

TREATMENT OF AGRICULTURAL WASTEWATER WITH CONSTRUCTED WETLANDS

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ABSTRACT

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Animal manure contains essential plant nutrients such as nitrogen, phosphorus and potassium along with high number of bacteria, viruses and parasites. Pollutants contained in manure enter water bodies both as diffuse or non-point source and as point source from concentrated livestock production systems. Pathogens may flow to the water bodies when manure is applied to fields prior to rainfall. Nutrients that reach water bodies cause eutrophication and pathogens pose health risk.

Two separate studies were conducted to evaluate the applicability of constructed wetlands in treating pollutants originating from animal manure. One constructed wetland system was spiked with high number of *E. coli* and bacteriophage P22 for a short period of time to simulated tile-drain flow and the number of *E. coli* and bacteriophage P22 in the effluent were monitored in winter and summer seasons. The other constructed wetland system was continuously supplied with diluted dairy wastewater and removal of pollutants and recovery of nutrients were measured.

On average, 0.54 and 0.69 log reduction of *E. coli* were obtained in summer and winter months, respectively from the surface flow (SF) wetlands subjected to pulse loading. With similar loading, 3.16 and 1.23 log reduction of *E. coli* were obtained from subsurface flow (SSF) wetlands in summer and winter months, respectively. *E. coli* removal in subsurface flow wetland was higher than in surface flow wetland in both seasons. Two models one based on the

convection dispersion equation (CDE) and the other based on colloid filtration theory did not adequately describe *E. coli* removal in constructed wetlands. Higher removal of bacteriophage P22 was observed in both SF and SSF wetlands in both winter and summer months in the wetlands subjected to pulse loading. P22 removal rates in SSF wetlands were 41 times the removal rate in SF wetlands in winter and 19 times in summer. The CDE model could accurately describe bacteriophage P22 removal in constructed wetlands.

In the wetland systems that were subjected to continuous manure loading, chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) and *E. coli* in influent and effluent were measured. Duckweed was harvested every week to explore the nutrient recovery potential. Average COD, TN and TP removal obtained in surface flow wetlands from dairy wastewater were 28%, 28% and 16% respectively. Average annual mass removal of COD, TN and TP in the wetlands were 2137 g COD/m²/year, 149.5 g N/m²/year and 10.3 g P/m²/year, respectively. First order removal model that includes background concentration was found more suitable than first order model or DUBWAT model for predicting effluent COD, TN and TP removal in constructed wetlands. On average, 0.3 log reduction of *E. coli* was obtained across all the wetlands. Average N and P recovered by harvesting duckweed across all the wetlands were 22.4 g N/m²/year and 5.6 P/m²/year, respectively.

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CHAPTER 1: INTRODUCTION

Animal manure is rich in nutrients, such as nitrogen, phosphorus and potassium. Along with nutrients, manure also contains high numbers of pathogens, such as viruses, bacteria and parasites (Venglovsky et al., 2009). Excess nutrients that reach surface water cause eutrophication of water bodies and pathogens create health hazards. The pollutants, nutrients and pathogens, often enter water bodies from diffuse or non-point sources and from point source like concentrated livestock production systems (Knight et al., 2000). In North America and other parts of the world, field application of manure is a common practice for nutrient recovery and manure disposal. In tile-drained fields, liquid manure can reach the tile lines through wormholes, root channels, soil cracks and other macropores (Harrigan and Northcott, 2007). Thus, bacterial concentration in tile drains are increased by liquid manure application (Fleming and MacAlpine, 1995). Rainfall may further increase pathogens in tile drains by accelerating pathogen transport. Irrespective of the application method, high concentrations of bacteria have been found in tile drains when heavy rainfall occurred soon after manure application (Samarajeewa, 2010). In such events, tile drains are pulsed with high number of pathogens for a short period of time. Thus, livestock farming and subsequent land application of manure cause health and environment risks by polluting water bodies with nutrient and pathogen loads.

Constructed treatment wetlands are engineered systems that are designed to mimic natural wetland systems. Treatment wetlands remove pollutants from wastewater through the interactions with wetland substrates, plants and associated microorganisms (USEPA, 1993a). Treatment wetlands are potential alternatives to chemical or traditional sanitary treatment technologies due to lower construction, operation and maintenance cost (Sundaravadivel and Vigneswaran, 2001). Constructed wetlands remove nutrients and pathogens by the combined

effect of physical, chemical and biological processes discussed in next chapter. Previous pathogen removal studies in constructed treatment wetlands have primarily focused on steady flow of bacterial indicators (Garcia et al., 2008; Green et al., 1997; Karim et al., 2004; Stottmeister et al., 2003). Moreover, seasonal variation in pathogen removal in constructed wetlands has rarely been compared. Hence there is a need to compare viral and bacterial removal efficiency of constructed wetlands that are subjected to pulse loads in both warm and cold weathers.

Apart from pollution reduction, highly productive and nutritive floating macrophytes, such as duckweed, can be grown in constructed wetlands and are easy to harvest and stock (Boyd, 1974). Harvested biomass can be used for composting and soil amendments, digested anaerobically for biogas production, processed for animal feed or mixed with solid manure to increase nutrient content (Sooknah and Wilkie, 2004). Previous studies (Casabianca-Chassany et al., 1992; Costa et al., 2000; DeBusk et al., 1995; Polprasert et al., 1992; Sooknah and Wilkie, 2004; Whitehead et al., 1987; Xu and Shen, 2011) have tried to use floating aquatic macrophytes, such as duckweed and water hyacinth, for nutrient recovery from pig, swine and dairy manure-based wastewater. However, data on the effect of influent concentration on nutrient recovery and nutrients and pathogen reduction from unprocessed dairy wastewater is lacking.

The study consisted of two separate constructed wetland system researches. One research focused on the evaluation of bacterial and viral removal efficiency of constructed wetlands subjected to simulated tile drainage. Specific objectives of the study were:

- ❖ To compare seasonal variation in *E. coli* and bacteriophage P22 removal efficiency of surface flow and subsurface flow constructed wetlands subjected to pulse loading

- ❖ To examine the applicability of convection-dispersion equation in *E. coli* and bacteriophage P22 removal estimation of pulse loaded wetlands
- ❖ To examine the applicability of the colloid filtration based model introduced by Khatiwada and Polprasert (1999) for *E. coli* removal estimation
- ❖ To evaluate the use of bacteriophage P22 as a biotracer for characterizing constructed wetlands

Second research focused on pollutants reduction and nutrient recovery potential of duckweed based constructed wetlands subjected to dairy wastewater. Specific objectives of the studies were:

- ❖ To quantify chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) removal potential of duckweed based constructed wetlands from dairy wastewater
- ❖ To evaluate first order, first order with background concentration, and DUBWAT models for COD, TN and TP removal from dairy wastewater
- ❖ To quantify indicator bacteria (*E. coli*) reduction potential of duckweed based constructed wetlands subjected to dairy wastewater
- ❖ To quantify nutrient recovery potential from dairy wastewater through duckweed harvesting

CHAPTER 2: LITERATURE REVIEW

2.1 Nutrients and pathogen in animal manure

In the United States, livestock produce 1.20 to 1.37 billion tons of raw manure each year (Rogers and Haines, 2005). Animal manure is rich in crop nutrients such as nitrogen, phosphorus and potassium. The forms and proportions of nutrients in manures depend on animal type, animal diet, storage condition, and moisture content (Maguire and Heckendorn, 2009). Apart from nutrients, animal feces contain large quantity of viruses, bacteria and parasites (Venglovsky et al., 2009). For example, each gram of cow and sheep feces contain 10^5 - 10^7 number of fecal coliforms and 10^6 - 10^8 fecal streptococci (Maier et al., 2000). These zoonotic pathogens have potential to cause disease to human beings. Zoonotic pathogens are transmitted from livestock animals to human beings through air, water and food, cause human diseases that result in considerable monetary loss and human suffering in United States each year (Rogers and Haines, 2005). Nutrients, especially nitrogen and phosphorus that reach water bodies increase primary productivity resulting in ecological imbalance.

Nutrients and pathogens contained in manures can enter surface water as a point source through direct disposal of manure from concentrated livestock production systems or as diffuse or non-point source with overland flow (Knight et al., 2000). In order to control water pollution that are related to livestock-farming, various control measures such as vegetated filter strips, detention ponds, lagoons, and constructed wetlands have been introduced. Among them, free-floating macrophyte-based constructed wetlands have two potential benefits. First, it can reduce nutrients and pathogens from dairy wastewater. Second, floating macrophytes can be harvested and the biomass can be used for composting and soil amendments, digested anaerobically for biogas

production, processed for animal feed or mixed with solid manure to increase nutrient content (Sooknah and Wilkie, 2004; Triscari et al., 2009).

In United States and around the world, land application of manure is a common practice for nutrient recovery and disposal. In tile drained fields, applied manure can pass to the drain either with irrigation and rainfall or directly through macropores. When liquid manure is applied, manure may take preferential flow paths such as wormholes, root channels, soil cracks and other macropores and reach tile line quickly (Harrigan and Northcott, 2007). With liquid swine manure application, bacterial concentrations in tile drains were found to increase 30 to 900 fold within two hours of application (Evans and Owens, 1972). Irrespective of the application method, tile drains were found to be loaded with high bacterial concentration in rainfall soon after manure application (Samarajeewa, 2010). In the worst case, tile drains are pulsed with high number of manure-borne pathogens when a rainfall occurs soon after manure application. These pathogens in tile drains ultimately end up in surface water, posing health risk to human and animal health.

2.2 Constructed treatment wetlands

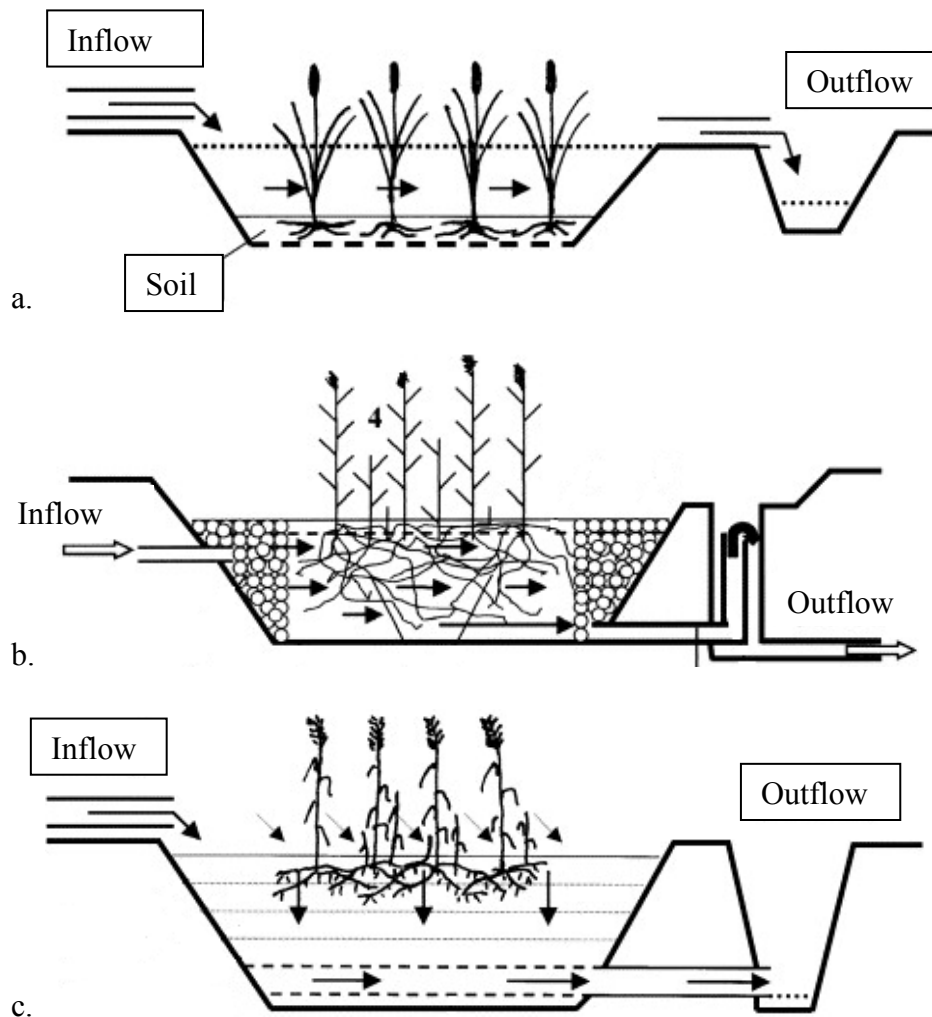
Constructed treatment wetlands are man-made treatment systems that treat wastewater by utilizing natural processes provided by wetland vegetation, soils, and associated microbial community (USEPA, 2004). Constructed wetlands are designed to mimic natural systems and take advantage of many natural wetland processes in a more controlled environment (Vymazal, 2005a). Natural wetlands have been used to treat wastewater for at least a century; however, constructed treatment wetlands started from a research conducted at Max Planck Institute in West Germany in 1952 and gained popularity worldwide since 1985 (Kadlec and Wallace, 2009). In North America, research on the efficacy of wetlands in treating wastewater began in

1970s when scientists from University of Michigan and University of Florida started in-depth studies on wetlands processes (Kadlec and Wallace, 2009). In early days, use of constructed wetlands was limited to domestic and municipal sewage treatment; however, after late 1980s, use of constructed wetlands have been widened and have been used in treating agricultural wastewater, food processing wastewater, heavy industry wastewater, mine drainage, landfill leachate, and runoff waters (Vymazal, 2005a). Constructed treatment wetlands offer several benefits, including low construction and operation cost, easy maintenance, reliable and effective wastewater treatment, stability under fluctuating hydrologic and pollutant loading rates and potential ancillary benefits such as aesthetics, wildlife habitats and recreational and educational areas (Hammer and Bastian, 1989).

Based on relative level of the water column and substrate bed, constructed wetlands can be broadly divided into two categories: surface flow (SF) and subsurface flow (SSF) constructed wetlands. In SF wetlands (Figure 2-1a), also known as free water surface wetlands, the water level is above the substrate bed resembling natural wetlands. The level of water column in SF wetlands is relatively shallow, often less than 0.4 m deep and the substrate bed is covered with dense vegetation (Sundaravadivel and Vigneswaran, 2001). SF wetlands are rarely used in secondary treatment due to potential human exposure to pathogens and most commonly used in polishing effluent from secondary and tertiary treatment processes (Kadlec and Wallace, 2009). Based on how macrophytes grow, SF wetlands are further divided into floating plant systems, submerged plant systems and emergent plant systems. In floating plant systems, free floating plants that do not require substrate for their growth float on the water surface. A wide variety of wetland macrophytes ranging from large plants with well-developed aerial, floating or submerged roots (such as water hyacinth, water lettuce and pennywort) to floating plants having

few or no roots (such as duckweed) can grow in floating plant systems (Brix, 1993). In submerged plant systems, aquatic macrophytes do not protrude beyond the water surface and have their photosynthetic tissues completely submerged in water. Submerged macrophytes include small low-productivity species such as *Isoetes lacustirs* and *Lobelia dortmanna* to larger, high-productivity species such as *Elodea canadensis* (Brix, 1993). Practical usefulness of submerged-plant system in treating wastewater is limited by algal growth and plant death or damage caused by anaerobic conditions (USEPA, 1988). In emergent macrophyte-based systems, wetland plants are attached to wetland substrate or sediments with their shoots or leaves protruding above water surface. Emergent macrophytes commonly used in constructed treatment wetlands include reeds, cattails, bulrushes and sedges (Sundaravadivel and Vigneswaran, 2001). In SSF wetlands, substrate bed is usually made of rock, gravel, sand or soil media and the water level is maintained below the surface of the substrate bed. The substrate used in the wetland also supports the growth of emergent vegetation. In horizontal SSF wetlands (Figure 2-1b), water flows from inlet to the outlet through the substrate in a horizontal direction. While passing through the wetlands, the wastewater comes in contact with the medium and root and rhizomes of the emergent vegetation and is cleaned by biological, chemical and physical processes (Vymazal et al., 1998). In vertical flow SSF wetlands (Figure 2-1c), wastewater flows from the top to the bottom of the wetlands. Suitable mechanisms are employed to make sure that the wastewater is evenly sprayed over the surface of the wetland. Bulrush, reeds and cattails are the emergent plants used in most of the constructed SSF wetlands in United States (USEPA, 1993b). In United States, the flow path of SSF constructed wetlands is usually horizontal (USEPA, 1993b).

Figure 2-1: Schematics of various constructed wetlands with emergent vegetation (Vymazal, 2007)



a) Surface flow, b) Horizontal subsurface flow, and, c) Vertical subsurface flow

The number of SF wetlands is twice the number of SSF wetlands in US, while, the number of SSF wetlands exceed the number of SF wetlands in Europe (Halverson, 2004). Compared to SF wetlands, SSF wetlands offer several advantages such as low risk of odors, exposure or insect vector due no standing water above substrate, smaller system requirement due to higher available surface for treatment and greater thermal protection in cold climates due to the insulation provided by substrate and accumulated plant debris (USEPA, 1993b). However, as the

wastewater in SSF wetlands need to pass through the porous medium, both horizontal and vertical SSF wetlands are susceptible to clogging (Kadlec and Wallace, 2009). Compared to SSF wetlands, SF wetlands cost less to design and build, but require more area to achieve same level of pollution reduction (Halverson, 2004).

2.3 Pathogen indicators and removal mechanisms

2.3.1 Wetland pathogen indicators

Domestic wastewater contains various pathogens which can be divided into bacteria, viruses, helminthes, protozoan, and fungi (Kadlec and Knight, 1996). Due to their diverse nature, pathogens in wastewater are difficult to detect and identify. Present detection methods are complex, lengthy, and require concentration and subsequent selective enrichment or amplification using molecular biology methods (Borrego and Figueras, 1997). Hence, indicator organisms that are easily monitored and correlate with pathogen population are frequently employed (Vymazal, 2005c). Ideal fecal coliform indicators are constantly present in feces, should not multiply outside the gastrointestinal tract, should show similar persistence in the environment as pathogens, and should be simple to analyze (Hurst and Crawford, 2002). Due to the variation in ecological and survival characteristics of pathogens under environmental conditions, the presence of all enteric pathogens cannot possibly be predicted by single indicator organism (Savichtcheva and Okabe, 2006). Though there is lack of single perfect indicator organism, scientists have long used coliform bacteria as the first choice for indicator organisms (Dufour, 1977).

Coliforms are found in intestinal flora of mammals and are used as indicators of fecal contamination in water. Coliform groups are either reported as total coliform (TC) or fecal coliform (FC). Total coliform includes rod-shaped, non-spore forming, stain Gram-negative

facultative anaerobes that ferment lactose and produce gas in 48 hr at 35°C (Kadlec and Knight, 1996). Total coliform includes various bacteria from the family *Enterobacteriaceae*. Many bacteria in coliform family do not originate from human or animals and are capable of surviving or even reproducing in soil, water and plants. Hence, coliform family represent the least specific indicator of human fecal contamination (Vymazal, 2005c). Fecal coliforms are mostly fecally derived coliforms, with some free-living bacteria. Main genera of bacteria fecal coliform group are *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter*. They are capable of fermenting lactose and produce gas in 24 hr at 44.5 °C. Fecal coliforms are better fecal contamination indicators as compared to total coliform but are also not specific as fecal coliform include free living bacteria, especially *Klebsiella* spp. (Kadlec and Knight, 1996). *E. coli* is found in the mammalian digestive tract in large quantities and is not generally found in other environments. Hence, *E. coli* is considered one of the best indicators of fecal contamination (Molleda et al., 2008). However, as *E. coli* also originates from other warm-blooded animals, detection of *E. coli* does not reflect human fecal contamination alone. *E. coli* constitutes 20 to 30% of the total coliform in raw and treated domestic wastewater and above 90% in human feces (Borrego and Figueras, 1997; Dufour, 1977)

Total coliform, fecal coliform, *E. coli* and *Enterococci* are the major waterborne pathogen indicators in the United States (National Research Council and Committee on Indicators for Waterborne Pathogens, 2004). In 1986, Environmental Protection Agency (EPA) formulated a recreational water quality criteria which allows a maximum geometric-mean concentrations of 126 *E. coli* and 33 *Enterococci* per 100 ml for freshwater and 35 *Enterococci* per 100 ml of marine water for full body contact use (USEPA, 1986). In many other countries *Escherichia coli* is also the preferred choice as indicator bacteria (Kadlec and Knight, 1996).

Other frequently used bacterial indicators are fecal streptococci and fecal enterococci. Fecal streptococci include species *S. faecalis*, *S. faecium*, *S. avium*, *S. gallinarum*, *S. bovis* and *S. equines*. Enterococci represent a subgroup of fecal streptococci and include *S. faecalis*, *S. faecium*, *S. avium*, *S. gallinarum* genera of streptococci and their variant (USEPA, 2006).

Enterococci are sometimes used as synonymous to streptococci. Enterococci are spherical and stain Gram-positive and grow in chain. Fecal streptococci are present in human feces as well as feces of other warm blooded animals and are not considered to multiply in water and soil (Kadlec and Knight, 1996). Compared to fecal coliform, fecal streptococci (FS) are more resistant to environmental stresses and may better represent wastewater-originated viruses that are more persistent in the environment. Consequently fecal streptococci are used as a second indicator of fecal contamination (Clausen et al., 1977). Sometimes FC to FS ratio is used to differentiate human and nonhuman coliform contamination. Animal waste contains higher FS, and hence the FC to FS ratio is less than 0.7; in contrast, the ratio of FC to FS in human waste is usually greater than 4.0 (Clausen et al., 1977). However, FC to FS ratio is applicable only within 24 hr of discharge as bacterial die-off affects the ratio (Vymazal, 2005c).

Coliforms are insufficient to represent presence of pathogens, particularly viruses and parasites (Payment and Franco, 1993). Under varying environmental conditions, the growth and die-off rates of *S. typhimurium* and coliform organism are also found to be different (Colwell, 1978). Many researchers have raised questions in the sole use of coliform, fecal coliform and *E. coli* to predict the virological and protozoal safety of water (Leclerc et al., 2001). Zhiwen et al. (2008) found significantly higher concentration of *Salmonella* in influent and effluent than *E. coli* in FWS constructed wetland; no significant correlation was observed between the indicator and pathogenic organisms.

Another bacterial indicator for fecal contamination is *Clostridium perfringens*. *Clostridium perfringens* is an anaerobic spore-forming bacterium that is always present in human feces. *C. perfringens* spores are very resistant to environmental conditions; survive longer than fecal coliform and vegetative forms do not appear to reproduce in aquatic environment (Molleda et al., 2008; Vymazal, 2005c). Cysts and oocysts of protozoa are also resistant to environmental stresses. *C. perfringens* count is the most suitable indicator of viral removal and inactivation and also can be used as indicator of cysts and oocysts removal and inactivation (Payment and Franco, 1993).

Compared to bacteria, viruses are more resistant to chlorination and inactivation, indicating that fecal coliforms are not suitable viral indicators (Colwell, 1978; Gersberg et al., 1987a; Kadlec and Knight, 1996). Certain types of bacteriophage are similar to enteroviruses in physical structure and can be detected with simple, fast, reliable and economical methods (Tanji et al., 2002). Bacteriophages are the viruses that infect specific bacteria but do not affect human, animal or plant cells (Rossi et al., 1998). Coliphages are viruses that infect fecal coliforms. Out of six groups, two coliphage families *Leviviridae* (single stranded RNA phages) and *Inoviridae* (single stranded DNA phages) infect only F⁺ male hosts and enter the hosts through F sex pilus (Cole et al., 2003). These two families of coliphages are known as FRNA and FDNA coliphages, respectively and have been used as viral indicators. Havelaar et al. (1993) found FRNA coliphages to be highly correlated with virus concentration in raw and partially treated drinking water, raw and partially treated wastewater and surface waters. Bacteriophage MS2 is a widely used FRNA viral indicator in pathogen removal studies. MS2 exhibits similar behavior to enteroviruses; both are single stranded DNA; are similar in size, and are resistant to UV light (Kapuscinski and Mitchell, 1983). But detection of FRNA does not indicate direct human fecal

contamination as FRNA coliphages are not frequently found in human feces (Bitton, 2005).

Stetler (1984) reported better correlation of enterovirus with coliphages than with other indicator organisms such as total coliform, fecal coliform or fecal streptococci.

Another common viral indicator is the bacteriophage PRD1. A number of gram negative bacteria act as host to PRD1; the most often used host in PRD1 production for environmental applications is *Salmonella typhimurium* (Harvey and Ryan, 2004). Due to its stability over a wide range of temperatures and lower attachment in aquifer sediments, bacteriophage PRD1 is popular in transport studies involving geologic media (Harvey and Ryan, 2004). Persistence of pathogenic viruses is reported to be better predicted by PRD1 than MS2 in ambient groundwater conditions (Blanc and Nasser, 1996). Bacteriophage P22, once confused with PRD1, infects smooth strains of *Salmonella typhimurium* and is a suitable indicator for complex freshwater systems (Shen et al., 2008).

2.3.2 Wetland pathogen removal mechanism

Constructed wetlands remove pathogens by the combined effect of physical, chemical and biological processes (Vymazal, 2005c; Werker et al., 2002). Physical processes include mechanical filtration, adsorption and sedimentation; temperature and solar radiation. Chemical processes are UV radiation, pH effect and oxidation. Biological processes are natural die-off, antibiosis, nematodes, protozoa and zooplankton predation and attack by lytic bacteria and viruses. Though there seems to be general agreement on the processes of pathogen removal, the most influential parameter has not yet been identified (Werker et al., 2002).

2.3.2.1 Filtration, adsorption and sedimentation

When pathogens are passed through the media smaller than their size, pathogens are filtered or strained. Filtration is affected by the size of porous media, size and shape of pathogen, clogging

and saturation of filter media (Stevik et al., 2004). In subsurface flow (SSF) wetlands, filtration may play a significant role since the pathogens are passed through the media, but in surface flow (SF) wetlands without vegetation, the filtration effect is thought to be limited. However roots of the macrophytes adsorb and filter the pathogens in vegetated wetlands. When the pores are larger than the pathogens, pathogens are not filtered but can adsorb to the media. Adsorption is affected by specific surface area of the porous media, flow velocity, ionic strength, temperature, pH and pathogen concentration (Stevik et al., 2004). In sedimentation, pathogens that are adsorbed to or trapped within settleable solids are removed through settling. In duckweed ponds, sedimentation is the primary removal mechanism and that filtration and adsorption to the plant biomass may also be a process of microbial removal (Gerba et al., 1999). Adsorption, sedimentation, and inactivation kinetics is variable depending upon the wastewater and wetland characteristics (Boutilier et al., 2009).

Microbes that are associated with less dense particles or are free floating remain more mobile, while the ones associated with solid particles, especially dense, tend to settle out quickly (Characklis et al., 2005). Vandonsel and Geldreich (1971) reported that fecal coliform concentrations were 100 to 1000 times greater in underlying mud than in water. *Salmonella* were recovered in higher numbers and frequencies from the underlying sediments than from the overlying water (Hendricks, 1971; Vandonsel and Geldreich, 1971). However, in a constructed wetland planted with duckweed and hyacinth, concentrations of fecal coliforms and coliphage numbers did not differ substantially between the water column and sediments (Karim et al., 2004). Similar concentrations of indicator organisms were attributed to the attachment of microbes to the root surface which would reduce the number of settled bacteria. However, higher die-off rates of bacteria and coliphage were observed in the water column than in sediments. In

another study, Boutilier et al. (2009) investigated the effect of sedimentation and adsorption of *E. coli* in constructed wetland fed with septic tank effluent, treated wetland effluent and dairy wastewater and found that approximately 50%, 20% and 90% of *E. coli*, respectively, were either free floating or associated with particles less than 5 µm in size indicating that settling did not contribute to the *E. coli* removal. Zhiwen et al. (2008) found significant concentrations of pathogens in sediments and reported that die-off rates of pathogens were greater in water than in the sediments. The above findings suggest that physical processes such as filtration, adsorption and sedimentation are important pathogen removal mechanisms in constructed wetlands; however, the significance of the processes depends on the type of wetlands and wastewater used.

2.3.2.2 Effect of temperature

Although some microbes survive extreme low or high temperatures, most microbes have narrow range of temperature for optimal growth. Chemical and enzymatic reactions rates become faster at higher temperatures, leading to increased growth. However, at elevated temperatures, proteins are irreversibly damaged and microbial inactivation rate increases. There exists minimum, optimum and maximum temperature for growth and survival of microbes. Some bacteria are inactivated in cold or experience cold injury at freezing temperatures. Initially, bacteria face nonlethal physical or metabolic injury, and as the exposure time increases, injury becomes more severe, which may even results in death of cells (Straka and Stokes, 1959). However, at above freezing temperatures, enteric bacteria survive longer at low temperature (Pundsack et al., 2001). Sampson et al. (2006) incubated *E. coli* at temperatures 4°C, 10°C, 14°C, and 25°C in lake water and found that *E. coli* die-off was highest at 14°C and lowest at 4°C. Under starved condition, *Salmonella* were also found to survive up to 24 weeks at 30°C, while the pathogen survived up to 58 weeks at 5°C (Sugumar and Mariappan, 2003). Longer survival at lower

temperature might be attributed to the slower reaction rates at low temperatures. Increasing the temperature by 10°C doubles the bacterial growth rate until optimum temperature is reached (Metcalf & Eddy et al., 2003). Mcfeters and Stuart (1972) examined the survival of fecal coliform in natural water and reported that temperature above 15°C was less critical for the survival of fecal coliform but decreasing the temperature below 15°C increased the survival rate. There are conflicting reports on the effect of temperature on pathogen removal in constructed wetlands, likely due to the complexity and variety of pathogen removal processes that include physical, chemical and biological processes. In a review on constructed wetlands, Werker et al. (2002) reported that temperature may or may not affect the pathogen removal. Higher temperature can extend the survival of bacteria but also supports the growth of its predators; physical processes are less sensitive to temperature (Vymazal, 2005c). Effect of temperature may vary depending on the pathogen (Bahlaoui et al., 1998; Mezrioui and Baleux, 1992). At lower temperatures, plant photosynthetic activity aboveground decreases leading to lower oxygen availability in the root zone in spite of increased oxygen solubility. Hatano et al. (1993) reported influence of plant type on temperature and reported that wetland planted with *Typha* did not show seasonal variation but wetlands planted with *Phragmites* had higher microbial population during summer months. Poorer pathogen removal at lower temperatures can be attributed to lower metabolic activity in root zone leading to lower bacterial grazing and antibiosis or lower dissolved oxygen concentration in root zone (Rivera et al., 1995).

Smith et al. (2005) studied fecal coliform removal in warm and cold Canadian climates in surface flow constructed wetland using dairy wastewater. The authors reported 96.8 to 99.7% in removal rates and mass reduction in both climates. Season variation, even the ice formation in winter months, did not reduce the removal efficiency of the wetlands. However, the results

should be interpreted cautiously as the hydraulic retention time of the wetlands was 95 days.

Cooper and Boon (1987) also did not observe an effect of temperature, ranging from just above freezing to 25°C, on pathogen removal. With a hydraulic loading rate of 7 days, a mean removal of 99.3% and 95.8% in summer (temperature reaching above 20°C) and winter (temperature reaching below 0°C) was reported by Kern et al. (2000) where the subsurface flow constructed wetland was fed with agricultural wastewater.

However, other researchers suggest that temperature affects pathogen removal. Coliforms are reported to survive longer at lower temperatures leading to greater concentrations in constructed wetland outflows in winter than in summer months (Gersberg et al., 1989). In oxidation ponds, Bahlaoui et al. (1998) reported seasonal distribution of fecal coliform and fecal streptococci and found higher abundance in winter than in summer. Zdragas et al. (2002) found lower coliform removal in winter in constructed wetlands treating municipal wastewater in Mediterranean climate, reporting maximum removal of coliform in the summer from a combined effect of high temperature and solar radiation. Zhiwen (2010) reported higher removal rates of *Salmonella* in summer than in winter, but higher *E. coli* were removed in spring than in autumn in temperate zone in China. Molleda et al. (2008) used three stage treatment system: deep lagoon, surface flow, and combination of surface flow and subsurface flow systems, planted with aquatic vegetation to treat human and livestock wastewater. With a hydraulic retention time of 13 days, they observed fecal coliform reductions of 1.42, 3.18, 1.42 and 0.6 log units in summer, autumn, winter and spring, respectively. A 100% reduction in Helminthes eggs and cysts and oocysts of *Giardia* and *Crystosporidium* was observed during all seasons.

2.3.2.3 Effect of solar radiation

The impact solar radiation on pathogen removal depends on the type of constructed wetland. In subsurface flow constructed wetlands with no standing water, the effect can be considered negligible as wastewater is not exposed to radiation. In surface flow wetlands, the impact of solar radiation on pathogen removal can be significant, but depends on the vegetation density (Vymazal, 2005c). In waste stabilization ponds, since only 1% of UVB, UVA and photosynthetically active radiation reaches to 12 cm, 27 cm, and 30 cm depth respectively (Sweeney et al., 2007), the effect of solar radiation is limited to the shallow depths. Similar trends are likely in surface flow constructed wetlands.

Specific wavelengths may be responsible for inactivation of specific pathogens. Davies-Colley et al. (1997) studied the effect of solar radiation in waste stabilization ponds and reported that ultraviolet B (UVB) (290–320 nm), ultraviolet A (UVA) (320–400 nm) and blue to green visible light (400–550 nm) contributed almost equally to the inactivation of enterococci and F-RNA phage but that UVB (290–320 nm) was primarily responsible for *E. coli* and F-DNA phage inactivation. Sinton et al. (2002) reported that wide range of wavelengths inactivated enterococci and F-RNA but shorter wavelengths (UVB) inactivated fecal coliforms and somatic coliphages, suggesting photooxidative and photobiological damages in fecal coliforms and somatic coliphages, respectively.

Solar radiation was found to have lethal effect on coliform and the severity depended on temperature; a more significant effect was observed at lower temperatures than at higher temperatures (Zdragas et al., 2002). The reason is that solar radiation not only provides UV radiation, but also supplies heat to increase the temperature thereby creating unfavorable

condition for the pathogens. Hence, higher ultraviolet-induced die-off can be expected at higher temperatures than at lower temperatures (Vymazal, 2005c).

Davies-Colley et al. (1999) summarized the effect different wavelength of solar radiation in terms of three mechanisms:

Mechanism 1: This mechanism is not dependent on oxygen and involves direct absorption of UVB contained in solar radiation causing damage to DNA. UVB affects all DNA-containing organisms but at low doses, microorganisms may repair the damage caused by the radiation.

Mechanism 2: In this mechanism, photo-sensitizers present in cells catalyze the production of reactive oxygen species, causing photo-oxidative damage. Single strand DNA breaks, damaging a range of internal targets. As the absorbance is high only at low wavelengths, the damage is primarily caused by UVB.

Mechanism 3: This mechanism is catalyzed by exogenous photo-sensitizers, particularly dissolved humic substances, which absorb wide range of wavelengths to produce reactive oxygen, resulting in photo-oxidation damage. However, the absorbance decreases with increasing wavelength from UV to visible spectrum. External structure of the microorganisms, such as membrane, is damaged by reactive oxygen species.

Zdragas et al. (2002) described that though the wavelengths up to 700 nm were harmful to coliforms, UVA and UVB had the most destructive effects. The authors categorized the effects as lethal and nonlethal. Lethal effects are due primarily to by lesions in the DNA. Nonlethal effects are growth inhibition and delay, reduced active transport, mutagenesis and induced enzyme synthesis inhibition.

Pathogens are capable of repairing damage from solar radiation through two mechanisms: photo-reactivation and dark repair (Sonntag et al., 2003). In photo-reactivation, UV-inactivated

microorganisms repair pyrimidine dimers in the DNA under near-UV and visible light and recover activity. Repair mechanisms other than photo repair, such as excision repair, are known as dark repair and do not require light. Some viruses may even use enzymes of host cell for repair. This is the reason why sampling should be done after photo repair, usually before full sunlight, while evaluating bacterial removal.

2.3.2.4 Effect of pH

Acidity or alkalinity of the surrounding environment directly affects pathogens as microbial cellular metabolic reactions, such as energy generation and ionic transport, are dependent on hydrogen ions (Mitchell, 1992). At high pH, molecular oxygen is excited to an ionic form, which is toxic (Awuah et al., 2002). Extreme high or low pH chemically alters the macromolecules and disrupts enzyme and transport functions, leading to inactivation. Indicator organisms cannot grow in extreme high or low pH. A pH 4-9 is required for the growth of most of the indicator organisms (Rheinheimer, 1992). Prescott et al. (1996) mentioned that the pH required for the indicators is even narrower and is between 5.5 and 8.

Macrophytes and algae create varying pH conditions depending upon the plant types used for treatment. Water lettuce, duckweed and algal ponds produce acidic, neutral and alkaline conditions, respectively (Awuah et al., 2002). In water stabilization ponds, especially in nutrient limited conditions, Pearson et al. (1987) found that pH 9.0 or above was found to increase fecal coliform die-off rate, and the rate increased with the increase in temperature but did not substantially change with dissolved oxygen level. Similar behavior was observed for *Streptococcus*, *Salmonella* and *Campylobacter* isolates. Parhad and Rao (1974) observed no *E. coli* growth in wastewater above the pH value of 9.2. Solic and Krstulovic (1992) noted that in sea water, the optimum pH for fecal coliform survival was between 6 and 7 and the population

declined sharply above or below the optimum values. Enterococci had lower tolerance to acidic conditions than to high (>9) pH conditions and survived at pH of 11, especially under dark conditions (Awuah et al., 2001). Awuah (2006a) effect of fluctuating and stable pH in *E. coli*, coliforms, *Salmonella* and other enterobacteria in domestic wastewater and reported that survival of *E. coli* and coliforms were more impacted by a stable pH than fluctuating pH (ranging from neutral to 11), with the exception of fluctuating pH between 4 and 9, whereas, fluctuating pH was more detrimental to *Salmonella* and other enterobacteria. The author attributed the observed effects to the possible ammonia toxicity.

2.3.2.5 Effect of dissolved oxygen

Oxygen concentration in constructed wetlands varies with time. In surface flow wetlands, wind-induced agitation and algal photosynthesis increase dissolved oxygen (DO) concentration, whereas, in subsurface flow wetlands, oxygen may leak through the root and rhizomes of the macrophytes. Enteric bacteria are either obligate anaerobes or facultative anaerobes and thus the presence of oxygen is usually detrimental to their survival. DO concentration is linked with other effects that lead to pathogen removal. Zooplanktons are the predator of *E. coli* and prefer high DO concentration. Hence, higher DO concentration is directly detrimental to *E. coli* and supports growth of microbial predators, leading to indirect reduction in *E. coli* concentration. Curtis et al. (1992) reported that oxygen alone did not contribute to *E. coli* removal under dark conditions, but under light, the effect of solar radiation on *E. coli* die-off increased with increasing DO concentration. In another experiment, Davies-Colley et al. (1999) reported that enterococci, *E. coli*, and F-RNA phage inactivation due to sunlight increased with increased in DO but increasing DO did not have any effect on F-DNA removal. Polioviruses, bacteriophages and Coxsackie virus B3 removal were also found to increase with aeration (Kaneko, 1997).

2.3.2.6 Effect of hydraulic retention time

Increasing hydraulic retention time or hydraulic residence time (HRT) increases pathogen removal. With longer HRT pathogens are exposed to unfavorable environments for longer time, thus resulting in higher pathogen removal. Diaz et al. (2010) reported HRT to have the greatest influence on pathogen removal efficiency in constructed wetlands: 66% of *E. coli* removed with 0.9 days of HRT and 91% with 11.6 days of HRT. However the reported relationships between HRT and pathogen removal efficiency varies over a wide range. Some reports suggest that removal rate increases linearly with the increase in HRT and eventually levels off. Further increase in HRT does not increase the removal rate. In a horizontal subsurface flow constructed wetland, Garcia et al. (2003) found that in 3 days, microbial inactivation reached saturation, and that increasing HRT above 3 days did not significantly increase the removal rate. Their conclusion was that when the HRT increases, pathogen removal cannot be accurately estimated as first order. In surface flow constructed wetlands, Toet et. al (2005) found increased *E. coli* removal with increased HRT but concluded that *E-coli* removal did not increase by increasing HRT above 4 days. However, other reports suggest that increasing HRT increases removal rates beyond 3 or 4 days. For example, Tanner (1995) reported that, in gravel-bed constructed wetland, increasing HRT from 2 to 7 days increased the FC removal from 76.2% to 95.3%. Similar to the finding, in FWS constructed wetland planted with *Cyperus papyrus*, Okrut and van Bruggen (2000) reported that FC removal rate increased up to 12 days of retention time. Above mentioned findings suggest that HRT plays a significant role in pathogen removal, and increases with increase in retention time but the optimum retention time to achieve desired removal rate differs from wetland to wetland. The difference can be explained by the fact that every wetland is unique and parameters such as hydraulic loading rate, configuration of wetland, pH,

temperature, predator population, vegetation type and all the affecting parameters vary significantly.

2.3.2.7 Effect of vegetation

Similar to the temperature effect, there are conflicting reports about the effect of vegetation on pathogen removal in constructed wetlands. On one hand, plants roots enhance filtration, plant rhizosphere enhance aerobic degradation and secrete anti-microbial compounds (Werker et al., 2002), all contributing to increased pathogen removal. For example, fecal indicator bacteria, such as *E. coli*, and pathogenic bacteria, such as *Salmonella*, may be killed by the root excretion of certain plants, including *Scirpus lacustris* and *Phragmites communis* (Seidel, 1976). On the other hand, free floating macrophytes may reduce UV penetration, provide favorable attachment sites, and reduce free oxygen exchange that is crucial for predator population (MacIntyre et al., 2006), contributing to decreased pathogen removal.

Wetland plants may increase the dissolved oxygen concentration in wetland either due to algal photosynthesis or oxygen leaks through roots and rhizomes. Old roots and rhizomes of *P. australis*, *Glyceria maxima*, *Typha latifolia* and *Iris pseudacorus* were not found to release oxygen but their subapical regions or young roots released oxygen; phragmites had tendency to release more oxygen than *Typha* from the roots (Brix and Schierup, 1990). Presence of oxygen creates unfavorable condition for enteric bacteria, as enteric bacteria are either facultative or obligate anaerobes (Vymazal, 2005c). Warren et al. (2000) found that unplanted gravel beds required twice the length of planted gravel beds to give the same ten-fold reduction in *E. coli* and the authors attributed the difference to the higher DO level created by the vegetation, generating unfavorable condition for the organism. Wetland vegetation alters pH and DO, modify the amount of solar radiation reaching to the pathogens, and their root may provide attachment sites

for pathogens. As mentioned previously, water lettuce, duckweed and algal ponds produce acidic, neutral and alkaline conditions, respectively (Awuah et al., 2002), and thus affect pathogen removal indirectly.

In gravel-bed wetlands, Gersberg et al. (1987a) found improved total coliform removal with wetlands planted with *Schoenoplectus validus* (great bulrush) as compared to unplanted ones, but Tanner et al. (1995) did not find any significant difference between unplanted gravel-bed wetlands and gravel-bed wetlands planted with *S. validus*. Compared to unplanted beds, ciliate predation appeared to be higher in planted beds (Decamp et al., 1999). In water lettuce ponds, presence of protozoa had significant effect in *E. coli* and *Salmonella*, whereas, the effect was not significant in algal or duckweed ponds (Awuah, 2006b). In mesocosm scale gravel bed wetlands, Hench et al. (2003) observed greatest microbial reduction in planted beds compared to unplanted beds.

MacIntyre et al. (2006) reported increase in *E. coli* removal efficiency of surface flow wetland after removal of floating *Lemna* spp. Their finding was that the duckweed was providing favorable condition for *E. coli* by reducing the UV radiation penetration, increasing attachment site and reducing the dissolved oxygen level of water, thereby producing unfavorable condition for its predator, zooplankton. *E. coli* is facultative anaerobe and can flourish in both high and low levels of DO but zooplankton better thrives in high DO levels.

2.3.2.8 Predation and competition

Predation is other important pathogen removal mechanism in constructed wetlands. Many organisms such as protozoa, especially ciliates, mini-metazoa, such as nematodes, rotifers and copepods feed on bacteria and pathogens (Decamp and Warren, 1998; Green et al., 1997; Song et al., 2008). Protozoa feed on different organisms such as algae, yeasts, bacteria and other

protozoa (Storer, 1979). In aerobic biological wastewater treatment processes, Ciliates (a group of protozoa) have been found to be dominant in removing dispersed bacterial growths by predation (Curds, 1992). In reed bed constructed wetland treating wastewater, Decamp and Warren (1998), using the root zone method, demonstrated that ciliates are capable of all observed *E. coli* removal. But Awuah (2006b) did not find any effect of protozoa in fecal bacteria removal in algal and duckweed ponds, but presence of protozoa significantly improved the removal rate in water lettuce ponds. In estuarine water, presence of protozoan predators determined the survival of *E. coli* but the lytic bacteria did not have any effect (Enzinger and Cooper, 1976). Some researchers have suggested that size-selective feeding may explain the protozoan predation and the grazing on bacteria is affected by the composition of protozoan communities (Ronn et al., 2002). In FWS constructed wetlands, Copepods were also found to reduce indicator and pathogenic microorganism, with rapid decrease in their population with higher concentration of copepods and vice versa (Song et al., 2008). *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have also been reported to inactivate poliovirus through predation (Kim and Unno, 1996).

Pathogens, like other microorganisms, require nutrients for their growth and survival. Though in smaller quantities, bacteria require water, minerals, vitamins and other carbon and nitrogen sources (Portier and Palmer, 1989). Presence of other bacteria and microorganisms, along with predation, create competition for the limiting nutrients, and thus, pathogens may starve. In open environments, available nutrient and energy sources restrict the growth and survival of *E. coli* (van Elsas et al., 2011). Furthermore, competition for nutrients creates starvation stress, which may lead to microbial sensitivity to secondary stresses (PostGate, 1967).

2.3.2.9 Natural die-off

Natural die-off is the common process of all living organism. Pathogens also have their self life and eventually die. The dying process is enhanced by other factors mentioned previously.

As long the inflow population is high, enteric bacterial removal in constructed wetlands is modeled using exponential decline equation (Kadlec and Knight, 1996):

$$\frac{C_o - C^*}{C_i - C^*} = \exp\left(\frac{-k_1}{q}\right) = \exp(-k_v \tau) \quad (2-1)$$

where,

C_o = bacteria concentration in outflow (CFU/100 ml)

C_i = bacteria concentration in inflow (CFU/100 ml)

C^* = background concentration (CFU/100 ml)

k_1 = area-based, first-order rate constant, m/d

q = hydraulic loading rate, m/d

k_v = volume-based, first-order decay rate, 1/d

τ = nominal retention time, d

Above equation incorporates the background concentration, introduced to the wetland system by wildlife and other means. If there is no such interference, it can be simplified to:

$$\frac{C_o}{C_i} = \exp\left(\frac{-k_1}{q}\right) = \exp(-k_v \tau) \quad (2-2)$$

However, there are reports that increasing the hydraulic retention time does not necessarily increase the removal rate beyond certain days (Garcia et al., 2003; Toet et al., 2005). The findings indicate that at higher HRTs, first-order microbial decay is no more valid.

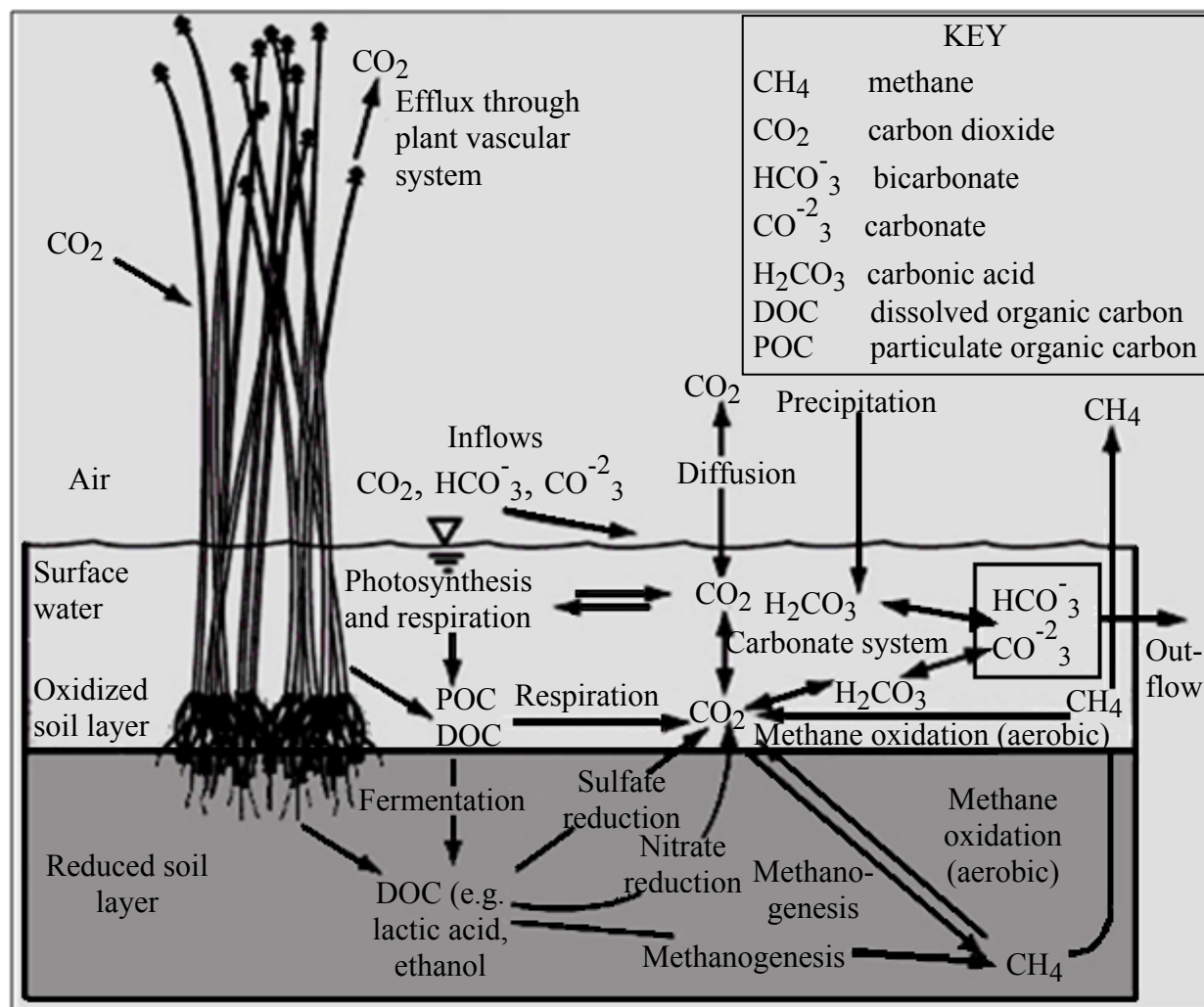
2.4 Carbon and nutrients processes and removal mechanisms

2.4.1 Carbon transformation

Total organic matter present in a given sample is measured as chemical oxygen demand (COD), biochemical oxygen demand (BOD), carbonaceous biochemical oxygen demand (CBOD) or total organic carbon (TOC). When organic matter is oxidized by using oxidant, usually potassium dichromate, the amount of oxidant consumed in oxidizing the organic matter is termed as COD. Oxygen consumed by microbes in oxidizing organic matter is termed as BOD. As the usual BOD test runs for five days, BOD test is also designated as BOD₅. COD is higher than BOD as COD oxidizes larger groups of compounds, and in wetland environment, presence of humic substances result in much higher COD than BOD (Kadlec and Wallace, 2009). In BOD test, if the nitrification is chemically inhibited, result is termed as CBOD. TOC is measured through CO₂ analysis after chemical oxidation.

Carbon constitutes almost half of the dry wetland plant and soil material (Kadlec and Wallace, 2009). Carbon is present in wetland environment in both organic and inorganic forms. Inorganic carbon consists of dissolved inorganic carbon and gaseous end products. Dissolved inorganic carbon includes carbonate, bicarbonate and carbon dioxide gas. Both inorganic gaseous end products, CO₂ and CH₄, are formed under anaerobic condition, while, CO₂ is the sole product under aerobic conditions (Reddy and DeLaune, 2008). Organic form of carbon in wetland environment is present as plant biomass, microbial biomass, particulate organic carbon (POC) and dissolved organic carbon (DOC). Figure 2-2 shows the carbon cycle and major pathways in wetlands.

Figure 2-2: Carbon cycle in wetlands showing major pathways (Mitsch and Gosselink, 2007)



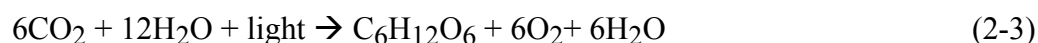
Organic matter is composed of a complex mixture of biopolymers, which can be labile or recalcitrant (Vymazal and Kropfelova, 2009). Proteins, carbohydrates, and lipids are labile compounds and are easily degraded, while, lignin and hemicelluloses are recalcitrant compounds and are not easily degraded. Organic matters are decomposed through aerobic as well as anaerobic processes. Aerobic degradation is fairly efficient in energy transfer, however, due to anoxic environment in wetlands, anaerobic process also occurs in the proximity to aerobic processes (Mitsch and Gosselink, 2007). Aerobic microorganisms are dominant in water column and a thin layer of surface soil and anaerobic microorganisms are dominant in the rest of the

wetland. In aerobic decomposition, organic matter is oxidized to CO₂ and 100% of energy is released; however, in anaerobic process, large part of the released energy is stored in reduced end products (Reddy and DeLaune, 2008).

Major carbon processes in wetlands are photosynthesis, respiration, fermentation, nitrate reduction, iron and manganese reduction, sulfate reduction, methanogenesis and methane oxidation (Kadlec and Wallace, 2009; Mitsch and Gosselink, 2007; Reddy and DeLaune, 2008).

2.4.1.1 Photosynthesis

Photosynthesis is a process by which macrophytes and algae convert inorganic carbon to organic carbon.



Photosynthesis, or net primary productivity, in wetland is higher than many terrestrial ecosystems and comparable with tropical rain forest and is variable depending on vegetation type, geographic location of the wetland, nutrient availability, and method of productivity determination (Reddy and DeLaune, 2008).

2.4.1.2 Aerobic respiration

In aerobic respiration, microorganisms use oxygen as terminal electron acceptor, producing CO₂ gas.



Aerobic degradation of the organic compounds is supported by the oxygen supplied through atmospheric diffusion or through the roots of the macrophytes. Oxygen availability limits the amount of aerobic biological oxidation, and if oxygen is unlimited, aerobic degradation is regulated by the amount of available active organic material (Vymazal et al., 1998).

2.4.1.3 Fermentation

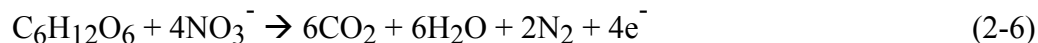
In fermentation, microbes use organic compound both as electron donor and acceptor. Organic substrates are only partially oxidized to simpler products and small amount of energy is released. Microbes convert soluble organic monomers, produced through hydrolysis or previously present in water, into volatile short-chain fatty acids (Garcia et al., 2010).



Fermentation is carried out by facultative and obligate anaerobes. Fermentation is a major process by which high-molecular-weight organic compounds are broken down to low-molecular-weight organic compounds and made available to other microbes (Mitsch and Gosselink, 2007). *Clostridium*, *Bacteriodes*, *Eubacterium* and *Peptostreptococcus* are involved in fermentation of organic compounds (Molongoski and Klug, 1976). Fermentation of organic compounds is favored by lack of oxygen, low number of competing aerobes and facultative anaerobes and low concentration of fermentation end products such as organic acids, alcohols and hydrogen (Reddy and DeLaune, 2008).

2.4.1.4 Nitrate reduction

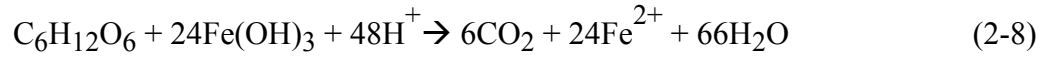
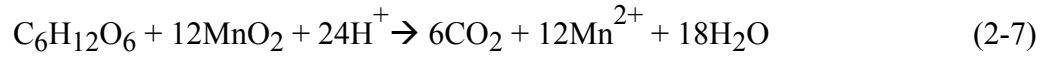
Nitrate reduction or denitrification occurs in anoxic or anaerobic zones where microbes use nitrate as terminal electron acceptor. Nitrate reduction process connects carbon cycle with nitrogen cycle as denitrifying bacteria use nitrate as terminal electron acceptor while bacteria obtain energy from organic compounds (Garcia et al., 2010).



Denitrification is carried out by many different groups of bacteria such as *Bacillus*, *Pseudomonas* and *Thiobacillus*. Denitrifiers can use a variety of organic substrates including fermentation end products and monomers (Reddy and DeLaune, 2008).

2.4.1.5 Manganese and iron reduction

In anoxic and anaerobic environments, in the absence of oxygen or nitrate as electron acceptor, facultative microbes can use oxidized forms of manganese (Mn^{4+}) and iron (Fe^{3+}) as electron acceptors to break down organic matter.

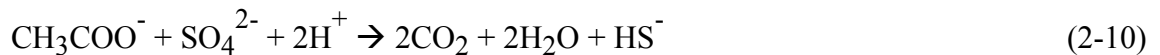


A model assumes that complex organic matters are degraded by hydrolysis, the products are then fermented producing simple organic compounds, which can then be oxidized by iron or manganese reducing microbes (Lovley, 1991). Manganese and iron reduction reaction is complete and results in the production of CO_2 and H_2O .

2.4.1.6 Sulfate reduction

In anaerobic zones, when other electron acceptors such as oxygen, nitrate, Mn and Fe are depleted, sulfate reducing bacteria can use sulfate as terminal electron acceptor to oxidize organic matter to CO_2 and reduce sulfate to sulphides (Widdel, 1988). Sulfate reducing bacteria are obligate anaerobes that cannot hydrolyze polymers and monomers, and hence, depend on fermenting bacteria to produce simple organic compounds such as lactate, acetate and alcohol (Reddy and DeLaune, 2008).

Chemical equations representing lactate and acetate oxidation are given by Equations 2-9 and 2-10, respectively.



Organic matter oxidation through sulfate reduction is dominant in saltwater wetlands where sulfur is abundant (Mitsch and Gosselink, 2007).

2.4.1.7 Methanogenesis

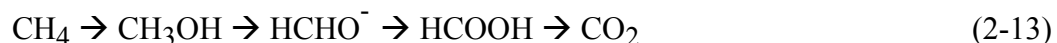
When other electron acceptors are depleted, methanogens can use CO₂ or low molecular weight compounds as terminal electron acceptors and produce methane gas. Methanogens are obligate anaerobes, function in wetlands when redox potential (Eh) is below <-200 mV and can be autotrophs or heterotrophs (Reddy and DeLaune, 2008). Methanogens cannot use higher-molecular-weight compounds and need to rely on hydrolytic, fermentative and hydrogen-producing acetogenic bacteria (Capone and Kiene, 1988).



Largest non-anthropogenic source of atmospheric methane emission are freshwater wetlands (Shoemaker and Schrag, 2010) and methanogenesis is the most important process of methane release from freshwater wetlands (Torres et al., 2005).

2.4.1.8 Methane oxidation

Methane oxidizing bacteria and nitrifying bacteria are capable of oxidizing methane under aerobic condition. Methane oxidation is an aerobic process that reduces atmospheric methane emission. In methane oxidation, methane is sequentially converted to CO₂ via methanol, formaldehyde route (Mitsch and Gosselink, 2007).

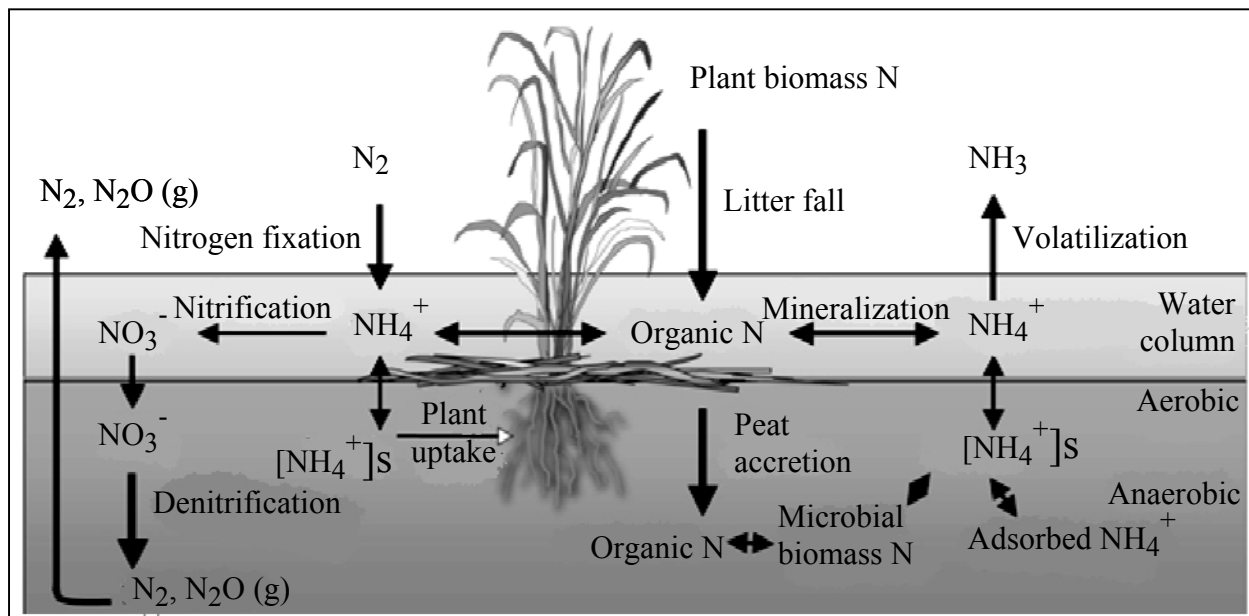


As methane oxidation requires oxygen, methane oxidation process mainly occurs in freshwater wetlands when wetland soil is exposed to air (Torres et al., 2005).

2.4.2 Nitrogen transformation

Nitrogen is present in wetlands in both inorganic and organic forms. Inorganic forms are ammonium (NH_4^+), ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), dinitrogen (N_2), nitric oxide (NO) and nitrous oxide (N_2O). Among all nitrogen forms, NH_3 , N_2 , NO and N_2O are gaseous forms and may be released to atmosphere from wetlands. NO_2^- is not a stable form of nitrogen as nitrite is readily oxidized to nitrate in aerobic or reduced to ammonium in anaerobic environments. NH_4^+ and NO_3^- are the most stable forms of inorganic nitrogen in wetlands environments. Organic forms of nitrogen include various compounds including proteins, nucleic acids, amino sugars, urea, uric acid, purines and pyrimidines (Kadlec and Wallace, 2009; Reddy and DeLaune, 2008). Figure 2-3 shows the various forms of nitrogen and major nitrogen transformation processes in wetlands.

Figure 2-3: Schematics of nitrogen transformation in wetlands (Reddy and DeLaune, 2008)



Major nitrogen transformation processes in constructed wetlands are ammonification, ammonia volatilization, nitrification, nitrate reduction, anaerobic ammonia oxidation (Anammox), plant and microbial assimilation, nitrogen fixation and peat accretion.

2.4.2.1 Ammonification

Ammonification, first step in mineralization of organic nitrogen, is the biological conversion of organic nitrogen to ammonium nitrogen (Reddy and Patrick, 1984). Ammonification is a complex and multi-step biochemical process that releases energy (Vymazal, 2007).

Ammonification occurs in both aerobic and anaerobic environments, however, anaerobic ammonification process is much slower than aerobic ammonification (Reddy and Patrick, 1984). However, as the depth of aerobic zone in saturated soils is less than 1 cm, aerobic mineralization contribution to overall N mineralization is very small as compared to the facultative anaerobic and obligate anaerobic mineralization (Reddy and Graetz, 1988). Typical formula of ammonification of urea can be given as (Mitsch and Gosselink, 2007):



In some instances, released energy is used by microbes and ammonia is assimilated into microbial biomass, immobilizing nitrogen. Net mineralization or immobilization of nitrogen depends on N requirement of anaerobic microbial populations, nature of organic material and various other soil and environmental factors (Reddy and Patrick, 1984). Anaerobic microbial communities in wetlands require lower nitrogen, which favors net mineralization (Reddy and DeLaune, 2008). Reported ammonification rates vary widely in literature with values ranging between 0.004 to 0.53 g N m⁻² day⁻¹ (Vymazal, 2007).

2.4.2.2 Ammonia volatilization

Ammonia volatilization is the release of ammonium nitrogen in the form of ammonia gas (NH_3) from wetland to the atmosphere. Ammonium in the wetland comes from external sources such as fertilizer or wastewater or produced internally through ammonification. Ammonia volatilization is a complex process that is controlled by various physical, chemical and biological factors (Reddy and DeLaune, 2008). Ammonium nitrogen is in equilibrium ionized form (NH_4^+) and gaseous form (NH_3). Ammonium ions are converted to ammonia gas as:



As can be seen from above equation, formation of NH_3 requires hydroxyl ion. Hence, at neutral or acidic pH, ammonium ion is predominant, and at alkaline pH, NH_3 gas is formed (Reddy and Patrick, 1984). At pH 9.3, ammonia and ammonium ion ratio reaches 1:1 (Vymazal, 2007). Hence, ammonia loss through volatilization are insignificant below pH 7.5 and not serious below 8.0, however, in the range of 8.5 to 10.0, losses via volatilization is significant (Reddy and Patrick, 1984). NH_3 gas formed at high pH diffuses from the wetland to the air water interface and is released to the atmosphere. Primary factors that regulate ammonia volatilization are pH and ammonium concentrations and both the factors can be used as ammonia volatilization indicators (Reddy and DeLaune, 2008). Nitrogen loss through ammonia volatilization can be as high as $2.2 \text{ g N m}^{-2} \text{ day}^{-1}$ (Stowell et al., 1981).

2.4.2.3 Nitrification

Nitrification is the microbial mediated process of formation of nitrate or nitrite from reduced nitrogen compounds using oxygen as the terminal electron acceptor (deGraaf et al., 1996). In

many wetland treatment systems, nitrification is the principle transformation mechanism that converts ammonium nitrogen into oxidized nitrogen compounds (Kadlec and Wallace, 2009). Nitrification is strictly an aerobic process and is restricted to aerobic water columns, aerobic soil-flood water interface and aerobic root zone (Reddy and DeLaune, 2008). Chemoautotrophic bacteria are predominantly responsible for nitrification; however heterotrophic nitrification also occurs and can be significant (Paul and Clark, 1996). Chemoautotrophic bacteria form nitrate in a two step process. First, chemoautotrophic bacteria from genera *Nitrosospira*, *Nitrosovibrio*, *Nitrosolobus*, *Nitrosococcus* and *Nitrosomonas* oxidize ammonia to nitrite. Second, facultative chemolithotrophic bacteria from genera *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira* oxidize nitrite to nitrate.



Ammonium oxidation is an energy releasing process and the released energy is taken up by the microbes as energy source to support their metabolic activities. Nitrification process is influenced by temperature, pH, inorganic carbon source, microbial population and concentrations of ammonium-N and dissolved oxygen (Vymazal, 1995).

In addition, methylotrophs, heterotrophic bacteria and fungi are also capable of oxidizing ammonium to nitrate (Reddy and DeLaune, 2008). Methane oxidizing bacteria that belong to the group methylotrophs are capable of cooxidizing ammonium to nitrite, nitrate or both.

Heterotrophic bacteria such as *Arthrobacter globiformis*, *Aerobacter aerogenes*, *Mycobacterium phlei*, *Streptomyces griseus*, *Thiosphaera*, and *Pseudomonas* spp. are also known to nitrify ammonia. However, ammonia nitrifying bacteria do not gain any energy from ammonia oxidation as the bacteria use organic substrate as energy source (Reddy and DeLaune, 2008).

Apart from bacteria, fungi, such as *Aspergillus flavus*, *Penicillium* or *Cephalosporium* are also capable of oxidizing ammonia (Paul and Clark, 1996). Reported values of nitrification rates in wetlands range from 0.01 to 2.15 g Nm⁻²day⁻¹ with a mean value of 0.48 g Nm⁻²day⁻¹ (Vymazal, 2007).

2.4.2.4 Nitrate reduction

Nitrate is reduced in wetland environmental by denitrification and dissimilatory nitrate reduction to ammonia (DNRA) processes (Reddy and DeLaune, 2008). In most type of wetlands, denitrification is a significant nitrogen removal process (Mitsch and Gosselink, 2007).

Denitrification is the microbial process that converts nitrite or nitrate into gaseous products such as nitrous oxide and nitrogen gas. Facultative heterotrophic microorganism, which can use both oxygen and nitrogen oxides as terminal electron acceptor during cell respiration, mediate nitrification reduction process (Kadlec and Wallace, 2009; Reddy and DeLaune, 2008). Various organisms such as organotrophs (*Pseudomonas*, *Alcaligenes* etc.), chemolithotrophs (*Thiobacillus*, *Thiomicrospira*, *Nitrosomonas* etc.), photolithotrophs (*Thodopseudomonas*), diazotrophs (*Rhizobium*, *Azospirillum* etc.), archaea (*Halobacterium*) and others such as *Paracoccus* or *Neisseria* are capable of carrying out denitrification (Kadlec and Wallace, 2009). Under anaerobic and anoxic conditions (Eh = +350 to +100 mV) and in the presence of available organic substrate, nitrate reducing heterotrophs use nitrate as electron acceptor instead of oxygen (Kadlec and Wallace, 2009). Denitrification is an irreversible process and is dissimilatory; the end product (nitrogen) is not assimilated by the microbes. Denitrification occurs in the following sequence: (1) diffusion of ammonium nitrogen to the aerobic zone, (2) nitrification of ammonium nitrogen, (3) diffusion of nitrate nitrogen to anaerobic zone, and (4) denitrification of nitrate nitrogen (Mitsch and Gosselink, 2007). Factors that directly or indirectly affect

denitrification are absence of oxygen, presence of readily available carbon, temperature, soil moisture, pH, presence of denitrifiers, redox potential, nitrate concentration, soil texture and presence of overlying floodwater (Reddy and Patrick, 1984; Vymazal, 2007). Reported denitrification rate in literature ranges from 0.003 to 1.02 g Nm⁻²day⁻¹ (Vymazal, 2007).

Although denitrification is considered the primary nitrate transformation process in wetlands, dissimilatory nitrate reduction to ammonia (DNRA) also occurs simultaneously. DNRA is carried out by obligate anaerobes that use mobile nitrate as alternative electron acceptor and produce less mobile ammonium during cellular respiration (Mitsch and Gosselink, 2007; Reddy and DeLaune, 2008). DNRA proceeds in two stages: nitrate is reduced to nitrite and then to ammonium (Meharg et al., 2004).



DNRA requires low redox potential and high electron donor (carbon) to acceptor (nitrate) ratio and the process decreases with the decrease in ratio (Reddy and DeLaune, 2008). Van Oostrom et al. (1994) reported that DNRA contributed 5% to the total nitrate removal in constructed wetlands.

2.4.2.5 Anaerobic ammonia oxidation (Anammox)

Anammox refers to the process where ammonium is oxidized to nitrogen gas using nitrite or nitrate as electron acceptor (Mulder et al., 1995). Equations below show the reaction in Anammox process:



Temperature, availability of organic matter, ammonium, nitrate and nitrite and presence of other oxidants (iron, manganese oxides etc.) regulate Anammox process (Reddy and DeLaune, 2008). Organisms responsible for Anammox have been found in many natural environments, however, the extent to which the reaction occurs in constructed wetlands is still unknown (Reddy and DeLaune, 2008; Vymazal, 2007). Anammox may be an important process of nitrogen removal in the wetlands where denitrification is limited by lack of carbon (Mitsch and Gosselink, 2007).

2.4.2.6 Nitrogen fixation

Nitrogen fixation refers to the process by which atmospheric nitrogen is converted to ammonia nitrogen. In wetland environment, nitrogen fixation is mostly carried out by organisms such as free-living bacteria (*Bacillus*, *Klebsiella*, *Clostridium* etc.), cyanobacteria (blue-green algae), *Rhizobium* bacteria and actinomycetes (such as *Frankia*) (Kadlec and Wallace, 2009). Nitrogen fixation can occur in periphyton mats in water columns, aerobic or anaerobic soil layer, in the plant rhizosphere or on the leaf and stem of plants (Mitsch and Gosselink, 2007; Reddy and DeLaune, 2008). During nitrogen fixation, high energy is required to break the triple bond of nitrogen and nitrogenase enzyme is required to catalyze the reaction. Since nitrogenase activity is suppressed by oxygen, low oxygen environments are favorable for nitrogen fixation (Mitsch and Gosselink, 2007). Nitrogen fixation in freshwater wetlands range from 0.03 to 12 g Nm⁻² day⁻¹, with most values are close to 1 g Nm⁻² day⁻¹ (Bowden, 1987).

2.4.2.7 Nitrogen assimilation

Plants uptake inorganic forms of nitrogen and convert into organic forms while building their tissues and cells. Free floating macrophytes uptake nutrients from water, while, emergent and rooted floating-leaved macrophytes uptake nutrients from sediments (Wetzel, 2001). Plants uptake nitrate and ammonium forms of nitrogen, however, preference depends on the forms

available in the soil (Lambers et al., 1998). Ammonium form of nitrogen is preferred over nitrate form by wetland plants, however, in nitrate rich waters, nitrate may be an important nitrogen source (Kadlec and Wallace, 2009). Duckweed (*Lemna minor*) has been found to uptake both nitrate and ammonium forms of nitrogen through roots and fronds (Cedergreen and Madsen, 2002). Cattails (*Typha latifolia*), algae and cultivated rice are also found to utilize both forms of nitrogen (Brix et al., 2002; Kronzucker et al., 2000; Naldi and Wheeler, 2002). Net productivity and nutrient concentration on plant tissue determine the potential nutrient uptake by plants (Vymazal, 2007). Nutrient content in wetland plants varies between plants parts, stages of maturity, plant species and plant stands (Bowden, 1987). Younger plants store more nitrogen per unit of their biomass and the nitrogen content decreases with maturity while total nitrogen storage in plant tissue increases with maturity (Reddy and DeLaune, 2008). Plants serve only as a temporary storage for nutrients. When plants die, biomass is returned to wetland for nutrient recycling and burial. Majority of plant biomass is decomposed and the nutrients are recycled, while, small fraction of the biomass is buried and stored as new soil and sediment. In temperate climates, plants translocate nutrients from leaves and shoots to roots before the plants senesce in fall. Translocation rate may reach more than 50% depending on plant species and environmental factors (Vymazal, 1995).

2.4.2.8 Sorption of ammonia

Oxidized forms of nitrogen are negatively charged, and hence are not bind to solid substrates but ammonium ion may be bind to solid substrates due to its positive charge (Kadlec and Wallace, 2009). Ammonium is only loosely bound to the substrates and is always in equilibrium with the surrounding ammonia concentration. The equilibrium implies that, when ammonium concentration in water column increases, sorption of ammonium also increases. When

ammonium concentration in water column decreases, ammonium is desorbed from substrates to establish new equilibrium. Various factors such as presence of other cations, organic matter content, clay mineral structure, soil porosity affect the amount of exchangeable ammonium sorbed to solid matrix (Reddy and DeLaune, 2008). Organic matter and clay particles increase cation exchange capacity (CEC), thus increasing ammonium sorption capacity of wetlands. Other cations, if present, occupy exchangeable sites or displace sorbed ammonium, reducing the amount of sorbed ammonium. If wetland oxygen level increases, ammonium sorbed to substrates is oxidized to nitrate and washed away. In SF wetlands, ammonium nitrogen sorbed to sediments and detritus is labile and is not very large (Kadlec and Wallace, 2009). Freundlich isotherm (Sikora et al., 1995) or Langmuir isotherm (Weatherley and Miladinovic, 2004) can be used to model ammonium sorption.

2.4.2.9 Peat accretion

Not all the dead plant material in wetlands is decomposed; part of the material is resistant to decay and buried through peat formation. Remnant of macrophyte root, stem, dead roots and rhizomes and undecomposable fractions of dead microflora and microfauna contribute to new sediment formation (Kadlec and Wallace, 2009). Murkin et al. (2000) reported $4.5\text{--}6.5 \text{ g Nm}^{-2} \text{ year}^{-1}$ of accretion in low nutrient mixed marshes. In marsh receiving strong agricultural runoff, Soto-Jimenez (2003) reported annual accretion of $11.3 \text{ g Nm}^{-2} \text{ year}^{-1}$. However, there is lack of data on the amount of nitrogen burial through peat accretion in constructed wetlands.

Not all the mechanisms mentioned above remove nitrogen from wetlands. Ammonification and nitrification do not remove nitrogen from wetlands but transform nitrate from one form for further processing. Nevertheless, all of the nitrogen transformation processes are very important

in nitrogen removal in constructed wetlands. Importance of each nitrogen removal process depends on type of wetlands. Table 2-1 shows relative importance of nitrogen removal mechanisms in various types of wetlands.

Table 2-1: Potential magnitude of various nitrogen transformation processes in various types of constructed wetlands (Vymazal, 2007)

	FWS	HSSF	VSSF
*Volatilization	Medium	Zero	Zero
Ammonification	High	High	High
Nitrification	Medium	Very low	Very high
Nitrate ammonification	-	-	-
*Denitrification	Medium	Very high	Very low
Nitrogen fixation	-	-	-
Microbial uptake	Low	Low	Low
*Plant uptake ^a	Low	Low	Low
Ammonia adsorption	Very low	Very low	Very low
*Organic nitrogen burial	Low	Low	Very low
*Annamox	-	-	-

^a With harvest

* Removal mechanisms

FWS: free water surface constructed with emergent vegetation, HSSF: horizontal subsurface flow wetland, VSSF: vertical subsurface flow wetland

Ammonia volatilization does not contribute to nitrogen removal in SSF wetlands but may be significant in SF wetlands depending on ammonium loading and pH. Unless there is high ammonium N concentration and high pH in soil water system, ammonia volatilization is not an important mechanism of N loss (Reddy and Patrick, 1984). If algae are present, diurnal pH may be very high due to algal photosynthesis increasing ammonia volatilization. Coupled nitrification-denitrification is the major nitrogen removal process in all types of constructed wetlands (Vymazal, 2007). Denitrification loss is not limited by nitrate diffusion but by ammonia diffusion and subsequent nitrification processes (Mitsch and Gosselink, 2007). Nitrate ammonification has not been well studied; however, nitrate ammonification process may be important if anaerobic condition exists in treatment wetlands (Vymazal, 2007). Nitrogen fixation adds nitrogen to the system. However, in nitrogen-rich systems, contribution of nitrogen fixation to the constructed wetland nitrogen cycle is not likely to be significant (Kadlec and Wallace, 2009). Harvesting plant biomass to recover nitrogen may be an option for nitrogen removal; however, only a small fraction of applied nitrogen can be recovered (Kadlec and Wallace, 2009). Harvesting may be effective in treatment wetlands with floating macrophytes where harvesting is easy or in the wetlands in tropical climates where seasonal translocation is minimal and harvesting is possible year round (Kadlec and Wallace, 2009; Vymazal, 2007). Ammonium nitrogen may be sorbed to detritus and inorganic sediments in SF wetlands and media in SSF wetlands. However, limited amount of nitrogen can be removed by sorption due to lower cation exchange capacity in SSF wetlands and inefficient interaction of water with substrate in SF wetlands (Reddy and DeLaune, 2008; Vymazal, 2007). Nitrogen removal through peat accretion is not very well studied (Kadlec and Wallace, 2009), and is limited to SF wetlands where peat accretion is an important process in nutrient removal (Vymazal, 2007).

2.4.3 Phosphorus transformation

Phosphorus is present in wetlands as soluble and insoluble compounds in both organic and inorganic forms. Inorganic forms include H_2PO_4^- , HPO_4^{2-} and PO_4^{3-} ions of phosphates, collectively known as orthophosphates (Mitsch and Gosselink, 2007). Their relative abundance depends on pH; at low pH H_2PO_4^- is predominant which is converted to HPO_4^{2-} and then to PO_4^{3-} as pH increases. Orthophosphate is the primary link between organic and inorganic phosphorus cycling in wetlands as algae and macrophytes are believed to utilize only the free orthophosphate form of phosphorus (Vymazal, 2007). Other inorganic phosphorous compounds are linearly condensed and cyclic polyphosphates (Vymazal, 2007).

Organic forms of phosphorus can be classified as easily decomposable (nucleic acids, phospholipids or sugar phosphates) and slowly decomposable (inositol phosphate or phytin) compounds (Dunne and KR, 2005). About 50-90% and 10-50% of total phosphorus is in organic form in wetlands dominated by organic soils and wetlands dominated by mineral wetland soils, respectively (Reddy and DeLaune, 2008).

Wetlands remove phosphorus by peat accretion, adsorption and precipitation, microbial uptake and plant uptake.

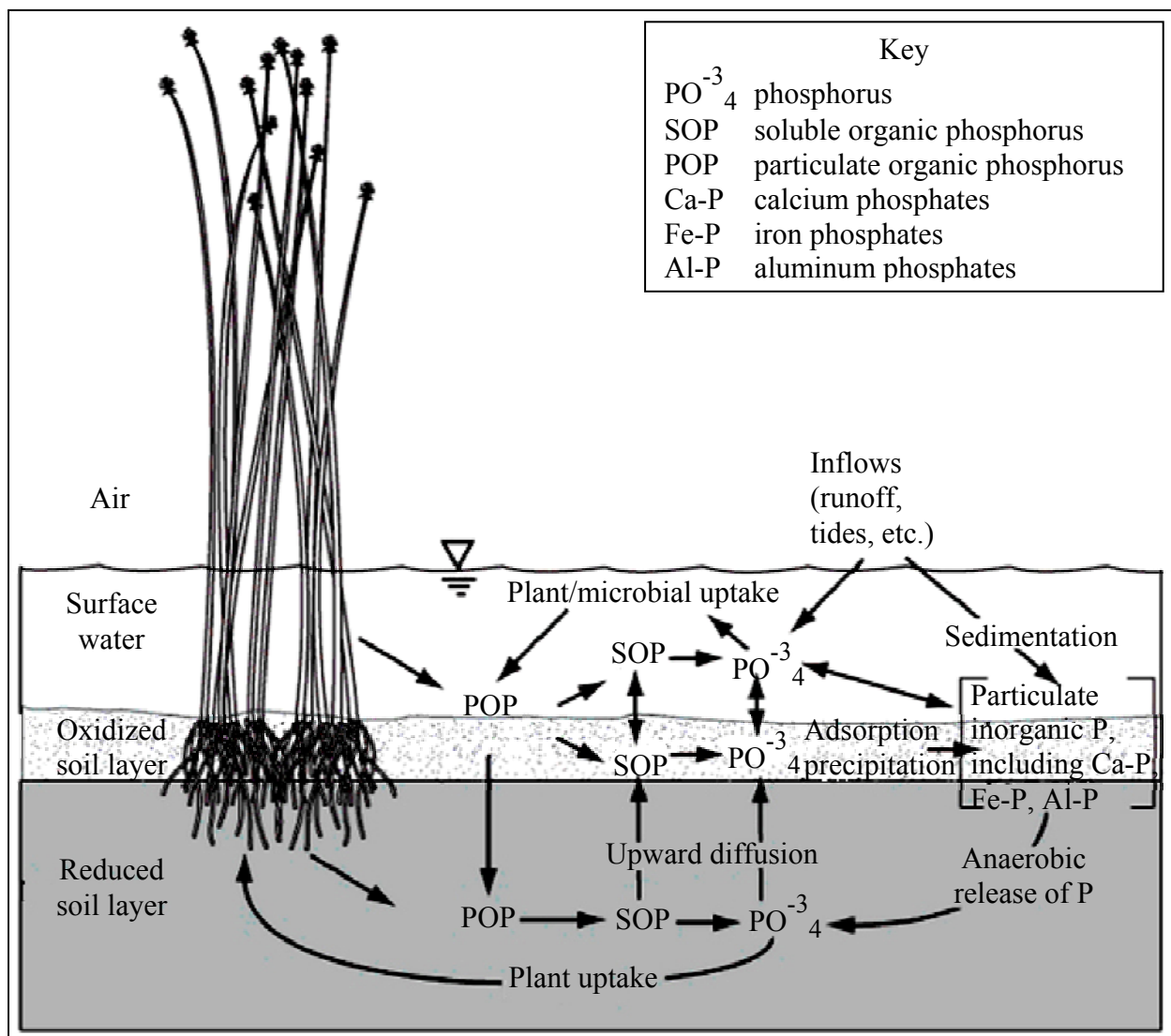
2.4.3.1 Peat accretion

Peat accretion is the accumulation of organic matter in the wetlands. Major fraction of phosphorus in wetlands is present in soil and sediments followed by plant and litter, accounting for more than 95% of phosphorus pool in natural wetlands (Kadlec and Wallace, 2009).

Phosphorus in soil or peat compartment is due to undecomposed litter from previous times, new

addition of phosphorus due to precipitation and adsorption, plant roots and new materials from the litter compartments (Richardson and Craft, 1993).

Figure 2-4: Phosphorus cycle in wetlands (Mitsch and Gosselink, 2007)



Though very slow, peat accumulation process is the most important long-term phosphorus removal process in wetlands (Vymazal, 2007). Phosphorus removal efficiency of wetlands due to peat accumulation depends on peat accretion rate and annual phosphorus storage rate in peats (Richardson and Craft, 1993). Vymazal (2007) reported that permanent storage of phosphorus in wetlands due to peat accretion is less than $1 \text{ gm}^{-2} \text{ yr}^{-1}$ and is around $0.5 \text{ gm}^{-2} \text{ yr}^{-1}$.

2.4.3.2 Adsorption and precipitation

Adsorption is the movement of soluble inorganic phosphorus from liquid phase to underlying soil mineral surface without penetrating it. Adsorption of phosphorus to wetland media is a reversible process, which depends on the phosphorus content of the pore water and phosphate retaining capacity of the solid phase. Phosphorus in soil mineral surface is always in equilibrium with pore water described by various isotherms such as linear isotherm, Freundlich isotherm, Langmuir isotherm and single-point isotherm (Reddy and DeLaune, 2008). When the phosphorus content of pore-water is high, there is a net movement of phosphorus from water to soil until equilibrium is reached. However, if the pore-water phosphorus content is low and soil particles are saturated, phosphorus moves from soil to water until new equilibrium is reached (Vymazal, 2007). Wetland solid media becomes saturated after few weeks or months after start-up and phosphorus removal by adsorption is severely reduced. Phosphates can also bind to humic particles produced by degradation of dead vegetation, which might be an intermediate step before chemical binding (Lüderitz and Gerlach, 2002). Sorption of phosphorus can be described as two step process (Kadlec and Ruthbun, 1984): rapid exchange of phosphorus between soil mineral surface and soil pore water (adsorption) followed by slow penetration into solid phases (absorption). In many wetland studies, adsorption and precipitation of phosphorus are not differentiated but are reported as a single process. Adsorption is followed by precipitation and then nucleation of a ‘surface precipitate’, and hence, it is difficult to distinguish between adsorption and precipitation (Rhue and Harris, 1999). In precipitation, two or more compounds form new solid compounds that settle out of the soil pore water. Phosphorus precipitation and retention in wetlands depend on acidity or alkalinity of the medium and availability of precipitating ions- Fe, Al and Ca. Insoluble complexes of phosphorus are formed by aluminum

or iron in acidic condition and by calcium in alkaline condition (Reddy and D'Angelo, 1994). Researchers have demonstrated that amendment with chemical substances such FeCl_3 , alum, $\text{Ca}(\text{OH})_2$, calcite, and dolomite as can stimulate precipitation and substantially reduce soluble phosphorus content (Garcia et al., 2010). Also, compared to calcium-rich soils, iron filings was more effecting phosphorus removal (Lüderitz and Gerlach, 2002). However, under reducing condition, phosphorus bound to ferric iron compounds may be released due to the conversion of ferric iron to soluble ferrous form (Kadlec and Wallace, 2009).

2.4.3.3 Microbial uptake

Microorganisms are involved in various phosphorus transformation processes in wetlands. Microbes use enzymatic hydrolysis and metabolic activities to mineralize organic phosphorus, immobilize inorganic phosphorus by assimilation and may alter physiochemical environment which may result in release of phosphorus from insoluble phosphate complexes (Reddy and DeLaune, 2008). While sediment or peat accretion controls the long-term phosphorus storage, short-term phosphorus uptake is dominated by microbes and algae (Richardson et al., 1996). Microbial phosphorus uptake is very fast due to their rapid growth; however, total amount of phosphorus uptake is very low (Vymazal, 2007). Microbial uptake is a reversible process and most of the phosphorus assimilated is released back to wetland when microbes decompose; only a fraction of uptake becomes part of peat accretion. Hence, high microbial uptake is expected in the start-up phase of constructed wetlands and as the initial phase is completed, net phosphorus removed by microbes is very low (Garcia et al., 2010). Immobilization and mineralization of phosphorus is also related to the nutrient content and dissolved oxygen level in wetlands. Microbes in low-nutrient wetlands store more phosphorus as compared to the microbes in eutrophic wetlands (Richardson et al., 1996). Edwards et al. (2006) reported that phosphorus

assimilation process was dominant in aerobic part of rhizosphere, while phosphorus mineralization was dominant in anaerobic part of wetland. Decomposition of organic phosphorus by heterotrophic microbes also depends on availability of labile organic carbon as energy source for the microbes (Reddy and DeLaune, 2008). In addition, microbial phosphorus under aerobic condition (20%) was found to be greater than that in anaerobic condition (<10%) (McLatchey and Reddy, 1998). In phosphorus treatment wetlands, 15-25% of total organic phosphorus in soils and flocks was microbial phosphorus (Reddy et al., 2002). After five years of operation in a horizontal SSF constructed wetland, Mander et al. (2003) found that only 4.4% of total phosphorus retention was due to microbial immobilization.

2.4.3.4 Plant uptake

Plant phosphorus concentration varies among plant types, nutrient availability and growth stage. Plants in the early stage of growth or grown in nutrient rich environment accumulate more phosphorus than mature plants or plants grown in low nutrient environment, where tissue phosphorus concentration (dry basis) in wetland plants range from less than 1 to 7 g/kg (Reddy and DeLaune, 2008). Phosphorus accumulation in vegetation can either be short-term or long-term depending on vegetation type, litter degradation rate, detrital leaching of phosphorus and translocation of phosphorus from above-ground biomass to below-ground biomass (Vymazal, 2007). Plants uptake nutrients while growing and if not harvested, decompose in wetlands returning nutrients to the wetlands. When plants die, decomposing above-ground biomass releases phosphorus to the water, whereas, below-ground biomass decomposes underground and returns refractory compounds to the soil and leachate to the pore water in the root zone (Reddy and DeLaune, 2008). As such, removal of phosphorus due to plants uptake is very low. Compared to nutrient mass input, nutrient removed by harvesting above-ground biomass of

macrophytes is insignificant as small fraction of passing nutrient is taken up by plants (Vymazal, 2004). Hence, very limited amount of phosphorus can be removed from wastewater using macrophytes even under optimum management conditions (Davies and Cottingham, 1993). Usually macrophytes are reported to remove less than 10% of the total phosphorus load. Davies and Cottingham (1993) reported that only about 6% of influent phosphorus load can be removed by wetland macrophytes; Edwards (2006) reported 1.5% of annual P load could be removed by *Phalaris arundinacea* in horizontal SSF CW; Tanner (2001) reported 6-13% removal by *Schoenoplectus tabernaemontani* in SSF wetlands. Vymazal (2005b) reported that phosphorus removed by plant uptake is more pronounced in tropical climates due to taller standing crops and absence of translocation in autumn. Moreover, harvesting plant biomass for phosphorus removal is expensive and has disposal problem, and hence, regardless of removal efficiency, harvesting has been rarely used except for the floating plants (Kadlec and Wallace, 2009).

Peat accretion and chemical precipitation are the sustainable phosphorus removal processes in constructed wetlands. Plant and microbial uptake represent temporary storage mechanisms from which phosphorus is returned to wetland unless removed by harvesting or buried as peat. As such, very little phosphorus is present in microbes, algae and water in wetlands with major fraction of phosphorus present in soil and sediments followed by plant and litter (Kadlec and Wallace, 2009). Table 2-2 shows the effectiveness of various wetland processes in phosphorus removal in different types of wetlands.

Table 2-2: Potential magnitude of phosphorus transformations in various types of constructed wetlands (Vymazal, 2007)

Type of CW	FFP	FWS	HSSF	VSSF
Soil accretion	Very low	High	Zero	Zero
Adsorption	Very low	Low	High ^a	High ^a
Precipitation ^b	Zero	Very low	Very low	Very low
Plant uptake ^c	Medium	Low	Low	Low
Microbial uptake	Low	Low	Low	Low

^a When special filtration materials are used

^b When washed gravel or crushed rock is used

^c With harvest

FFP: free-floating plants, FWS: Free water surface wetland, HSSF: Horizontal sub-surface flow wetland, VSSF: Vertical sub-surface flow wetland

CHAPTER 3: MATERIALS AND METHODS

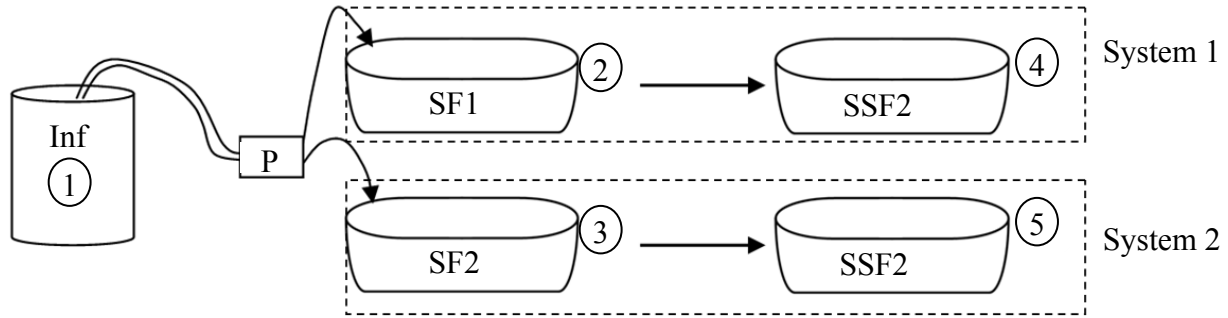
The study consisted of two separate constructed wetland systems. One system was designed to study pathogen removal efficiency of constructed wetlands subjected to simulated tile drainage, while other system was designed to study the pollutant reduction and nutrient recovery potential of duckweed-based constructed wetlands. This chapter provides details of each system design, sampling procedure and analysis methods. Short forms for methods are provided in each of the following sections. Models used to describe obtained data are also discussed in respective chapters.

3.1 Pathogen removal from simulated tile drainage

3.1.1 Experimental setup

Constructed wetlands used in pathogen removal studies were constructed using oval shaped, opaque, low density polyethylene tanks. Each tank was 0.84 m × 0.66 m × 0.30 m in length × width × height with total volume of 138 liters. Two surface flow and two subsurface flow wetlands were used in the experiment and were connected as shown in Figure 3-1. The configuration produced two parallel systems, each consisting of surface flow and subsurface flow wetlands connected in series. The wetlands were kept inside a greenhouse in the open space behind Farrall Hall at Michigan State University (East Lansing, MI). In winter months, the greenhouse was heated with electric heaters and temperature in the wetlands was maintained above 1°C so as to prevent the wetlands from freezing solid.

Figure 3-1: Schematic of constructed wetland systems used in pathogen removal studies



Inf: influent tank, SF: surface flow wetland, SSF: subsurface flow wetland, P: pump. Arrows show direction of flow and numbers denote sampling locations.

Since their construction in May 2009, the wetlands were supplied with groundwater supplemented with half-strength duckweed growth media as described in Standard Methods (Eaton et al., 2005). SF wetlands were planted with Duckweed (*Lemna minor*), while, SSF wetlands were planted with bulrush (*Scirpus lacustris*). Though the wetlands were kept inside greenhouse, seasonal effect on plant coverage was obvious. In warmer months, SF wetlands were covered with thick duckweed mat, while the wetlands were barren after the duckweed senesced in winter months. Bulrush grew and covered the SSF wetlands in warmer months and similar to duckweed, senesced during winter months. However, dead standing biomass remained throughout the winter months in SSF wetlands.

Flow to the SF wetlands from influent tank was provided through opaque tubing (Masterflex Norprene L/S 14, Cole-Parmer, IL) connected to peristaltic pumps (Masterflex HV-07014-20, Cole-Parmer, IL) operated by variable speed pump drives (Masterflex, HV-77200-12, Cole-Parmer, IL). Gravitational force maintained the flow from SF wetlands to SSF wetlands. Water was free drained at end of the SSF wetlands. During each pathogen removal study, nominal hydraulic retention time (HRT) was 8.3 days for SF wetlands and 1.5 days for SSF

wetlands. At other times, HRT was approximately 15 days for SF wetlands and 5 days for SSF wetlands.

3.1.2 Environmental parameters monitoring

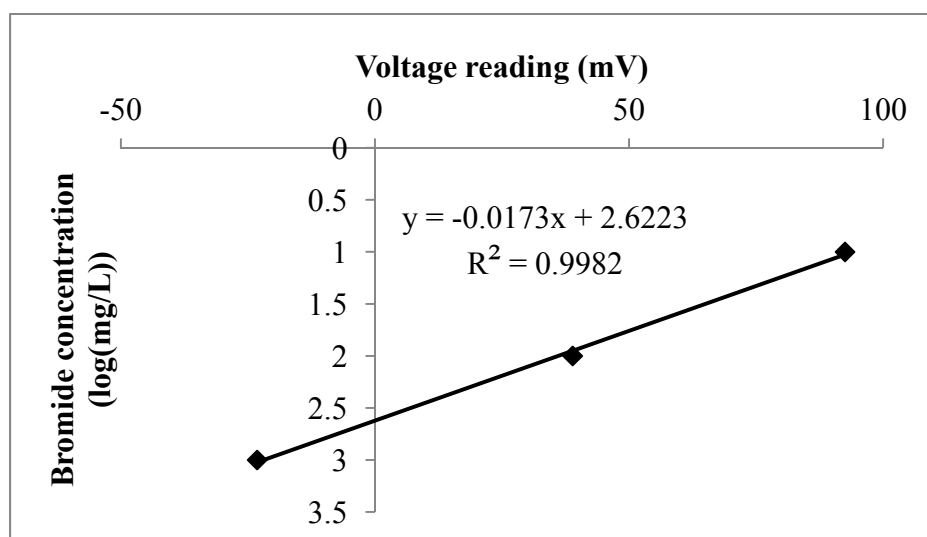
Ambient temperature and temperature in both SF and SSF wetlands were continuously monitored using temperature sensors (107, Campbell Scientific, Inc., Logan UT). Solar radiation above the below the water-air interface in SF wetlands was continuously monitored by placing solar radiation sensors (CS300, Campbell Scientific, Inc., Logan UT) immediately above and below the water-air interface. Hourly temperature and solar radiation data were recorded by a data logger (CR-10, Campbell Scientific, Inc., Logan UT) connected to the sensors. The pH in SF wetland was measured at each sampling time using handheld pH meter (pH/Con 10, Oakton Instruments, Vernon Hills, IL).

3.1.3 Tracer studies, sampling and analysis

Conservative tracer studies were conducted in March 2011, February 2012 and August 2012 to estimate the transport parameters of the wetlands. Multiple tracer studies were conducted so as to update transport parameters that affect pathogen transport properties of the wetlands. Bromide was chosen for tracer study due to its nonreactive nature, ease of analysis and lower background concentration (Kung, 1990). During each tracer study, each wetland system was spiked with 0.72 L (a total of 1.44 L) of 0.125 M bromide (KBr) solution obtained by dissolving potassium bromide in deionized water. For bromide solution injection, tubes carrying influent to the wetlands were taken out of the influent tank and placed in beaker containing bromide solution. When the beaker ran empty, the tubes were placed back to the influent tank. Samples were collected from each wetland outlet and preserved at 4°C until bromide concentration was measured. Samples were collected every six to eight hour in the beginning of the experiment and

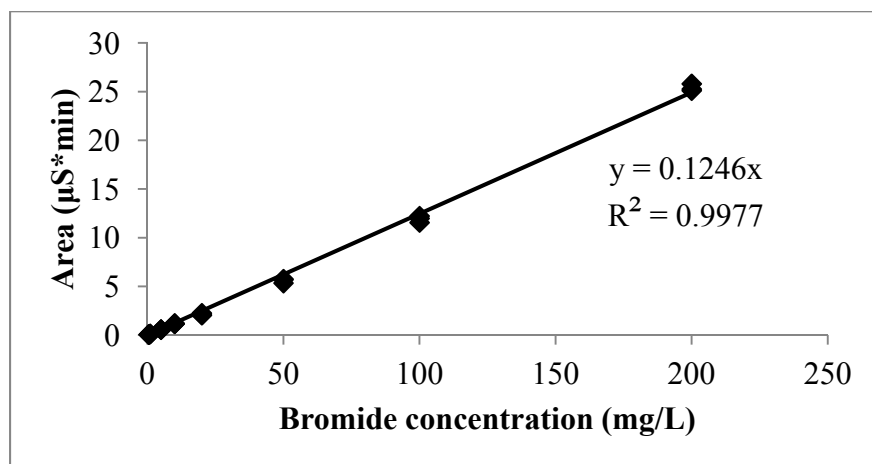
as the bromide concentration reached peak, samples collection frequency was reduced. Bromide concentration was measured with a bromide ion selective electrode (YO-27502-05, Cole-Parmer, Vernon Hills, IL). When the electrode is connected to a voltmeter, voltage output is inversely proportional to the bromide concentration of the solution in logarithmic scale. To adjust the ionic strength of solution, 2 ml of ionic strength adjuster (5M NaNO₃) solution was added to standards and samples prior to measurement. As suggested in the electrode manual, three-point calibration was completed and bromide concentrations in the samples were obtained from the calibration curve. Figure 3-2 shows a calibration curve for the bromide ion specific probe.

Figure 3-2: Calibration curve for bromide ion specific probe



In the third tracer study, bromide concentrations in the samples were measured with ion chromatography (IC) ICS 5000 (Dionex Corporation, Sunnyvale, CA). The IC was fitted with IonPac AS22 carbonate eluent anion-exchange column, AS22 guard and 4 mm suppressor column. Eluent used in the analysis was 4.5 mM sodium carbonate/1.4 mM sodium bicarbonate with a flow rate of 1 ml/min. Multipoint calibration was done prior to sample analysis. Figure 3-3 shows a calibration curve for ion chromatographic analysis of bromide concentration.

Figure 3-3: Calibration curve for ion chromatic analysis of bromide concentration



3.1.4 Pathogen removal studies, sampling and analysis

E. coli strain C3000 (ATCC No. 15597) was chosen as bacterial indicator due to its wide use as an indicator organism (Kadlec and Knight, 1996) and ease of detection and enumeration.

Likewise, the bacteriophage P22 was chosen as viral indicator due to its similarity to widely used bacteriophage PRD1 and its previous use as a biotracer (Shen et al., 2008; Steiner, 2009).

Bacteriophage P22 was obtained from The Water Quality, Environmental, and Molecular Microbiology Laboratory, Department of Fisheries and Wildlife, Michigan State University.

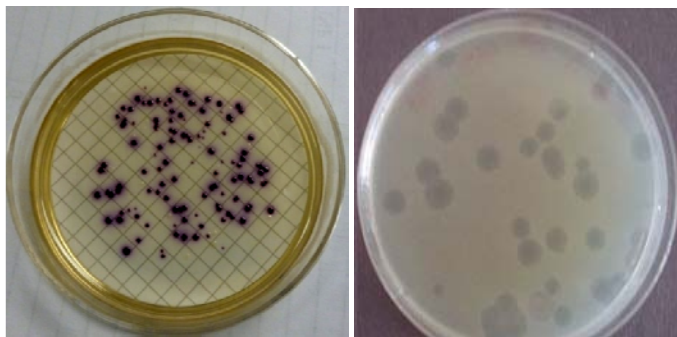
Bacterial removal studies were conducted in July 2010, July 2011 and January 2012, and viral removal studies were conducted in January 2011, July 2011 and January 2012. Bacterial and viral removal studies were conducted simultaneously by filling 113 L influent tank with bacterial and viral indicators to achieve approximate concentration of 10^6 colony forming unit (CFU) or plaque forming unit (PFU) per 100 ml of each indicator. During July 2010 and January 2011 experiments, there were difficulties with enumeration of bacteriophage P22 and *E. coli*, respectively and hence respective data were not available for the experiments. During the

experiments, samples were taken from influent, SF effluent and SSF effluent from the locations shown in Figure 3-1. At each sampling time, a 50 ml of sample was collected from each sampling location, preserved at 4°C and plated within 24 hours of sample collection. Culture-based methods were used to enumerate *E. coli* and bacteriophage P22. Prior to plating, the samples were diluted serially using sterilized phosphate buffer solution (0.5 M monopotassium phosphate /0.5 M dipotassium phosphate) to achieve an acceptable colonies of 10 to 100 CFU or PFU/plate.

E. coli stock solution was prepared from stock culture using tryptic soy broth (TSB). To prepare TSB solution, 30 g of tryptic soy broth powder was added to a liter of water, heated to dissolve, autoclaved at 121°C for 15 minutes and cooled to room temperature. One ml of Stock *E. coli* was added to 200 ml of TSB solution and incubated at 37°C for 48 hours. Two flasks of *E. coli* stock solution, each containing 200 ml, were added to influent tank in each experiment. *E. coli* were enumerated using membrane filtration coupled with modified mTEC agar method (USEPA, 2009). *E. coli* produce β -D-glucuronidase that catabolizes chromogen (5-bromo-6-chloro-3-indolyl- β -D-glucuronide) to glucuronic acid and other compound that is red or magenta in color and by counting the red or magenta colonies, number of *E. coli* can be known (USEPA, 2009). To prepare media for *E. coli* enumeration, 45.6 g of modified mTEC powder was dissolved in 1 L of water, boiled to dissolve, cooled in 56 °C water bath. pH of the solution was adjusted to 7.3 ± 0.2 with 1N NaOH, sterilized by autoclaving at 121°C for 15 minutes, cooled in 56°C water bath and dispensed 5-7 ml into Petri plate. The plates were then put back into sleeves and stored at 4°C until ready to use. To analyze *E. coli* in a sample, sample was filtered through 0.45 μ m membrane filter and the filter membrane was placed in Petri dish containing modified mTEC media. All dilutions of the same samples were filtered with single funnel, starting from the most

diluted one. Filter funnels were always sterilized after completing each sample. To ensure the reliability of the method, negative and positive controls were included with each batch of samples. The Petri dishes were then incubated at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 2 ± 0.5 hours and then at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 22 ± 2 hours. After the incubation period, red or magenta colonies of *E. coli* were counted and reported as CFU per ml.

Figure 3-4: *E. coli* (left) and P22 (right) after incubation (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.)



Stock P22 solution preparation and analysis P22 were done using Salmonella as host cells.

Salmonella in log-phase was prepared by inoculating 5 ml of TSB solution with salmonella stock solution, incubating overnight at 37°C , adding 1 ml of overnight culture to 30 ml of TSB solution and incubating at 37°C for 4-6 hours. One ml of stock P22 solution was added to 25 ml of salmonella in log-phase solution and incubated for 37°C for four hours. After four hours, 0.1 ml of 50 mg/ml of lysozyme and 0.75 ml of 0.5 M EDTA were added and centrifuged for 10 minutes at 4000 RPM. The solution was then filtered using 0.4 μm membrane filter and stored at 4°C . About 30-40 ml of stock P22 solution was added to influent tank during each experiment. Cultural based methods were used to enumerate bacteriophage using the double agar layer (DAL) method (Adams, 1959). Agar overlay tubes and tryptic soy agar (TSA) plates were

prepared prior to sample analysis. To prepare agar overlay tubes, 1% weight/volume of bacto agar was added to TSB solution, melted by boiling, dispensed 2.5 ml to cultural tubes and autoclaved for sterilization. TSA plates were prepared by adding 40 g of TSA powder to 1 lit of water, boiling the mixture, autoclaving at 121°C for 15 minutes and dispensing 20 ml to Petri dishes. Both agar overlay tubes and TSA plates were stored at 4°C until ready to use. To analyze the samples, melted one percentage agar overlay tubes were inoculated with 0.3 ml of *Salmonella* host cells in log phase. After mixing, 1 ml of the sample was added to the overlay tube, mixed thoroughly and dispensed to TSA plates. The plates were inverted and incubated at 37 °C for 24±2 hours and circular zones of clearing in the bacterial host lawn were counted as plaques and reported as plaque forming unit (PFU)/ml. Negative and positive controls were included with each batch of samples to ensure the reliability of the method.

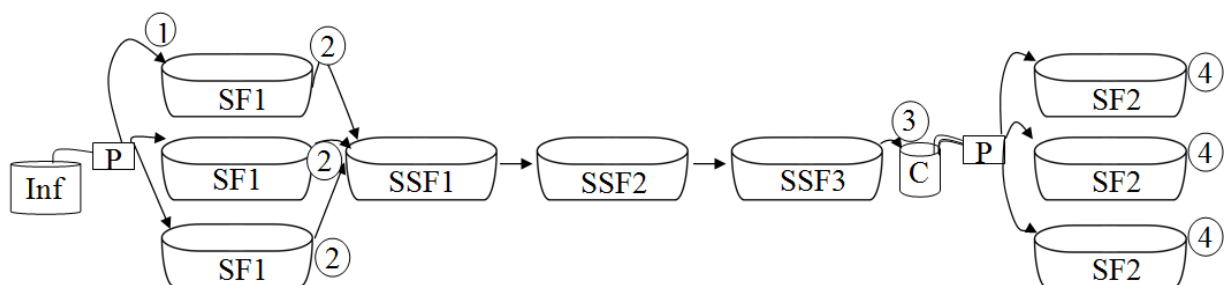
3.2 Pollutants reduction and from dairy manure

3.2.1 Experimental setup

Constructed wetlands used in the study consisted of surface flow (SF) and subsurface flow (SSF) wetlands connected as shown in Figure 3-5. The wetlands were constructed from oval shaped, opaque, low density polyethylene tanks with overall dimensions of 0.84 m × 0.66 m × 0.30 m (length × width × height). SF wetlands were planted with duckweed (*Lemna minor*). SSF wetlands were constructed by filing the polyethylene tanks with pea gravel and planting with Beggar-Ticks (*Bidens comosa*) and bulrush (*Scirpus lacustris*). Flow to the SF wetlands from influent tank and from collection tank to the SF wetlands were provided through opaque tubing (Masterflex Norprene L/S 18, Cole-Parmer, IL) connected to peristaltic pumps (Masterflex HV-07018-20, Cole-Parmer, IL) operated by variable speed pump drives (Masterflex L/S variable-speed modular drive, Cole-Parmer, IL). Gravitational force maintained the flow from SF

wetlands to SSF wetlands and to the collection tank. Water was free drained at end of the SF wetlands.

Figure 3-5: Schematic of constructed wetlands system used in nutrient and pathogen removal studies



Inf: influent tank, SF1: primary surface flow wetland, SSF: subsurface flow wetland, SF2: secondary surface flow wetlands, C: collection tank, P: pump. Arrows show direction of flow and numbers denote sampling locations.

The wetlands were kept inside Anaerobic Digestion Research and Education Center (ADREC) building at Michigan State University (East Lansing, MI). The building was heated in winter, and hence, seasonal variation was minimized. Light was provided by fluorescent lights operated 16 hours daily. Flow rate was maintained to achieve approximate theoretical hydraulic residence time (HRT) of 9 days. Dairy manure slurry after solid separation was obtained from Car-Min-Vu Farm, Webberville, MI and used as influent after dilution. The manure was stored in air-tight buckets until used. Sample analysis focused on steady-state condition on target chemical oxygen demand (COD) of 250 mg/l (low), 500 mg/l (medium) and 1000 mg/l (high). Appropriate dilutions were made to achieve target influent COD.

3.2.2 Sample collection and analysis

Sampling began after sufficient time to allow the wetlands to come to steady-state. Steady-state of the wetland system was determined by collecting and analyzing samples for COD. When

COD of the influent and effluent samples were stable for at least 2 weeks, the system was assumed to have come to steady state. Samples were collected once a week for total N, total P and *E. coli* analysis and twice a week for COD analysis from the sampling locations indicated in Figure 3-5. COD was measured using high range HACH COD digestion vials. The method uses potassium dichromate ($K_2Cr_2O_7$) to oxidize organic compounds in the presence of sulfuric acid (H_2SO_4). When organic compounds are oxidized, dichromate ion $Cr_2O_7^{2-}$ is reduced to green chromic ion (Cr_2^{3+}). Total COD was determined by measuring the amount of chromic ion colorimetrically using DR2800 spectrophotometer in 620 nm wavelength against a blank sample. *E. coli* were analyzed using the same method described previously.

For analyzing nitrate, phosphate and ammonium in the samples, the samples were simply filtered with 0.2 μm syringe filter and placed in sample vials and analyzed with ion chromatographic method. Total nitrogen and total phosphorus were analyzed using persulfate digestion method. Reagents used in digestion were 0.148 M potassium persulfate ($K_2S_2O_8$) and 0.1 M sodium hydroxide (NaOH) solutions. Fifteen ml of sample was placed in digestion vial and five ml of $K_2S_2O_8$ solution was added to the sample followed by 250 μl of NaOH solution. The vials were covered with aluminum foil, capped tightly and inverted to mix. The vials were autoclaved at 110°C in a liquid cycle for half an hour, cooled and 250 μl of NaOH solution was added to it. The samples were filtered with 0.2 μm syringe filter and analyzed by ion chromatographic method. Total phosphorus was analyzed using slightly different method. Digestion reagent was 0.074 M $K_2S_2O_8$ /0.075M NaOH solution and borate buffer solution was 1M boric acid (H_3BO_3)/0.2 M NaOH solution. To a digestion vial containing 10 ml of sample, 5 ml of

digestion reagent was added, covered with aluminum foil, capped and inverted to mix. The vials were autoclaved at 110°C in a liquid cycle for half an hour, cooled and 1 ml of borate buffer solution was added to it. Persulfate digestion converted all forms of nitrogen into nitrate and all forms of phosphate into phosphate. The samples were filtered with 0.2 µm syringe filter and analyzed by ion chromatographic method for nitrate and phosphate, respectively.

Duckweed was harvested from 50% of SF wetland area each week. Fresh weight was measured after draining harvested duckweed for 20 min. A small sample was taken out of the harvested duckweed, dried at 65°C for 48 hours and weighed to calculate moisture content. For total nitrogen and phosphorus in duckweed, dry duckweed was ground with mortar and pestle. Known amount of ground duckweed was placed in digestion vial and DI water added to make the volume same as the liquid sample volume. After that, same digestion and analysis procedure mentioned above was applied.

3.2.3 Ion chromatic analysis

Dionex ICS 5000 chromatography system (Dionex Corporation, Sunnyvale, CA) was used to analyze nitrate, phosphate, total nitrogen, total phosphorus and ammonium in samples. After filtration, the samples were placed in vials and injected by AS-AP autosampler. Anions were separated using Ionpac AS22 carbonate eluent anion-exchange column and measured by conductivity detector. Eluent used in the analysis was 4.5 mM sodium carbonate/1.4 mM sodium bicarbonate solution. Flow rate was maintained at 1.2 ml/min and pressure limit of the system was 200-2900 psi. Total run time for each sample was 15 minutes.

Before analyzing each batch, eluent was passed through the system and sufficient time was allowed for the system to reach a stable reading. Multipoint calibration curve was created using standard solution. Linear calibration was used for all anions using concentrations as shown in

Table 3.1. Figures 3.6 and 3.7 illustrate the calibration curves for total nitrogen and total phosphorus.

Table 3-1: Calibration standard curve for total N and total P

Standard	Total nitrogen mg/l	Total phosphorus mg/l
1	0.5	0.5
2	1	1
3	2	2.5
4	5	5
5	10	10
6	20	25
7	50	50
8		
R^2	99.84	99.66
Rel. std. dev.	9.86	7.92
R^2 : coefficient of determination		

Rel. std. dev.: relative standard deviation

Figure 3-6: Total nitrogen calibration curve

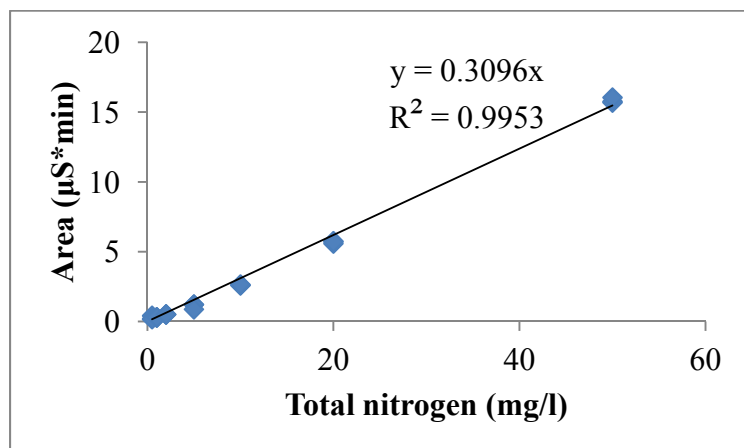
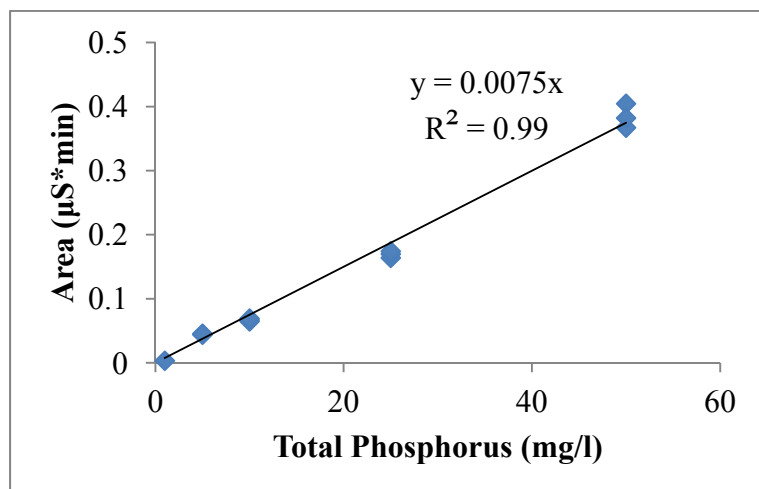


Figure 3-7: Total phosphorus calibration curve



CHAPTER 4: *E. COLI* REMOVAL IN CONSTRUCTED WETLANDS SUBJECTED TO PULSE LOADING

4.1 Introduction

Several major disease outbreaks related to agricultural activities have been reported in the past decades. In 2000, *E. coli* O157:H7 and campylobacter outbreak in Walkerton, Ontario, Canada resulted in the death of seven people and infection of 2,300 people. The source of bacteria was traced to groundwater supply contaminated by cattle manure. In 1999, two deaths and 781 infections in Washington County Fair, New York was caused by *E. coli* O157:H7 which was leached to well from manure storage area. In 1996, estimated 2000 people in Branbrook, Canada suffered gastroenteritis and the source of contamination was reported to be animal manure (Hrudey and Hrudey, 2004). In Michigan, 20% of the monitored beaches in 2009 and 25% of the monitored beaches in 2010 exceeded *E. coli* limits, which resulted in beach closures (MIDEQ, 2011).

In many North American farm operations, liquid manure is applied to fields for effective use and disposal (Joy et al., 1998), where large portion of the agricultural land is tile drained (Jamieson et al., 2002). Significant number of bacteria can pass through the soil to the sub-surface tile drain and contaminate receiving surface water (Joy et al., 1998). Evans and Owens (1972) reported a 30- to 900-fold increase in bacterial concentrations in tile drainage within two hours of liquid swine manure application. Irrespective of the application method, high concentration of bacteria has been found in tile drains after heavy rainfall that occurred soon after manure application (Samarajeewa, 2010). In order to control the negative impacts of leaching of bacteria to water bodies, a number of control measures including vegetated filter strips, detention ponds, and constructed wetlands have been evaluated.

Fecal coliform removal in vegetated filter strips have been reported to be up to 100% from the runoff from cattle-manure treated plots (Lim et al., 1998), 74% from the runoff from poultry litter applied fields (Coyne et al., 1995) and 87% from the runoff from livestock manure storage facilities (Fajardo et al., 2001) and the efficiency is affected by various factors such as length and slope of VFS, vegetation type, inflow concentration etc. (Parajuli et al., 2008). In an study that included 186 detention ponds worldwide, Von Sperling (2005) reported median fecal coliform removal efficiency of 98% for primary facultative ponds, 90% for secondary facultative ponds and 94% for maturation ponds and the removal efficiency depended on pond depth and associated factors such as solar radiation penetration, photosynthesis, pH, DO etc. Vymazal (2005c) reported that fecal coliform removal in constructed wetlands was comparable with activated sludge process, trickling filters and slow sand filtration processes. The authors reported 95 to >99% removal of fecal coliform and reported that hydraulic loading rate and resultant hydraulic residence time were the major influencing factors.

Several studies have attempted to model fecal coliform or *E. coli* transport in various best management practices (BMPs) such as vegetated filter strips (Guber et al., 2009; Parajuli et al., 2008) and waste stabilization ponds (Bahlaoui et al., 1998; Mayo, 1995; Polprasert et al., 1983; Von Sperling, 1999; Von Sperling, 2005). However, limited studies are available addressing fecal coliform, including *E. coli*, fate in constructed wetlands. In general, coliform removal is usually modeled using first order area-based or volume-based models that describes effluent in terms of influent concentration, retention time and first order removal rate constant with or without background concentration (Kadlec and Wallace, 2009). Khatiwada and Polprasert (1999) modeled *E. coli* removal in surface flow wetlands using dispersed flow equation by dividing first order rate coefficient into temperature, solar radiation, and sedimentation, adsorption and

filtration coefficients for a cattail-based surface flow wetland. Boutilier et al. (2011) used Water Quality Analysis and Simulation Program (WASP) to develop *E. coli* transport model in surface flow wetlands and reported that the model was good in predicting average effluent *E. coli* concentration but did not adequately forecast minimum and maximum values. Carleton (2002) proposed Damkohler number (*Da*) distribution model (DND) for predicting contaminant transport in treatment wetland but the model requires several sets of inlet-outlet concentrations data at different hydraulic loading rates before the model can be applied. All of the above models were developed under steady state condition and the models are not evaluated for their validation in pulse loaded wetlands.

The overall aim of this study was to evaluate the efficacy of constructed wetlands in removing *E. coli* from simulated tile-drain flow. The specific objectives of this study were: 1) compare the seasonal (winter and summer) variation in *E. coli* removal in pulse-loaded surface flow and subsurface flow constructed wetlands; 2) examine the applicability of convection-dispersion equation in *E. coli* removal estimation of pulse loaded wetlands; and 3) examine the applicability of the colloid filtration based model introduced by Khatiwada and Polprasert (1999) for *E. coli* removal estimation.

4.2 Materials and Methods

4.2.1 Experimental design and environmental parameter monitoring

Two identical set of duckweed-based surface flow constructed wetlands were made of low density polyethylene tanks with a dimension of 0.84 m × 0.66 m × 0.30 m length × width × height (Figure 3-1). Starting from their construction in May 2009, the wetlands were supplied with groundwater with added half-strength duckweed media as described in Standard Methods (Eaton et al., 2005). The wetlands were kept inside a greenhouse which was heated in winter

months to prevent the wetlands from freezing. Influent was supplied to the wetlands through opaque tubing connected to peristaltic pumps operated by variable speed pump drives as shown in Figure 3-1. Temperature and solar radiation above and below the wetland-air interface were recorded every hour using sensors and data logger. pH in the wetlands was measured whenever samples were taken for *E. coli* enumeration. Wetland vegetation was not harvested from the wetlands during experiment.

4.2.2 Tracer studies

Tracer studies were conducted in February 2011, February 2012 and August 2012 to estimate the transport parameters of the wetlands in winter and summer conditions, respectively. In each study, wetlands were spiked with 0.72 L of 0.125 M bromide obtained by dissolving potassium bromide in deionized water. Bromide concentration in the effluent was collected and preserved at 4°C until analysis. Bromide concentration in February 2011 and February 2012 experiment was measured with bromide ion specific probe (YO-27502-05, Cole-Parmer, Vernon Hills, IL) while the concentration in August 2012 experiment was measured with ion chromatography using a ICS 5000 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with AS22 carbonate eluent anion-exchange column, AS22 guard and ASRS 4 mm suppressor. Eluent used in the analysis was 4.5 mM sodium carbonate/1.4 mM sodium bicarbonate with a flow rate of 1.2 ml/min.

4.2.3 *E. coli* removal studies

E. coli (strain C3000, ATCC No. 15597) removal studies were conducted in summer of 2010, summer of 2011 and winter of 2012. Stock *E. coli* solution was prepared by inoculating tryptic soy broth with stock culture and incubating at 37°C for 48 hours. During each experiment, influent tank was filled with water, duckweed growth media (Eaton et al., 2005) and stock *E. coli*

solution to the influent tank. Samples were taken from the top of the influent tank every 6-8 hours until the tank was empty. When tank was empty, tank was rinsed and refilled with water and duckweed growth media and no more samples were taken thereafter. Wetland sampling continued until *E. coli* number in the effluent diminished as compared to the peak. In all experiments, samples were taken from both the wetlands every 6-8 hours for first 110 hours and less frequently thereafter. In summer 2010, sampling was done for 228 hours and a total of 18 samples were collected. In summer 2011, sampling continued for 426 hours and a total of 27 samples were collected. In winter, sampling continued for 354 hours and a total of 26 samples were collected. At each sampling time, 50 ml of sample was collected from influent tank and wetland outlets. Samples were preserved at 4°C and were processed within 24 hours of sample collection. Prior to plating, samples were diluted serially with phosphate buffer to obtain acceptable colonies (10 to 100 CFU) per plate. *E. coli* were enumerated by membrane filtration technique using modified mTEC agar as a growth medium (USEPA, 2009).

4.2.4 Modeling

4.1.1.1 Convection-dispersion equation (CDE) model

Observed tracer and pathogen removal data were fitted to the one dimensional convection-dispersion equation (CDE) given as:

$$R \frac{\partial C}{\partial t} = \frac{\partial^2 (DC)}{\partial x^2} - \frac{\partial (uC)}{\partial x} - \mu C \quad (4-1)$$

where, R is the retardation factor, C is the tracer or bacteriophage concentration (mg/L for bromide and CFU/ml for *E. coli*), t is the time (d), D is the dispersion coefficient (m²/d), u is the pore water velocity (m/d), μ is the first order removal coefficient (1/d), and x is the distance from inlet (m). For conservative tracers, there is no retardation (R=1) or removal ($\mu=0$). Hence, the Equation (4-1) simplifies to

$$\frac{\partial C}{\partial t} = \frac{\partial^2 (DC)}{\partial x^2} - \frac{\partial (uC)}{\partial x} \quad (4-2)$$

The CXTFIT model (Toride et al., 1995) in the Studio of Analytical Models (STANMOD)(PC-Progress, Prague, Czech Republic) software was used to fit the data to the model. First, the tracer study data were used to calculate the pore water velocity (u) and dispersion coefficient (D) using Equation (4-2). The fitted values of u and D were used in subsequent *E. coli* removal studies using Equation (4-1) to calculate the first order removal coefficient (μ) and retardation factor (R). A pulse input was used to model the SF wetlands, whereas, output from the SF wetland was converted to successive multiple pulse outputs using the trapezoidal method and used as input to the SSF wetlands.

4.1.1.2 Colloid filtration theory based model

Colloid filtration theory based constructed wetland fecal coliform removal model developed by Khatiwada and Polprasert (1999) was used to estimate *E. coli* kinetics in the wetlands. The model uses the dispersed flow equation and divides overall removal rate coefficient into removal rate coefficients due to temperature, solar radiation and adsorption, filtration and sedimentation. Dispersed flow equation describes effluent and influent fecal coliform concentration by following equation:

$$\frac{C_e}{C_o} = \frac{4a_1 e^{\frac{1}{2d}}}{\left(1 + a_1\right)^2 e^{\frac{a_1}{2d}} - \left(1 - a_1\right)^2 e^{-\frac{a_1}{2d}}} \quad (4-3)$$

Where,

$$a_1 = \sqrt{1 + 4ktd} \quad (4-4)$$

C_e = effluent fecal coliform concentration, most probable number (MPN)/100 ml

C_o = influent fecal coliform concentration, MPN/100 ml

d = dispersion number

t = hydraulic retention time, days

k = overall removal rate coefficient, day^{-1}

Overall removal rate coefficient is given by

$$k = k_T + k_i + k_f \quad (4-5)$$

Where,

k = overall removal rate coefficient, day^{-1}

k_T = removal rate coefficient due to temperature, day^{-1}

k_i = removal rate coefficient due to solar radiation, day^{-1}

k_f = removal rate coefficient due to adsorption, filtration and sedimentation, day^{-1}

Removal coefficient due to temperature is modeled using Arrhenius equation

$$k_T = k_{t,20} \phi^{(t-20)} \quad (4-6)$$

Where,

k_T = removal rate coefficient at t °C (1/day)

ϕ = temperature coefficient

T = water temperature (°C)

$k_{t,20}$ = removal rate coefficient at 20 °C (1/day)

Removal coefficient due to solar radiation is modeled using the following equation:

$$k_i = \varphi \times I_{avg} \quad (4-7)$$

$$I_{avg} = \frac{I_0}{\tau h} (1 - e^{-\tau h}) \quad (4-8)$$

Where,

I_0 = incident solar radiation received at the wetland surface, $\text{cal/m}^2 \cdot \text{day}$

φ = light mortality constant, m^2 / cal

I_{avg} = average solar radiation, $\text{cal/m}^2 \cdot \text{day}$

τ = vertical light extinction coefficient, $1/\text{m}$

h = depth of wetland bed, m

Removal coefficient due to sedimentation, adsorption and filtration is modeled using following equation:

$$k_f = \frac{4}{\pi} \eta \alpha \frac{u(1 - \theta)}{d_c} \quad (4-9)$$

Where,

η = Single collector removal efficiency

α = sticking efficiency

u = velocity of flow (m/day)

θ = porosity of the wetland bed

d_c = collector diameter (m)

Average temperature recorded in previous 24-hours and total solar radiation received in wetland in previous 24-hours was used in modeling. Solar radiation recorded above and below the wetland surfaces were used separately in the modeling; using separate vertical light extinction coefficient as shown in Table 4-1.

Since the model as developed by Khatiwada and Polprasert (1999) was based on steady-state flow, collected data were transformed to a series of steady-state loads prior to fitting the model. To prepare data, detention time distribution (DTD) function was created using following equation (Kadlec and Wallace, 2009) based on tracer study results:

$$f(t) = \frac{C}{\int_0^{\infty} C dt} \quad (4-10)$$

Where,

$f(t)$ = DTD function, 1/hour

C = Outlet tracer concentration, mg/L

dt = time step, hour

The time interval of total *E. coli* input was broken down to the time intervals equal to tracer application time (0.8 hr). Number of *E. coli* flowing to the wetlands at each time interval was estimated using linear interpolation between two measured points. This interval was chosen in order to create series of steady state conditions from the single pulse used in this study.

Meanwhile, the total number of *E. coli* expected in the outlet with no wetland degradation was calculated by summing the product of the number of *E. coli* input in each time interval and the DTD function.

Other parameters used in the model were obtained from sensor data, tracer study results and from literature. Table 4-1 shows the parameters used in the model along with their sources.

Experimental wetlands consisted of two identical wetlands receiving influent from the same tank. The wetlands were kept in the same greenhouse and were therefore subjected to similar environmental conditions. For calibration, model was developed for SF1 using the solar radiation and temperature data from SF1. For validation, model output from calibration was compared with the output from SF2. For overall model evaluation, model developed for SF1 was compared with the outputs from both SF1 and SF2.

Table 4-1: Parameters used in the model and their sources

Parameter	Symbol	Unit	Value	Source
Removal rate coefficient at 20 °C	$k_{T,20}$	1/day	0.047	Khatiwada and Polprasert, 1999
Temperature coefficient	ϕ	-	1.07	Khatiwada and Polprasert, 1999
Temperature	T	°C	Continuous	Temperature sensors
Light mortality constant	φ	m^2/cal	0.0103	Khatiwada and Polprasert, 1999
Incident solar radiation	I_0	$cal/(m^2 \cdot day)$	Continuous	Solar radiation sensors
Vertical light extinction coefficient	τ	1/m	25 (with duckweed) 1 (without duckweed)	Khatiwada and Polprasert, 1999
Wetland depth	h	m	0.3	Measured
Sticking efficiency	α	-	0.003	Khatiwada and Polprasert, 1999
Porosity	θ	-	Summer: 0.98 Winter: 1	Calculated from the volume of duckweed No vegetation in winter
Boltzmann constant	K_B	J/K	1.38×10^{-23}	Khatiwada and Polprasert, 1999
Viscosity	μ	(N.S)/ m^2	Continuous	Kestin et al., 1978
<i>E. coli</i> diameter	d_p	m	1×10^{-6}	Khatiwada and Polprasert, 1999
Duckweed root diameter	d_c	m	1.76×10^{-4}	Cedergreen and Madsen, 2002
<i>E. coli</i> density	ρ_p	kg/m^3	1050	Khatiwada and Polprasert, 1999
Density of water	ρ	kg/m^3	1000	Khatiwada and Polprasert, 1999
Gravitational constant	g	m/s^2	9.8	
Hydraulic resident time	t	day	SF1: sum=6.46, win=9.27 SF2: sum=7.53, win=7.98	Calculated from tracer data by fitting into one dimensional convection-dispersion equation
Dispersion number	d	-	SF1: sum=0.65, win=0.36 SF2: sum=0.73, win=0.15	Calculated from tracer data by fitting into one dimensional convection-dispersion equation
Velocity of flow	u	m/day	SF1: sum=0.13, win=0.091 SF2: sum=0.113, win=0.105	Calculated from tracer data by fitting into one dimensional convection-dispersion equation

4.3 Results

4.3.1 Tracer study

Data collected from each tracer experiment were fitted to CDE model as shown in Equation (4-2) and flow parameters were estimated. Figures 4-1 to 4-3 show the tracer study results along with CDE model fitted lines.

Figure 4-1: Tracer study data February 2011 along with CDE model fitted lines

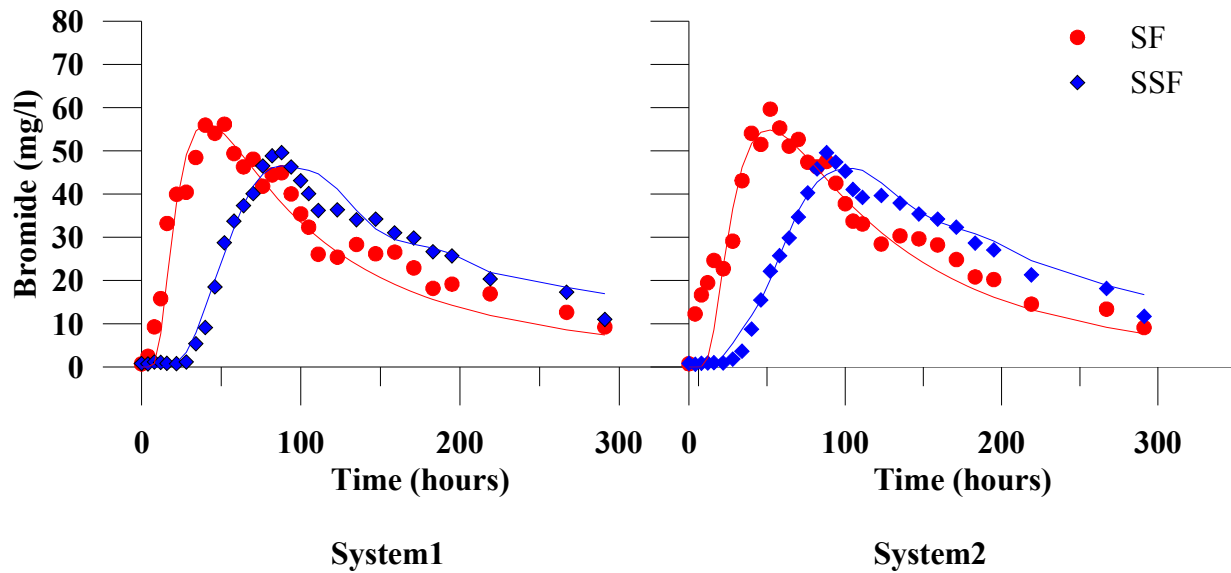


Figure 4-2: Tracer study data February 2012 along with CDE model fitted lines

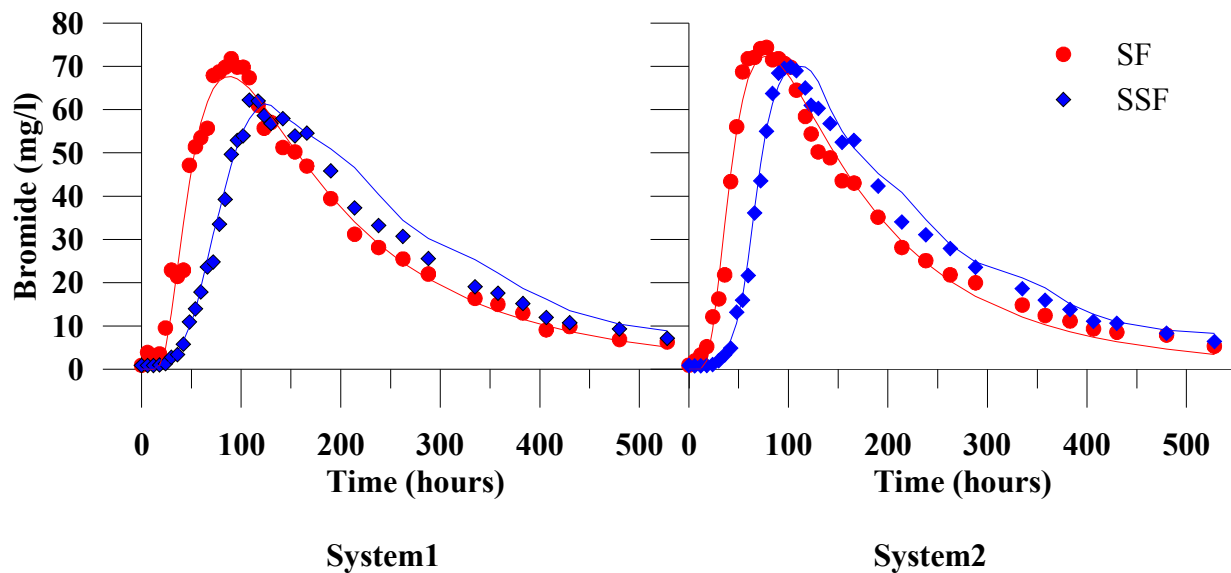


Figure 4-3: Tracer study data August 2012 along with CDE model fitted lines

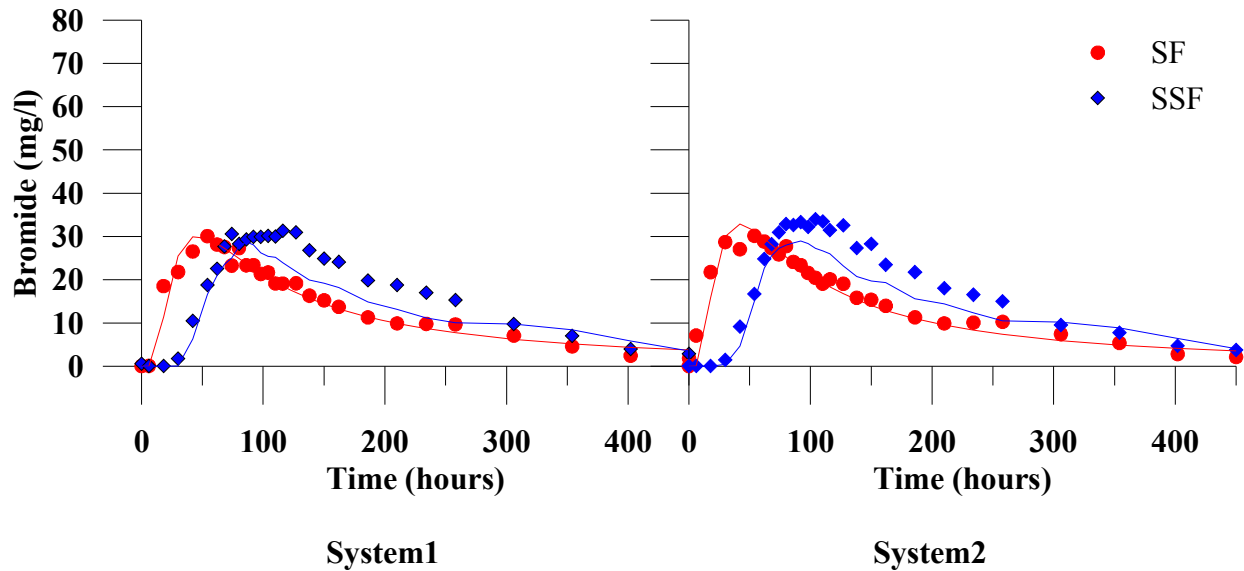


Table 4-2 shows the estimated flow parameters of the wetlands based on tracer study results.

Table 4-2: One dimensional convection dispersion model estimated parameters for tracer studies

	Wetland	u (cm/day)	D (cm ² /day)	Pe (unitless)	τ (unitless)	R ²
Winter 2011	SF1	10.4±0.7	667±30.2	1.3±0.1	8.1±0.6	0.90
	SF2	12.0±0.8	505±31.0	2.0±0.2	7.0±0.5	0.83
	SSF1	59.4±2.4	916±191.5	5.4±1.2	1.4±0.1	0.97
	SSF2	53.0±2.3	1210±223.9	3.7±0.7	1.6±0.1	0.97
Winter 2012	SF1	9.1±0.5	273±8.7	2.8±0.2	9.3±0.5	0.98
	SF2	10.5±0.5	307±9.1	2.9±0.2	8.0±0.4	0.98
	SSF1	61.1±4.1	2930±652.3	1.8±0.4	1.4±0.1	0.97
	SSF2	89.4±3.5	1672±395.0	4.5±1.1	0.9±0.0	0.99
Summer 2012	SF1	12.0±0.4	616±22.4	1.6±0.1	7.0±0.2	0.95
	SF2	12.0±0.5	685±28.0	1.5±0.1	7.0±0.3	0.99
	SSF1	88.8±11.8	83±377.3	90.1±410.3	0.9±0.1	0.85
	SSF2	69.5±7.8	72±183.3	81.0±206.5	1.2±0.1	0.86

u: flow velocity, D: dispersion coefficient, Pe: Peclet number, τ : hydraulic residence time, R²:

coefficient of determination

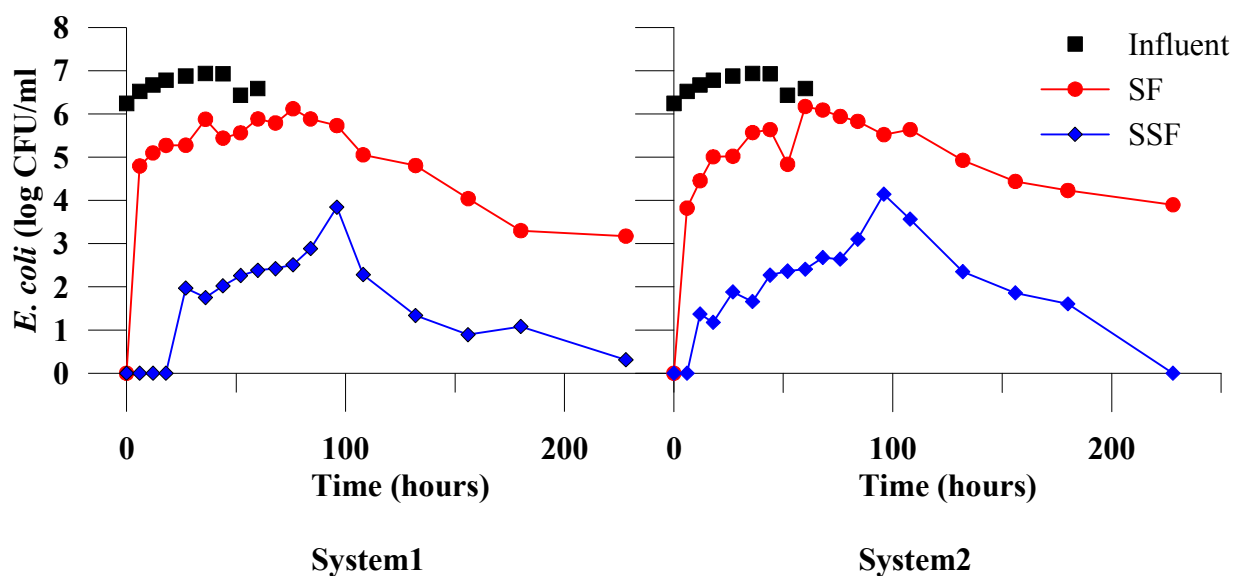
The results indicate that there was not major change in Peclet number and hydraulic residence time in winter and summer tracer studies except for the dispersion coefficient for subsurface flow wetlands in summer. Hydraulic residence time for SF wetlands ranged from 7.0-9.3 days and

Peclet number ranged from 1.3-2.9. For the SSF wetlands, residence time ranged from 0.9 to 1.6 days. In summer months, dispersion coefficient for SSF wetlands was lower, resulting in higher Peclet number suggesting that in summer months hydraulic transport in SSF wetlands was mainly due to convection and that dispersion was lower. SSF wetlands were covered with actively growing plant biomass in summer, while no actively growing plants were observed in winter. The plant root present in summer months might have caused lower dispersion in summer months.

4.3.2 *E. coli* removal

In summer 2010, 32.7% and 35.3% of influent *E. coli* was recovered in 228 hours from surface flow wetlands 1 and 2, respectively. In the same period, 0.19% and 0.45% of *E. coli* were recovered from subsurface flow wetlands 1 and 2, respectively. Overall, 99.94% and 99.84% of *E. coli* were removed by systems 1 and 2, respectively. Figure 4-4 shows influent and effluent *E. coli* in surface and subsurface flow wetlands along with influent *E. coli*.

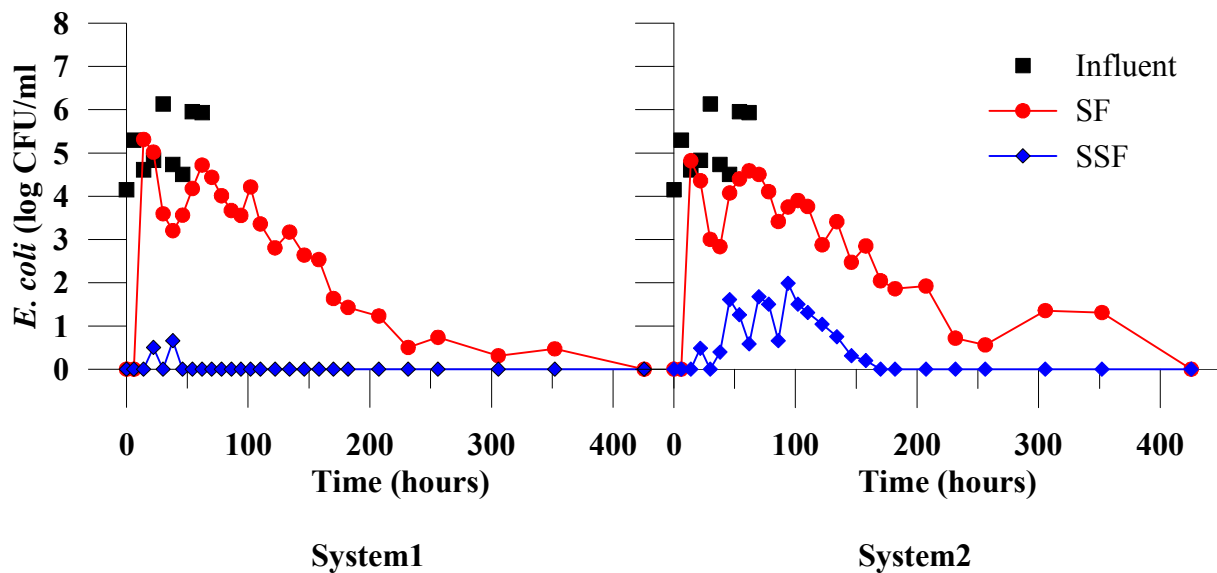
Figure 4-4: Number of *E. coli* in influent and effluent samples in summer 2010 experiment



SF: surface flow wetland, SSF: subsurface flow wetland

In summer 2011, 34.1% and 18.0% of *E. coli* were recovered from surface flow wetlands 1 and 2, respectively. In the same period, 0.002% and 0.14% of *E. coli* were recovered from subsurface flow wetlands 1 and 2, respectively. Overall removal of *E. coli* and system 1 and 2 were 99.999% and 99.97%, respectively. Figure 4-5 shows influent and effluent *E. coli* in surface and subsurface flow wetlands along with influent *E. coli*.

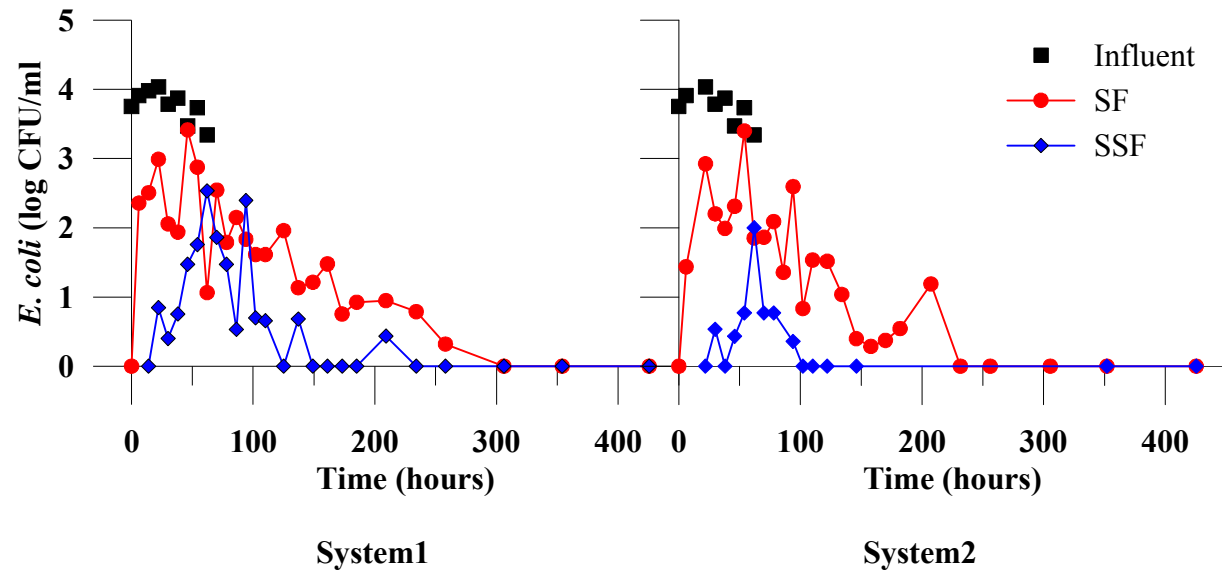
Figure 4-5: Number of *E. coli* in influent and effluent samples in summer 2011 experiment



SF: surface flow wetland, SSF: subsurface flow wetland

In winter 2012, number of influent *E. coli* was less than the number of influent *E. coli* in summer. A total of 22.3% and 18.3% of *E. coli* were recovered from surface flow wetlands 1 and 2, respectively. In the same period, 13.6% and 2.5% of *E. coli* were recovered from subsurface flow wetlands 1 and 2, respectively. Overall removal of *E. coli* and system 1 and 2 were 96.98% and 99.53%, respectively. Figure 4-6 shows influent and effluent *E. coli* in surface and subsurface flow wetlands along with influent *E. coli*.

Figure 4-6: Number of *E. coli* in influent and effluent samples in winter 2012 experiment



SF: surface flow wetland, SSF: subsurface flow wetland

4.3.3 Modeling *E. coli* removal

Obtained *E. coli* removal data were fitted to CDE model as shown in Equation (4-1). For the fitting, u and D in the model were obtained from tracer experiment and R and μ were estimated by minimizing the mean squared error. Table 4-3 shows the results obtained from fitting the curve. The results showed retardation factor ranging from 0.00 to 4 and first order degradation constant of 0.63 to 12.98. However, coefficient of determination ranged from -0.12 to 0.67, which suggest that the model was not optimum fit for the data.

Table 4-3: CDE model estimated *E. coli* transport parameters from the data

	Wetland	R	μ (1/day)	MSE	R^2
Summer 10	SF1	0.65	0.76	8.72E+10	0.40
	SF2	0.64	0.75	1.02E+11	0.52
	SSF1	0.48	6.66	1.67E+06	0.40
	SSF2	0.90	4.38	3.81E+06	0.67
Summer 11	SF1	0.00	1.90	2.19E+09	-0.11
	SF2	0.00	2.26	2.85E+08	-0.12
	SSF1	0.70	12.98	7.30E-01	0.35
	SSF2	0.95	5.92	2.68E+02	0.47
Winter 12	SF1	0.00	0.63	2.77E+05	0.08
	SF2	0.00	0.80	2.51E+05	0.06
	SSF1	0.74	2.91	3.69E+03	0.46
	SSF2	4.00	8.00	3.89E+02	0.02

R: retardation factor, μ : first order removal rate constant, MSE: mean squared error, R^2 : coefficient of determination

In absence of light, pathogens are capable of repairing themselves the damage caused by solar radiation through dark repair (Sonntag et al., 2003). Hence, to eliminate the diurnal fluctuation of *E. coli* removal due to solar radiation effects, only the data collected at 6-9 am were fitted to the model using the same flow parameters. Table 4-4 show results obtained from the fitting.

Coefficient of determination for the fitting ranged from -0.21 to 0.71 suggest that the fitting approach was also not optimum for the data collected.

Table 4-4: CDE model estimated *E. coli* transport parameters only using the morning hour data

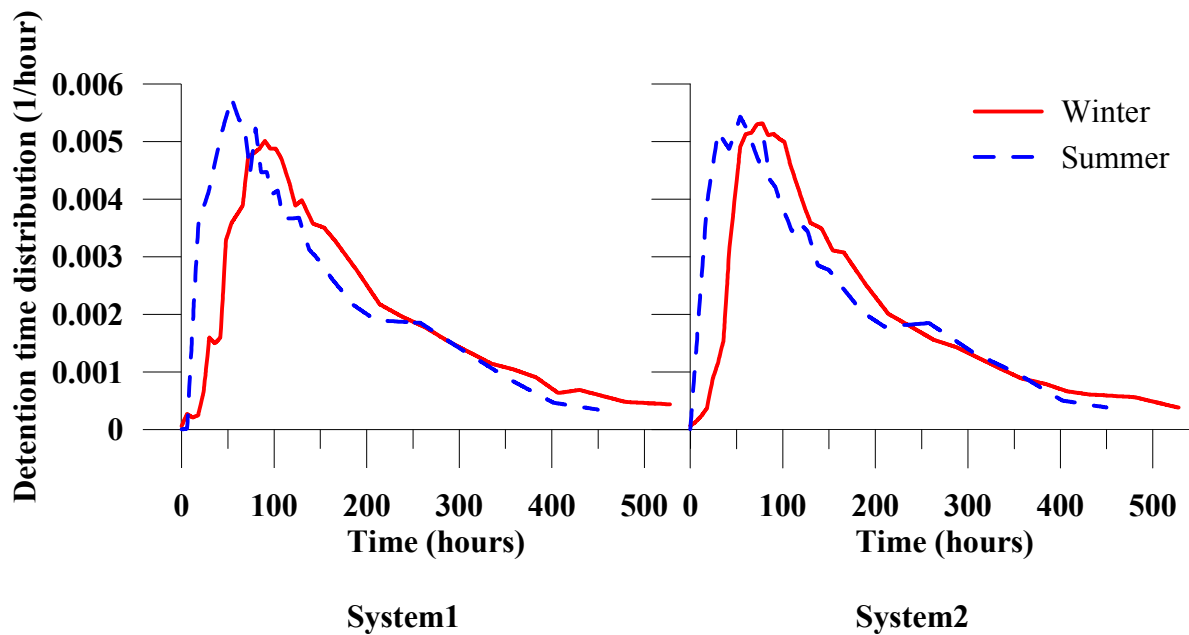
	Wetland	R	μ (1/day)	MSE	R^2
Summer 10	SF1	0.00	1.26	1.42E+11	-0.05
	SF2	0.00	1.12	2.19E+11	0.12
	SSF1	0.00	8.61	2.77E+04	0.57
	SSF2	0.29	5.45	4.35E+05	0.71
Summer 11	SF1	0.00	3.84	3.43E+09	-0.12
	SF2	0.00	4.05	4.51E+08	-0.18
	SSF1	0.00	12.76	8.38E-01	0.43
	SSF2	1.05	7.39	2.04E+01	0.33
Winter 12	SF1	0.00	1.62	1.02E+04	-0.21
	SF2	0.00	1.90	1.04E+04	-0.16
	SSF1	0.00	0.14	4.78E+03	0.47
	SSF2	0.00	4.64	7.39E+02	0.04

R: retardation factor, μ : first order removal rate constant, MSE: mean squared error, R^2 :

coefficient of determination

To explore the applicability of the colloid filtration theory based model as mentioned previously, DTD function was created from the tracer study results. Figure 4-7 shows the DTD function for both the wetlands for winter and summer experiments.

Figure 4-7: Detention time distribution functions from summer and winter tracer experiments for surface flow wetlands



Using DTD function, influent *E. coli* was calculated for the colloid filtration theory as mentioned in section 4.1.1. Based on the calculated input, expected output using the model were calculated and compared to the observed data. Solar radiation recorded above and below the wetland surfaces were used separately in the model to predict *E. coli* output. Table 4-5 shows the results of comparison. Results show that the coefficient of determination between predicted and observed data ranged from 0.01 to 0.23 suggesting that the model did not adequately predicted the *E. coli* output. However, there was little or no difference in using the solar radiation above or below the wetland surface as long as proper light extinction coefficient was used.

Table 4-5: Mean squared error and coefficients of determination for model fitting, calibration and validation of the colloid filtration theory based model for *E. coli* removal data

	Solar above	Solar below	Solar above	Solar below	Solar above	Solar below	Solar above	Solar below
	SF1	SF1	SF2	SF2	Calibration	Calibration	Validation	Validation
Summer 2010								
MSE	3.E+11	3.E+11	4.E+11	3.E+11	5.E+11	4.E+11	3.E+11	3.E+11
R^2	0.04	0.05	0.01	0.01	0.01	0.01	0.02	0.02
Summer 2011								
MSE	4.E+09	2.E+09	2.E+09	4.E+08	2.E+09	4.E+08	3.E+09	1.E+09
R^2	0.14	0.17	0.16	0.23	0.17	0.23	0.12	0.15
Winter 2012								
MSE	4.E+05	5.E+05	3.E+05	5.E+05	4.E+05	4.E+05	4.E+05	5.E+05
R^2	0.14	0.14	0.09	0.06	0.08	0.08	0.11	0.11

Solar above: solar radiation recorded above wetland surface used in modeling

Solar below: solar radiation recorded below wetland surface used in modeling

SF1: surface flow wetland 1, SF2: surface flow wetland 2

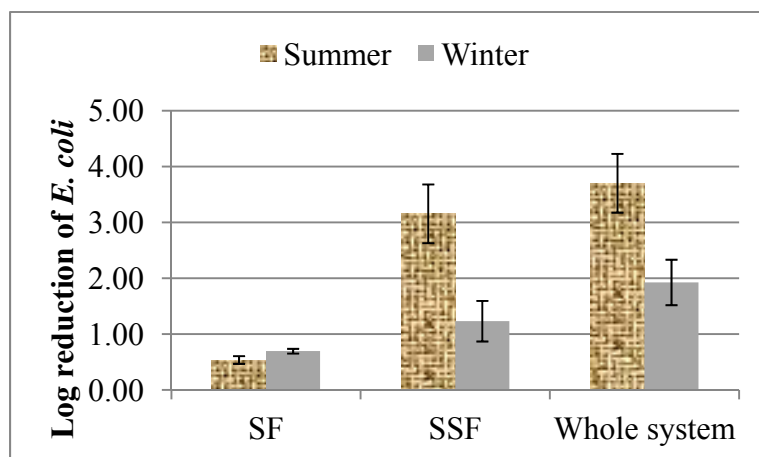
MSE: mean squared error, R^2 : coefficient of determination

4.4 Discussion

Based on *E. coli* recovered, log reduction of *E. coli* in SF wetlands ranged from 0.45 to 0.74 in summer and 0.65 to 0.74 in winter experiments. The mass removal observed in two seasons were very close. However, the removal rates observed in SSF wetlands in two seasons differ substantially. In SSF wetlands, log reduction of *E. coli* ranged from 2.35 to 5.2 in summer

months, while the range for winter months was 1.52 to 2.33. Overall, 0.54 ± 0.07 and 0.69 ± 0.04 log reduction of *E. coli* were obtained from SF wetlands in summer and winter experiments, respectively. Irrespective of the season, *E. coli* reduction in SSF wetlands were always higher in SSF wetland than in SF wetlands. Mean log reduction of *E. coli* in SSF wetlands were 3.16 ± 0.53 and 1.23 ± 0.36 for summer and winter experiments, respectively. SSF wetlands provide more surface area for contact and biofilm formation. As a result, higher contamination removal per unit area is obtained than SF wetlands (Halverson, 2004). Due to the higher *E. coli* removal obtained in SSF wetlands in summer, overall system performance in *E. coli* reduction was better in summer months as compared to the winter months. Overall *E. coli* log reduction of the combine systems were 3.70 ± 0.53 and 1.93 ± 0.41 for winter and summer months, respectively. These findings are in agreement with the findings by several authors (Bahlaoui et al., 1998; Molleda et al., 2008; Zdragas et al., 2002) that pathogens are removal rates are higher in summer than in winter months. Figure 4-8 shows the comparison of the log reductions obtained in summer and winter experiments.

Figure 4-8: Comparison of *E. coli* removal in summer and winter experiments



Major parameters that affect pathogen removal in constructed wetlands are filtration, adsorption and sedimentation, temperature, solar radiation, pH, dissolved oxygen, hydraulic retention time, vegetation and predation and competition. Median pH in SF wetlands in summer and winter experiments were 7.1 and 7.8, respectively. In pH range of 6-8, inactivation of coliphages is lowest (Feng et al., 2003), and thus it can be concluded that pH was not a significant factor in variation in *E. coli* removal. Physical processes such as filtration, adsorption and sedimentation are less sensitive to temperature (Vymazal, 2005c) and therefore should have changed minimally in winter and summer months. Hydraulic retention time remained similar in both the seasons, and hence should not have substantial impact in the difference in removal rates. Higher removal in SSF wetlands in summer months as compared to winter months and similar performance of SF wetlands in both seasons can be explained by the change in other parameters brought about by seasonal variation. Solar radiation cannot penetrate SSF surface, and hence its effect is likely minimal in SSF wetlands. Coliforms survive longer in lower temperature, and thus, higher outflow concentrations can be expected in winter months (Gersberg et al., 1989). Median temperature in SSF wetlands were 4.7 °C in winter months and 28.4°C in summer months. Temperature might have played a significant role in SSF wetlands in winter months, leading to lower removal. In both types of wetlands, actively growing vegetation was present in summer, while, no actively growing vegetation was present in winter. Oxygen is known to leak to the wetlands through roots and rhizomes of vegetation. Hence, though dissolved oxygen were not measured, higher dissolved oxygen level can be expected in SSF wetlands in summer months as compared to winter months. *E. coli* are facultative anaerobes and favor lower oxygen availability, whereas, zoonotic predators flourish better in higher oxygen availability. Lower

predation, as a result of lower oxygen availability, might also have played a role in lower *E. coli* removal in SSF wetlands.

Lower removal rates caused by lower temperature and vegetation effect might have been offset by the solar radiation effect. Median temperature in SF wetlands were 5.2 °C in winter months and 27.0°C in summer months. Solar radiation is lethal to coliforms. Zdragas et al. (2002) reported that up to 700 nm wavelength contained in solar radiation is harmful to coliforms, with UVA and UVB having most destructive effects. Median solar radiation at noon above SF wetland surface were 72.5 and 393.5 W/m² for winter and summer months, respectively.

However, median solar radiation at noon that actually reached SF wetlands were 18.0 and 0.5 W/m² for winter and summer months, respectively. Difference in solar radiation indicates that duckweed growing on the SF wetland in summer months blocked the solar radiation from reaching the wetlands. MacIntyre et al. (2006) also found similar effect and reported that number of effluent *E. coli* sharply declined after removing duckweed from the wetlands. Thus, the higher amount of solar radiation reaching the wetlands might have neutralized the temperature and vegetation effects, resulting in similar removal in both the seasons.

Both CDE model and colloid filtration theory based model did not adequately represent the *E. coli* transport mechanisms in constructed wetlands. CDE model estimates *E. coli* removal rate based on the hydraulic transport parameters estimated from tracer data. The reason for poor fitting may have been caused by different transport mechanism of *E. coli* from tracer transport mechanism. The colloid filtration theory based model was developed under steady flow condition. Poor correlation between model predicted data and observed data indicates that the model was not a good fit for pulse loaded situations.

4.5 Conclusions

E. coli removal in surface flow wetlands were similar in summer and winter seasons. Subsurface flow wetlands were superior to surface flow wetlands in *E. coli* removal in both winter and summer months. Seasonal variation in *E. coli* removal was observed in SSF wetlands, where removal rates were higher in summer than in winter season. Convection-dispersion equation and colloid filtration theory based models did not adequately describe *E. coli* removal kinetics in constructed wetlands. New modeling approach should be explored to better describe *E. coli* transport mechanism in pulse loaded wetlands.

CHAPTER 5: MODELING SEASONAL VARIATION IN BACTERIOPHAGE REMOVAL IN CONSTRUCTED WETLANDS USING CONVECTION-DISPERSION EQUATION

5.1 Introduction

Animals produce 1.20 to 1.37 billion tons (wet weight) of manure each year in the United States (Rogers and Haines, 2005). Animal manure contains valuable crop nutrients such as nitrogen, phosphorus and potassium in forms and proportions that depend on animal type, animal diet, storage condition and moisture content (Maguire and Heckendorn, 2009). Apart from nutrients, animal feces also contains microbial contaminants such as viruses, bacteria and parasites (Venglovsky et al., 2009). Each gram of cow, sheep, pig and poultry feces contain 10^5 - 10^7 fecal coliforms and 10^6 - 10^8 fecal streptococci (Maier et al., 2000). In most North American farm operations, liquid manure is applied to cropland for nutrient recovery and disposal (Joy et al., 1998). Up to 20,000 CFU of fecal coliforms per 100 g of soil have been found at a depths of 70 cm in soil after manure application (Dean and Blackie, 1991) and liquid manure application has been shown to increase bacterial concentration in tile drainage effluent (Fleming and MacAlpine, 1995). Bacterial concentrations in tile drains are correlated with rainfall and high concentrations have been observed when heavy rainfall occurs soon after manure application, irrespective of the application method (Samarajeewa, 2010). Likewise, as both bacteria and viruses are present in manures, high viral concentrations can be expected in tile drains. Hence, there is a need to treat agricultural return flow so as to minimize transport of pathogens to the water bodies.

Constructed wetlands are characterized by relatively low cost installation, operation and maintenance and thus, provide alternate treatment systems for polluted waters in both developed and developing countries (Sundaravadivel and Vigneswaran, 2001). Due to natural die-off and hostile environmental conditions, wetlands have been found to reduce pathogen numbers

significantly, but with varying degrees of effectiveness (Kadlec and Wallace, 2009). Constructed wetlands remove pathogens by the combined effect of physical, chemical and biological processes (Vymazal, 2005c; Werker et al., 2002). Physical processes include mechanical filtration, adsorption and sedimentation, while chemical processes include UV radiation, pH effect and oxidation. Biological processes include natural die-off (as affected by temperature), antibiosis, predation by nematodes, protozoa, and zooplankton, and attack by bacteria and viruses. A review of the literature indicates that constructed wetlands with emergent vegetation can remove 95 to above 99% of total and fecal coliforms, and 80 to 95% of fecal streptococci (Vymazal, 2005c). Since enteric bacterial removal in constructed wetlands is comparable with an activated sludge process, trickling filters and slow sand filtration (Vymazal, 2005c), they can potentially be used to treat tile drain effluent from manure-applied cropped land. However, previous constructed wetland research has focused on steady-state flow and bacterial pathogens. There is a need to evaluate the ability of constructed wetlands to reduce viral concentrations when subjected to pulse loading.

Bacteriophages are viruses that infect bacteria and are generally considered to be more representative of microbe-dynamics in hydrological studies than traditional anionic tracers such as bromide (Hodgson et al., 2003). MS2 and PRD1 are the frequently used bacteriophages in biotracer and survival studies (Vidales-Contreras et al., 2012; Yahya et al., 1993). Both are suitable for viral studies as they are not infectious to humans, can be detected inexpensively, rapidly, and easily, and can be prepared in high concentrations (Vidales-Contreras et al.). PRD1 persists longer in the environment, and hence, is a better model for studying enteric viruses than MS2 (Blanc and Nasser, 1996; Vidales-Contreras et al., 2006). Similar to PRD1, bacteriophage P22 uses smooth strains of *Salmonella typhimurium* as its host (Shen et al., 2008) and has been

previously used as biological tracer in column studies and surface water systems (Shen et al., 2008; Steiner, 2009).

Studies were conducted to evaluate the removal rate and applicability of constructed wetlands in treating tile drain effluent. Two-stage, tub-scale wetlands with surface flow (SF) and subsurface flow (SSF) were spiked with bacteriophage P22 in cold and warm environments to simulate a heavy rainfall soon after a manure application to cropland. Specific objectives of this research were to: (i) compare seasonal variation in bacteriophage P22 concentrations in constructed wetlands subjected to pulse loading, (ii) evaluate the convective-dispersive equation as a model to describe viral transport and retention in constructed wetlands, and (iii) evaluate the use of bacteriophage P22 as a biotracer for characterizing constructed wetlands.

5.2 Materials and methods

5.2.1 Experimental setup

Two two-stage wetlands, each consisting of a surface flow (SF) and a subsurface flow (SSF) systems connected in series, were constructed (Figure 3-1). The wetlands were constructed from oval shaped, opaque, low density polyethylene tanks with overall dimensions of 0.84 m × 0.66 m × 0.30 m (length × width × height) and a total volume of 138 liters. The wetlands were maintained inside a greenhouse and were continuously supplied with groundwater supplemented with half-strength duckweed growth media as described in Standard Methods (Eaton et al., 2005) beginning in May 2009. Duckweed (*Lemna minor*) and bulrush (*Scirpus lacustris*) were planted in the SF and SSF wetlands, respectively. The SF wetlands were covered with a thick mat of duckweed during summer experiments, but were barren after the duckweed senesced during the winter. The bulrush senesced during the winter in the SSF wetlands, but a standing biomass remained throughout the winter.

Influent water was delivered to the surface flow wetlands through opaque tubing (Masterflex Norprene L/S 14, Cole-Parmer, IL) connected to peristaltic pumps (Masterflex HV-07014-20, Cole-Parmer, IL) operated by variable speed pump drives (Masterflex, HV-77200-12, Cole-Parmer, IL). Flow from SF wetland to SSF wetland and from SSF wetland to the outlet was maintained by gravity. The flow rate during each trial was maintained to achieve a nominal hydraulic retention time (HRT) of 8.3 days for the SF wetlands and 1.5 days for the SSF wetlands. Between trials, nominal HRTs of approximately 15 days and 5 days were maintained for the SF and SSF wetlands, respectively. During the winter, the greenhouse was heated with electric heaters to maintain a minimum temperature of 1°C to prevent the wetlands from freezing solid.

5.2.2 Environmental parameters monitoring

Environmental ambient and water temperature was continuously monitored using temperature sensors (107, Campbell Scientific, Inc., Logan UT) installed in all the wetlands and recorded with data logger (CR-10, Campbell Scientific, Inc., Logan UT). Solar radiation sensors (CS300, Campbell Scientific, Inc., Logan UT) were placed above and below the water-air interface of SF wetlands. Solar radiation and temperature were recorded hourly. The pH in the SF wetland was monitored with a handheld pH meter at each sampling time (pH/Con 10, Oakton Instruments, Vernon Hills, IL).

5.2.3 Tracer study

Tracer studies were conducted in March 2011 and February 2012 to evaluate the hydraulic properties of the wetlands. The wetlands were spiked with 1.44 L of 0.084 M of potassium bromide (KBr) obtained by dissolving KBr in deionized water. The bromide concentration in the effluent of the SF and SSF wetlands was monitored every six hours initially and then less

frequently after the peak was observed. Samples were preserved at 4°C until analyzed with a bromide ion specific probe (YO-27502-05, Cole-Parmer, Vernon Hills, IL).

5.2.4 Virus removal studies

The bacteriophage P22 was chosen as viral indicator due to its similarity to widely used bacteriophage PRD1 and its previous use as a biotracer (Shen et al., 2008; Steiner, 2009). Bacteriophage P22 was obtained from The Water Quality, Environmental, and Molecular Microbiology Laboratory, Department of Fisheries and Wildlife, Michigan State University. Bacteriophage studies were conducted in January 2011, July 2011 and January 2012. During each trial, a 113 liter influent tank was spiked with the bacteriophage to achieve an approximate concentration of 10^6 CFU (PFU)/100 ml. The flow rate of the system during the each spike was maintained so as to achieve a nominal HRT of 8.0 days for the SF wetlands and 1.2 days for the SSF wetlands. When the influent tank was empty the tank was refilled with ground water and duckweed media and no additional samples were taken from the influent tank. Samples were collected for a minimum of 426 hours (17.75 days).

5.2.5 Sample collection and analysis

Samples were taken from the influent tank, surface flow effluent and subsurface flow effluent and analyzed for bacteriophage. A fifty ml sample was collected from each sampling point and were preserved at 4°C and analyzed within 24 hours after sample collection. Cultural based methods were used to enumerate bacteriophage using the double agar layer (DAL) method (Adams, 1959). *Salmonella typhimurium* (strain LT2, Felix d'Herelle Collection, Laval University, Canada) was used as the host organism. Prior to sample analysis, 5 ml of Tryptic Soy Broth (TSB) was inoculated with *Salmonella* host cells and incubated overnight at 37°C. Thirty ml of TSB was inoculated with 1 ml of overnight culture and incubated for 4 hours at 37°C to

prepare *Salmonella* host cells in log phase. Melted one percentage agar overlay tubes were inoculated with 0.3 ml of *Salmonella* host cells in log phase. Samples were diluted serially in phosphate buffer solution to obtain an acceptable number of plaques (10 to 100) per plate. After mixing, 1 ml of the diluted sample was added to the overlay tube, mixed thoroughly and dispensed to 1.5% Tryptic Soy Agar (TSA) plates. The plates were inverted and incubated at 37 °C for 24±2 hours and circular zones of clearing in the bacterial host lawn were counted as plaques. Negative and positive controls were included with each batch of samples to ensure the reliability of the method.

5.2.6 Parameter Estimation

Observed tracer and pathogen removal data were fitted to the one dimensional convection-dispersion equation (CDE),

$$R \frac{\partial C}{\partial t} = \frac{\partial^2 (DC)}{\partial x^2} - \frac{\partial (uC)}{\partial x} - \mu C \quad (5-1)$$

where, R is the retardation factor, C is the tracer or bacteriophage concentration (mg/L for bromide and PFU/ml for P22), t is the time (d), D is the dispersion coefficient (m²/d), u is the pore water velocity (m/d), μ is the first order removal coefficient (1/d), and x is the distance from inlet (m). For conservative tracers, there is no retardation (R=1) or removal ($\mu=0$). Hence, the Equation (5-1) simplifies to

$$\frac{\partial C}{\partial t} = \frac{\partial^2 (DC)}{\partial x^2} - \frac{\partial (uC)}{\partial x} \quad (5-2)$$

The CXTFIT model (Toride et al., 1995) in the Studio of Analytical Models (STANMOD)(PC-Progress s.r.o., Prague, Czech Republic) software was used to fit the data to the model. First, the tracer study data were used to calculate the pore water velocity (u) and dispersion coefficient (D) using equation Equation (5-2). The fitted values of u and D were used in subsequent pathogen

removal studies to calculate the first order removal coefficient (μ) and retardation factor (R) using Equation (5-1). A pulse input was used to model the SF wetlands, whereas, output from the SF wetland was converted to successive multiple pulse outputs using the trapezoidal method and used as input to the SSF wetlands.

Mean resident time was calculated as:

$$\tau = \frac{L}{u} \quad (5-3)$$

where, τ is the mean resident time (d) , L is the length of the wetland (m).

Peclet number was calculated using

$$P_e = \frac{uL}{D} \quad (5-4)$$

where, P_e is the Peclet number (dimensionless) and L is the length of the wetland (m).

Total mass recovery was calculated from the breakthrough curve by estimating the area under the curve by the trapezoidal method.

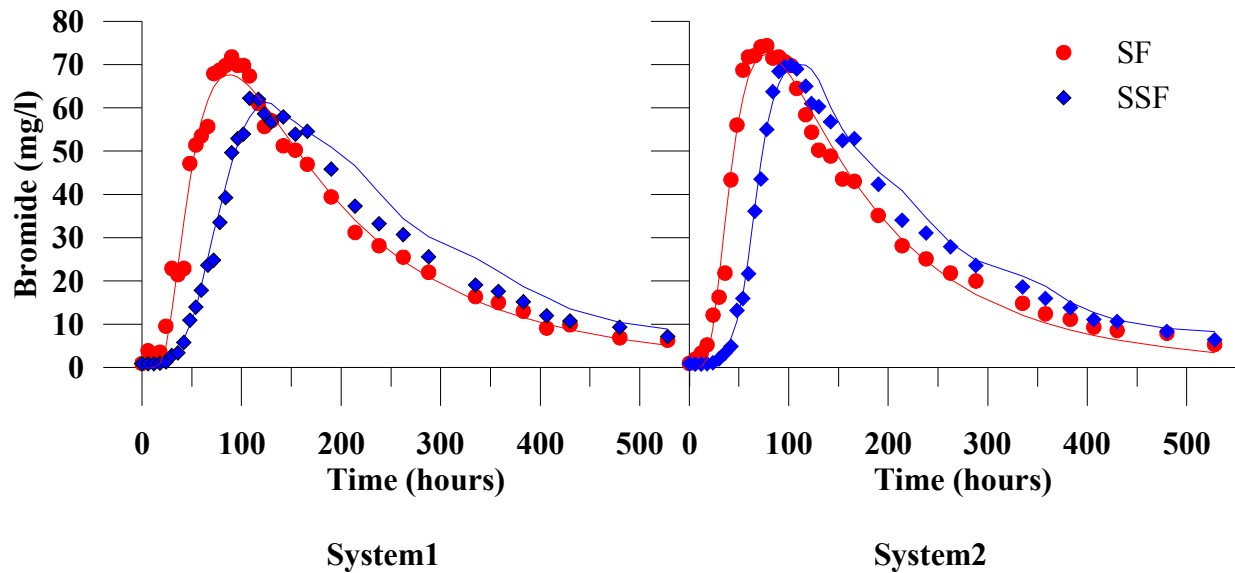
5.3 Results

5.3.1 Tracer study

For the first tracer study (March, 2011), samples were collected for 291 hours (12.1 days).

During the sampling period, 101% and 104% of injected bromide was recovered from SF1 and SF2, respectively. Observed recoveries in subsurface wetlands were 93% and 91% (94% and 95% of main influent) for SSF1 and SSF2, respectively. In the second tracer study (February 2012), sample collection continued for 528 hours (22 days). During the period, 70.4% and 74.3% of injected bromide was recovered from SF1 and SF2, respectively. Likewise, 93.9% (66.1% of the main influent) and 98.5% (73.2% of the main influent) of bromide was recovered from SSF1 and SSF2 respectively.

Figure 5-1: Bromide concentrations in SF and SSF effluent when spiked with bromide in the tracer study February 2012



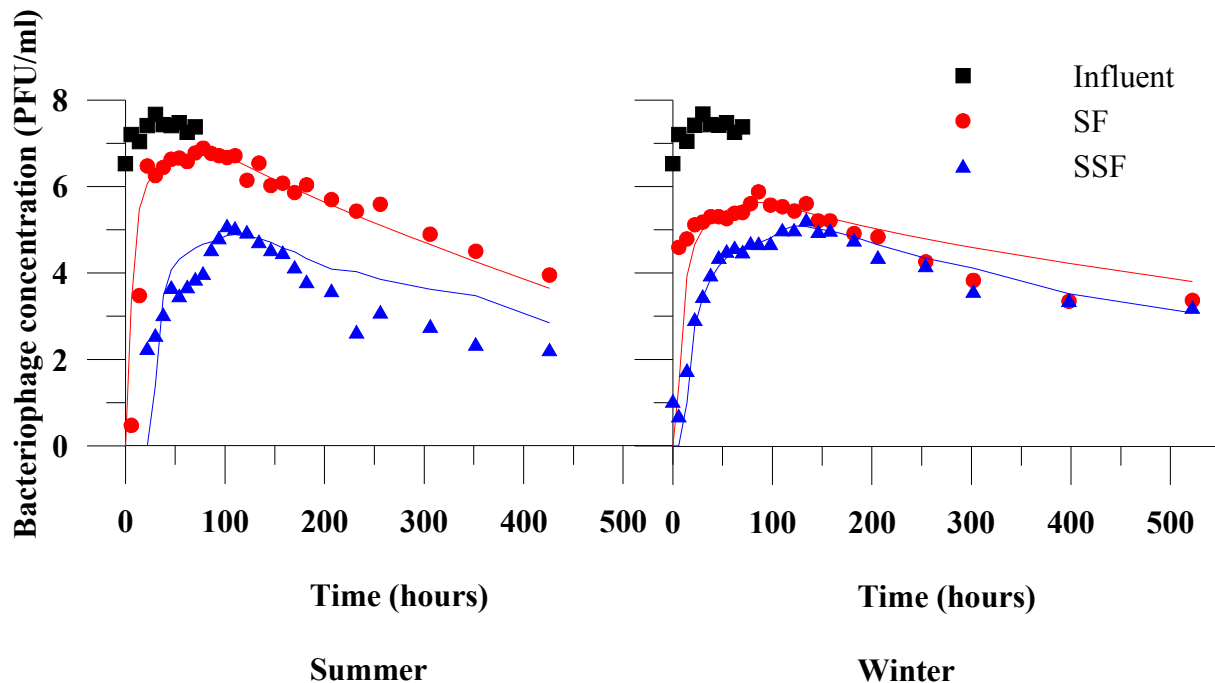
Lines are the fitted values in deterministic equilibrium CDE model. (SF: surface flow, SSF: subsurface flow)

The data were fitted to the CDE equation using the CXTFIT deterministic equilibrium model (Figure 5-1) to calculate values of u and D in Equation (5-1). Table 4-4 shows CDE estimated pore water velocity (u), dispersion coefficient (D), calculated hydraulic residence time (τ), and Peclet number (Pe) along with standard errors. Fitted values were in good agreement with the observed values ($R^2 > 0.83$). Calculated Peclet number for all the wetlands was within the range (2.7-14.3) reported by Kadlec and Knight (1996), except for two instances. The estimated Pe value indicates that dispersion was an important transport process in both SF and SSF wetlands.

5.3.2 Bacteriophage removal

Three bacteriophage experiments were conducted in the winter 2011, summer 2011 and winter 2012. Variable bacteriophage recovery (after 426 hours) was observed in SF as well as SSF wetlands. In summer, bacteriophage recoveries ranged from 26-37% for SF wetlands and 0.4-0.6% for SSF wetlands. In winter, higher numbers of bacteriophage were recovered in 426 hours in both SF and SSF wetlands. Winter bacteriophage recoveries ranged from 59-86% for SF wetlands and 4-25% for SSF wetlands.

Figure 5-2: Bacteriophage removal in SF1 and SSF1 during summer and winter spike



Sum: summer spiking, Win: winter spiking, SF: surface flow, SSF: subsurface flow

Data obtained from the bacteriophage removal experiments were fitted to CDE equation to obtain the retardation factor (R) and first order removal coefficient (μ). Values of u and D obtained from 2011 tracer study were used to model bacteriophage removal data from winter 2011 and summer 2011 experiments, while, u and D from 2012 tracer experiments were used to model bacteriophage removal data from winter 2012. Figure 5-2 shows examples of observed

and CDE fitted data for system 2 during the summer 2011 and winter 2011 experiments. Table 5-1 shows the estimated parameters obtained by fitting the bacteriophage removal experiments data to the CDE model. Results showed that the retardation factor (R) for both the SF and SSF wetlands in all experiments were different from R=1. In case of the SF wetlands, the values were less than one. For SSF wetlands, average R values were always higher than one and were slightly higher in summer than in winter.

Table 5-1: Bacteriophage removal properties of the wetlands obtained by fitting observed data to CDE model (values after \pm are standard errors)

	R	μ (1/day)	R^2
Winter 2011			
SF1	0.92 \pm 0.09	0.06 \pm 0.03	0.74
SF2	0.77 \pm 0.06	0.04 \pm 0.02	0.77
SSF1	1.42 \pm 0.15	1.16 \pm 0.06	0.90
SSF2	1.83 \pm 0.19	1.23 \pm 0.07	0.85
Summer 2011			
SF1	0.77 \pm 0.05	0.24 \pm 0.02	0.89
SF2	0.61 \pm 0.04	0.33 \pm 0.02	0.90
SSF1	1.91 \pm 0.39	5.58 \pm 0.19	0.74
SSF2	2.73 \pm 0.17	5.14 \pm 0.10	0.92
Winter 2012			
SF1	0.46 \pm 0.03	0.06 \pm 0.01	0.80
SF2	0.37 \pm 0.01	0.10 \pm 0.00	0.98
SSF1	1.10 \pm 0.32	3.71 \pm 0.18	0.79
SSF2	1.86 \pm 0.27	4.57 \pm 0.17	0.83

R: retardation factor, μ : first order removal coefficient, R^2 : coefficient of determination

As demonstrated by the first order removal coefficient (μ), the P22 removal rate was greater in summer than in winter in both the SF and SSF wetlands. Average P22 removal rates were 0.29 and 0.06 1/day for SF wetlands and 5.36 and 2.67 1/day for SSF wetlands for the summer and winter experiments, respectively. The removal rate was 4 times higher in the summer than in the winter for SF wetlands and 2 times higher in the summer than in the winter for SSF wetlands. Additionally, the P22 removal rate was higher in SSF wetlands than in SF wetlands both in

summer and winter seasons. On average, the bacteriophage removal rate in SSF wetlands was approximately 19 times the bacteriophage removal rate in SF wetlands in summer and 42 times in winter. The coefficient of determination (R^2) ranged from 0.74 to 0.98 in all the fitted parameters, which indicates that the model was a good fit to the observed data.

To evaluate the bacteriophage P22 as a biotracer, the data obtained from the experiments were fitted to CDE model using CXTFIT by keeping $\mu=0$ and $R=1$. Only the SF bacteriophage data obtained during the winter 2011 experiments fit the model. Only the data from first winter 2011 experiments predicted a HRT where the tracer study results were within standard error range of the calculated values.

5.4 Discussion

The retardation factors for SF wetlands were below one, which indicates that the P22 bacteriophage reached the peak concentration before the bromide tracer. Several other researchers have found similar results: for MS2 in sand columns (Keller et al., 2004), for F-RNA and MS2 in gravel aquifers (Sinton et al., 1997) and for PRD1 in subsurface flow constructed wetlands (Vidales-Contreras et al., 2012). There are two potential explanations for the observed retardation factors of less than 1: bacterial motility and size exclusion. As the bacterial hosts for P22 were not destroyed, P22 was introduced to the wetlands with *S. typhimurium*. A flagellated bacterium, *S. typhimurium* is able to achieve velocities of 19.8 – 39.5 $\mu\text{m/s}$, even in nutrient-rich medium (Garcia et al., 2011). Consequently, bacterial motility could have contributed to increase velocity of P22 in the SF wetlands. Additionally, previous studies have attributed retardation factors less than 1 to size exclusion. Similar to colloids, bacteria and bacteriophages are greater in size than soluble bromide. Colloids tend to travel only through pore spaces that are several times greater than colloidal diameter (Keller et al., 2004). Under these conditions,

colloids travel more rapidly in straighter paths with smaller dispersion coefficients. However, approximately less than 10% of total volume in SF wetlands was occupied by duckweed and dead organic matter. Consequently, size exclusion likely only accounted for a fraction of the increased velocity of bacteriophage through the SF wetland.

In SSF wetlands, retardation factor was always greater than one. Retardation was greater in summer months (2.32 ± 0.28) than in winter months (1.55 ± 0.23). Retardation of microbes occurs when they are sorbed to the media through which they pass. Solid organic matter present in the media may increase the attachment rate of virus through hydrophobic binding (Schijven and Hassanizadeh, 2000). Roots and rhizomes of bulrush in the SSF wetlands might have increased the sorption of virus, and thus increased the retardation factor. As compared to the winter, active growth of the plants in the summer might have increased the amount of organic matter present in the wetlands, causing higher retardation in summer.

A few researchers have reported first order removal rates for viruses or bacteriophages in constructed wetlands. In a SF constructed wetlands with a HRT of 9 ± 3 days, Chendorain et al. (1998) estimated μ to be 0.44/day for MS2 for both winter and summer experiments. Vidales-Contreras et al. (2006) calculated μ for PRD1 to be 0.30 1/day in SF wetlands in winter months. Vidales-Contreras et al. (2012) reported μ to be 0.96 1/day for PRD1 in SSF wetlands. Gersberg et al. (1987b) reported μ for MS2 to be 1.06 to 1.25 1/day for SSF wetlands with a HRT 5.5 days. In SSF wetlands treating secondary wastewater, Vidales et al. (2003) reported PRD1 inactivation rate in the range of 0.16 to 1.17 for spring months. Our average μ values for SF wetlands (0.285 for summer and 0.065 1/day for winter) are lower than these reported values. For SSF wetlands, our average μ values (5.36 for summer and 2.67 for winter) are greater than

those mentioned in previous work. However, our results are consistent with current literature in indicating higher removal of viruses in SSF wetlands than in SF wetlands.

The results of this work demonstrated seasonal variation in viral removal in the two-stage constructed wetlands. High humidity, low temperature, low to no exposure to sunlight and neutral or near neutral pH favors organism survival (WHO, 2006). Hence, it is commonly assumed that lower pathogen removal can be expected in winter, which is consistent with the estimated removal rates. Other important factors that fluctuated seasonally in the wetlands were plant cover, solar radiation and temperature. Both the SF and SSF wetlands were covered with vegetation in summer and without active vegetation in winter. Plants in wetlands contribute to pathogen removal as their roots enhance filtration, enhance aerobic degradation and secrete anti-microbial compounds; however, there is no consensus on a positive effect of plants on pathogen removal (Werker et al., 2002). MacIntyre et al. (2006) even reported that free floating macrophytes may reduce UV penetration and provide favorable attachment sites, reducing pathogen removal efficiency. However, other researchers have found positive effects of vegetation on pathogen removal in constructed wetlands (Gersberg et al., 1987a; Gersberg et al., 1987b; Hench et al., 2003; Warren et al., 2000). To further elucidate the reasons for lower bacteriophage removal in winter trials, the impacts of solar radiation, pH, and temperature on P22 removal were examined.

Table 5-2: Environmental parameters for bacteriophage removal studies

	Winter1	Summer	Winter2
Surface flow			
Median temperature (°C)	5.2	27.0	7.0
Median pH	7.9	7.1	7.8
Rad-Abo 12* (W/m ²)	72.5	393.5	101.0
Rad-Bel 12** (W/m ²)	18.0	0.5	27.0
Fresh duckweed density (gm/cm ²)	0.0	0.8	0.0
Subsurface flow			
Median temperature (°C)	4.7	28.4	6.6

* Median solar radiation received above duckweed mat in SF wetlands at noon

* Median solar radiation received below duckweed mat in SF wetlands at noon

The median pH in the SF wetland during the summer and winter experiments were similar; about 7.8 in winter and 7.1 in summer (Table 5-2). Furthermore, maximum diurnal fluctuation in pH in SF wetlands during all experiments was 0.2. These values are in agreement with the findings that duckweed does not influence acidity or alkalinity of the system and produces neutral condition (Awuah et al., 2002). Human enteric viruses are stable within a pH range of 3-10, and survival is generally considered greatest near neutral pH (Charles et al., 2008). Inactivation of coliphages has been reported to be lowest within the pH range of 6-8 in various temperatures (Feng et al., 2003). Therefore, it is unlikely that pH had a significant effect on variation in bacteriophage removal.

Variability in solar radiation, both ambient and that which penetrates the water, has the potential to affect P22 removal, especially in the SF wetlands. Solar radiation enhances pathogen removal as ultraviolet A and B in solar radiation are harmful to pathogens (Zdragas et al., 2002). The SF wetland was covered with duckweed the in summer and despite higher ambient solar radiation intensity, the solar radiation penetrating the duckweed mat was very low (Table 5-2). Maximum ambient solar radiation was 648.2 W/m^2 in summer, but maximum radiation that penetrated the duckweed mat was only 7.8 W/m^2 . During winter months, both the SF and SSF wetlands were without vegetation. As a result, higher solar radiation reached the SF wetland surface, though ambient solar radiation was less than that received during summer experiments. Maximum ambient solar radiation during the winter 2011 experiment was 286 W/m^2 and the maximum solar radiation recorded beneath the wetland surface was 80 W/m^2 . While solar radiation is only effective in shallow water due to poor penetration to deeper sections (Mayo, 1995), the higher removal rates between winter trials is most likely due to the higher solar radiation during Winter 2 (27 W/m^2) than during Winter 1 (18 W/m^2).

In viral survival studies in aquatic environments, temperature is usually considered the most important factor (Olson et al., 2005). Higher temperature releases viral RNA as a result of viral capsid damage caused by denaturation or microbial degradation (Nasser and Oman, 1999). As a result, viruses survive longer at lower temperature and inactivation increases with increase in temperature (Yates et al., 1987). Yates et al. (1985) incubated three viruses (poliovirus 1, echovirus 1, and MS-2 coliphage) in groundwater at different temperatures (12-32°C) and reported that temperature was the only factor that correlated with all three virus decay rates. Vidales et al. (2003) did not find any difference in PRD1 removal rates in SSF wetlands treating

secondary wastewater in spring and summer when the same average temperature of 16.3°C was reported for both seasons. These findings suggest that temperature is an important parameter in viral inactivation in aquatic environments. In our experiments, wetland temperatures during the summer 2011 experiments ranged from 21 to 34°C and 1 to 10°C during the winter. Median temperature during all the experiments ranged from 5-7°C in winter and 26-28°C in winter. Consequently, higher temperatures likely contributed to higher removal rates obtained in summer months.

Although bacteriophages MS2 and *Enterobacter cloacae* have been used as a biotracer to estimate retention time in constructed wetlands (Hodgson et al., 2003), our study results suggests that bacteriophage P22 should not be used biotracer in wetland systems because of the high die-off of the P22, especially in summer months. Hydraulic retention time estimates using bacteriophage are only accurate if the decay rate is negligible (Vidales-Contreras et al., 2012). Moreover, estimated retardation factors for all bacteriophage P22 studies were less than one for SF wetlands and more than one for SSF wetlands. This would lead to an erroneous estimation of wetland HRT if P22 was solely used as the biotracer. Hence, we conclude that bacteriophage P22 is not a reliable biotracer for wetland studies.

5.5 Conclusions

SF and SSF constructed wetlands that were subjected to pulse loading reduced the concentration of the P22 bacteriophage substantially, but did not eliminate it. The one dimensional CDE model was successful in describing P22 dynamics in the constructed wetland. The results of this work demonstrated seasonal variations in P22 removal in two-stage constructed wetlands. For both SF and SSF wetlands the P22 removal rate in the summer was two to four times greater than in the winter. The SSF wetland was more effective in removing the P22 than the SF wetland. The

SSF removal rate was approximately 41 times greater than the removal rate in SF wetlands in winter and 19 times in summer. Higher removal rates in summer were attributed to higher temperature, while higher removal rates between two winter trials was attributed to higher solar radiation. Bacteriophage could not be used as biotracer to describe hydraulic characteristics of the wetlands due to high degradation and the dependence of retardation factor on wetland type.

CHAPTER 6: USE OF DUCKWEED BASED CONSTRUCTED WETLANDS FOR NUTRIENT RECOVERY AND POLLUTANTS REDUCTION FROM DAIRY WASTEWATER

6.1 Introduction

Animal manure contains various nutrients such as nitrogen, phosphorus and potassium along with pathogens such as bacteria, virus and parasites (Maguire and Heckendorn, 2009; Venglovsky et al., 2009). The pollutants contained in manure often contaminate surface water by entering as diffuse or non-point source associated with surface water and as point source from concentrated livestock production systems (Knight et al., 2000). A variety of management practices such as oxidation ponds, facultative lagoons, vegetated buffer strips, constructed wetlands, storage pond, land spreading, composting and aerobic and anaerobic digestion are used to treat excess nutrients and pathogens from animal manure (NRCS, 1999; Rogers and Haines, 2005).

Constructed wetlands provide alternate wastewater treatment systems for both developing and developed countries due to their low installation, operation and maintenance cost (Sundaravadivel and Vigneswaran, 2001). Nutrients in constructed wetlands are removed through a variety of processes. Major nitrogen removal processes are ammonia volatilization, nitrification-denitrification, plant and microbial uptake, anaerobic ammonia oxidation and burial. Phosphorus is primarily removed through sorption, precipitation, plant and microbial uptake and peat or soil accretion (Vymazal, 2007). Pathogen removal mechanisms include natural die-off, attack by lytic bacterial and bacteriophages, predation, filtration, adsorption and subsequent sedimentation, chemical oxidation and inactivation by UV radiation (Vymazal et al., 2006).

Duckweed, a small free-floating aquatic macrophyte from the family *Lemnaceae*, have worldwide distribution. There are four common genera (*Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella*) and 40 species of duckweed (Rusoff et al., 1980). Duckweed propagates through vegetative reproduction, produces biomass more rapidly than most other plants and can double its weight every 2 or 3 days (Landolt, 1986; Rusoff et al., 1980). Duckweed's preferential uptake of ammonium to nitrate (Cedergreen and Madsen, 2002) makes duckweed suitable candidate to be grown in dairy wastewater where ammonium is the dominant form of nitrogen. Duckweed provides additional surface for bacterial growth and supply additional oxygen enhancing organic materials degradation (Korner et al., 2003). Cultivation of floating macrophyte such as duckweed has advantage of high productivity, high nutritive value and ease of harvesting and stocking (Boyd, 1974). Harvested biomass can be used for composting and soil amendments, digested anaerobically for biogas production, processed for animal feed or can be mixed with solid manure to increase nutrient content (Sooknah and Wilkie, 2004).

In the past, few researchers have attempted to quantify nutrient reduction and recovery potential of floating aquatic macrophyte based systems from anaerobically digested flushed dairy wastewater (Sooknah and Wilkie, 2004) and from dairy lagoon wastewater (DeBusk et al., 1995; Tanner, 1996; Tripathi and Upadhyay, 2003). Moreover, these previous studies did not include pathogen reduction potential of the systems.

The present study was designed to investigate pollutant reduction and nutrient recovery potential of duckweed based constructed wetlands. Specific objectives were: 1) To quantify chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) removal potential of duckweed based constructed wetlands from dairy wastewater; 2) To evaluate first order, first order with background concentration and DUBWAT models for COD, TN and TP removal from

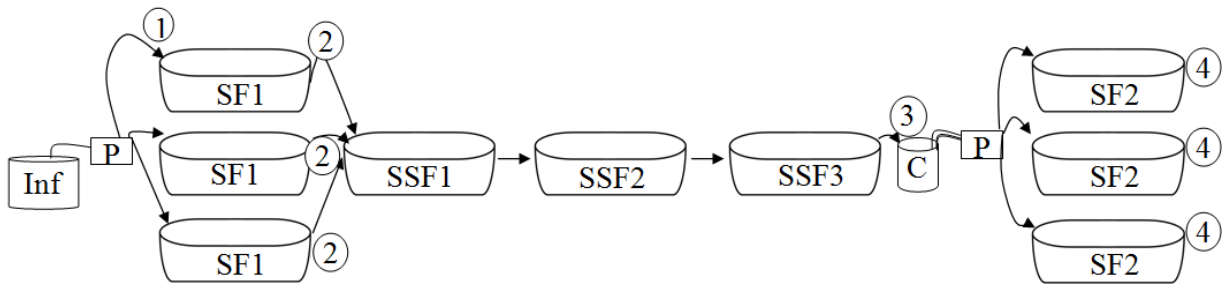
dairy wastewater; 3) To quantify *E. coli* reduction potential of duckweed based constructed wetlands subjected to dairy wastewater; and 4) To quantify nutrient recovery potential from dairy wastewater through duckweed harvesting.

6.2 Materials and methods

6.2.1 Experimental setup

Constructed wetlands used in the study consisted of surface flow (SF) and subsurface flow (SSF) wetlands connected as shown in Figure 6-1.

Figure 6-1: Schematics of the constructed wetland system



Inf: Influent, SF: Surface flow, SSF: Subsurface flow, P: Pump, C: collection bucket. Numbers denote sampling locations

The wetlands were constructed from oval shaped, opaque, low density polyethylene tanks with overall dimensions of 0.84 m × 0.66 m × 0.30 m (length × width × height). SF wetlands were planted with duckweed (*Lemna minor*). SSF wetlands were constructed by filling the polyethylene tanks with pea gravel planted with Beggar-Ticks (*Bidens comosa*) and bulrush (*Scirpus lacustris*). The wetlands were kept inside a building which was heated in winter, and hence, no seasonal variation was realized. Light was provided by fluorescent lights operated 16 hours daily, operated by automatic timers. Flow rate was maintained to achieve theoretical hydraulic residence time (HRT) of 9 days. Diluted dairy manure slurry after solid separation was

obtained from a dairy farm and used as influent. *E. coli* measured in the experiment were the *E. coli* contained in the wastewater; no additional *E. coli* was added the system.

6.2.2 Sampling and analysis

Sample analysis focused on steady-state condition on approximate chemical oxygen demand (COD) of 250 mg/l (low), 500 mg/l (medium) and 1000 mg/l (high). Sufficient time was allowed for the system to attain steady-state condition. When influent and effluent COD were stable, samples were taken twice for COD analysis and once for nutrients and *E. coli* analysis. COD was measured using high range HACH COD digestion vials (HACH Company, Loveland, CO). *E. coli* were analyzed using membrane filtration technique using modified-MTEC agar as growth medium (USEPA, 2009).

Nutrients analyzed were total nitrogen and total phosphorus. Modified persulfate digestion method (Ebina et al., 1983) were used to convert nitrogen compounds to nitrate and phosphorus compounds to phosphate. For total nitrogen analysis, 5 ml of 0.148 M potassium persulfate ($K_2S_2O_8$) and 250 μ l of 0.1 M sodium hydroxide (NaOH) solutions were added to 15 ml of sample and autoclaved for 30 minutes at 110°C in a liquid cycle. After digestion, 250 μ l of 0.1 M sodium hydroxide (NaOH) solution was added, filtered through 0.2 μ m syringe filter, and analyzed with IC. For total phosphorus analysis, 5 ml of digestion reagent (0.074 M $K_2S_2O_8$ /0.075M NaOH solution) was added to 10 ml of sample and autoclaved for 30 minutes at 110°C in a liquid cycle. After digestion, 1 ml of borate buffer solution was added, filtered through 0.2 μ m syringe filter, and analyzed with IC.

A 50% SF wetland area was harvested for duckweed each week. Harvested duckweed was drained for 20 minutes and fresh weight was measured. Sample was taken from harvested duckweed, dried at 65°C for 48 hours and dry weight was measured. Dry duckweed was ground

in mortar and pestle. Deionized water was added to a known weight of ground duckweed, digested and analyzed using the same procedures as mentioned previously.

Ion chromatography system used to analyze samples was Dionex ICS 5000 chromatography system (Dionex Corporation, Sunnyvale, CA). The system was equipped with AS-AP autosampler and Ionpac AS22 column for anions separation. Prior to analysis, calibration curves were prepared by using respective standard solutions. Anions were eluted with 4.5 mM sodium carbonate/1.4 mM sodium bicarbonate solution at a flow rate of 1.2 ml/min and quantified with conductivity detector.

6.2.3 Curve fitting

Three models were evaluated for chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) removal data obtained from the experiments. First model evaluated was first order reaction model as shown in Equation 6.1.

$$\ln \frac{C_e}{C_o} = -kt \quad (6-1)$$

Where,

C_e = effluent concentration (mg/l)

C_o = influent concentration (mg/l)

k = first-order rate constant (1/day)

t = hydraulic residence time (day)

Second model evaluated was first order reaction model with background concentration as shown in Equation 6.2.

$$\ln \frac{C_e - C_*}{C_o - C_*} = -kt \quad (6-2)$$

Where,

C^* = background concentration (mg/l)

Other parameters are same as previous. Third model evaluated was duckweed-based wastewater treatment (DUBWAT) model developed by Khatiwada and Polprasert (2008). The model describes effluent concentration in terms of influent concentration, organic loading rate and duckweed density as shown in Equation 6.3.

$$\ln \frac{C_e}{C_o} = -k\lambda^x \beta^y \theta^{(T-20)} t \quad (6-3)$$

Where,

k = temperature dependent, first-order rate constant (1/day)

λ = OLR or organic loading rate (kg COD/(ha-d))

β = SD or stocking density (kg/m²)

T = temperature (°C)

θ, x, y = coefficients

t = hydraulic residence time (day)

The value of θ is 1.05 (Polprasert and Agarwalla, 1995).

MATLAB R2009b (The MathWorks, Inc., Natick, MA) was used to fit the experimental data to the models.

6.3 Results

6.3.1 COD, TN and TP removal

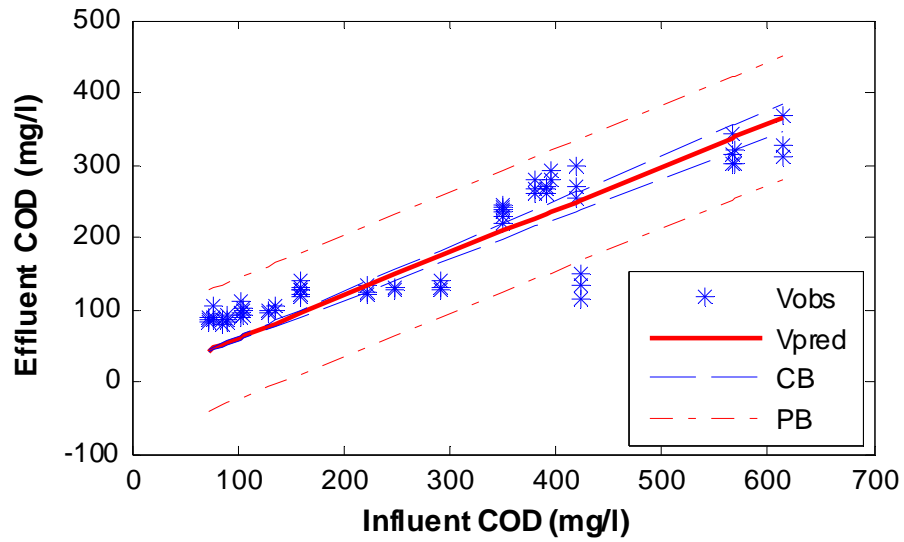
On average, 27.9% of COD was removed by the wetlands. On average, primary wetlands removed 43.6% of COD, while secondary wetlands removed 12.1% COD. Multiple linear regression showed that the removal rates depended on influent COD concentration ($p < 0.01$).

There was also significant different in removal rates between wetland types (primary or

secondary) ($p < 0.01$). Interaction between influent COD and wetland type was also significant ($p < 0.01$) indicating that the slope between influent COD and removal rate was different between two wetland types. Mean area-based removal rates were higher for primary wetlands than secondary wetlands. Average annual removal rate was $2137 \text{ g COD/m}^2/\text{year}$, while the values were 3869 and $405 \text{ g COD/m}^2/\text{year}$ for primary and secondary wetlands, respectively. TN removal rate was not dependent on influent TN ($p = 0.33$) or wetland types ($p = 0.60$). Overall TN removal in the wetlands was 28.3% , while primary wetlands removed 29.3% of TN and secondary wetlands removed 27.3% of TN. Overall annual TN removal rate for the wetlands was $149.5 \text{ gTN/m}^2/\text{year}$. On average, whole wetlands system could remove 16.4% of TP from the influent. TP removal rates were 31.7% and 7.6% for primary and secondary wetlands, respectively. Multiple linear regression showed that influent TP significantly affected the removal rate ($p < 0.01$), while wetland type did not significantly affect TP removal rate ($p = 0.76$). Primary and secondary wetlands could remove 194.9 and $104.1 \text{ gTN/m}^2/\text{year}$. Overall annual TP removal in the wetlands was $11.3 \text{ gTP/m}^2/\text{year}$, which was lower than TN removal. Average annual TP removal in the primary and secondary wetlands was 13.0 and $10.3 \text{ g TP/m}^2/\text{year}$, respectively.

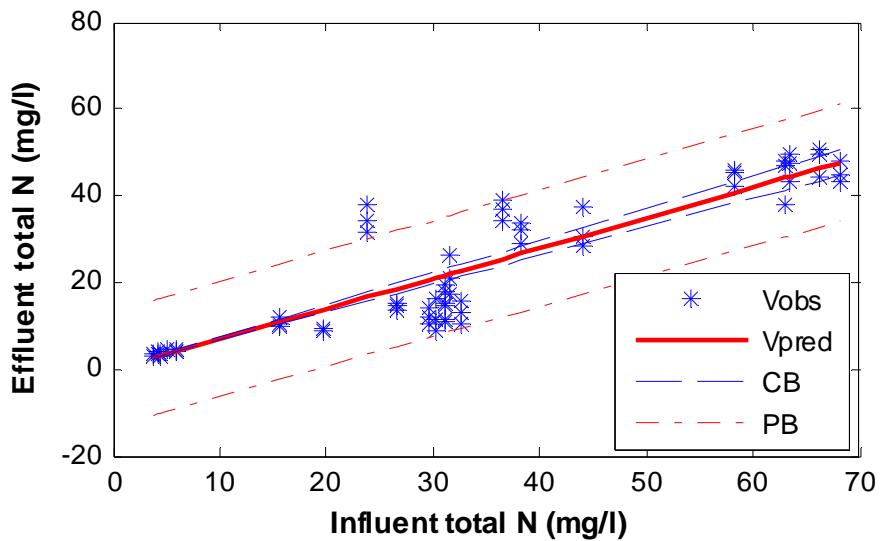
Three models mentioned previously were fitted to the collected COD, TN and TP removal data. During high loading, duckweed did not grow in primary SF wetlands. Since duckweed density is a predictor variable in DUBWAT model, COD, TN and TP data collected during the experiment period from primary SF wetlands were excluded so as to compare the models. Figure 6-2 to 6-4 show observed and predicted data with first order model along with 95% confidence intervals.

Figure 6-2: Observed influent and effluent COD along with first order model fitting



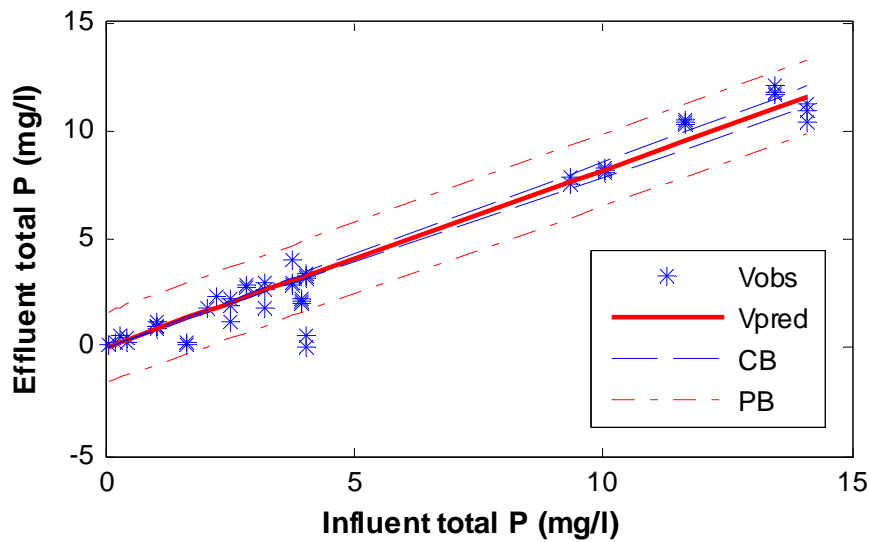
Vobs: observed data, Vpred: model prediction, CB: 95% confidence band, PB: 95% prediction band

Figure 6-3: Observed influent and effluent total N along with first order model fitting



Vobs: observed data, Vpred: model prediction, CB: 95% confidence band, PB: 95% prediction band

Figure 6-4: Observed influent and effluent total P along with first order model fitting



Vobs: observed data, Vpred: model prediction, CB: 95% confidence band, PB: 95% prediction band

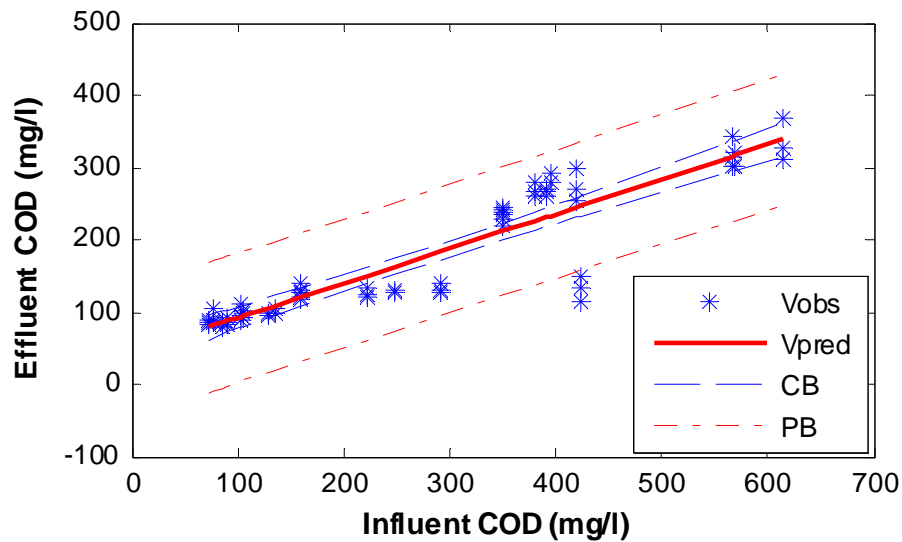
Table 6-1 show the first order rate constant along with 95% confidence interval and standard error. The p-value shows that the fittings were all significant.

Table 6-1: Estimated values and other fitting parameters for COD, TN and TP removal data fitted with first order model

	R^2	Parameter	Estimate	95% confidence interval		Standard error	p-value
				Lower	Upper		
COD	0.78	k (1/day)	0.058	0.052	0.064	0.003	<0.01
Total N	0.83	k (1/day)	0.040	0.033	0.047	0.003	<0.01
Total P	0.96	k (1/day)	0.020	0.017	0.023	0.002	<0.01

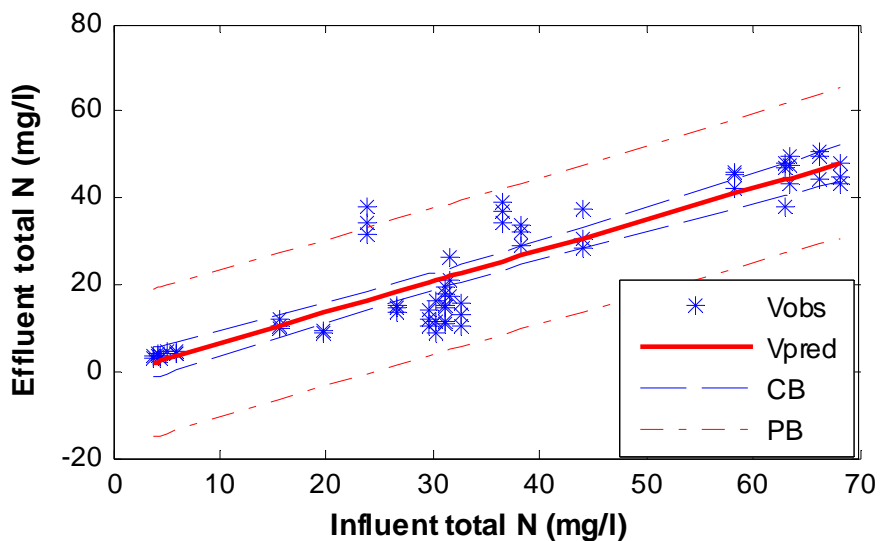
The data was again fitted to first order with background concentration model. Figures 6-5 to 6-7 show observed along with fitted data.

Figure 6-5: Influent and effluent COD along with first order with background concentration model fitting



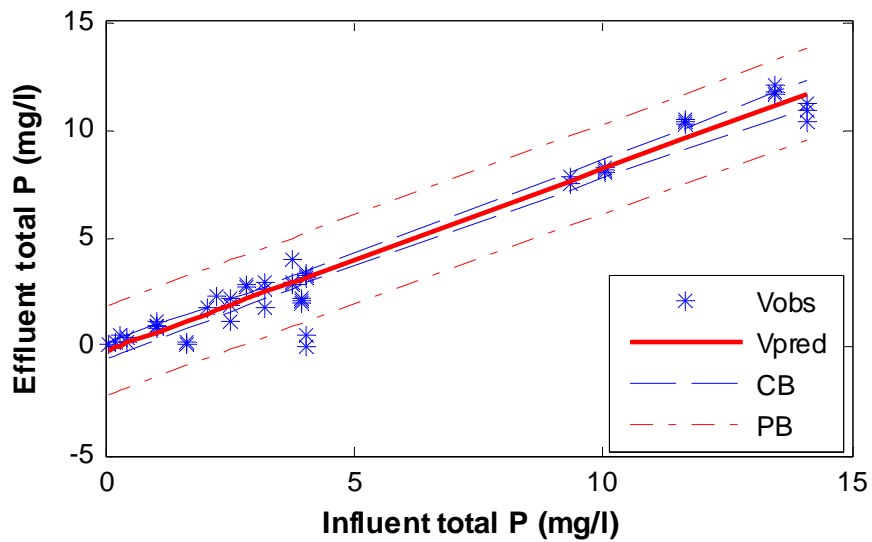
Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

Figure 6-6: Influent and effluent total N along with first order with background concentration model fitting



Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

Figure 6-7: Influent and effluent total P along with first order with background concentration model fitting



Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

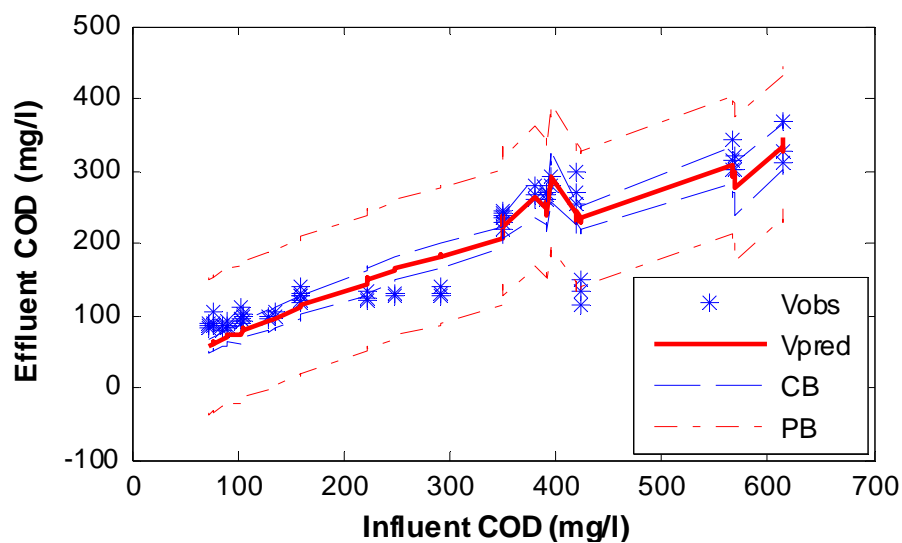
Table 6-2 shows the fitting parameters. The p-value indicates that background concentration was significant for COD but not significant for TN and TP removal.

Table 6-2: Estimated values and other fitting parameters for COD, TN and TP removal data fitted with first order model with background concentration

			95% confidence		Standard error	p-value	
R ²	Parameter	Estimate	interval				
			Lower	Upper			
COD	0.84	k (1/day)	0.082	0.070	0.093	0.006	<0.01
		Cb	84.0	59.6	108.5	0.15	<0.01
Total N	0.83	k (1/day)	0.037	0.025	0.049	0.006	<0.01
		Cb	-2.730	-13.832	8.373	-2.038	0.31
Total P	0.96	k (1/day)	0.021	0.016	0.026	0.003	<0.01
		Cb	0.141	-1.456	1.737	5.627	0.43

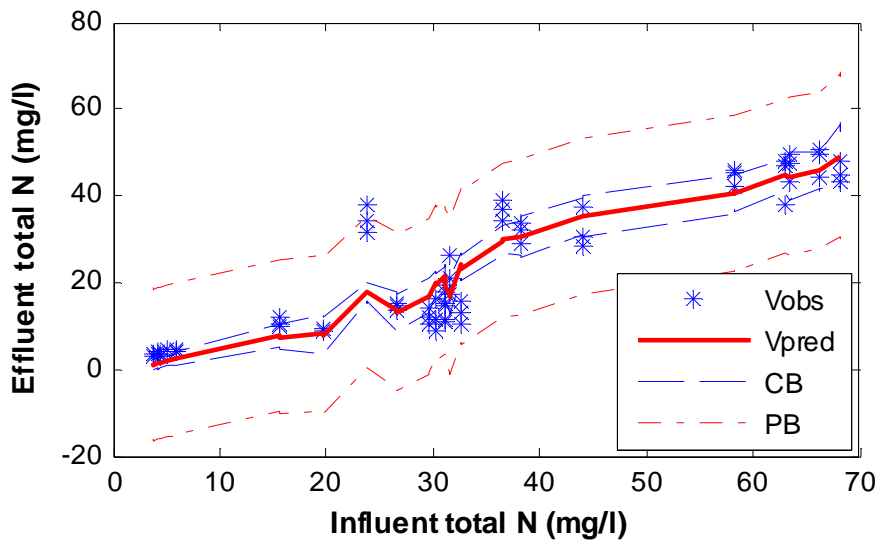
The data was then fitted to DUBWAT model. Figures 6-8 to 6-10 show observed along with DUBWAT model fitted data.

Figure 6-8: Influent and effluent COD along with DUBWAT model fitting



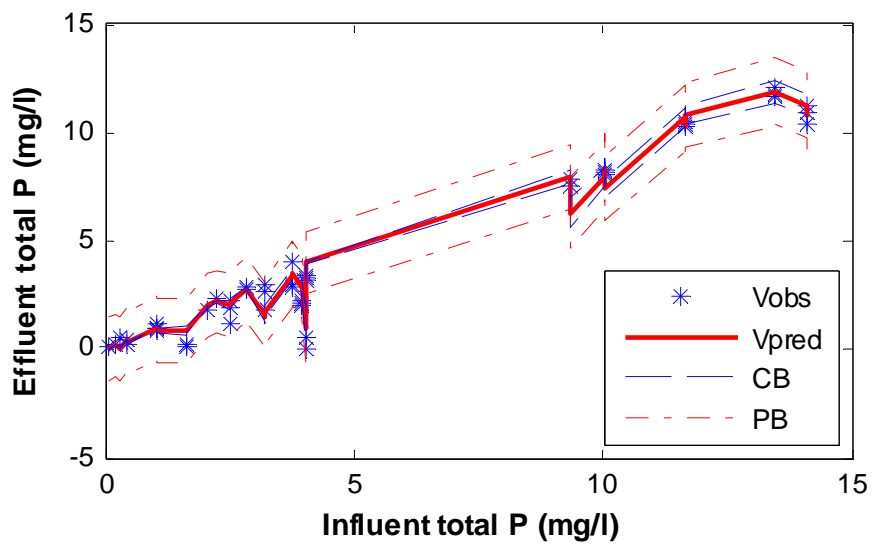
Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

Figure 6-9: Influent and effluent total N along with DUBWAT model fitting



Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

Figure 6-10: Influent and effluent total P along with DUBWAT model fitting



Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

Table 6-3 shows the fitting parameters for the model. The p-values indicate that first order rate constant (k) value were not significant for all COD, TN and TP removal data. Moreover, duckweed density was also not significant for TN removal.

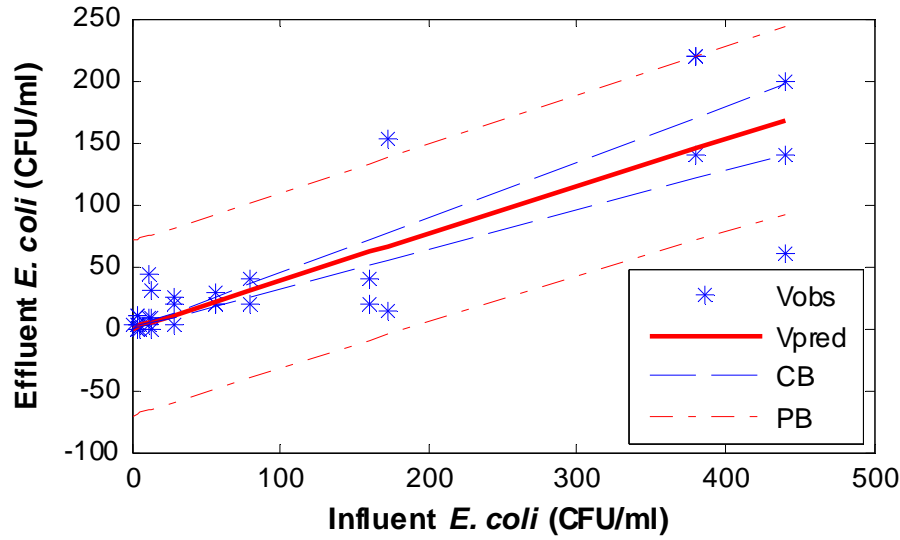
Table 6-3: Estimated values and other fitting parameters for COD, TN and TP removal data fitted to DUBWAT model

	R^2	Parameter	Estimate	95% confidence interval		Standard error	P-value
				Lower	Upper		
COD	0.87	k (1/day)	0.013	-0.004	0.029	0.008	0.06
		x	0.366	0.119	0.613	0.124	<0.01
		y	0.250	0.146	0.355	0.052	<0.01
Total N	0.86	k (1/day)	1.907	-5.179	1.366		0.12
		x	-0.801	-1.188	-0.414	0.194	<0.01
		y	0.023	-0.147	0.194	0.085	0.39
Total P	0.98	k (1/day)	0.000	0.000	0.000	0.000	0.30
		x	2.058	1.287	2.828	0.385	<0.01
		y	0.762	0.584	0.940	0.089	<0.01

6.3.2 *E. coli* removal

E. coli were not detected in most instances. *E. coli* counts in influent ranged from none to 440 CFU/ml. Log *E. coli* removal obtained in the wetlands ranged from -0.57 to 1.11 with a mean value of 0.30. A simple first order equation, as shown in Equation 6-1, adequately described the relationship ($R^2=0.71$). First order rate constant for the relationship was 0.11 ± 0.02 1/day. Figure 6-11 shows the relationship between influent and effluent *E. coli*.

Figure 6-11: Relationship between influent and effluent *E. coli* in constructed wetlands



Vobs: observed data, Vpred: model prediction, CB: asymptotic 95% confidence band, PB: asymptotic 95% prediction band

6.3.3 Duckweed production and nutrient recovery

Duckweed production did not correlate with any of the influent parameters except for the influent N concentration. Relationship between influent duckweed production and influent total N concentration could be satisfactorily described by the following second order polynomial equation (adjusted $R^2=0.62$).

$$DW = p_1 + p_2 \times X + p_3 \times X^2 \quad (6-4)$$

Where,

DW = duckweed production (g dry wt)

X = influent total N concentration (mg/l)

p_1, p_2, p_3 = coefficients

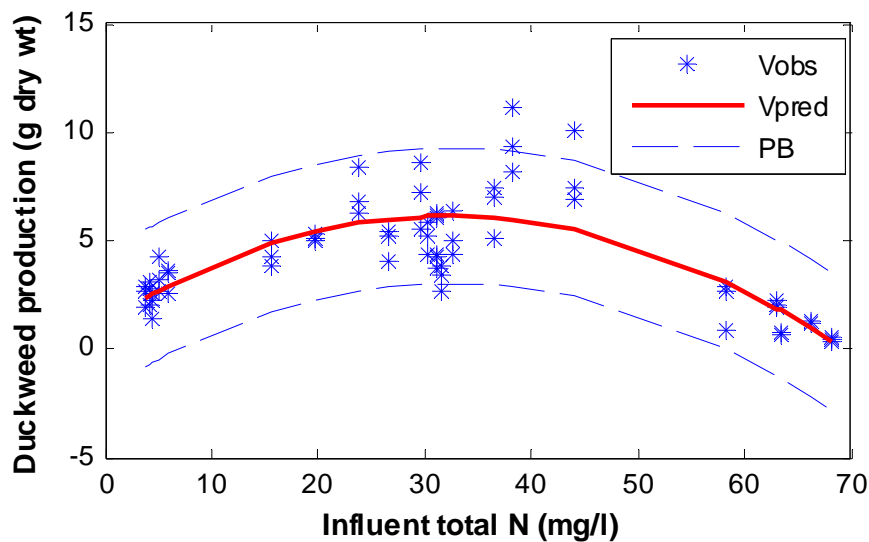
Table 6-4 shows the fitted values along with their 95% confidence interval.

Table 6-4: Second order polynomial fitting parameters for duckweed production as a function of influent total N

Parameter	Value	95% confidence interval	
		lower	upper
p ₁	1.317	0.3302	2.304
p ₂	0.29	0.23	0.36
p ₃	-0.0045	-0.0054	-0.0037

Figure 6-12 shows observed and predicted data along with asymptotic 95% confidence interval.

Figure 6-12: Observed and second order polynomial fitted data for duckweed growth

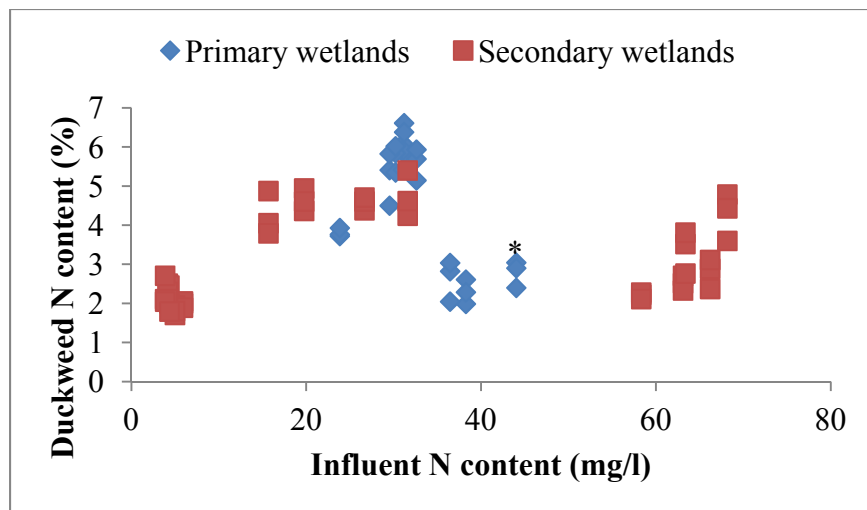


Vobs: observed data, Vpred: model prediction, PB: asymptotic 95% prediction band

N concentration in duckweed was negatively correlated with influent N content in primary wetlands ($r=-0.57$), while it was positively correlated with influent N concentration in secondary wetlands ($r=0.22$). Duckweed P content in primary wetlands was slightly correlated with influent P concentration ($r=0.33$), while duckweed P content in secondary wetlands was strongly

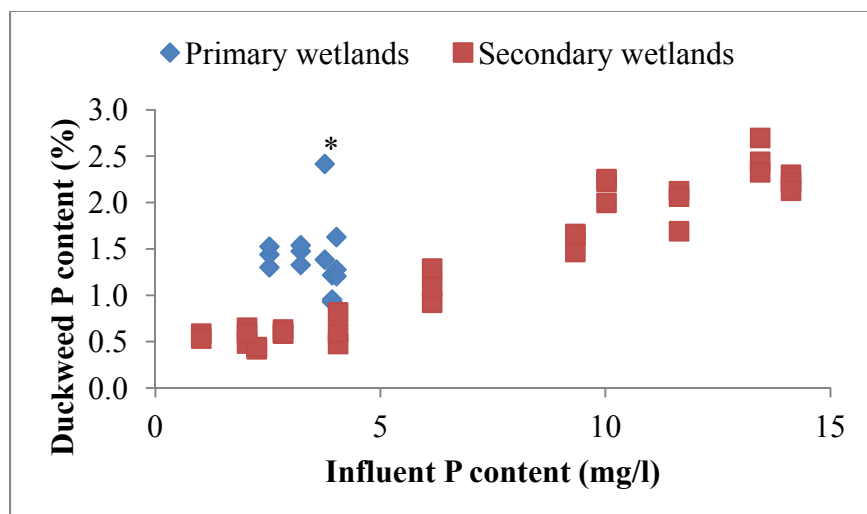
correlated with influent P content ($r=0.96$). Multiple linear regression showed that duckweed P content was not influenced by influent P content ($p=0.10$) but by wetland type ($p<0.01$). Figures 6-13 and 6-14 show relationship between influent N and P concentrations with duckweed N and P concentrations.

Figure 6-13: Influent N content and duckweed N content



*: concentration of influent N above which no duckweed growth occurred

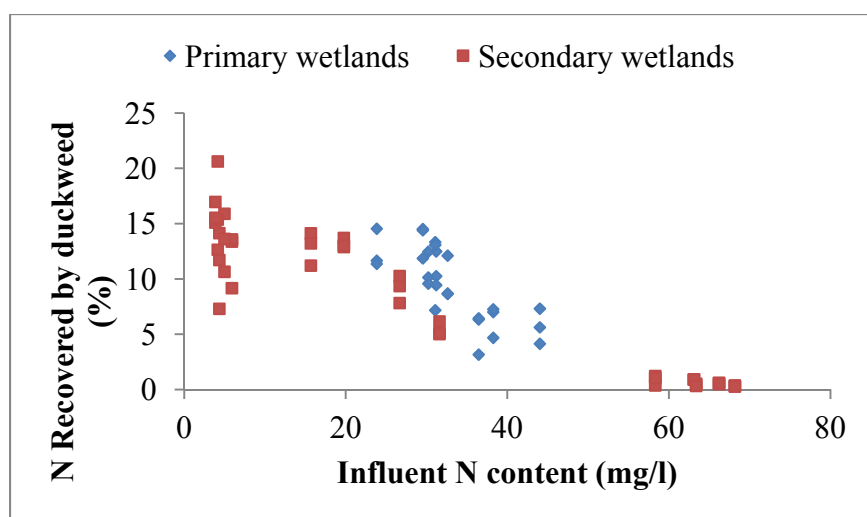
Figure 6-14: Influent P content and duckweed P content



*: concentration of influent N above which no duckweed growth occurred

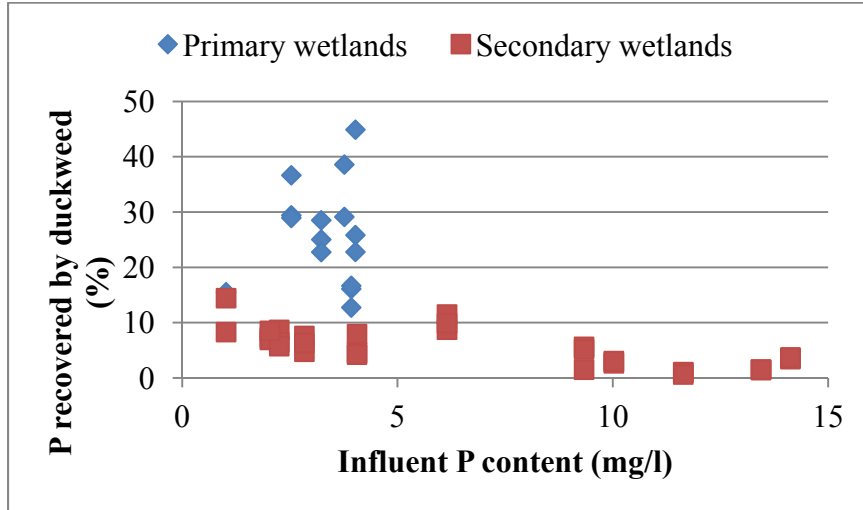
Total N recovery by duckweed ranged from 0.3 to 20.6% depending on the influent concentration. At lower influent concentrations, large fluctuation in the percentage recovery was observed. The reason for the variation might be that wetlands reach steady state after certain influent loading is reached. Total N recovery by duckweed harvesting decreased linearly with the increase in influent total N. Figure 6-15 shows the relationship between influent total N and percentage N recovered by duckweed.

Figure 6-15: Percentage N recovered by duckweed



Total P recovery by duckweed ranged from 0.6 to 45%, depending on the influent P concentration. P recovery by duckweed also showed decreasing trend; however, the relationship was not as strong as that for the total N. Figure 6-16 shows the relationship between influent total P and percentage P recovered by duckweed harvesting.

Figure 6-16: Percentage P recovered by duckweed



6.4 Discussion

Kadlec and Wallace (2009) reported removal rates of 2325 to 9975 g COD/ m²/year with a median value of 3427 COD/ m²/year for the surface flow wetlands loaded with >200 mg/l of influent COD. Total COD loading in primary SF wetlands ranged from 159 to 2089 mg/l. COD removal rates found in this study for primary wetlands (3869 g COD/ m²/year) were well within the range and comparable to the median value reported. However, COD removal obtained in the secondary wetlands (405 g COD/ m²/year) were well below the range reported by Kadlec and Wallace (2009) for the COD loading. In this study, COD loading in secondary wetlands ranged from 64 to 395 mg/l. The result obtained were comparable to the values reported by Kadlec and Wallace (2009) for secondary wetlands (30 to 100 mg/l COD). The authors reported removal range of 69 to 3230 g COD/ m²/year with a median value of 485 COD/ m²/year for the surface flow wetlands. Secondary wetlands used in the experiment received wastewater that was treated by primary wetlands and subsequent three stage SSF wetlands. Thus, settleable and easily

degradable organic matters were removed from the wastewater before reaching secondary wetlands and leaving behind recalcitrant organic matter. For the reason, though the COD loading in the wetlands was comparable to primary loading, secondary treatment wetlands behaved as lightly loaded wetlands.

In a review of constructed wetlands, Vymazal (2007) reported 40 to 55% removal of total N and 250-630 g N/ m²/year total N load removal in constructed wetlands. In another review, Kadlec and Wallace (2009) reported a annual TKN (total Kjeldahl nitrogen) removal rates ranging from 6 to 4683 g TKN/m²/year with a median value of 207 g TKN/m²/year. Overall removal percentage obtained in this study was lower than the rates reported, nonetheless comparable to the annual N load removal rates. Vymazal (2007) reported average TP removal of 40 to 60% with an annual total P load removal of 45-75 g P/m²/year. Kadlec and Wallace (2009) reported annual phosphorus load removal of -31 to 474 g P/m²/year with a median value of 6 g P/m²/year. Annual removal rates obtained in this study for both primary and secondary wetlands were lower than the value reported by Vymazal (2007) both in terms of percentage removal and annual P load removal. However, the annual load removal of phosphorus obtained in this study (10.1 g TP/m²/year for primary and 3.0 g TP/m²/year for secondary) were comparable and higher than the rates reported by Kadlec and Wallace (2009). These findings suggest that COD, TN and TP removal kinetics in constructed wetlands subjected to dairy wastewater were not significantly different from other constructed wetlands found in literature.

Table 6-5 shows comparison of three models fitted to the data. Based on AIC criteria, DUBWAT model appears to be the best fit to the data as compared to other two models. However, as can be seen from MSE, DUBWAT model improvement over other two models were not dramatic.

Moreover, DUBWAT model requires additional parameters (organic loading rate and duckweed stocking density) and as shown in Table 6-3, many fitting parameters in the model were not significant. Based on AIC criteria, MSE and number of parameters required, first order models with and without background appear to be similar except for COD removal. First order model with background appears to perform better than the model without background concentration. Hence, it was concluded that first order model with background concentration was the optimum model for the data.

Table 6-5: Comparison of different model fitting parameters for COD, TN and TP removal in constructed wetlands

	DUBWAT	First order	First order model with background concentration
COD			
Adjusted R ²	0.87	0.78	0.84
AIC	486	518	496
MSE	1052.15	1748.88	1234.73
RMSE as % of Y scale	11.22	14.47	12.16
Total nitrogen			
Adjusted R ²	0.86	0.83	0.83
AIC	255	265	267
MSE	36.93	44.25	44.73
RMSE as % of Y scale	12.76	13.97	14.05
Total phosphorus			
Adjusted R ²	0.98	0.96	0.96
AIC	-76	-25	-23
MSE	0.25	0.62	0.63
RMSE as % of Y scale	4.12	6.53	6.56

R²: coefficient of determination, MSE: mean squared error, AIC: Akaike information criterion,

RMSE: root mean squared error

In a review of various fecal coliform removal data, Kadlec and Wallace (2009) reported -2.79 to 3.16 log reduction in constructed wetlands. Mean log removal (0.30) obtained in this study and

the range was well within the range reported by the authors. Moreover, influent and effluent data satisfactorily fitted first order removal kinetics. These results suggests that bacterial removal kinetics in constructed wetlands subjected to dairy wastewater is similar to the other treatment wetlands usually found in literature.

Duckweed production in this experiment was a function of influent total N. Landesman et al. (2005) observed a similar relationship between duckweed growth and influent N content and modeled duckweed growth in terms of influent N concentration, temperature and solar radiation. However, in their experiment, Landesman et al. (2005) observed peak duckweed production at about 8 mg/l of influent N. In this experiment, raw manure was used and total available nitrogen was not as much as measured total nitrogen. As a result, duckweed production continued to increase up to nearly 30 mg/l of influent total N. As influent total N increased, there was sharp decline in duckweed production potentially due to ammonia toxicity. The trend explains why there was no duckweed growth at higher influent loadings. Nitrogen content in harvested duckweed ranged from 1.7 to 6.6% of dry duckweed biomass. Phosphorus contained in harvested duckweed ranged from 0.4 to 2.7% of dry duckweed biomass. Phosphorus content in the duckweed harvested from secondary wetlands showed high correlation with influent phosphorus content ($r=0.96$), while primary wetland did not show such correlation ($r=0.33$). Nitrogen content in duckweed was not strongly correlated with influent N content ($r=-0.57$ for primary wetlands and $r=0.22$ for secondary wetlands). Influent total N concentration ranged from 3.9 to 68.2 mg/l while influent total P concentration ranged from 0.05 to 14.1 mg/l. This might be due to high influent nitrogen and phosphorus concentration, so that there was above optimum nitrogen and phosphorus content in the influent.

Higher amount of nitrogen and phosphorus could be recovered by harvesting duckweed from primary wetlands as compared to the secondary wetlands. Total N and total P recovered from primary wetlands were $31.1 \text{ g N/m}^2/\text{year}$ and $10.1 \text{ g P/m}^2/\text{year}$, respectively. Total N and total P recovered from secondary wetlands were $10.2 \text{ g N/m}^2/\text{year}$ and $3.0 \text{ g P/m}^2/\text{year}$, respectively. Overall, $22.4 \text{ g N/m}^2/\text{year}$ and $5.6 \text{ g P/m}^2/\text{year}$ could be recovered by harvesting duckweed from the wetlands. Kadlec and Wallace (2009) reported that 50 to $150 \text{ g N/m}^2/\text{year}$ and $<20 \text{ g P/m}^2/\text{year}$ could be recovered by harvesting duckweed. Nitrogen recovered by harvesting in this experiment was below the value reported, while phosphorus removal was comparable to the value reported.

6.5 Conclusions

Primary wetlands were superior to secondary wetlands in COD, TN and TP removal. In an average, 28% of COD, 28% of total N and 16% of total P could be removed in duckweed based surface flow constructed wetlands. Average annual mass removal of COD, TN and TP in the wetlands were $2137 \text{ g COD/m}^2/\text{year}$, $149.5 \text{ g N/m}^2/\text{year}$ and $11.3 \text{ g P/m}^2/\text{year}$, respectively. First order with background concentration model was found to be the better predictor of effluent COD, TN and TP removal than simple first order model or DUBWAT model. Mean log *E. coli* reduction of 0.30 obtained in this experiment were within the range reported in literature. Duckweed production in the wetlands could be adequately described as a second order polynomial function of total influent nitrogen. More N and P could be recovered from primary wetlands as compared to secondary wetlands. Average N and P recovered by harvesting duckweed across all the wetlands were $22.4 \text{ g N/m}^2/\text{year}$ and $5.6 \text{ g P/m}^2/\text{year}$, respectively.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

Chapter 4 described *E. coli* removal performance of surface and subsurface flow wetlands subjected to pulse loading.

- Surface flow wetlands that were subjected to pulse *E. coli* loading did not show substantial difference in *E. coli* removal; 0.54 and 0.69 log reductions were obtained in summer and winter months, respectively.
- Seasonal variation in *E. coli* removal existed in SSF wetlands subjected to pulse loading; summer and winter log reductions for *E. coli* were 3.16 and 1.23, respectively.
- Subsurface flow wetlands were superior to surface flow wetlands in *E. coli* removal in both winter and summer months.
- Two models, one based on colloid filtration theory and the other based on convection-dispersion equation failed to adequately describe *E. coli* removal kinetics in constructed wetlands.

Chapter 5 described bacteriophage P22 removal performance of surface and subsurface flow wetlands subjected to pulse loading.

- Seasonal variation in bacteriophage P22 removal existed in both SF and SSF wetlands and the removal rates were higher in summer than in winter season.
- The convection-dispersion equation model adequately described P22 dynamics in the constructed wetland.
- Summer removal rates of bacteriophage P22 in the wetlands were two to four times higher than the winter removal rates in both types of wetlands.

- The SSF wetlands were more effective in removing the P22 than the SF wetland; SSF wetland removal rates were 19 times and 42 times higher in summer and winter months, respectively.
- Bacteriophage P22 was not a suitable biotracer for describing the hydraulic characteristics of the wetlands due to lower recovery.

Chapter 6 described pollutants removal and nutrient recovery in duckweed based surface flow constructed wetlands subjected to steady load of diluted dairy wastewater.

- Surface flow wetlands treating primary dairy wastewater were superior to the wetlands treating secondary wastewater in terms of COD, TN and TP removal.
- Average annual mass removal of 2137 g COD/m²/year, 149.5 g N/m²/year and 10.4 g P/m²/year were obtained from the wetlands treating dairy wastewater.
- First order removal model that includes background concentration was a better predictor of effluent COD, TN and TP removal than first order model or DUBWAT model.
- Duckweed production in the wetlands was adequately described by a second order polynomial function of total influent nitrogen.
- On average, 22.4 g N/m²/year and 5.6 g P/m²/year were recovered from harvested duckweed.
- *E. coli* removal from the dairy wastewater was adequately described by a first order removal model.

Recommendations for future research

- New modeling approach should be explored to better describe *E. coli* transport mechanism in pulse loaded wetlands.

- More research is needed to better understand the effect of nutrients and organic matter on the removal of *E. coli* and bacteriophage in pulse loaded constructed wetlands.
- To optimize the nutrient recovery potential of duckweed from constructed wetlands treating dairy wastewater, more research is needed with different harvesting frequencies and hydraulic retention time.

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