CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Estrogens are a group of steroid compounds that function as the predominantly primary female sex hormones. Estrogens are important for controlling the development of breasts, skin, brain, and reproductive system. They can be divided into two groups; natural estrogens and synthetic estrogens. Natural estrogens are naturally produced by living organisms including human and animals while synthetic estrogens are manufactured and are commonly used as active ingredients for contraceptive pills for birth control and growth promoter. Estrogens as pollutants or contaminants in the environment can be clustered under the group known as endocrine disrupting chemicals (EDCs).

EDCs are exogenous substances that interfere with the endocrine system and disrupt the reproductive system of humans and animals. The US Environmental Protection Agency (EPA) defines endocrine disruptors as:

> "……an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that is responsible for the maintenance of homeostasis, reproduction, development, and/or behavior."

Apart from estrogens, some other well-known examples of EDCs are dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), furans, and phenols.

2.2 Natural Estrogens

Common estrogenic steroid hormones are estrone (E1), 17β-estradiol (E2), and estriol (E3). The IUPAC name of E1, E2, and E3 are 3-hydroxyestra-1,3,5[10]-trien-17-one, 1,3,5[10]-estratriene-3,17β-diol, and 1,3,5[10]-estratriene-3,16α,17β-triol, respectively. E2, E1, and E3 have the highest to the least potency of estrogenic activity, respectively (Khanal et al., 2006). E1, E2, and E3, are composed of four rings: a phenol

(ring A), two hexacyclic (rings B and C), and a cyclopentane (ring D) (See Figure 2.1). The differences between these compounds are at C16 and C17 positions. E2 has a hydroxyl group on C17 pointed upward of the molecular plane. The chemical structures of E1, E2, and E3 are presented in Figure 2.2.

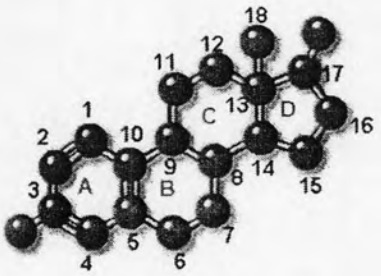
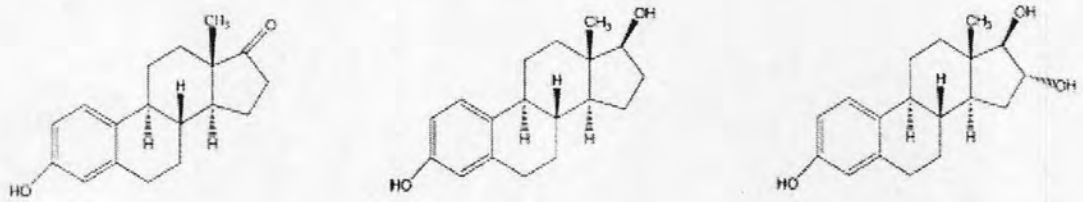


Figure 2.1 Basic structure of estrogens



Estrone (E1) Estradiol (E2) Estriol (E3)

Figure 2.2 Structures of E1, E2, and E3

The physical-chemical properties of E1, E2, and E3 are presented in Table 1. E1, E2, and E3 have vapor pressures ranging from 2.3×10^{-10} to 6.7×10^{-15} mm Hg, indicating low volatility. They are moderately hydrophobic compounds with log octanol-water partition coefficient (log K_{ow}) of about 2.8 - 4.0. All these natural estrogens have a solubility of approximately 13 mg/L at 20°C. From the physical-chemical properties, it is expected that natural estrogens are likely to adsorb on the solid phase such as sludge and floc/biofilm in wastewater treatment. Khanal et al. (2006) suggested that natural estrogens are mainly removed from the aqueous phase by adsorption process.

Table 2.1 Physical-chemical properties of natural estrogens[a]

Compound	MW[b]	Water solubility (mg/L at 20°C)	Vapor pressure (mm Hg)	log K_{ow}[c]
E1	270.37	13	$2.3x10^{-10}$	3.43
E2	272.39	13	$2.3x10^{-10}$	3.94
E3	288.39	13	$6.7x10^{-15}$	2.81

[a] Lai et al. (2000), [b] Molecular weight, [c] Octanol-water partition coefficient

Humans excrete estrogens mainly in urine as inactive compounds, sulfate and glucuronide conjugates. Conjugated forms of E2 can occur by esterification at the 3 or 17 carbon positions which can be written as the following: E2-3SUL, E2-17G, where the 3 and 17 refers to the position on the molecule and SUL for sulfate and G for glucuronide. Conjugated estrogens are hydrolyzed by fecal bacteria, *Eschericia coli* (*E.Coli*) (Belfroid et al., 1999) present in wastewater. Fecal bacteria express glucuronidase and sulfatase enzymes which can hydrolyze these conjugates back to their original bioactive forms, resulting in an increase in total estrogenic potency. Ternes et al. (1999a) found that two glucuronides of E2 were cleaved by diluted activated sludge accompanied by appearance of E2. Inactive polar conjugate can be reactivated to active form (Figure 2.3). De-conjugation of E2 occurs at C_3 and C_{17} position by glucuronidation or sulphatation. Estrogens would become an active form when the C_3 position is de-conjugated.

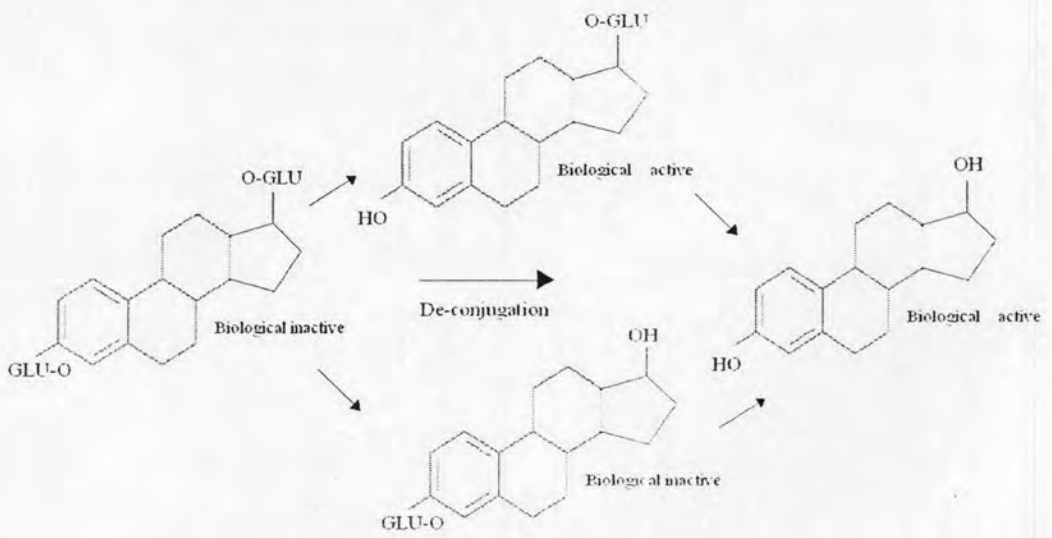


Figure 2.3 De-conjugation of E2 (Flemming and Bent, 2003).

2.3 Sources of E2

All humans as well as animals excrete estrogens from their bodies. Estrogens from animals and human being are excreted in the urine and feces irrespective of their sex and age. For example, laying hens and roosters excrete 533 and 93 µg of E2/day per 1,000 kg live animal weight, respectively (Shore et al., 1995). The daily excretion of estrogens from human males and females vary widely as seen in Table 2.2. E2 excretion by males is 1.6 µg/day while menstruating and menopausal females excrete 8 and 4 µg of E2/day, respectively. More importantly, pregnant women excrete as high as 259 µg of E2/day. However, data on daily excretion of estrogens from different domestic animals are still lacking. Additional possible sources of E2 may come from animal manures used as fertilizers in agricultural practices. A report conducted by Shemesh and Shore (1994) indicate that poultry waste contained 44 ng of E2/g dry weight.

Table 2.2 Daily excretion of estrogenic steroids by humans (Johnson et al., 2000)

Category	E2 (µg/day)	E1 (µg/day)
Males	1.6	3.9
Menstruating females	3.5	8
Menopausal females	2.3	4
Pregnant women	259	600

2.4 Adverse Effects of E2

The presence of estrogenic compounds in the environment has raised considerable concern worldwide because they may interfere with the reproduction of human, livestock, and wildlife. Intake of estrogens by humans via food or drinking water may cause male fertility disorder, decreased sperm count, and increased incidents of testicular cancer (Sharpe and Skakkeback, 1993). Recent studies indicated that there is a potential for humans being to be exposed to sludge-amended soils with estrogens. In some countries, pond sludge and sediments are dredged and used to prepare soil for crop production, thereby, spreading the exposure and risk of estrogens to terrestrial organisms.

Although E2 can be degraded by microbes to nanogram levels, this concentration still has potential estrogenic effect that can impact aquatic ecosystem and human health.

This potential exists for natural estrogens present in sewage biosolids and animal manures which may be transferred to the environment.

E2 can exert hormone-like effects or impedes hormonal function in aquatic organisms; induces abnormality in reproductive function and behavior, demasculinization; and decreased hatching success. Contamination in waterways at levels between 10-100 ng/L in water can adversely affect the reproductive biology of vertebrate species such as fish, turtles, and frogs via the disruption of the normal functions of endocrine systems (Hanselman et al., 2003).

Routledge et al. (1998) found that adult male rainbow trout (*Oncorhynchus mykiss*) and adult roach (*Rutilus rutilus*) exposed via water to environmentally relevant concentrations of E2 of more than 100 ng/L for 21 days resulted in a significant elevation of plasma vitellogenin levels. The elevated level of vitellogenin which is an egg yolk precursor protein, is normally produced only by adult females and would have accompanied by the inhibition of testicular growth. Desbrow et al. (1998) indicated that effluents with E2 ranging from 1 ng/L up to almost 50 ng/L may be responsible for the observed induction of vitellogenin synthesis in male fish. Panter et al. (1998) showed that low concentrations of E2 have profound effects on plasma vitellogenin and testicular inhibition on male fish.

Furthermore, estrogens may interfere with the normal functioning of endocrine systems of wildlife affecting reproduction and development (Jobling et al., 1998). Hormonal steroids in the environment may affect plants according to Shore et al. (1995). They reported that alfalfa irrigated with runoff from agricultural land amended with poultry manure, which contained E2, resulted in elevated levels of phytoestrogen in alfalfa crops.

2.5 Level of E2 in Wastewater Treatment System

E2 was detected with average concentrations of 21 ng/l and 48 ng/l in the influent wastewater of the Brazil and Netherland wastewater treatment plants (WWTPs), respectively (Ternes et al., 1999a; Johnson et al., 2000). E2 has been detected in the effluents of municipal WWTPs in different countries. Desbrow et al. (1998) found that E2 was present in the effluent of seven municipal WWTPs at concentrations ranging from approximately 1 ng/L up to 50 ng/L. Routledge et al. (1998) showed that E2 was present at concentrations in the 10 ng/L range in the final effluent discharge of several municipal

WWTPs. In Canadian WWTPs, E2 was determined in 9 of 10 effluent samples with maximum concentrations of 64 ng/L (Ternes et al., 1999a).

2.6 Fate of E2 in Wastewater Treatment System

Sewage discharge has been linked to the occurrence of estrogenic compounds in the environment. Municipal WWTPs are a major source of estrogens in the environment. Removal of estrogens compounds in municipal WWTPs are mainly achieved through adsorption and degradation. However, due to the diverse microbial groups in municipal WWTPs, about 84% of E2 are mineralized within 24 hours (Khanal et al., 2006). In contrast, a study by Layton et al. (2000) showed that with industrial WWTPs sludge, only 4% mineralization of E2 was achieved.

2.6.1 Sorption

The behavior and fate of estrogens in the environment depend on their physical-chemical properties and the environmental media. It is expected that E2 will be adsorbed to the activated sludge. Andersen et al. (2005) found that the linear adsorption coefficient, K_D for E2 adsorbed onto activated sludge was 476±192 L/kg in a batch study using 4 g/L of activated sludge. The sorption behavior of steroidal estrogens has been modeled using the Freundlich isotherm. About 66±13% E2 was estimated to be sorbed during activated sludge treatment. Clara et al. (2004) showed that log K_D of E2 for activated sludge was 2.84. Lai et al. (2000) published a log K_{oc} value of 3.50 for E2. However, Ren et al. (2007) indicated that the adsorption of estrogens in activated sludge process was independent of their hydrophobic characteristics. The Freundlich adsorption (K_F) of E2 was 12.46 ($\mu g^{1-1/n}.L^{1/n}.g^{-1}$) at 20°C while the 1/n value was 0.79 and was lower than other kinds of natural estrogens, E1 and E3 which had K_F values of 14.25 and 1985.64 ($\mu g^{1-1/n}.L^{1/n}.g^{-1}$), respectively. Table 2.3 provides some adsorption coefficients for E2 and EE2.

Table 2.3 Some equilibrium adsorption coefficients of E2 and EE2

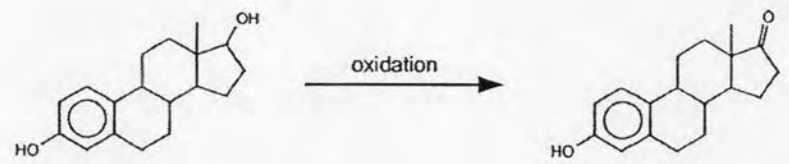
Adsorption Coefficients	Media	E2	EE2	Reference
Log K_D	sludge	2.84	2.84	Clara et al., 2004
Log K_D	sludge	2.68	2.77	Andersen et al., 2005
Log K_D	sludge	-	2.54	Ternes et al., 2004
Log K_{oc}	sludge	3.50	3.80	Lai et al., 2000

A study conducted by Desmes et al. (2008) showed that under anaerobic conditions in upflow anaerobic sludge blanket (UASB), adsorption which accounted for 32-35% of removal probably played an important role on the loss of total E1+E2.

2.6.2 Degradation

DEPA (2004) indicated that free estrogens can be degraded through biotic route rather than abiotic route for an estrogen level at 500 ng/L as E2 equivalent in a column study. Investigations on the removal of estrogens in wastewater treatment plants have demonstrated the potential of E2 converting to E1 followed by subsequent degradation of E1 under aerobic conditions (Joss et al., 2004; Lee and Liu, 2002; Ternes et al., 1999b; Vader et al., 2000; Shi et al., 2004). In many cases, the loss of E2 typically results in an accumulation of E1. Conversion of E2 to E1 would decrease the estrogenicity as E1 has less estrogenic activity than E2. However, in a wastewater treatment plant E1 can potentially be converted back to E2 (Dytczak et al., 2008). Therefore, decrease in the total E1+E2 would result in the total loss of estrogenic activity in the effluent.

As indicated earlier, conversion of E2 to E1 is possible, but there are very few studies documenting the subsequent removal of E1 (Joss et al., 2004; Lee and Liu, 2002). Since Joss et al. (2004) suggested that E1 could be partly transformed back to E2 under anaerobic conditions, there is an increase risk that E2 would be released into the environment under anaerobic conditions. Ternes et al. (1999b) investigated the persistence of E2 in aerobic batch experiments and found that E2 was oxidized to E1 (see Figure 2.4) by more than 95% after a period of 1-3 hours. After 5 hours neither E2 nor E1 were found in their study.



17β-estradiol estrone

Figure 2.4 The oxidation of E2 to E1 (Ternes et al., 1999b)

Lee and Liu (2002) found that bacteria present in wastewaters were capable of degrading estrogenic compounds into harmless products. They proposed a pathway for the degradation of E2 by sewage bacteria (Figure 2.5). The presence of *E.coli*, *Pseudomonas fluorescens*, and *Bacillus thuringiensis* in municipal sludge improve E2 degrading capacity as reported by Yu et al. (2005).

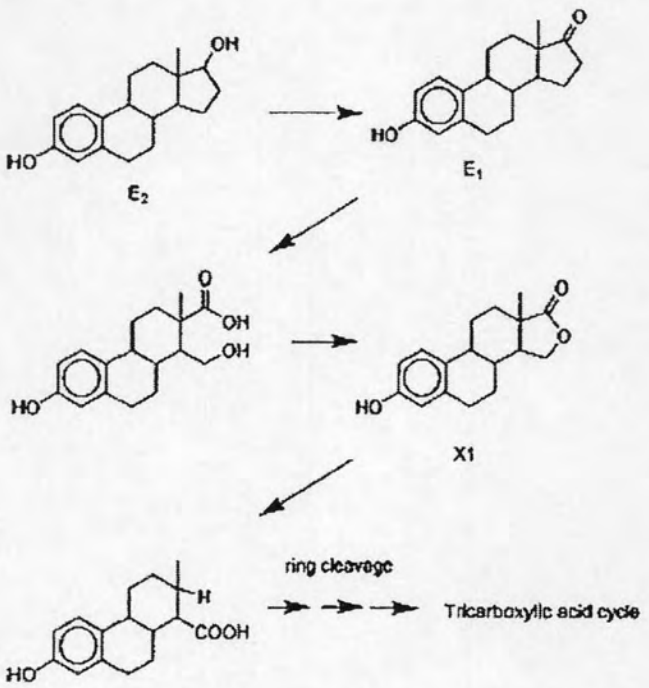


Figure 2.5 E2 biodegradation pathway by sewage bacteria (Lee and Liu, 2002)

According to Figure 2.5, biodegradation of E2 appeared to be initiated at the hydroxyl group at C-17 of ring D, leading to the formation of a keto group at the same position which is the major metabolite, E1. By the further oxidation of E1 and the cleavage of ring D, labile metabolite, X1 was observed. Apparently, ring A of E2 was not the most favored attack site for the pathway.

Joss et al. (2004) proposed a model for the biological degradation and sorption of E1 and E2 as shown in Figure 2-6 below:

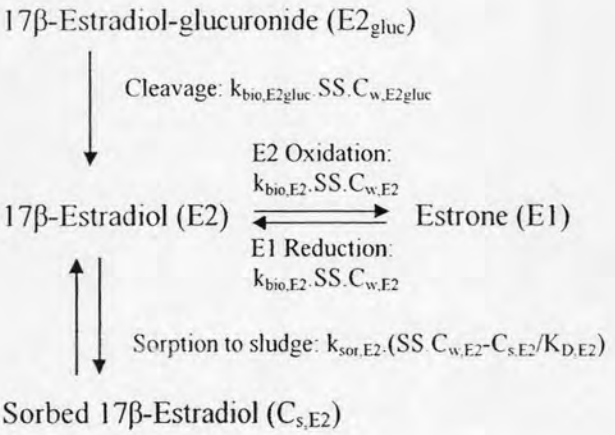


Figure 2.6 Model of biological degradation and sorption of E2 (Joss et al., 2004) Abbreviations: k_{bio} and k_{sor}, pseudo-first-order reaction rate constant; SS, suspended solids; C_w, bulk soluble concentration; C_s, sorbed concentration per reactor volume; K_D, sorption coefficient.

According to Figure 2.6, the reactions that are involved in the degradation and sorption of E2 are as follows: (1) cleavage of the conjugates (glucuronides and sulfates), (2) oxidation of E2 to E1, (3) reduction of E1 to E2 under anaerobic condition, and (4) sorption of E2 to sludge.

Khanal et al. (2006) suggested that E2 adsorbed onto the floc/biofilm are further degraded by microbes within the floc/biofilm. They also speculated that estrogen concentrations in the secondary treatment are governed by three factors: (a) solid retention time (SRT), (b) estrogen partition coefficient ($K_{d,SS}$), and (c) biodegradation rate constant (k_{bio}) and mixed liquor volatile suspended solids (MLVSS).

The higher the SRT, the lower was the concentration of estrogen in the effluent. Ternes et al. (1999) indicated that when the SRT of the activated sludge systems was increased from 6 to 11 days, the removal efficiency of E2 increased from 75 to 96%. Because of the high $K_{d,SS}$ for estrogen, higher removal efficiency can be attained with higher mixed liquor suspended solids (MLSS). Kikuta and Urase (2003) reported that E2 concentrations in the secondary effluent decreased from 7.9 to 2.2 ng/L when the MLSS was increased from 1,000 to 10,000 mg/L in a bench-scale experiment. An increase in k_{bio}

and an increase in MLVSS would decrease the estrogen concentration in sludge. DEPA (2004) described estrogen removal rate as follows:

$$\frac{dC}{dt} = k_{bio}.MLVSS.t$$

A recent work by Dytczak et al. (2008) using two sequencing batch reactors (SBRs) with aerobic conditions and alternating anoxic/aerobic conditions showed that E2 was readily converted to E1. The transformation under aerobic condition was faster than anoxic conditions. E1+E2 removal was 50% under aerobic conditions and was similar for alternating anoxic/aerobic reactor. They found that E1+E2 percent removal increased with respect to increasing nitrification percent removal which supports the hypothesis that nitrifying biomass could remove estrogens which was consistent with previous observation (Shi et al., 2004; Vader et al., 2000). Interestingly, a metabolite, 17α-estradiol was found to form under denitrifying anoxic conditions by Dytezak et al. (2008) and this compound subsequently disappeared under aerobic conditions. The metabolite found was consistent with the study by Czajka and Londry (2006).

Vader et al. (2000) indicated that estrogen removing capability was mainly attributed to the presence of nitrifying bacteria via nitrification route. Servos et al. (2005) showed that operations with nitrification tended to have higher estrogen percent removal than operations that are non-nitrifying. Shi et al. (2004) showed that nitrifying activated sludge and ammonia-oxidizing bacterium, *Nitrosomonas europaea,* could degrade E1 and E2 with zero order biodegradation rates of 0.0022 and 0.0016 mg/L/hour, respectively. The rate of degradation of E1 and E2 were correlated to rates of ammonia consumption and were 1.5 mgNH_4-N/L/hour for E1 and 1.45 mgNH_4-N/L/hour for E2.

Organic concentration has been considered a major factor affecting the nitrification (Wheaton et al., 1994). Many factors influence the growth of nitrifying bacteria. These factors include pH, dissolved oxygen, and temperature (Metcalf and Eddy, 1991). Easily biodegradable organic matters, elevated dissolved oxygen, and unlimited space support the growth of heterotrophic bacteria which will compete with the growth of nitrifiers. Heterotrophic bacteria typically have a maximum growth rate of about five times more and yield of two to three times more than nitrifiers (Grady and Lim, 1980).

An experiment conducted by Boller et al. (1994) showed that increased water and air flow rates could lead to higher nitrification in biofilters. In addition, Lee and Liu (2002) and Furuichi et al. (2006) showed that degradation of estrogens under anaerobic conditions was significantly lower than under aerobic process. Zhu and Chen (2001) showed that lower nitrification was associated with higher organic loading rate in fixed film biofilters. This trend confirmed that heterotrophs will out compete nitrifiers for available organic carbon. However, the potential impact on nitrifiers became less and less when the carbon concentration became sufficiently high. This can be explained by Monod kinetics where when the nutrient loading and oxygen are adequate, the heterotrophs growth rate increases as the organic loading increases until it reaches a saturation level.

2.7 Removal of E2 by Attached Growth System

Furuichi et al. (2006) studied the treatment of swine wastewater with an up-flow anaerobic sludge blanket (UASB) followed by a trickling filter and found that estrogenic activity was efficiently removed at >97%. The majority of estrogenic activity in this study from E1 and E2 were significantly removed by the trickling filter. The removal efficiencies of specific estrogenic compounds ranged from 44-90%. In addition, significant reduction in estrogenic activity was obtained under aerobic conditions.

Joss et al. (2004) found that E2 removal efficiency was ≥90% in the fixed-bed reactor with a short hydraulic retention time of 35 min. They suggested that HRT seems to have little impact on estrogen removal capability and the long sludge age of the fixed-bed reactor was a likely reason for the good performance. Lorenzen et al. (2004) found a significant reduction in the estrogenic activity and estrogen compounds by aerobic treatment involving trickling filter.

2.8 Measurement of E2 by Gas Chromatography (GC-MS)

To assess the environmental impact of estrogenic compounds, reliable analytical methods are required. In all cases, the method consists of an extraction and pre-concentration step followed by analysis using gas or liquid chromatography (GC or LC) coupled with mass spectrometry (MS).

Sample preparation for estrogens analysis is accomplished by several approaches based on the type of samples. For aqueous phase samples, pretreatment procedures including extraction and concentration the estrogens with solid-phase extraction (SPE). When GC is used as a separation technique, estrogens need to be derivatized prior to

analysis in GC. For derivatization, silylation agents such as MTBSTFA, BSTFA, and acetic anhydride have been employed. The agents could be used alone or in combination with a small amount of catalyzers, such as TMCS.

Normally, the compounds in the water samples are derivatized immediately before analysis. Apparent degradation of silylated reagents was not detected after 2 months of storage at -20°C (Quintana et al., 2004). They also found that samples derivatized at neutral pH value have the advantage of cleaner extracts. pH adjustment is typically unnecessary, but if a yellowish extract was obtained, a clean up step should be mandatory.

2.9 Enhanced Biological Phosphorus Removal (EBPR)

Due to more stringent water and wastewater quality standards, substantial improvements in removing pollutants such as phosphorus from the wastewater must be optimized. The removal of phosphorus by biological means is known as enhanced biological phosphorus removal (EBPR). EBPR utilizes certain heterotrophic bacteria which are capable of sequestering high levels of phosphorus as intracellular poly P as an energy storage material (Mino et al., 1998). Biological methods have been used successfully at municipal and industrial levels to remove this pollutant. In general, the percent removal of phosphorus by conventional wastewater treatment plants usually does not exceed 30% (Sommariva, 1996). The remaining phosphorus has to be removed chemically by adding chemicals such as lime which adds to the fixed costs of the treatment. The principal advantages of biological means are reduced chemical costs and less sludge production as compared to chemical precipitation. Accordingly, EBPR is an accepted economical and environmental sustainable process for the removal of phosphorus from wastewater.

The common parts of an EBPR system include an anaerobic tank followed by an aeration tank as shown in Figure 2.7 below:

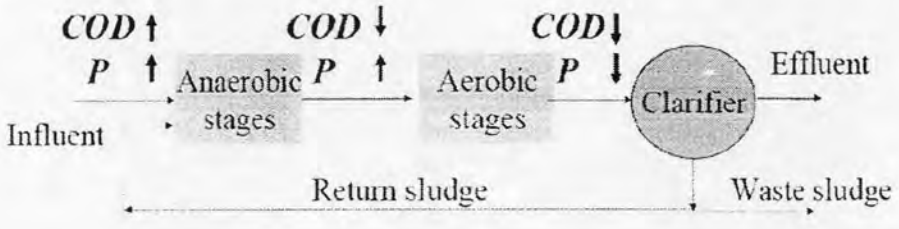


Figure 2.7 Typical A/O process for phosphorus removal

2.9.1 Process Occurring in the Anaerobic Zone

In anaerobic zone, growth of PAOs capable of uptake and storage of volatile fatty acids (VFAs) such as acetate, propionic acid and other fermentation products are enhanced (Fuhs and Chen, 1975). In this condition, no electron acceptors are involved, PAOs are able to sequester the electron and carbon in insoluble intracellular solids such as poly-β-hydroxybutyrate (PHB). During polymerization, the cells require an active chemical in the form of acetyl coenzyme A (HSCoA). Formation of HSCoA is an energy consuming step, and the energy (transported as adenosine triphosphate (ATP)) comes from the hydrolysis of poly P. The hydrolysis of poly P results in the release of phosphate to the cellular P pool. The increase in phosphorus concentration in wastewater under anaerobic condition is taken as an indication of phosphorus release.

2.9.2 Process Occurring in the Aerobic Zone

In aerobic phase, there is ample supply of electron acceptors (O_2) and PAOs used the PHB as a carbon source. The electron storage material, PHB, is hydrolyzed to HSCoA which is then oxidized in the tricarboxylic acid (TCA) cycle. The released electrons carried by nicotinamide adenine dinucleotide ($NADH_2$) are used for ATP synthesis through respiration with O_2 or NO_3^- as electron acceptor. Some of the ATPs generated are invested in the synthesis of poly P. Inorganic phosphate must be imported for poly P synthesis. The uptake of P by the bacteria in aerobic condition is larger than the release of P under anaerobic condition resulting in a net uptake of phosphorus from wastewater. This will generally lead to low concentrations of P in the treated effluent. The simplified biochemical diagram of PAOs is shown in Figure 2.8. The release of phosphate can occur even without acetate uptake and after extended contact time in the anaerobic phase which is termed as "secondary release" (Barnard, 1984) leading to lower phosphorus removal efficiency. Figure 2.9 shows the fate of soluble BOD and orthophosphorus over time in phosphorus removal reactor under anaerobic and aerobic conditions.

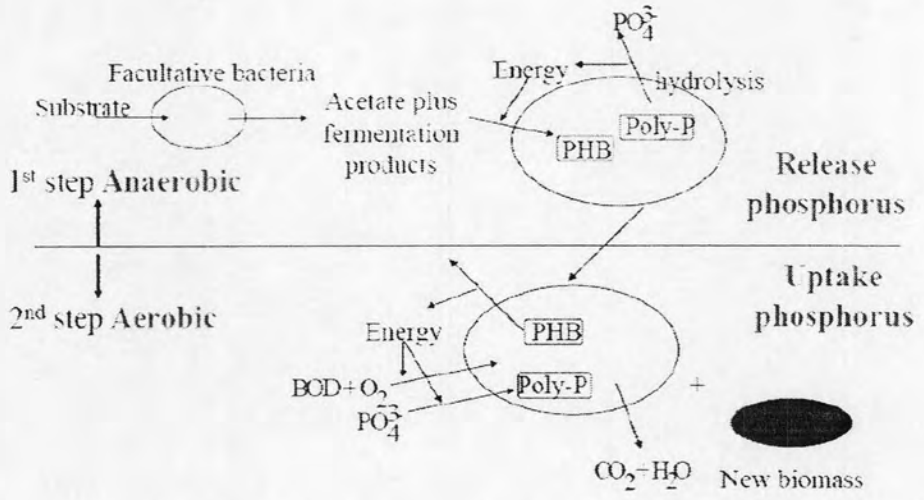


Figure 2.8 Simplified biochemical diagram of PAOs

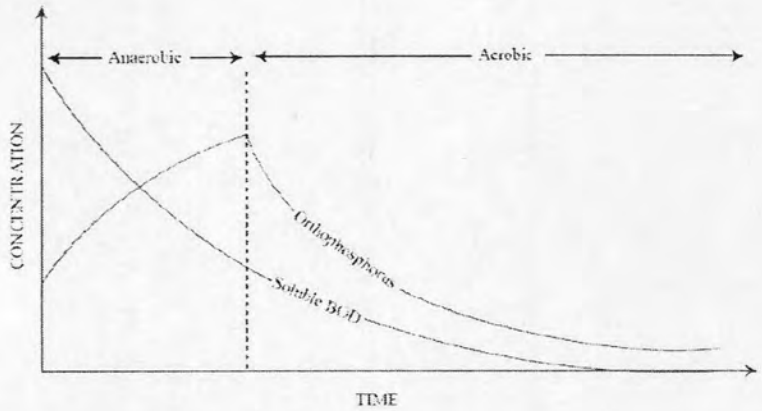


Figure 2.9 Fate of soluble BOD and phosphorus (Metcalf and Eddy, 2003)

The phosphorus fraction of phosphorus accumulating biomass in EBPR system is between 4-8% as opposed to 3% of the biomass in conventional activated sludge system as shown in Table 2.4. This polyphosphate-rich biomass is then separated from the treated water such as sludge wasting to remove the phosphorus from the system.

Table 2.4 Comparison of P content of sludges in different processes. (Sommariva, 1996)

Process	Concentration (%)
Conventional activated sludge	0.03
Bardenpho	0.04-0.07
A/O	0.05-0.08
Phostrip	0.04-0.07

A study conducted by Zeng et al. (2003a) found that the most abundant PAO analysed by FISH was *Candidatus Accumulibacter phosphatis* which was found in 41% of total biomass. Post-FISH staining with DAPI confirmed that poly-P accumulated in the cells. Also, they found that PAOs took up acetate faster than denitrifying phosphorus accumulating organisms (dPAOs) at the rate 2.62 and 1.87 Cmmol/gVSS. This could be partially caused by aggregation of the dPAOs biomass into large granules leading to limited granule surface area. In the PAOs SBR, typical flocculant growth was observed. During normal condition, acetate uptake was accompanied by release of orthophosphate, consumption of glycogen, and production of polyhydroxyalkanoate (PHA) which then subsequently followed by aerobic and anoxic cellular growth, replenishment of intracellular glycogen, and use of poly-P with concomitant decrease in extracellular phosphate. When the anaerobic-anoxic SBR, enriched with dPAOs, was converted to anaerobic-aerobic condition, aerobic uptake of phosphorus occurred immediately, but when the anaerobic-aerobic SBR, enriched for PAOs, exposed to anoxic operation, a 5 hour lag period elapsed before denitrification and phosphorus uptake proceeded. However, phosphorus uptake was at a much lower rate than during normal cycle since PAOs require a lag time to synthesize the necessary enzymes for anoxic EBPR metabolism. An earlier study also reported that *Candidatus Accumulibacter phosphatis* was found, comprising 53% of all the sludge bacteria, and was organized in regular spherical clusters, cocci, at the size of 1.0-1.5 μm (Levantesi et al., 2002).

2.10 Effect of Phosphorus and Nitrogen

When phosphorus and nitrogen are not removed from wastewater, eutrophication is accelerated resulting in the dramatic growth of algae in water resources. The concentration of phosphorus necessary to support an algal bloom is only 0.005 to 0.05 mg P/L (McGhee, 1991). Toxic levels of ammonia as low as 0.01 mg/L have been reported (EPA, 1973).

2.11 Removal of Phosphorus by Attached Growth System

In this study, an alternating anaerobic/aerobic attached growth system was applied for enhanced removal of phosphorus from wastewater. By this anaerobic/aerobic configuration, PAOs are selectively enriched and grown in the process.

Attached growth treatment process is a complex aggregation of microorganisms growing on solid substrate. Microbes are attached to the support packing materials, such

as gravel, plastic, or wood to maintain a high population. Using attached growth, the biomass is fixed. In a typical A/O system, the biomass migrates from one reactor to another and is then returned by recycling the sludge. In an attached growth system, enrichment of PAOs requires alternating conditions. Attached growth systems are attractive in term of cost and space needed, of compactness of system (high biomass concentration, 4-5 times concentrated than activated sludge system (Bacquet et al., 1991), require no clarification), operational flexibility (modularity), and ease of operation (automatic). In biofilter, it is possible to achieve strict anaerobic conditions as compared to activated sludge. And the anaerobic tank can be placed ahead of the activated-sludge aeration tank which can also provide contact with the return activated sludge and influent wastewater.

Goncalves and Rogalla (1992) used fixed biological bed up-flow reactors to remove phosphorus. They reported that PAOs can be grown in fixed biofilm through an alternating two biofilter system and that removal of phosphorus was 93 % using wastewater containing 14.3 mg P/L. Choi et al. (1996) investigated phosphorus removal by combining in a single reactor anaerobic and aerobic conditions using 32.9 mg TP/L of synthetic wastewater. The percent removal efficiencies were 92% for TP. The phosphorus removal efficiency decreased as the N/P ratio increase. A study by Rovatti et al. (1995) investigated the feasibility of excess phosphorus uptake using fluidized bed with synthetic wastewater containing 7.5 mg P/L. They found that the phosphorus uptake was 70 % under strictly anaerobic conditions and showed that phosphorus removal using alternating anaerobic and aerobic conditions is possible.

Tay et al. (2003) showed that the use of a single upflow fixed-bed filter with anaerobic, anoxic and aerobic zones and recirculating effluent of treated and partially treated wastewater can remove nitrogen and phosphorus efficiently, with removal efficiencies at 87 and 76%, respectively, at COD:N:P ratio of 300:5:1. Phosphorus removal yields were higher than 90% by operating a dual system under alternating unaerated/aerated conditions with an HRT of 6 hours by feeding wastewater containing phosphorus levels up to 100 mg/L (Sommariva et al., 1997; Converti et al., 1995).

Shanableh et al. (1997) found that varying the cyclic duration (CD) of the two biofilters had significant effect on phosphorus uptake/release, nitrification, denitrification, and organic removal. Short CD (less than 6 hours) was not suitable for denitrification as opposed to longer CD (12 hours), which permitted denitrification. However, their work was limited to an COD of 50 mg/L.

Pak and Chang (2000) found that the factors affecting phosphorus removal in alternating systems were HRT, organic loading, SS, nitrogen loading, and biomass wasted during backwash. High organic loading may inhibit the microbial phosphorus release/uptake in the system. An increase in COD/TP can negatively affect the phosphorus uptake as opposed to an increase of SS and HRT which can positively affect phosphorus removal. Nitrate and nitrite remaining from aerobic process can inhibit microbial phosphorus release when its condition is switched to anaerobic. A recent work by Broughton et al. (2008) showed that using SBR to treat synthetic wastewater with COD_{VFA} of 800 mg/L (raw COD of 3,000 mg/L) by various readily biodegradable COD (rbCOD), rbCOD:P loading of 25:1, 15:1, and 10:1, achieved phosphorus removal of 99% for rbCOD:P ratios of 25:1 and 15:1, and 82% for a ratio of 10:1. Additionally, as long as DO concentration in the system was above 1 mg O_2/L, the system performance was not affected. Also, pH values below 6.5 was found to significantly affect the removal efficiency (Sedlek, 1991).

EBPR, operated as a dual system under unaerated/aerated conditions can obtain achievable phosphorus removal (Wentzel et al., 1988; Goncalves and Rogalla, 1992) since phosphorus content in the biomass increased to approximately 0.093 mg P/mg VSS as compared to activated sludge which is only 0.03 mg P/mg VSS (Wentzel and Ekama, 1997).

Biofilm systems are another commonly used treatment system for the removal of carbonaceous matter. In biofilm systems, bacteria grow on the surface of a support media. Oxygen is provided by aeration and diffusion through the void spaces. The wastewater flows over the bacteria as shown in Figure 2.10. Nutrients and oxygen are transferred to the fixed water layer, and the end products from the biofilm entered to the bulk liquid after diffusion across the stagnant film. As the bacteria on the filter surface metabolize the waste and reproduce, they will gradually cause an increase in the depth of the slime layer. The biomass can be very dense and vary in density and depth. With thickening of the biological layer, the bacteria in the interior layers may find themselves in a nutrient-limited situation since the organic matter and oxygen are utilized near the surface. Eventually the organisms on the inside die and become detached from the media, causing a portion of the "slime" layer to "slough off" and be carried from the filter by the waste flow. The solids in the filter effluent are removed from the flow.

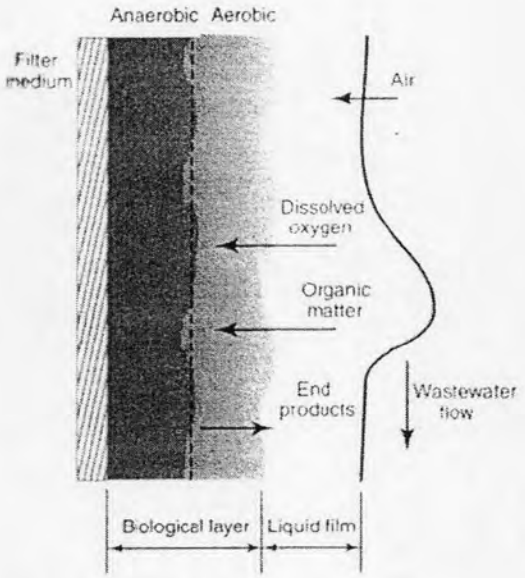


Figure 2.10 Details of attached growth biofilm (Metcalf and Eddy, 2003).

2.12 Simultaneous Nitrification, Denitrification, and Phosphorus Removal

Biological COD, nitrogen, and phosphorus removal are achieved by manipulating three biochemical reactions under which three groups of organisms: ordinary heterotrophic organisms (OHOs); PAOs; and autotrophic organisms (AOs), nitrifiers, can be favored to perform the nitrification, denitrification, and EBPR processes.

It is possible to achieve the removal of both nutrients simultaneously and there are several processes that have been developed to achieve this end. For example, processes include, Modified Bardenpho, UCT process, Phostrip II process, and A2/O process. Figure 2.11 shows the flow schematic of a typical three-stage A2/O process. Each stage is divided into equally sized, completely mixed compartments. Mixed liquor is recycled from the end of the aerobic stage to the anoxic stage for denitrification. However, both N and P removal processes require COD, which is typically limited in the domestic wastewater.

Recirculation is generally practiced since it provides a more uniform hydraulic and organic load, increases the mass of biological solids in the system, continuously reseeds the filter with sloughed bacteria, dilutes the influent with better quality water, and thins the biological slime layer.

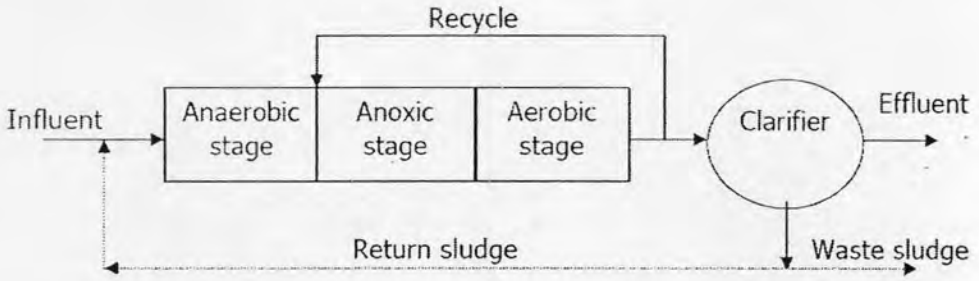


Figure 2.11 A2/O process for phosphorus removal

2.12.1 Process Occurring in the Anoxic Zone

Under anoxic condition where no oxygen is present but nitrate is available, phosphorus uptake by denitrifying phosphorus accumulating organisms (dPAOs) may also occur because dPAOs can perform the same metabolism under aerobic conditions. However, Hu et al. (2002) concluded that the growth yield of PAO under anoxic conditions was reduced to about 70% of that under aerobic conditions implying that dPAOs have a significantly lower EBPR performance and can use rbCOD in influent less efficiently than PAOs. Their results also indicated that the main factor influencing the occurrence of dPAOs is nitrate loading. If nitrate loading exceeds the denitrification potential of OHOs, PAOs would use the excess nitrate in the system. In practice, dPAOs capability can achieve EBPR and denitrification at the same time since dPAOs can utilize nitrate as electron acceptor.

Compared with PAOs, dPAOs are 40% less efficient in generating energy, and thus have a 20% to 30% lower cell yield (Kuba et al., 1994). Therefore, the use of dPAOs in biological nutrient removal (BNR) systems is highly beneficial in terms of lower COD demand, reduced aeration cost, and less sludge production.

Successful simultaneous nitrification, denitrification, and phosphorus removal have been reported in SBR operating in alternating anaerobic/aerobic mode with low dissolved oxygen concentration of 0.5 mg O_2/L (Zeng et al., 2003b). In this investigation, phosphorus in the effluent was reduced to lower than 0.5 mg/L and nitrogen was removed via nitrite, not nitrate because nitrous oxide (N_2O) was found as the major final product of denitrification rather than nitrogen gas (N_2). Additionally, denitrifying glycogen accumulating organisms (dGAOs) were responsible for the denitrification activity, not denitrifying phosphorus accumulating organisms (dPAOs).

A study by Hamamoto et al. (1997) using anaerobic-aerobic conditions in a single reactor to remove nitrogen and phosphorus simultaneously showed that the average nitrogen and phosphorus removal in the full-scale plant were 96 and 93%, respectively, for an average 7.3 mg TP/L. Kerrn-Jespersen et al. (1994) obtained phosphorus removal in a fixed-film reactor by alternating anaerobic-anoxic conditions. However, the coexistence of different microorganisms presented a competitive relationships for oxygen between PAOs and nitrifying bacteria and the phosphorus uptake was 2 mg PO4-P/mg NO3-N. Puznava et al. (2001) found that at low dissolved oxygen concentration from 0.5 to 3 mg O_2/L, the biofilm in biological aerated filter designed for nitrogen removal was not fully penetrated by oxygen.

Garzón-Zúñiga and González-Martínez (1996) conducted simultaneous nitrogen and phosphorus removal experiments in SBRs with an operation phase of 10 hours of anaerobic, 20 hours of aerobic, 3 hours of anoxic, and 3 hours of aerobic. They found their removal of COD, phosphates, and ammonia nitrogen were 89±1%, 75±15%, 87±10%, respectively.

Summary

Many wastewater systems are currently implementing phosphorus and nitrogen removal by using several tanks with the anaerobic and aerobic conditions. Attached growth filters have been developed which can retain high phosphorus accumulating biomass concentration over the suspended growth process. In addition, no return sludge is needed, the microbes are immobilized, it can be compact with a small footprint which will be useful for small wastewater systems. Although, the alternating filters are optimized for phosphorus and nitrogen removal, they are not optimized for removal of EDCs, such as estrogens which are becoming increasingly of concern. The longer HRT (also long SRT) has been known to remove E2 from wastewater.

Not much is known about the CD and the various COD, P, N loadings on phosphorus and nitrogen removal for alternating biofilters and the fate of estrogenic compounds in attached growth systems and in anaerobic systems. Although information is available on the degradation of estrogens under aerobic conditions in activated sludge plants, not much is available for anaerobic conditions. For alternating filters that are being developed to remove phosphorus, there is a possibility that estrogenic compounds may not be treated in one of the cycles.