CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Framework

The overall experimental framework for this study is presented in Figure 3.1.

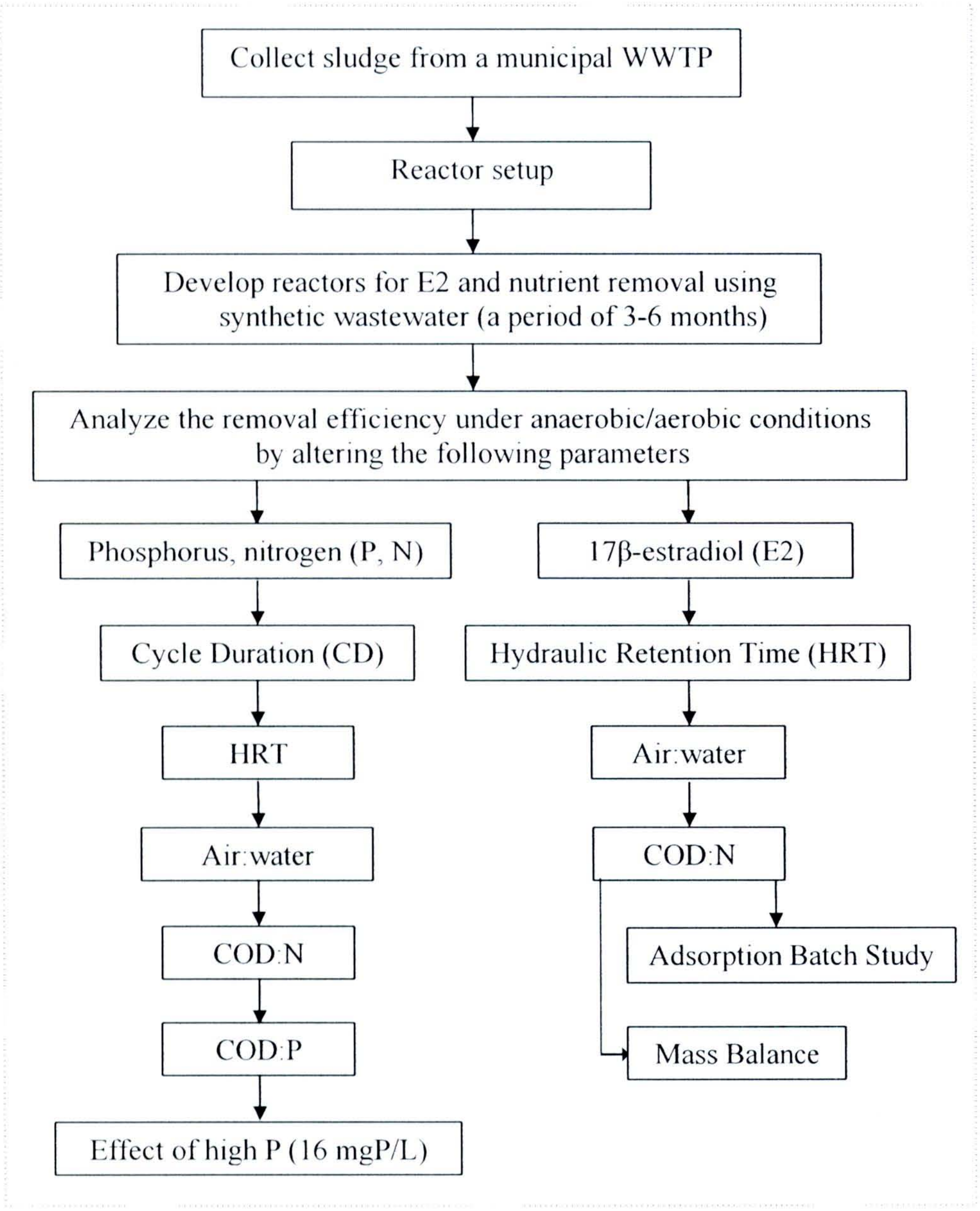

Figure 3.1 Experimental framework

This study focuses mainly on the removal of E2 and phosphorus in wastewater. Experiments are divided into 2 major parts: phosphorus removal and E2 removal (Figure 3.1). The effectiveness of the alternating attached growth filters was assessed under various operating conditions by adjusting the following parameters: cycle duration (CD), hydraulic retention time (HRT), air:water ratio, COD:N ratio, COD:P ratio, and P loading at 16 mg P/L. Mass balance and the assessment of adsorption capacity of E2 onto biofilm were determined.

3.2 Materials and Apparatus

3.2.1 Chemicals

E2 ($C_{18}H_{24}O_2$), MW: 272.4 g/mol (CAS 50-28-2, >98%) and E1 ($C_{18}H_{22}O_2$), MW: 270.37 g/mol (CAS 53-16-7, >99%) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Hach analytical reagents for analysis of total nitrogen (TN), total phosphorus (TP), ammonia, nitrite, and nitrate were purchased from Hach Company (Loveland, CO, USA). Solid phase extraction (SPE) cartridge LiChrolut EN® was purchased from Merck (Whitehouse Station, NJ, USA) and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) was ordered from Sigma Aldrich. Chemical reagents, Potassium dichromate ($K_2Cr_2O_7$), mercuric sulfate ($HgSO_4$), silver sulfate (Ag_2SO_4) for COD test were purchased from Fisher Scientific (Pittsburgh, PA, USA). Stock solutions of E2 were prepared in methanol HPLC grade at the concentration of 2,000 mg/L. Acetone, methanol HPLC grade, and dichloromethane (DCM) for E2 sample preparation were purchased from Merck (Whitehouse Station, NJ, USA).

3.2.2 Equipment

Two columns made of polyvinyl chloride (PVC) pipe with a diameter of 75 mm and approximately 1.70 m of packed bed were used as alternating attached growth columns (Figure 3.2). Synthetic wastewater was used and fed by a peristaltic pump from the bottom into anaerobic column. The effluent from the anaerobic column was then pumped into the aerobic column from the bottom. Aerobic conditions were achieved by injecting air through a diffuser at the bottom of the reactor. These conditions were maintained for a certain period and then the conditions for each reactor were alternated where the anaerobic becomes aerobic and aerobic becomes anaerobic. Each time this was done, the anaerobic column received the influent of the synthetic wastewater. The time or duration between the change over is known as the CD. Five equally spaced sampling ports were installed along the depth of each biofilter. The biofilters were backwashed from the bottom of the reactors.

3.2.3 Media

Gravel with an average diameter of 5-10 mm was used as the packed media in both columns. The media was packed to a depth of 1.70 m with a packing density of 1.63 kg/m^3. The media was supported by a layer of gravel of size 25-30 mm.

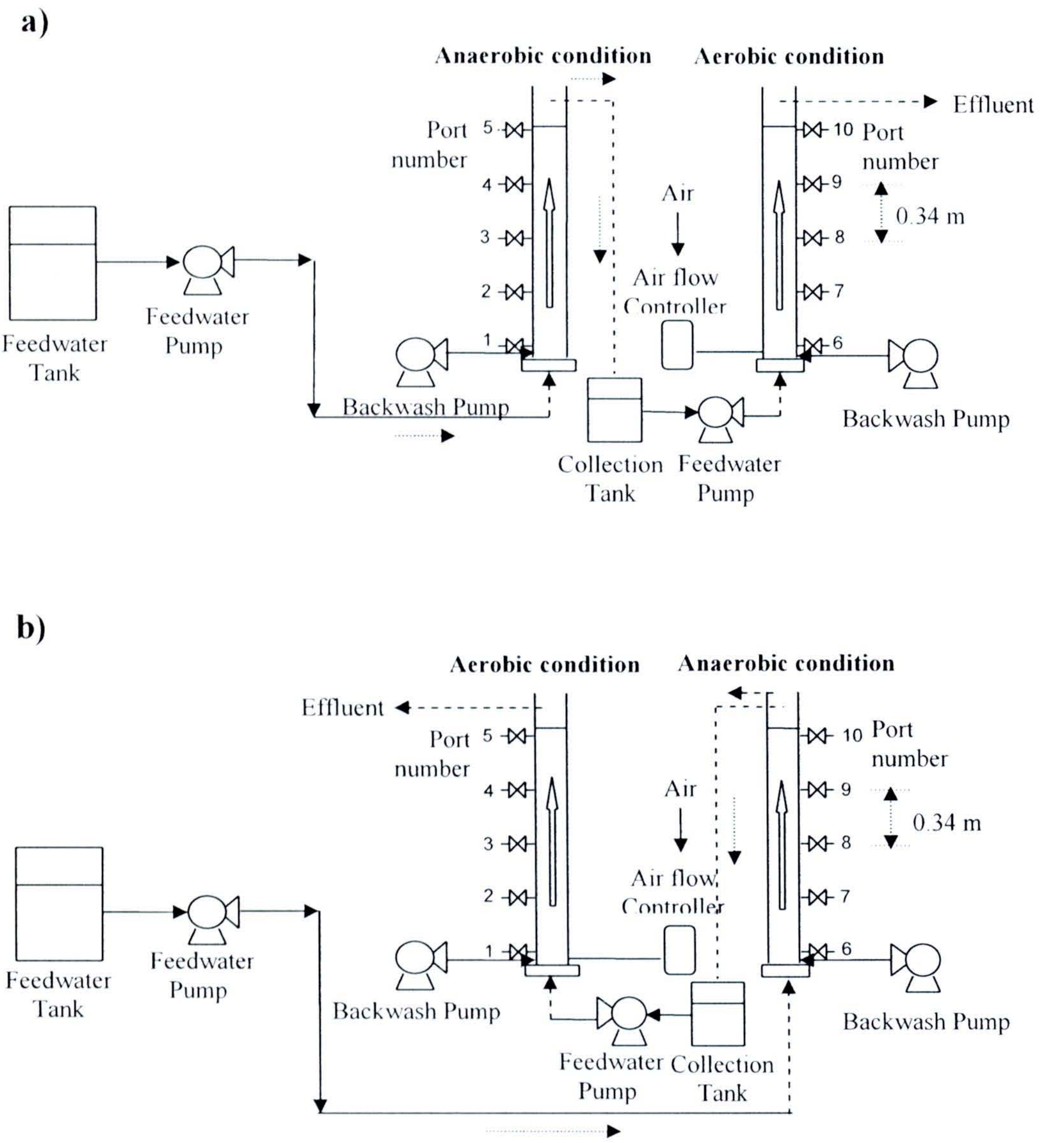

Figure 3.2 Flow diagram of the alternating attached growth filters with biofilters 1 and 2 under (a) anaerobic/aerobic conditions and (b) aerobic/anaerobic conditions

3.2.4 Enrichment

The alternating filters were seeded using activated sludge from the aeration tank of a municipal wastewater treatment plant in Boone, IA, USA. The biofilters were initially operated in batch mode for 17 days by adding synthetic wastewater until the biofilm was sufficiently established as indicated by COD removal of 80%. The columns were then operated in series by switching from anaerobic to aerobic mode and vice versa at a CD of 24 hrs and an HRT of 6 hrs until PAOs were developed within the two columns as shown by phosphorus removal of 80%. COD removal was also monitored but typically reached steady state conditions earlier than phosphorus removal.

3.2.5 Wastewater

The composition of the synthetic wastewater used in the experiments is presented in Table 3.1.

Table 3.1 Composition of synthetic wastewater

Ingredient	Concentration (mg/L)
Calcium Chloride ($CaCl_2$)	40
Magnesium Sulfate ($MgSO_4$)	6
Ferric Chloride ($FeCl_3$)	4
Sodium Biphosphate (NaH_2PO_4)	31
Sodium Bicarbonate ($NaHCO_3$)	100
Potassium Chloride (KCl)	8
Sodium Citrate ($Na_3C_6H_5O_7$)	440 (COD = 266)
Nutrient Broth	42 (COD = 28)
Ammonium Chloride (NH_4Cl)	95
Similac	(COD = 56)*
Estrogens (E2)	0.1
sCOD	350
Total phosphorus (TP)	8
Total nitrogen (TN)	28
Ammonia (NH_4-N)	22
Nitrite (NO_2-N)	0.01
Nitrate (NO_3-N)	3

*1 L Stock solution in 400 L of synthetic wastewater. Stock solution consists of 57 mL liquid Similac in 1 L of distilled water.

3.3 Sample Preparation

Unless specified, all wastewater samples were filtered through Whatman® glass microfiber filters, grade GF/C before analysis. To measure E2, the following procedures were used:

3.3.1 Solid Phase Extraction (SPE)

Fifty μL of a surrogate standard, [$^2H_3$]17β-estradiol-d3 (10 mg/L E2-d3) was added to 200 mL of the filtrate. The filtrate was extracted with disposable 3 mL SPE cartridge columns (119870) packed with ethinylbenzene-divinylbenzene copolymer LiChrolut EN®. The cartridge was preconditioned using 3 mL of acetone followed by 3 mL of HPLC-grade methanol and 10 mL of nanopure water. The filtrate was pumped into the cartridge at a flow rate of around 3 mL/min and the water level in cartridge was maintained over the packed area. The cartridge was then dried under vacuum (2,000 psi) for 20 min. The adsorbed E2 was then collected into test tubes by eluting 5 mL of acetone followed by 5 mL of methanol through the cartridge at a flow rate of 1 mL/min.

3.3.2 Derivatization (Silylation of Hydroxyl Groups)

The eluent was then dried using nitrogen gas and derivatized by adding 60 μL of BSTFA. The sample in the test tubes was then vortexed, capped, and incubated at 65°C for 60 min.

3.3.3 Internal Standard

The derivatized sample was then dried using nitrogen gas. 500 μL of methylene chloride (DCM) and 5 μL of internal standard (10 mg/L 5α-cholestane) were then added and the solution transferred to GC vials using glass Pasteur pipettes.

Concentrations of E2 in spiked and non-spiked samples were determined using E3-d2 as surrogate standard and 5α-cholestane as internal standard throughout the whole analytical procedure. Calibration curves were built by plotting the ratio of analytes peak area versus the E2 concentration.

3.4 Analytical Methods

3.4.1 Measurement of Water Quality Parameters

Parameters measured throughout the study include pH, temperature, oxidation-reduction potential (ORP), soluble chemical oxygen demand (sCOD), dissolved oxygen (DO), volatile suspended solids (VSS), suspended solids (SS), total nitrogen (TN), total phosphorus (TP), ammonia, nitrite, nitrate, phosphorus content of biomass waste, and E2. Grab samples were used for all the analysis. Analyses were conducted according to Standard Methods for the Examination of Water and Wastewater (APHA, 2003). ORP and DO concentration were measured by ORP (Orion 290A) and DO meter (Orion 830), respectively. Hach test kits were also used for the measurements of TN, TP, ammonia, nitrite, and nitrate as presented below:

Table 3.2 Hach test kit measurements

Parameter	Hach method	Catalogue Number	Method type	Range
TN	10071	26722-45	Persulfate digestion method	0-25 mg/L N
TP	10127	27672-45	Molybdovanadate method with acid persulfate digestion	0-33 mg/L PO_4^{3-}-P
Ammonia	10031	26069-45	Salicylate method	0-50 mg/L NH_3-N
Nitrite	8507	21071-69	Diazotization	0-0.3 mg/L NO_2^--N
Nitrate	8039	14034-99	Cadmium reduction method	0-30 mg/L NO_3^--N

3.4.2 Measurement of Phosphorus Content in Biomass

Nitric acid and sulfuric acid digestion method (APHA, 1989) in Methods of Phosphorus Analysis for Soils, Sediments, Residuals, and Waters was used to measure the total phosphorus content in the backwash biomass from the biofilters. After a backwash, the biomass was allowed to settle. A volume of 50 mL of the settled solids was added into a micro-kjeldahl flask. One mL of concentrated H_2SO_4 followed by 5 mL of concentrated HNO_3 were added and the solution digested to a volume of 1 mL. The digestion continued until the solution became colorless to remove HNO_3. The solution was allowed to cool down to room temperature before 20 mL of distilled water was added. A drop of phenolphthalein indicator solution was added and followed by approximately 15 mL of 1 M NaOH until a faint pink color was obtained. Distilled water was then added to bring the volume of the solution to 100 mL. The solution was tested for TP using Hach method 10127.

3.4.3 Measurement of COD using Closed Reflux, Colorimetric Method

COD was analyzed in accordance with Standard Methods for the Examination of Water and Wastewater. Using 1.5 mL dichromate digestion solution, 3.5 mL sulfuric acid reagent, and 2.5 mL of the sample solution were added to the digestion vessel with a volume of 12 mL. A blank sample was prepared by substituting DI water for the sample. The digestion vessel was heated for 2 hrs at a temperature of 150°C. The digested solution was then allowed to cool down to room temperature before the absorption of each sample using spectrophotometer (Hach Model, DR-2010) at wavelength 600 nm. Five standard solutions using potassium hydrogen phthalate (KHP) giving CODs ranging from 0 to 500 mg COD/L were prepared for the calibration curve.

3.4.4 Measurement of E2 by GC-MS

Derivatized estrogens were determined using an Agilent Technologies 6890N Network GC system equipped with a HP-5MS 5% Phenyl Methyl Siloxane (30 m×250 μm i.d., d_f : 0.25 μm) column and connected to an Agilent mass spectrometer 5973 Network Mass Selective Detector. 2.5 μL of the sample were injected into the GC-MS operating in a splitless mode. The injection temperature was at 20°C.

The oven was operated at 1 min at 50°C, first ramp at 30°C/min to 140°C (140°C held for 1 min), second ramp at 10°C/min to 240°C (240°C held for 3 min), third ramp at 3°C/min to 300°C (300°C held for 27 min) with an overall runtime of 64 min. The ion trap temperature was set at 280°C. Mass spectra were obtained with m/Z interval ranging from 200-550, using electron impact ionization. Retention times and m/Z ratios were used for quantitative purposes, in MS detection and are given in Table 3.3.

Table 3.3 Retention times (RT), MS detection parameters

Compound	Function	RT(min)	MS detection, quantification ion (m/Z)
E1	Analyte	20.8	342
E2	Analyte	21.4	416
E3-d2	Surrogate	24.6	506
5α-cholestane	Internal standard	24.2	217

The scan mode was used to find the retention time of the peaks and the major ion spectra. In scan mode, the mass filter was set to sequentially pass a range of masses and is used to determine general unknown compounds. The select ion monitoring (SIM) mode was used to quantify the estrogen concentration since in the SIM mode, the mass filter was set to pass at one selected m/z and is used when the analyst has prior knowledge of what ions to expect. The limits of detection of the method for E1 and E2 were 3 μg/L.

3.4.5 Measurement of E2 in sludge

To quantity E2 in sludge, E2 was extracted by adding a volume of methanol equal to the volume of the sample. Volumes of sample used were 5 mL. The solvent and solid phases were vortexed for 5 mins, and then the supernatant was removed. 5 mL of methanol was then added to the same sludge, vortexed for 5 mins, and then the supernatant was removed. This was repeated by adding methanol again. All the supernatants were then collected in a test tube and used for E2 analysis.

3.5 Methodology

The sequence of operation of the anaerobic/aerobic alternating biofilters is illustrated in Figure 3.1. The lead biofilter was operated in anaerobic condition while the lag biofilter was operated under aerobic condition. At the end of each CD, the condition for each biofilter were reversed with the influent. This alternating effect promotes the growth of PAOs in the biofilters.

3.5.1 Operating Conditions

Under typical operating conditions, the system was operated in series with one column under anaerobic condition to stimulate PHBs accumulation and release of phosphorus while the other column was operated under oxic condition for the uptake of phosphorus. The second biological filter (aerated) received the effluent of the first to complete the treatment cycle. Both biofilters were operated in an upflow direction and the aeration rate was maintained at approximately 3.0 L/min. The system was operated until the phosphorus removal in the second column tapered off. At this point, the air in the second column was turned off to receive the influent wastewater (reaching anaerobic condition) while the first column was aerated to receive the effluent from the anaerobic column. The two columns were alternated from anaerobic to aerobic to remove phosphorus by the PAOs. Phosphorus was removed from the system by backwashing the phosphorus-rich biomass every 7 days.

3.6 Operational Parameters

The effectiveness of the alternating attached growth filters was assessed under various operating conditions by adjusting the following parameters: CD, HRT, air:water ratio, COD:N ratio, and COD:P ratio. The columns were run at room temperature at a temperature of 20±5°C

3.6.1 Cycle Duration (CD)

After PAOs were developed within the two columns, the CD, the total time where the first column is re-exposed to the anaerobic condition, was altered in order to find its effect on total phosphorus, total nitrogen, ammonia, nitrate, and COD removal. Controlling the CD creates the environmental conditions to enhance or inhibit biological activity for phosphorus and nitrogen removal. Accordingly, CD can significantly impact the performance of the system due to the length of time the columns are exposed to unaerated/aerated conditions.

The CD assessed were 24, 12, 6, and 3 hrs. The COD and TP were maintained at 350 mg/L and 8 mg P/L, respectively for these CDs. Effluent samples were taken at the end of each cycle for analysis. According to the work of Shanableh et al. (1997), their system stabilized after a period of 3 days when the columns were switched to different conditions.

3.6.2 Hydraulic Retention Time (HRT)

HRT influenced the system efficiency as long HRT tends to provide sufficient time to allow biofilter system to facilitate the removal of TP and TN biologically. This study aimed to investigate the effects of the variation in HRT. The removal of E2, phosphorus, and nitrogen by the system were assessed to determine the optimal conditions. HRT was varied from 6 hours to as low as 2 hours while COD and TP remained fixed at 350 mg/L and 8 mg P/L, respectively. The HRT reported in this study is equal to the HRT of each individual biofilter.

3.6.3 Air:Water Ratio

High DO and sufficient sludge age are normally required for complete nitrification in the aerobic zone. Aeration is a fixed cost for the treatment and if the same removal efficiency of the system can be obtained with less aeration costs, this would be more economical and an environmental sustainable process.

The objective here was to determine the effects of DO concentrations on the biological phosphorus and nitrogen and also E2 removal. In order to investigate the optimum oxygen concentration, air:water ratios were tested at 0.024, 0.048, and 0.072. To ensure biological uptake of phosphorus, aerobic condition with adequate oxygen were needed. It is likely that E2 would be removed under nitrification conditions, thus adequate DO is required.

3.6.4 COD:N Ratio

Biological nitrification is accomplished by nitrifiers under aerobic conditions. The ratio of the organic concentration to the nitrogen concentration is a major factor affecting nitrification (Wheaton et al, 1994). Estrogen removing capability has been found to be attributed to the presence of nitrifiers (Shi et al., 2004; Vader et al., 2000). Therefore, varying N concentration may influence the growth of nitrifying bacteria. Additionally, it has been shown that nitrates can inhibit microbial phosphorus release under anaerobic conditions (Wentel and Ekama, 1997). Nitrites are known to inhibit the phosphorus release even further (Pak and Chang, 2000). This experiment examined the effects of COD:N ratio on simultaneous nitrogen and phosphorus removal and consequently E2 removal. Wastewater with varying N concentrations of COD:N of 8, 12, and 16 were used.

3.6.5 COD:P Ratio

Influent COD:P ratios of 30, 45, and 60 were used by fixing phosphorus at 8 mg P/L and changing the COD concentration accordingly. Typically, EBPR systems require 2.0-2.5 mg PO_4-P per 100 mg COD in the influent with a COD:P ratio of 40-50 as stated by WEF (1998). Apart from this, Randall et al. (1992) reported that for phosphorus removal of municipal wastewater, 50 mg COD was required for removing 1 mg P. Varying COD:P ratio will provide information on the impact of COD:P ratio on phosphorus removal. However, the optimal COD:P ratio for phosphorus may not be necessarily be the optimal ratio for E2 removal.

Additionally, this experiment attempted to study the impact of higher influent phosphorus concentrations at 16 mg P/L on phosphorus removal. In this case, the COD was kept constant at 350 mg/L while the phosphorus concentration was increased to 16 mg P/L to give a COD:P ratio of approximately 22.

3.7 Sorption Test

Batch sorption tests were carried out to examine the adsorption of E2 onto the biomass in the reactors. Biomass from the biofilters was collected from the backwash water (approximately 0.6 g/L for 1 L of backwash water) from each anaerobic and aerobic column. Backwash was performed at the end of each operational condition under steady state conditions. The biomass was allowed to settle for 30 min before the supernatant was carefully decanted. The biomass was sterilized using 0.5 mL/L mercury (II) sulphate (Hg_2SO_4, 200 g/L) according to Manfred et al. (2004) without consuming the oxygen in the slurry.

3.7.1 Adsorption kinetics

Adsorption equilibrium time was determined over a period of 2-3 days. In this test, a flask of 500 mL were used with 250 mL of slurry. E2 stock solution was added to obtain an initial concentration of 200 μg/L. The SS concentration used was 0.3 g/L. The samples were agitated at 120 rpm and at times of 0, 8, 16, 24, and 48 hrs, 100 mL of aqueous samples were collected. pH was maintained at approximately 7. Control sample (no biomass present) was also set up and samples collected and analyzed. For the control, synthetic feedwater was used but modified by omitting the organic carbon sources. $CaCl_2$ solution contained in synthetic wastewater can improve separation of solids from the aqueous solution and can minimize cation exchange (ionic strength).

The samples collected were immediately centrifuged at 1600 rpm for 10 min. Prior to analysis, the supernatant was filtered through a Whatman GF/C glass microfiber filters and then a sample volume of 200 mL was analyzed using the described GC-MS method.

3.7.1.1 Batch Adsorption Experiment

In the adsorption experiment, the biomass concentration was fixed at 0.3 g/L. A series of 500 mL flasks with 250 mL slurry were used. E2 concentration, C_{inf}, of approximately 10, 50, 250, 500, and 1000 μg/L were investigated.

The solutions used were synthetic feedwater free of organic carbon. To prepare 250 mL with 0.3 g/L of SS, a volume of 12.5 mL concentrated biomass of 6 g/L was added to 250 mL of synthetic wastewater. The flasks were loosely capped. The pH was adjusted to 7 by the addition of NaOH or HCl, if necessary. Aliquots of E2 standard solution were added to achieve different concentrations. The test was carried out in duplicate.

The flasks were agitated at 120 rpm in an incubator at 20°C and 200 mL of supernatant was collected at a predetermined steady state time and filtered for subsequent GC-MS measurement. In addition, control samples without biomass were examined in a manner similar to that described above.

3.7.2 Adsorption Isotherm

The adsorption behavior can be modeled using a linear sorption isotherm given by:

$$K_D = C_s^{ads}{}_{(eq)} / C_{aq}^{ads}{}_{(eq)}$$

Where; K_D = Adsorption coefficient (L/g.SS)

C_s^{ads} = Mass of E2 adsorbed on the biomass (mg/g.SS)

C_{aq}^{ads} = Concentration of the E2 in the aqueous phase (mg/L)

If the isotherm was non-linear, the Freundlich model can be used and is given by:

$$\log C_{s.ads\,(eq)} = \log K_F + 1/n \log C_{aq.ads\,(eq)}$$

where; K_F = Freundlich adsorption coefficient

$1/n$ = exponent where n was a constant

3.8 Mass Balance of E2

In the biofilter, the two mechanisms of removal of E2 are by adsorption onto the biofilm and SS and by biodegradation process. A mass balance approach was used to avoid difficulties in measuring the amount of E2 adsorbed on biofilm and SS. To approximate the masses in the system, the following assumption was made (steady state was reached, there was no chemical reaction, and all the newly biomass growth will be scoured during backwash and will represent the biomass for adsorbed E2). Backwashes were performed at the end of each operating condition. The mass balance of E2 can be modeled as follows:

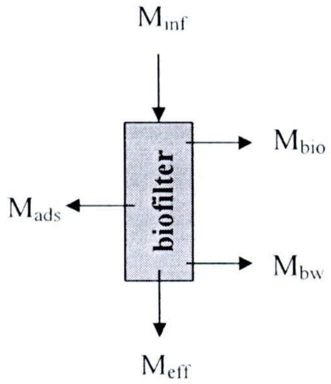

Figure 3.3 Mass balance of E2

Total mass input into the system (M_{inf}) = Mass exiting (M_{eff}) + mass biodegraded (M_{bio})
+ mass in the biomass in the backwash (M_{bw})
+ mass adsorbed (M_{ads})

Putting the terms together:

M_{inf} = $M_{eff} + M_{bio} + M_{bw} + M_{ads}$

M_{eff} = mass in suspended solids ($M_{ss,eff}$) + mass in the aqueous phase ($M_{aq,eff}$)

M_{bw} = mass in the backwash water ($M_{aq,bw}$)

Assuming $m_{biomass} = M_{biomass,bw}$

M_{ads} = mass in the biomass of backwash ($M_{biomass,bw}$)

Therefore,

$$M_{inf} = [M_{ss,eff} + M_{aq,eff}] + M_{bio} + [(M_{biomass,bw} + M_{aq,bw}] + M_{ads}$$

For a CD of t_{cycle} and flow of Q_{inf} we have,

$$t_{cycle}.Q_{inf}.C_0 = [t_{cycle}.Q_{eff}.SS_{eff}.C_s + (t_{cycle}.Q_{eff}.C_e)] + M_{bio} + [(t_{bw}.Q_{bw}.C_{aq,bw})] + [(t_{bw}.Q_{bw}.C_{ss,bw}.BS_{bw})]$$

where; M_{inf} = initial amount of E2

M_{eff} = final amount of E2

M_{bio} = amount of E2 removed by biodegradation

M_{ads} = amount of E2 sorbed in biofilm in reactor

M_{bw} = amount of E2 removed by adsorption

$M_{ss,eff}$ = amount of E2 on SS in effluent

$M_{aq,eff}$ = amount of E2 in aqueous phase in effluent

$M_{biomass,bw}$ = amount of E2 on biofilm from backwashing

$M_{aq,bw}$ = amount of E2 in aqueous phase from backwashing

Q_{inf} = flow rate of influent

Q_{eff} = flow rate of effluent

Q_{bw} = backwash flow rate

SS_{eff} = concentration of SS in effluent

SS_{bw} = concentration of biomass from backwashing

C_{inf} = initial concentration of the E2 in influent

C_{ads} = content of the E2 adsorbed on the SS in effluent

C_{eff} = concentration of the E2 in the effluent

$C_{ads,bw}$ = content of the E2 adsorbed on the biomass from backwashing

$C_{aq,bw}$ = concentration of the E2 in the backwash water

t_{cycle} = operating time

t_{bw} = backwashing time

$m_{biofilm}$ = amount of biofilm onto packed media

Using the above approach, the relative magnitude of biodegradation and sorption can be estimated and one can infer whether biodegradation or sorption was the dominant mechanism for E2 removal.