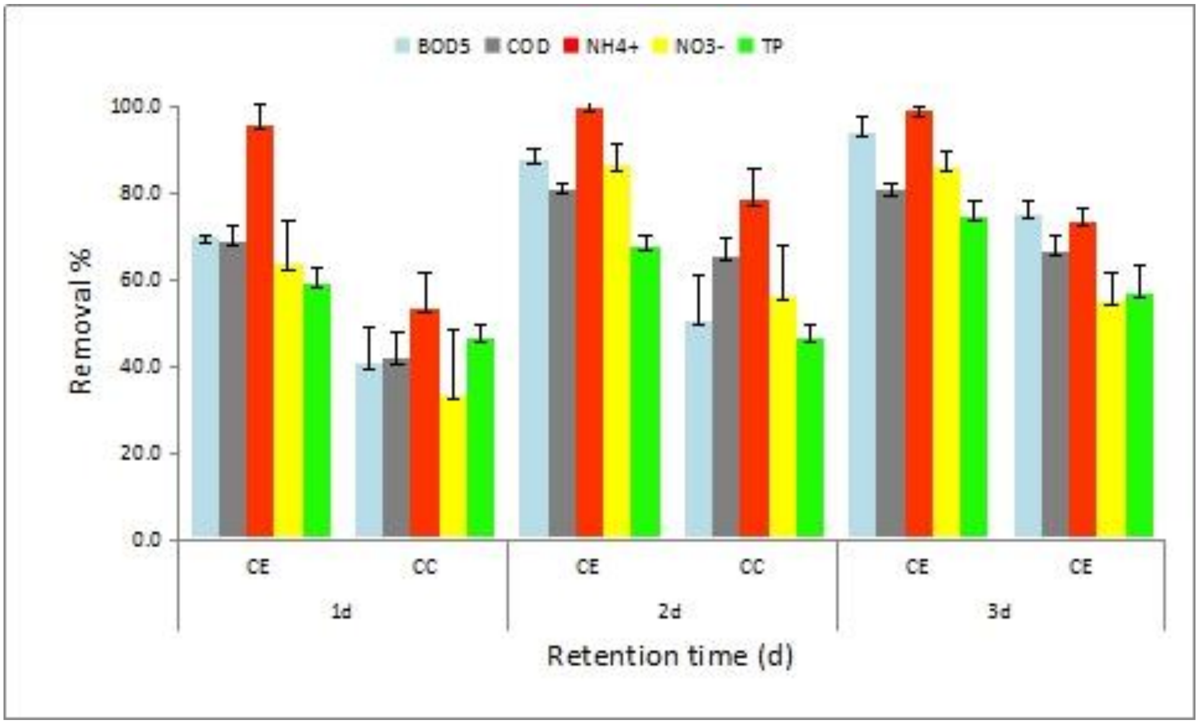


Fig. 5.4 Effects of retention time on removal efficiencies of biological oxygen demand (BOD_5), chemical oxygen demand (COD), ammonium (NH_4^+), nitrate (NO_3^-) and total phosphorus (TP) in CWs without EM (CC) and with EM (CE).

5.4. Conclusion

Based on our experimental results, the EM bio-augments in HSSFCWs could enhance organic matter and nutrients removal from the wastewater at field conditions. EM bio-augmentation in CWs increases the sediment bacterial density which positively correlated with the increased removal of organic matter and nutrients. Compared to the corresponding control, EM bio-augmentation in CWs can also help to shorten the time required for bioremediation of organic matter and nutrients from wastewater. Generally, this study gives a highlight on the use of EM bio-augments in CWs as an alternative wastewater treatment technology, particularly in floriculture industry.

References

APHA (2012). *Standard Methods for the Examination of Water and Wastewater*. 27^{st} ed. Washington DC, USA.

Berhan Teklu, Amare Hailu, Wiegant, D.A., Scholten, B.S. and Van den Brink, P.J. (2016a). Impacts of nutrients and pesticides from small and large scale agriculture on the water quality of Lake Ziway, Ethiopia. *Env Sci Pollut Res* http://dx.doi.org/10.1007/s11356-016-6714-1

Gentry, J. T, Rinsing, C.and Pepper, L. I. (2004). New Approaches for Bio-augmentation as a Remediation Technology. *Critical Review Environment Science Technology* **34**:447–494.

Gerhardt, P., Murray, R.G.E., Wood W.A. and Krieg, N.R. (1994). Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, D.C.

Herrero, M. and Stuckey, D.C. (2015). Bio-augmentation and its application in wastewater treatment: A review. *Chemosphere* **140**:119–128.

Higa, T. (1996). *An Earth Saving Revolution: A Means to Resolve Our World's Problems through Effective Microorganisms (EM).* Sunmark Publishing Inc. Tokyo, Japan.

Higa T. and Parr, J.F. (1994). *Beneficial and Effective Microorganisms for a Sustainable Agriculture and Environment.* International Nature Farming Research Center Atami, Japan

Karamany, H.M., El Shatoury, S.A., Ahmed, D.S. and Saleh, I.S. (2013). Potential of effective microorganisms (EM) for conventional activated sludge upgrade. *Journal of international water Technology* **3 (2)**:78-87.

Lananan, F., Hamid, S.H.A., W Din, W. N. S., Ali, N., Khatoon, H., Jusoh, A. and Endut, A. (2014). Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing Effective Microorganism (EM-1) and microalgae (Chlorella sp.). *International Biodeterioration and. Biodegradtion* **95**:127-134.

Maalim, K.M., Mgana, S.M., Mbuligwe, S.E., Kassenga, G.R. and Mohamed, S.M. (2013). Treatment of tropical domestic wastewater by USASB reactor inoculated with effective microorganism's consortium for COD and suspended solids removal. *Proceedings of the 13th international conference of environmental science and technology* Athens, Greece, 5-7.

Malefia Tadele (2009). Environmental impacts of floriculture industry on Lake ziway with particular reference to water quality, A Thesis Report Submitted to Addis Ababa University School of Graduate Studies as a Partial Fulfillment of MSc. Degree in Environmental Science, Addis Ababa University.Mandalaywala, H. P., Patel, P.V.and Trivedi, R. (2017). Introduction and use of effective microorganisms for bioremediation processes, A Review. *Ijsrm*. **7 (3)**:41-50.

Meng, P., Pei, H., Hu, W., Shao, Y.and Li, Z. (2014). How to increase microbial degradation in constructed wetlands: influencing factors and improvement measures. *Bioresources Technology* **157**:316–326.

Monica, S., Karthik, L., Mythili, S. and Athiavelu, A. (2011). Formulation of Effective Microbial Consortia and its Application for Sewage Treatment. *Journal of Microbia land Biochemical Technol*ogy **3(3)**:051-055.

Namsivayam, S., Narendrakumar, G. and Arvind, K. (2011). Evaluation of Effective Microorganism (EM) for treatment of domestic sewag. *Journal of Experimental Science* **2(7)**:30-32.

Priya, M., Meenambal, T., Balasubramanian, N. and Prumal, B. (2015). Comparative study of treatment of Soga wastewater using HUSAB reactor in the presence and absence of effective microorganisms. *Procedia Earth and Planetary Science* **11**: 483 – 490.

Rashed, E. M. and Massoud, M. (2015). The effect of effective microorganisms (EM) on EBPR in modified contact stabilization system. *JHBRC*. **11**:384-392.

Tadele Yeshiwas and Melkamu Alemayehu (2018). Social, economical and environmental issues of floriculture sector development in Ethiopia. *Review of Plant Studies* **5(1)**:1-10.

Victoria, J. and Uma Maheswari, N. (2016). Effective microorganisms: an incredible alternative for wastewater treatment. *International Journal of Development Research* **6(9)**:9399-9403.

Vymazal, J. (2011). Constructed wetlands for wastewater treatment: five decades of experience. *Environment Science Technol*ogy **45**:61–69.

Wu, H., Zhang, J., Ngo, H.H., Guo, W., Hu, Z., Liang, S., Fan, J. and Liu, H. (2015). A review on the sustainability of constructed wetlands for wastewater treatment: design and. *Bioresources Technol*ogy **175**:594–601.

Zhan, Q.Q., Yang, G.F., Zhang, L., Zhang, Z.Z., Tian, G.M. and Jin, R.C. (2017). Bio-augmentation as a useful strategy for performance enhancement in biological wastewater treatment undergoing different stresses: Application and mechanisms. *Critical reviews in environmental science and technology* **47(19)**:1877–1899.

Zhao, C., Xie, H., Mu, Y., Xu, X., Zhang, J., Liu, C., Liang, S., Ngo, H.H., Guo,W., Xu, J. and Wang, Q. (2014). Bioremediation of endosulfan in laboratory-scale constructed wetlands: effect of bio-augmentation and Bio-stimulation. *Environmental Science Pollution Research* **21**:12827–12835.

Zhou, X., Li, Z., Zheng, T., Yan, Y., Li, P., Odey, E.A., Mang, H.P. and Uddin, S.M.N. (2018). Review of global sanitation development. *Environment Internional* **120**:246–261.

6. Evaluating Endosulfan Removal in a Horizontal Subsurface Flow Constructed Wetlands from Wastewater in Flower Farm, Bishoftu Town, Ethiopia

Abstract

The presence of residual endosulfan in wastewaters is of great concern due to its persistent nature and adverse effects on the receiving environment. The purpose of this study was to investigate endosulfan removal through the use of a horizontal subsurface flow constructed wetlands (HSSFCW) in relation to its inflow concentrations, retention times, Bio-stimulation by sugar cane molasses (MS) and bio-augmentation of effective microorganisms (EM). Wetland inflow and outflow samples were analyzed to determine removal efficiency of endosulfan isomers. Sediments, plant tissues and water samples from the treatment zone of the HSSFCWs were also analyzed to predict the fate of endosulfan. The amounts of endosulfan isomers were examined by using GC-MS and density of wetland bacteria was monitored by the viable plate count technique. At a fixed retention time of 4 days, the mean removal of endosulfan was significantly higher (> 90%) from 0.5 μg/L inflow concentrations compared to (< 65%) from 10 μg/ L. Extending the retention time from 4 to 10 days, improved endosulfan removal efficiency by 6%, 12%, 21% and 23% at inflow concentrations of 0.5, 1, 5, and 10 μg /L respectively. Additions of sugar cane molasses (MS) and effective microorganisms (EM) to the CW units enhanced endosulfan removal by > 15.0% and 19.0% respectively compared to the corresponding control from 10 μg/ L at retention time of 10 days. This higher removal paralleled with increased bacterial densities in the treatment zone of the CW units. In our experiment, high removal efficiency of endosulfan without any accumulation of plants and low sediment sorption may suggest biodegradation as removal mechanism. The results of this study revealed a high potential of relatively cheap and eco-friendly HSSFCWs for bioremediation of low residual *endosulfan emanating from agriculture activities, especially when accompanied with molasses Bio-stimulation and EM bio-augmentation.*

Keywords: Effective Microorganisms, Endosulfan removal, Horizontal subsurface flow constructed wetland, Inflow concentrations, Molasses, Retention time

6.1. Introduction

Wastewater treatment in constructed wetlands (CWs) offers a sustainable, eco-friendly and economical technology than others conventional wastewater treatment system (Vymazal and Březinová, 2015). They provide physical, chemical and biological processes of removal of pollutants. It includes processes of sedimentation, hydrolysis, adsorption, microbial degradation and direct uptake by plants.

Horizontal subsurface flow CWs (HSSFCWs) are increasingly popular, in particular for providing different redox conditions in which pollutants get into contact with a network of aerobic, anoxic and anaerobic zones during their passage through it (Vymazal and Březinová, 2015). Furthermore, they are reported to offer a high purification efficiency of organic matter including numerous pesticides (Matamoros *et al*., 2007). However, studies are limited in assessing the feasibility of this technology for removal of priority pollutants (substances which threaten human health or ecosystems) given by the European Water Framework Directive at field conditions.

Endosulfan is one of the selected chlorinated pollutants prioritized by the European Water Framework Directive due to its persistent nature and many adverse effects in the environment. It has been widely used in agriculture to control pests of a wide range of crops, i.e. cereals, tea, coffee, cotton, rice, sorghum, cashew, soy, fruits and vegetables. As a consequence, endosulfan is one of the most commonly detected pesticides in surface waters emanating from agricultural activities in Ethiopia (Berhan Teklu *et al*., 2015; Hanssen *et al*., 2015; Berhan Teklu *et al*., 2016a; Berhan Teklu *et al*., 2016b). It is of great concern due to its persistence and semi-volatile nature (Weber *et al.*, 2010) and severe neurotoxicity (Silva and Beauvais, 2010). Thus, an effective management on its application sites is of critical importance.

Technical grade endosulfan is commercially available as a mixture, typically containing >95% of two diastereoisomers, known as α- and β-endosulfan in ratios from 2:1 to 7:3 in dependence of the technical mixture (Herrmann, 2002). The two isomers are subject to degradation resulting in endosulfan sulfate via oxidation or endosulfan diol via hydrolysis in aquatic systems (Weber *et al*., 2010). Similar to other persistent organic pollutants, levels of endosulfan isomers are usually higher than their allowed threshold in treated effluents (Gregoire *et al*., 2009). The removal processes assumed to be influenced by different factors including pesticide inflow concentrations, pesticide retention time and availability of nutrients or activities of microbial community.

Bio-stimulation via nutrient supply leads to stimulating indigenous microorganisms (Garcia-Blanco *et al*., 2007) whereas bio-augmentation adds effective catabolic microbes into an indigenous microbial community (Nzila *et al*., 2016). Both strategies are known to improve removal of various pollutants in different treatment systems (Zhao *et al*., 2014; Nzila *et al*., 2016).

For instance, sucrose biostimulated in lab-scale CWs related with a higher removal of endosulfan isomers (Zhao *et al*. 2014). Molasses as biostimulants is also reported to improve pollutants removal through enhancing processes of bioremediation (Lamichhane *et al*., 2012). Sugar cane molasses primarily provide a carbon and energy source for microbes as well as trace elements that are required for the production of microbial enzymes involved in the mineralization of pollutants. However, sugar cane molasses as a relatively cheap biostimulant for the removal of endosulfan in CWs have not been studied.

The EM consortia based on the original formulation of Higa and Parr (1994) implicated to offer a feasible treatment options for removal of contaminants from wastewaters (Monica *et al*., 2011; Mgana *et al*., 2013; Rashed and Massoud, 2014). However, there are limited studies showing its applicability for bioremediation of persistent pollutants such as endosulfan via bio-augmentation. Thus, the major aim of the present study was to determine endosulfan removal efficiencies in pilote scale HSSFCWs in relation to selected endosulfan inflow concentrations, retention times, sugar cane molasses supplementation and EM bio-augmentation at field conditions.

6.2. Materials and Methods

6.2.1. Description of the treatment system

Fig. 6.1 represents a schematic sketch of the four *Typha* planted horizontal subsurface flow constructed wetlands (HSSFCWs) units (C1, C2, C3 and C4) that were used for this specific experiment. The CW units were dosed with different endosulfan concentrations but fixed amount sugar cane molasses and EM.

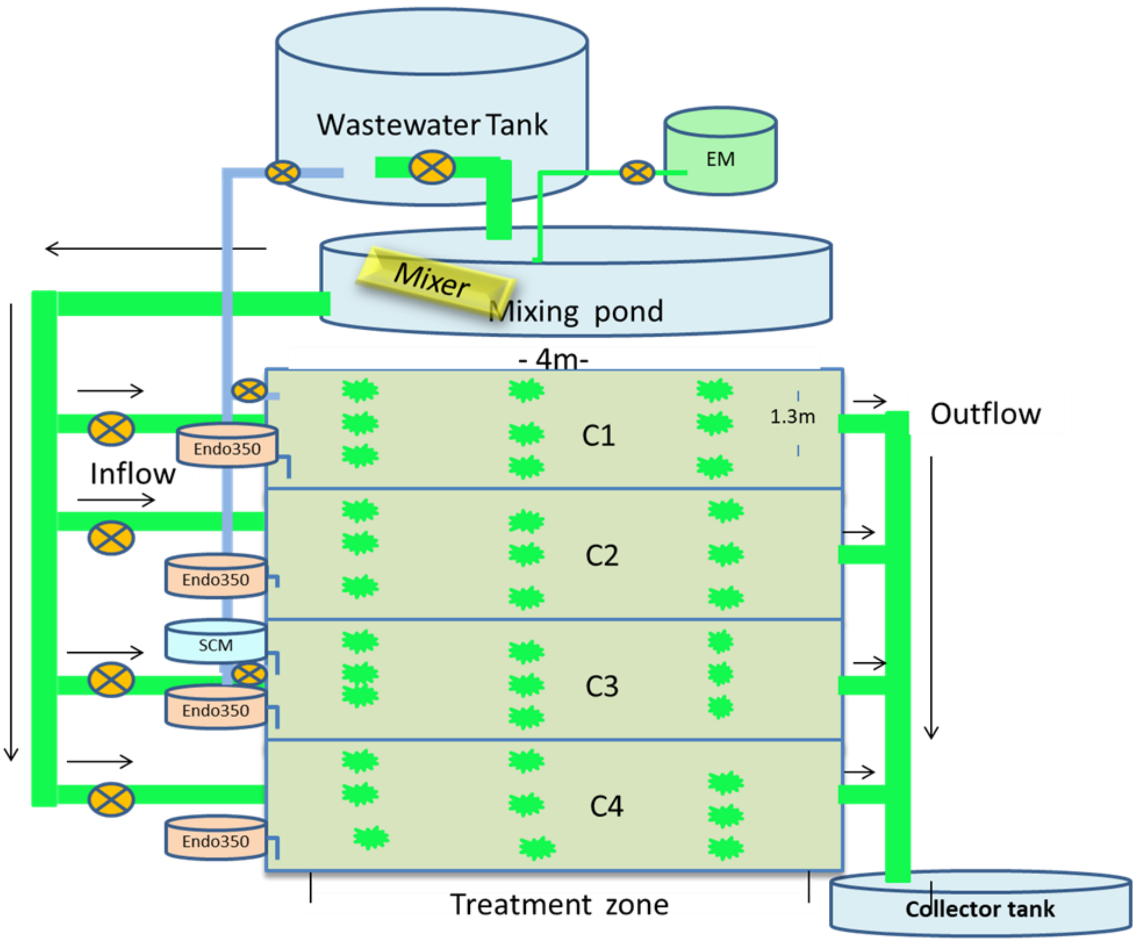


Fig. 6.1 Schematic representation of the four experimental CW units designated from C1 to C4, EM = Effective Microorganisms, Endo350 = Inflow endosulfan, SCM = Sugar Cane Molasses.

6.2.2. Experimental procedures

Endosulfan containing wastewater was prepared by mixing technical-grade endosulfan (Ensosulfan 350 EC) with the wastewater collected from the drainage system of the flower farm. The wastewater was apparently stable in physicochemical compositions with the following average inflow wastewater parameters of pH: 7.78±0.2, temperature: 23.1±1.7 ^{0}C, electrical conductivity: 0.91±0.02 mS/cm, BOD_5: 82.6±0.9 mg/L, COD: 148.5±1.5 mg/L, NH_4^+: 4.5±0.7mg/L, NO_3^-: 11.3±0.9 mg/L, TP: 8.6±5.5 mg/L and SO_4^{2}: 44.6±0.5 mg/L. The endosulfan was mixed with the wastewater in different ratios in order to get the final endosulfan inflow concentrations of 0.5, 1.0, 5.0, and 10.0 μg/L in the synthetic wastewater.

The effects of both initial endosulfan concentrations and retention time on endosulfan removal performance of the CW units were evaluated using four different endosulfan concentrations and retention times. The dose of 0.5 to 10 μg/L used in this study was selected to provide some degree of relevance to the concentration that commonly encountered in untreated floriculture wastewater. The prepared concentrations (0.5, 1.0, 5.0, and 10.0 μg/L) were also analogous to the range of the residual endosulfan concentrations detected in surface waters of the study area (Berhan Teklu *et al*., 2016b).

The 0.5, 1.0, 5.0, and 10.0 μg/L endosulfan-wastewater mixtures were allowed to enter the treatment zones of C_1, C_2, C_3 and C_4 respectively that were operating in continuous mode (Fig. 6.1). The effects of four hydraulic retention times (4, 6, 8, and 10 days) on removal efficiency of endosulfan isomers was also evaluated for each of the four endosulfan concentrations (0.5, 1.0, 5.0, and 10.0 μg/L). The retention time for the treatment system was adjusted by a flow controlled valves and electromagnetic flow meter.

Furthermore, influences of addition of sugar cane molasses as biostimulate (MS) and EM as mixed bioaugment on the removal of endosulfan isomers were evaluated. A controlled experiment in C_1 without MS and EM was conducted for comparison. MS were added from the supplementation tank to the wastewater containing 10 μg/L endosulfan in a ratio of molasses: wastewater 1:40 in C_3, which was the lowest amount molasses that was reported to provide effective degradation of organic compounds in Lamichhane *et al*. (2012).

EM was purchased from WOLJEEJII private EM supplies in Bishoftu town. Then, the EM solution was prepared by mixing 1 volume of EM to 1 volume of sugar cane molasses and 18 volumes of chlorine-free water following the manufacturer's protocol (Higa, 1996). EM, molasses and water were well mixed in a clean airtight plastic container and incubated at room temperature for around 15 days by an intermittent release of the fermentation gas produced. The pH of the product shifted from initially 6.8 to 3.7 indicated the completion of the activation process. Microbial cell density was monitored by viable cell counts, which approximately yielded $5.8+10^8$ CFU/ml. Finally, the prepared EM solution was added to C_4 CW unit in a proportion of 1: 40 (V:V) EM solution to wastewater containing 10 µg/L endosulfan.

6.2.3. Sampling processes

Inflow and outflow water samples from each CW units (C_1, C_2, C_3 and C_4) were collected once per two weeks for analysis of the levels of physicochemical parameters and endosulfan isomers during monitoring the experimental set up for four months. Wetland sediments, water and plant tissues samples were also collected from the treatment zones of each CW unit to examine the fate of endosulfan in the late phase of the experiment. The samples were collected at a distance of 1, 2, and 3 m intervals (in longitudinal direction, 4 m total CW length) and at a distance of 0.3, 0.6, and 0.9 m (crosswise direction, 1.3 m width CW) (Fig. 6.1). Water samples were collected from these 9 selected sites of the treatment zone. Similarly, sediments (fine gravels and accumulated organic matter) were collected at a depth of 25-35 cm from the plant roots of treatment zones of each CW unit which was apparently dominated by roots of the wetland plant.

The aerial tissues of plants with similar growth conditions were also collected from the 9 selected spots of each CW units. Thereafter, the 9 sub-samples were mixed and homogenized in order to generate one composite representative samples. The samples were all run in triplicate on interval of three days of sampling. The collected water, plant and sediment samples were kept in a cooler and immediately transported to the laboratory for analysis.

6.2.4. Endosulfan extraction and determination

The endosulfan in the water samples (1000 ml) was extracted according to the EPA Method 3510C using 60 ml of methylene chloride. The extraction procedure was repeated 6 times and concentrated to 1 ml using a Kuderna-Danish Apparatus.

Sediments and chopped plant tissue samples (15 grams) were extracted according to the AOAC Official Method 2007.01 using 15 ml of 1% Acetic Acid (HOAc) in Acetonitrile (MeCN), 6 grams of Anhydrous Magnesium Sulfate, and 1.5 grams of Anhydrous Sodium Acetate via shaking for 1 minute in 50 ml centrifuge tubes and centrifugation at 4000 rpm for 1minute.

A portion of the MeCN extract (8 ml of the upper layer) was transferred into a 50 ml centrifuge tube and mixed with 400 mg of Primary Secondary Amine (PSA), 400 mg of C-18, 400 mg Graphitized Carbon Black (GCB), and 1.2 g of Magnesium Sulfate were added and then shaken for 1 minute and centrifuged at 4000 rpm for 1 minute.

About 1 ml of the upper layer was transferred to a GC auto-sampler vial for analysis by Gas Chromatography/Tandem Mass Spectrometry (GC/MS). Endosulfan isomers were determined by injecting 1 μL of the sample at 250 ^{0}C using an Agilent 7890 Gas Chromatograph coupled with an Agilent 7000C Triple Quadrupole Mass Spectrometer equipped with an Agilent J and W DB-1701P column (30 m X 0.25 mm X 0.25 μm). Hydrogen was used as a carrier gas and nitrogen (1.5 ml/min) as a collision gas.

6.2.5. Monitoring bacterial density

The microbial load of water and sediment samples was estimated using total viable plate count technique procedure as described previously (APHA, 2012; Calheiros *et al.*, 2009a). Samples were prepared to appropriate dilutions; (sediment samples to 10^{-6}-10^{-12} and water samples to 10^{-1} to 10^{-9} in normal saline solutions (0.85%), from which 0.1 ml of each was inoculated on nutrient agar plates. The cultures were incubated for 48 hours at 37^0C. Upon appearance of bacterial colonies, plates with identifiable and countable colonies were selected and counted. All results obtained were expressed on a log basis per gram sediment or ml of sample water.

6.2.6. Data analysis

To assess treatment performance of each HSSFCW, endosulfan removal efficiencies were calculated on the basis of endosulfan concentrations in the inflow and outflow. The removal percentage was determined as shown below: where R% is removal percentage, C is the endosulfan concentration in mg/L, subscript i and o represent inflow and outflow samples, respectively.

$$R\% = \frac{(\mathrm{Ci} - \mathrm{Co}) * 100}{\mathrm{Ci}}$$

Graphical analyses were performed using Microsoft Origin, Minitab and Past software. Statistical analysis of the data was performed by using the Past software (version 2.08). Data of means with standard deviation were used in graph presentation. One-way analysis of variance (ANOVA) was used for statistically difference between means of observation. Differences in performance were considered to be statistically significant if $P \leq 0.05$.

6.3. Results and Discussion

6.3.1. Effects of endosulfan concentrations and retention time

Endosulfan removal potential of *Typha* planted CW units with a retention time of 4 days was evaluated using 4 different concentrations (0.5, 1.0, 5.0, and 10.0 μg/L) of endosulfan. In average more than 90.0% of each α- and β-isomers of endosulfan was degraded when the initial endosulfan inflow concentration was 0.5 μg/L (Fig. 6.2a). The removal efficiency was 61.0% and 55.8 % for the α- and β-isomers respectively when the initial endosulfan inflow concentration was increased to 10 μg/L. The removal performance showed declining trend as feed concentration increase (Fig.6.2a) and removal efficiency was statistically higher ($P < 0.05$) at inflow concentration of 0.5 μg/L compared to the inflow of 1.0, 5.0, and 10.0 μg/L

The decrease of the removal efficiency as increase of inflow concentrations could suggest remarkable effect of inflow concentration on remediation processes in treatment zone of the CW units. In Zhao *et al*. (2014) endosulfan removal efficiency > 85 % on 4 days of retention time was obtained from feed of 100 μg/L in laboratory-scale batch-operated vertical-flow CWs. The low removal of endosulfan from low concentration in our study could be the difference in treatment configuration as ours is continuous operated horizontal CWs at field conditions which may be influenced by uncontrolled environmental variabls such as oxygen tension and temperature.

The presence of gravel, plant roots, biofilm and organic matter complexes at anoxic conditions are reported to allow the HSSFCW to eliminate endosulfan by the hydrolysis path way (Braeckevelt *et al*., 2007; Matamoros *et al*., 2007). However, detection of endosulfan sulfate in the outflow samples along with the reduction of the α- and β-isomers of endosulfan in this study implicated degradation of endosulfan by oxidation in the treatment zones of the CW units (Fig.2ab).

Aerobic micro-sites in close proximity to plant roots was formed by the wetland plant through oxygen transfer systems from the atmosphere to the inside of the gravel beds may consider facilitating the observed oxidative degradation of endusulfan. Since we could not determine the endosulfan intermediates produced via hydrolysis, it remains uncertain whether oxidation represented the sole elimination pathway in this study. Though endosulfan sulfate was reported to be more stable in aqueous system (Kong *et al*., 2013), the relatively low amount of endosulfan sulfate detected along with a remarkable removal of the α- and β-isomers and the opposite at lower removal of endosulfan (Fig. 2ab) does not necessarily support a low biodegradability of endosulfan sulfate in our study.

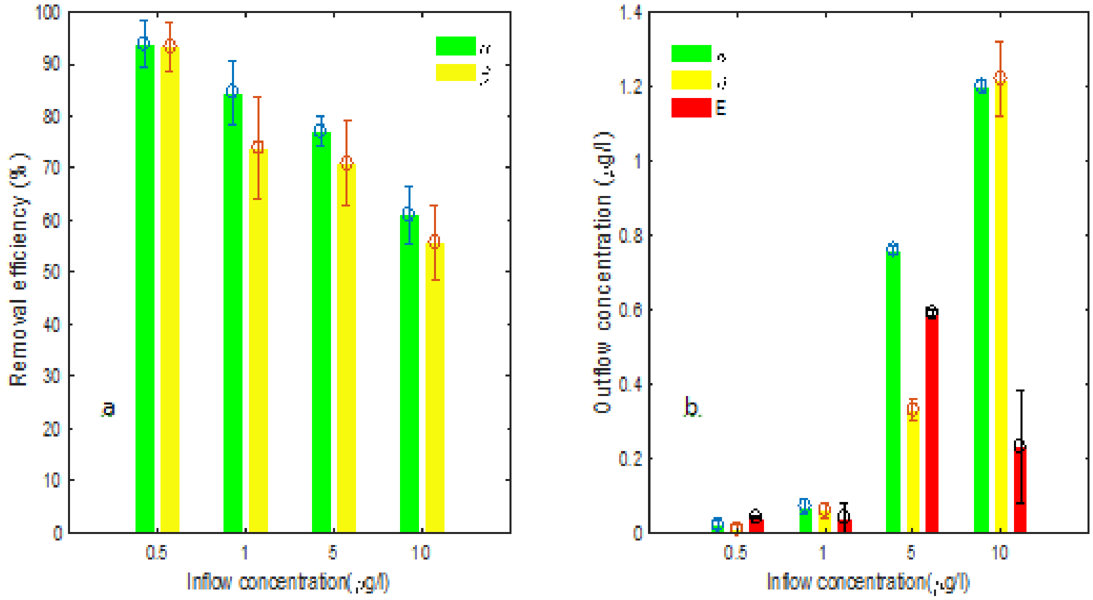


Fig. 6.2(a) Removal efficiency of α-isomer (α) and β-isomer (β) in CW units (b) outflow concentration of α-isomer (α) and β-isomer and its oxidative product Endosulfan sulfate(E), n=3, Error bars indicate the standard deviation

Fig. 6.3ab reveals the effects of various retention times, i.e. 4, 6, 8 and 10 days on endosulfan removal efficiency in the HSSFCW units. Extending the retention time from 4 days to 10 days, the removal of α-isomer of endosulfan increased by 5.9, 12.4, 9.6 and 23.2% for endosulfan inflow concentrations of 0.5, 1.0, 5.0 and 10.0 µg/L respectively (Fig. 3a). A similar pattern was observed for the endosulfan β-isomer removal, which increased by 5.6, 24.3, 21.4 and 26.1%, respectively (Fig. 6.3b).

In general, an increasing in retention time resulted in an increasing trend in removal efficiencies for both α- and β-isomers. The statistical analysis also showed a significantly higher removal of both isomers at a retention time of 10 days compared to 4 days, regardless of the tested inflow concentrations. However, as the increase of endosulfan inflow concentration, the effect of retention time became more pronounced (Fig. 6.3ab). Similar trends were found in Zhao *et al.* (2014), which both showed enhanced endosulfan removal efficiencies as a result of extending in retention time in their experimental CWs.

Hydraulic retention time affects the time water stays in the CWs and thus flow depth, substrate porosity and potential contact of microbial community with the pesticide. Thus, extending retention may lead to prolonged contact of endosulfan degrading wetland microbial consortia with the pesticide and may accounted for improved removal of endosulfan and its degradation product. Althogh insignificant from 8 days, the results obtained at retention time of 10 days in our CWs showed enhanced microbial degradation of endosulfan isomers, whereby the degradation efficiency greatly decreased with the increase in the pesticide's incoming concentration.

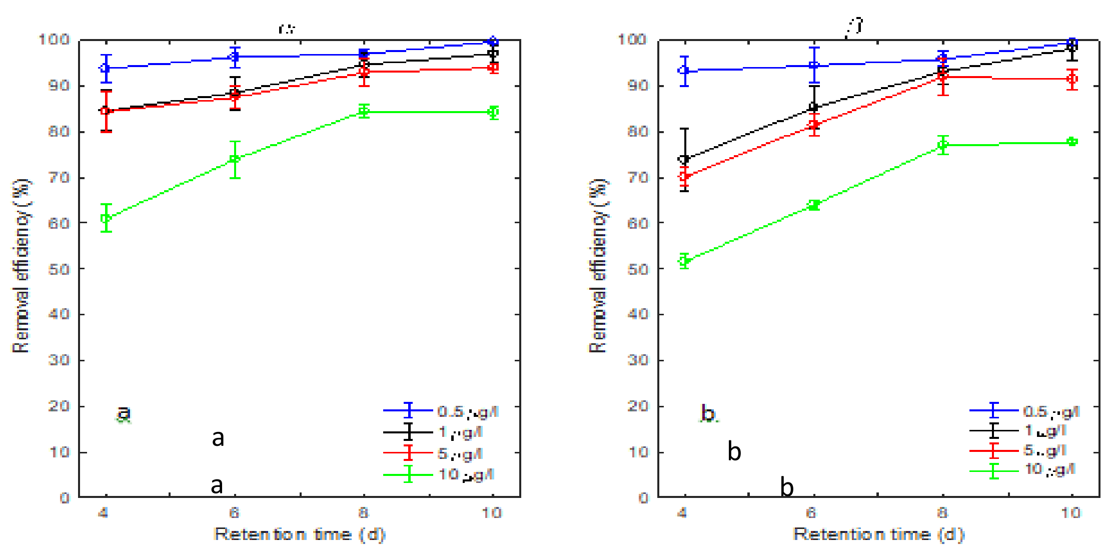


Fig. 6.3 Removal efficiency of HSSFCW for α- isomers (a) and β-isomer (b) in dependence of hydraulic retention time.

6.3.2. Effects of bio-stimulation and bio-augmentation

In this study, MS and EM as mixed microbial inoculums were evaluated for their bioremediation potential of endosulfan. Removal of endosulfan improved to 94.3±1.3% and 92.3±6.7% for α- and *β*-isomers respectively under supplementation of molasses from inflow endosulfan of 10.0 μg/L and a retention time of 10 days (Fig. 6.4a). It is higher by 10.1% and 14.6% for α-and *β*-isomers respectively compared to removal efficiency without addition of MS (Fig. 6.4a). This finding supports previous findings indicating that addition of an auxiliary carbon source to a treatment system significantly increases microbial biodegradation efficiency of organic pollutants (Goswami *et al.*, 2009b; Kumar and Philip, 2006; Meng *et al.*, 2014; Zhao *et al*., 2014). MS suppose to stimulate wetland microbial consortia for increasingly consuming the organic contaminants directly as a carbon source or indirectly via co-metabolism (Abraham and Silambarasan, 2014; Kumar *et al*., 2007; Lamichhane *et al*., 2012; Semrany *et al*., 2012; Singh and Singh, 2011). Likewise, MS assumed to enhance endosulfan bioremediation in our study by increasing bacterial density (Fig. 6.5) and stimulating their activities through provision of carbon and certain trace elements.

The use of EM bio-augmentation further improved the removal efficiency of endosulfan by > 5.0% compared to MS at same operational conditions (Fig. 6.4a). The increase in removal efficiency in the CW unit was also accompanied with the greatly reduced outflow concentrations of both α-and β-isomers as well as endosulfan sulfate (Fig. 6.4b) suggesting EM-bio-augmentation could positively impact on removal efficiency of endosulfan the CW units. A similar effect of bioaugmention was observed in Zhao *et al*. (2014), which improved removal by 5.9 and 7.6% for α- and β-endosulfan respectively compared to the corresponding control. However, differences of CWs design, endosulfan concentration and bioaugments may account for a higher removal of α- and β-endosulfan by more than 15.0% and 19.0% compared to the corresponding control in our study.

In this study, the removal efficiency of endosulfan was also paralleled with wetland bacterial density as determined by viable cell counts (Fig. 6.5). The higher removal efficiency of endosulfan linked with the higher bacterial density following EM-bio-augmentation (Fig. 6.5). On the other hand, the lower endosulfan removal efficiency correspond to the lower bacterial density in non-MS and EM-bioaugemented CW units (Fig. 6.5) suggest wetland microbial density as important aspect in transformation of endosulfan.

Due to their dynamic and inducible complex enzymatic systems which enable the efficient degradation of xenobiotics, wetland microbial consortia along with the EM-bioaugment assumed to play a key role in endosulfan removal. The bacterial cell density in our CW units (Fig. 6.5) was not significantly different from the result reported in Lananan *et al*. (2014) that ranged between 5.24 $x10^6$ and 9.14 x 10^9 CFU/mL when EM inoculated for treatment of aqaculture wastewater in bioreactors. In this study, the observed highest bacterial density in the EM-bioagmentation may not only result in the highest degradation of α- and β-isomers, but also of their oxidation product endosulfan sulfate (Fig. 6.4). Thus, it verifies the potential importance of EM-bioaugment for successful bioremediation of residual endosulfan in wastewater emanating from flower farm when using in continuous operating HSSFCW system at field conditions.

In general, previous studies indicate that both Bio-stimulation and bio-augmentation improve the removal of various organic pollutants by 0–12 % as compared to the respective corresponding control (Calvin *et al*., 2017; Taccari *et al*., 2012; Wu *et al*., 2011b; Zhao *et al*., 2014). Similarly, MS and EM bioagmentation greatly improved the endosulfan removal efficiency by > 10 and

15% respectively compared to the control (with no MS and EM) in this study which may be due to difference of treatment conditions. In our study, the average levels of endosulfan isomers in outflow were also reduced to a levels close to the permitted discharge limit of 0.1 μg/L (Fig. 6.4b), through the application of EM- bioagmentation. Thus, our result verifies the potential importance of sugar cane molasses and EM-bioaugment for successful bioremediation of residual endosulfan in wastewater emanating from agricultural activities when using in continuous operating HSSFCW system at field conditions.

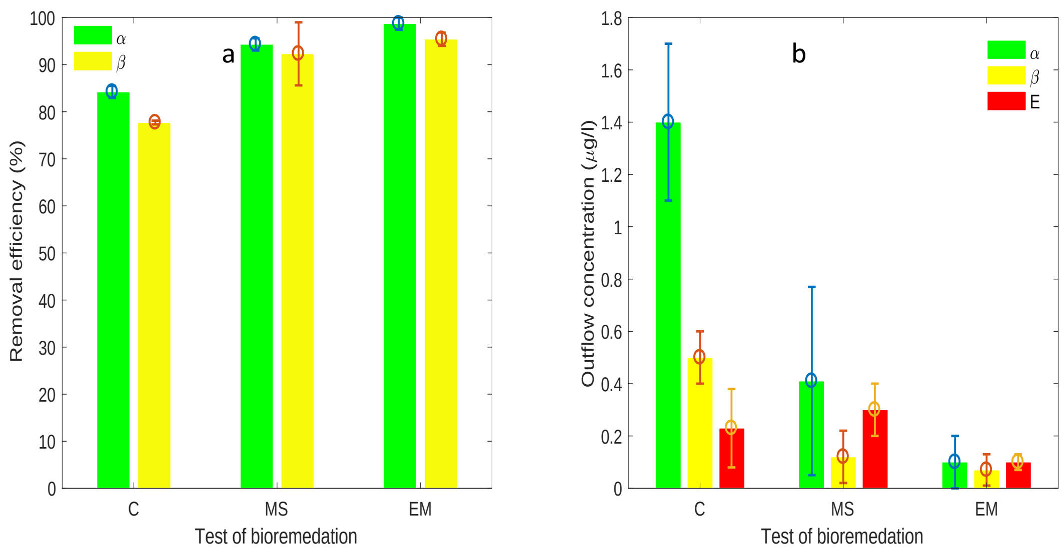


Fig. 6.4 Bistimulation and bio-augmentation on removal of of endosulfan (a) and out flow concentration of α-isomer (α) and β-isomer (β) of endosulfan, and its oxidative product E (Endosulfan sulfate) (b); C = control, *Typha* planted unit with no molasses and EM addition, MS = Molasses stimulation, EM = Effective microorganisms.

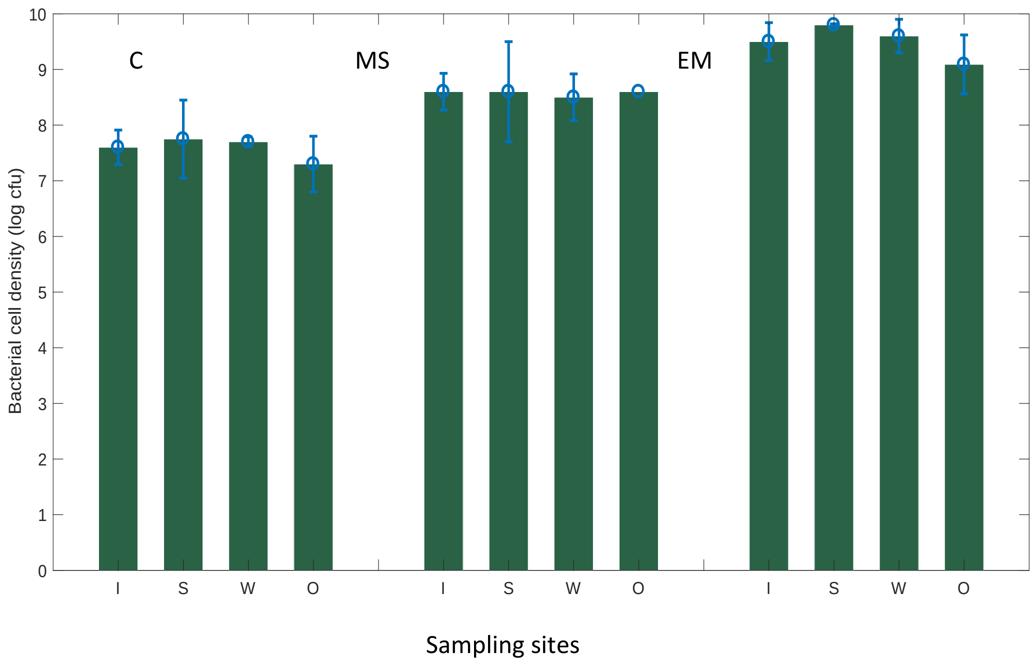


Fig. 6.5 Bacterial cell density following MS and EM additions; I = inflow, S = sediment, W = water of treatment zone and O = outflow samples

6.3.3. Mass distribution of endosulfan in the CW units

To determine the fate of endosulfan isomers in the CW units, a mass proportion analysis was conducted by using water, plant and sediment samples from the treatment zones of C_3 and C_4, which showed relatively better endosulfan removal. Endosulfan is reported commonly to be absorbed by plants, accumulate by plant tissues and is released via evapotranspiration (Hwang *et al*., 2015; Zhao *et al*., 2014). However, none of the endosulfan isomers were detected in plant samples in the present study and thus, the effect of phytoremediation were not clear. On the other hand, different proportions of endosulfan isomers were detected from sediments and water samples of the CW units (Fig. 6.6a). In average, reduced percentages of incoming α- and β-endosulfan (< 8.5% and < 2.5% in sediment and < 36% and < 13.5% in water samples, respectively) were found in the treatment zone of the CW units. The low detection of pollutants such as endosulfan in the CW units' effluent accompanied with low concentrations in the gravel bed of the CW units suggested that biodegradation represents the major elimination pathways for the pollutants (Matamoros *et al.*, 2007). Furthermore, the detection of endosulfan sulfate (Fig. 6.6b) paralleled with low proportions of the α- and β-isomers of endosulfan could support biotransformation of endosulfan in the treatment zone of the HSSFCW units in this study.

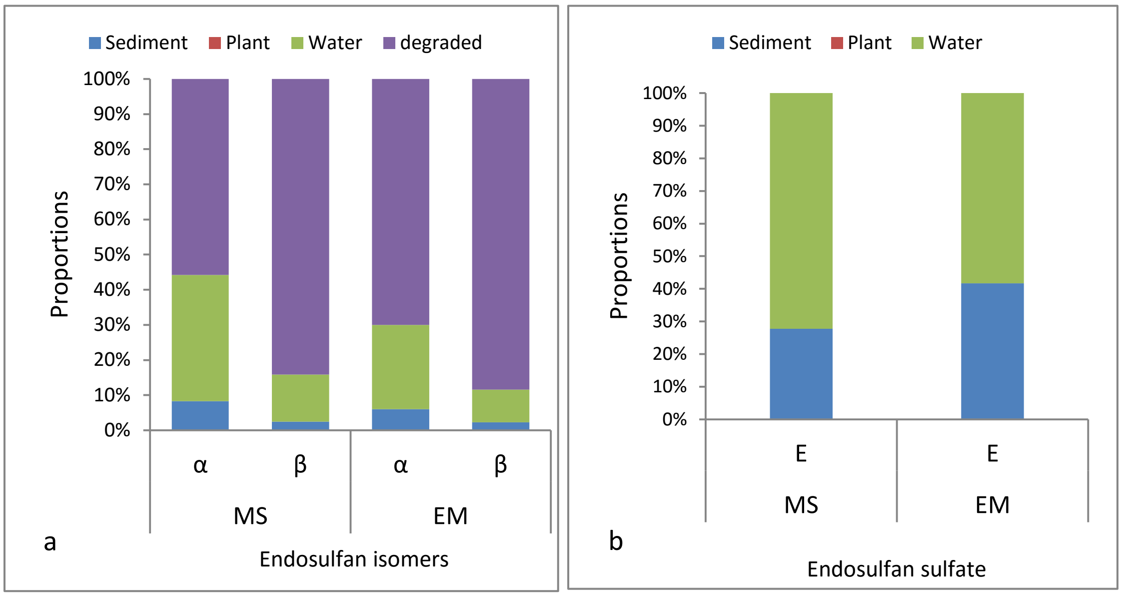


Fig. 6.6 Proportions of α- isomers, β-isomers of endosulfan (a) and its oxidative product endosulfan sulfate (b) in sediment, plant tissue and water samples in MS and EM treatments; α = α- isomers, β = β-isomers, E = endosulfan sulfate.

6.4. Conclusions

This study investigated removal of endosulfan isomers from wastewater in HSSFCWs. Thereby, the removal efficiency of endosulfan isomers was found to be influenced by inflow endosulfan concentration. Extending retention times also led to a greatly improved removal of endosulfan isomers, regardless of the inflow concentrations. Furthermore, our results confirmed the positive effects of MS Bio-stimulation and EM bio-augmentation for bioremediation of endosulfan isomers. The use of EM as bioaugmente lead to the removal of endosulfan isomers close to the discharging limit of 0.1 μg/L from 10 μg/L inflow endosulfan at a retention time of 10 days. The improved removal efficiency was linked with increased bacterial densities in the treatment zone of the HSSFCW units, particularly after EM addition. The reduction of endosulfan α-and β-isomers with detection of endosulfan and without any accumulation of plant tissues and a low substrate sorption suggests that biotransformation represents the main removal pathway for endosulfan in the treatment zone of the CW units. In general, our results revealed a high potential of a continuous operating HSSFCWs for bioremediation of residual endosulfan emanating from floriculture industries, in particular when used in combination with MS and EM-bio-augmentation.

References

Abraham, J. and Silambarasan, S. (2014). Biomineralization and formulation of endosulfan degrading bacterial and fungal consortiums. *Pesticide Biochemistry and Physiology* **116**:24–31.

APHA (2012). Standard Methods for the Examination of Water and Wastewater, 22nded. Washington DC, USA.

Berhan Teklu, Paulien, I., Adriaanse, P. I. and Van den Brink, P.J. (2016b). Monitoring and risk assessment of pesticides in irrigation systems in Debra Zeit, Ethiopia. *Chemosphere* **161**:280-291.

Berhan Teklu, Adriaanse, P.I., Horst, M.M.S., Deneer, J.W. and Van den Brink, P.J. (2015). Surface water risk assessment of pesticides in Ethiopia. *Science of the Total Environment* **508**:566-574.

Berhan Teklu, Amare Hailu, Wiegant, D.A., Scholten, B.S. and Van den Brink, P.J. (2016a). Impacts of nutrients and pesticides from small and large scale agriculture on the water quality of Lake Ziway, Ethiopia. *Env Sci Pollut Res http://dx.doi.org/10.1007/s11356-016-6714-1*.

Braeckevelt, M., Rokadia, H, Imfeld, G, Stelzer, N, Paschke, H, Kuschk, P., Kastne, M., Richnow, H.H. and Weber, S. (2007). Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland. *Environ Pollut doi:10.1016/j.envpol12.008*.

Calheiros, C.S.C., Duque, A. F., Moura, A., Henriques, I. S., Correia, A., Rangel, A. O.S. S. and Castro, P. M. L. (2009a). Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment. *Bioresources and Technology* **100(13)**:3228-3235.

Calvin, N.N., Onyedika, N.C., Emmanue, G.C. and Nkiruka, I. B. (2017). Biodegradation of endosulfan by mixed bacteria culture strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus. Science World Journal* **12(1)**:9-12.

Fahrenfeld, N., Zoeckler, J., Widdowson, M.A. and Pruden, A. (2013). Effect of biostimulants on 2, 4, 6-trinitrotoluene (TNT) degradation and bacterial community composition in contaminated aquifer sediment enrichments. *Biodegradation* **24**:179–190.

Garcia-Blanco, S., Venosa, A. D., Suidan, M. T., Lee, K., Cobanli, S., Haines, J. R., (2007). Bio-stimulation for the treatment of an oil-contaminated coastal salt marsh. *Biodegradation* **18**:1–15.

Goswami, S. and Singh, D.K. (2009). Biodegradation of α and β endosulfan in brothmedium and soil microcosm by bacterial strain *Bordetella sp. B9*. *Biodegradation* **20**:199–207.

Gregoire, C., Elsaesser, D., Huguenot, D., Lange, J., Lebeau, T., Merli, A., Mose, R., Passe-port, E.., Payraudeau, S., Schutz, T., Schulz, R., Tapia-Padilla, G., Tournebize, J., Trevisan, M. and Wanko, A. (2009). Mitigation of agricultural nonpoint-source pesticide pollution in artificial wetland ecosystems. *Environment Chemistry Letter* **7**:205–231.

Hanssen, V. M., Moen, B. E. and Magne Bra Tveit, M. B. (2015). High prevalence of respiratory and dermal symptoms among Ethiopian flower farm workers. *Archives of Environmental and Occupational Health* **70:**204–213.

Herrmann, M. (2002). *Preliminary risk profile of endosulfan*. Umweltbundesamt, Berlin Germany.

Hwang, J.I., Lee, S.E. and Kim, J. E. (2015). Plant uptake and distribution of endosulfan and its sulfate metabolite persisted in soil. *PLoS ONE 10(11):* *https://doi.org/10.1371/journal.pone.0141728*

Kong, L., Zhu, S., Zhu,. L., Xie, H., Su, K., Yan, T., Wang, J., Wang, J., Wang, F. and Sun, F. (2013). Biodegradation of organochlorine pesticide endosulfan by bacterial strain *Alcaligenes faecalis JBW4. Journal of Environmental Science* **25(11):**2257–2264.

Kumar, K., Devi, S.S., Krishnamurthi, K., Kanade, G.S. and Chakrabarti, T. (2007). Environment and isolation of endosulfan degrading and detoxifying bacteria. *Chemotherapy* **68(3):**479-488.

Kumar, M., and Philip, L. (2006). Bioremediation of endosulfan contaminated soil and water–Optimization of operating conditions in laboratory scale reactors. *Journal of Hazardous Materials* **36(2)**:354–364.

Lamichhane, K. M. (2012). Molasses enhanced phyto and bioremediation treatability study of explosives contaminated Hawaiian soils. *Journal of Hazardous Materials* **243**:334–339.

Lananan, F., Hamid, S.H.A., Din. W.N.S., Ali, N., Khatoon, H., Jusoh, A. and Endut, A. (2014). Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing Effective Microorganism (EM-1) and microalgae (*Chlorella sp*.). *Journal of International Biodeterioration and Biodegradation* **95**:127-134.

Matamoros, V., Puigagut, J., Grace, J., Josep, M. and Bayona, P. (2007). Behavior of selected priority organic pollutants in horizontal subsurface flow constructed wetlands: A preliminary screening. *Chemosphere* **69**:1374–1380.

Meng, P., Pei, H., Hu, W., Shao, Y., Li, Z. (2014). How to increase microbial degradation in constructed wetlands: influencing factors and improvement measures. *Bioresources Technology* **157**:316–326.

Mgana, S. M., Mbuligwe, S. E., Kassenga, G. R. and Mohamed, S. M. (2013). Treatment of tropical domestic wastewater by USASB reactor inoculated with effective microorganisms' consortium - COD and suspended solids removal. In: *Proceedings of the 13th international conference of environmental science and technology Athens*, pp. 5-7, Athens, Greece.

Monica, S., Karthik, L., Mythili, S., and Athiavelu, A. (2011). Formulation of Effective Microbial Consortia and its Application for Sewage Treatment. *Journal of Microbial and Biochemical Technology* **3(3)**:051-055.

Nzila, A., Razzak, S. A. and Zhu, J. (2016). Bio-augmentation: An emerging strategy of industrial wastewater treatment for reuse and discharge. *International Journal of Environment Research and Public Health* **13(846):**1-20.

Rashed, E. M., and Massoud, M. (2015). The effect of effective microorganisms (EM) on EBPR in modified contact stabilization system. J*HBRC*.**11**:384-392.

Silva, M. H., and Beauvais, S. L. (2010). Human health risk assessment of endosulfan: toxicology and hazard identification. *Regulatory Toxicology and Pharmacology* **56**:4–17.

Singh, D. K. and Singh, N. (2011). Biodegradation of endosulfan and endosulfan sulfate by Achromobacter *xylosoxidans strain C8B* in broth medium. *Biodegradation* **22**:845–857.

Taccari, M., Milanovic, V., Comitini, F., Casucci, C. and Ciani, M. (2012). Effects of Bio-stimulation and bio-augmentation on diesel removal and bacterial community. Journal of *International Biodeterioration and Biodegradation* **66:**39–46.

Vymazal, J.and Brˇezinová, T. (2015). The use of constructed wetlands for removal of pesticides from agricultural runoff and drainage: *A review Environment and International* **75**:11–20.

Weber, J., Halsall. C. J., Muir, D., Teixeira, C., Small, J., Solomon, K., Hermanson, M., Hung, H. and Bidleman, T. (2010). Endosulfan, a global pesticide: A review of its fate in the environment and occurrence in the Arctic. *Science of the Total Environment* **408**:2966–2984.

Wu, Z., Dong, H., Zou, L., Lu, D. and Liu, Z. (2011b). Enriched microbial community in bio-augmentation of petroleum-contaminated soil in the presence of wheat straw. *Applied Biochemistry and Biotechnol*ogy **164**:1071–1082.

Zhao, C., Xie, H., Mu, Y., Xu, X., Zhang, J., Liu, C., Liang, S., Ngo, H. H., Guo, W., Xu, J. and Wang, Q. (2014). Bioremediation of endosulfan in laboratory-scale constructed wetlands: effect of bio-augmentation and Bio-stimulation. *Environmental Science and Pollution Research* **21**:12827–12835.

7. Conclusion and Recommendations

7.1 Conclusion

The findings of this study give a picture of wastewater treatment performance of a horizontal subsurface flow constructed wetland systems (HSSFCWs) with different wetland planting and different hydraulic conditions operated in the prevailing climatic conditions from flower farms at Bishoftu, Ethiopia. It also helps to consider the application of cane molasses Bio-stimulation (MS) and Effective microorganisms (EM) bio-augmentation in the CWs as alternative strategies for bioremediation of residual agro-chemicals from flower wastewater.

Based on the results obtained from this study, HSSFCWs mixed planted with *Typha latifola*, *Cyprus papyrus* and *Pennisetum purpureum* and single planted with *Typha latifola*, operating at four days of retention time can be applied for reduction of organic matter (BOD_5 and COD) and nutrients (NH_4^+, NO_3^- and TP) from flower wastewater below the discharging standards set by Ethiopian Environmental Protection Authority (EEPA, 2003). Furthermore, performance of the CWs was obviously influenced by seasonal changes where better reductions of pollutants obtained during the warm seasons ($\geq 20^0$C) which mostly corresponded from March to July through the experimental study. Hence, potential effect of pollutants concentration in out flow due to seasonal changes should be considered to make sure of effective purification

Study of the wetland microbial communities through the use of RISA fingerprints highlighted that the wetland planting and hydraulic conditions seems to have a major influence on the established sediment bacterial communities where their diversity positively related with pollutant removal performance in the CWs. Further study of bacterial community of a differently planted CW unit (Unplanted, single and mixed planted) in relation to their organic matter and nutrients removal performance by 16S rDNA Illumina Miseq sequencing substantiated the sediment bacterial communities are influenced by CW planting condition. Thus, the planted CWs could outperform as they maintain higher bacterial community compositions which positively related with their pollutant removal performance.

With regard to approaches of bio-augmentation in CWs, adding effective microorganisms (EM) in the CW units showed an effort to enhance the ability of the wetland microbial community to better reduce organic matter (BOD_5 and COD) and nutrients (NH_4^+, NO_3^- and TP) from the wastewater with refrence to the corresponding control. In view of that, EM bio-augmentation in CWs can help to shorten the hydrualic retetion time and then reduce the size and cost of land in wetland construction required for bioremediation of organic matter and nutrients from wastewater in the ever-expanding flower farms in Ethiopia.

The study of removal for endosulfan as a trial pesticide, the results demonstrated a high potential of the eco-friendly HSSFCWs for bioremediation of low residual endosulfan emanating from floriculture activities, especially when accompanied with molasses Bio-stimulation (MS) and EM bio-augmentation. The use of EM (1:40 EM solution to wastewater) indicate a potential of removal of endosulfan isomers close to the discharging limit of 0.1 μg/L from as high as 10 μg/L inflow endosulfan at a retention time of 10 days along increasing the bacterial densities in the treatment zone of the HSSFCW units.

Overall, it can be concluded that the treatment performance of the pilot-scale HSSFCWs single planted with *Typha latifola* or mixed plantation with equal proportion of *Typha latifola, Cyprus papyrus* and *Pennisetum purpureum* applied at selected flower farm was very promising for the promotion and application of the CWs as an alternative wastewater treatment system to protect the environment and public health. Ethiopia has favorable climatic conditions for the implementation of CW systems and hence, the technology can continue as competent solution to alleviate the inherent environmental problems associated with discharging of untreated wastewaters.

7.2 Recommendations

Based on findings of this study, the following recommendations are forwarded

- The CWs with mixed plantation achieved reduction of the selectly studied water quality parameters below the discharge standard set by Ethiopian Environmental Protection Authority (EEPA, 2003). However, further studying the problem of keeping the system with mixed planting in a long term run as aggressive plants might out compete the less agrressive one is recommended
- Further studying performance of the system for management of commoly applying agro-chemicals (nutrients and pesticides) with different spatio-temporal conditions suggested in order to publicizing the technology for other flower and related farms.
- More detailed research works related to investigating the interation of wetland microbial community, wetland plants and wetland substrates in relation to removal of residual nutrients and pestisides is recommended to exploit the scenarios for improving the biodegradation of pollutants of the wastewater
- Mollases Bio-stimulation and EM-bio-augmentation is an attractive strategy for successful management of wastewater is recommended to be further evaluated in relation to performance and cost for removal of other pesticides before scaling up.