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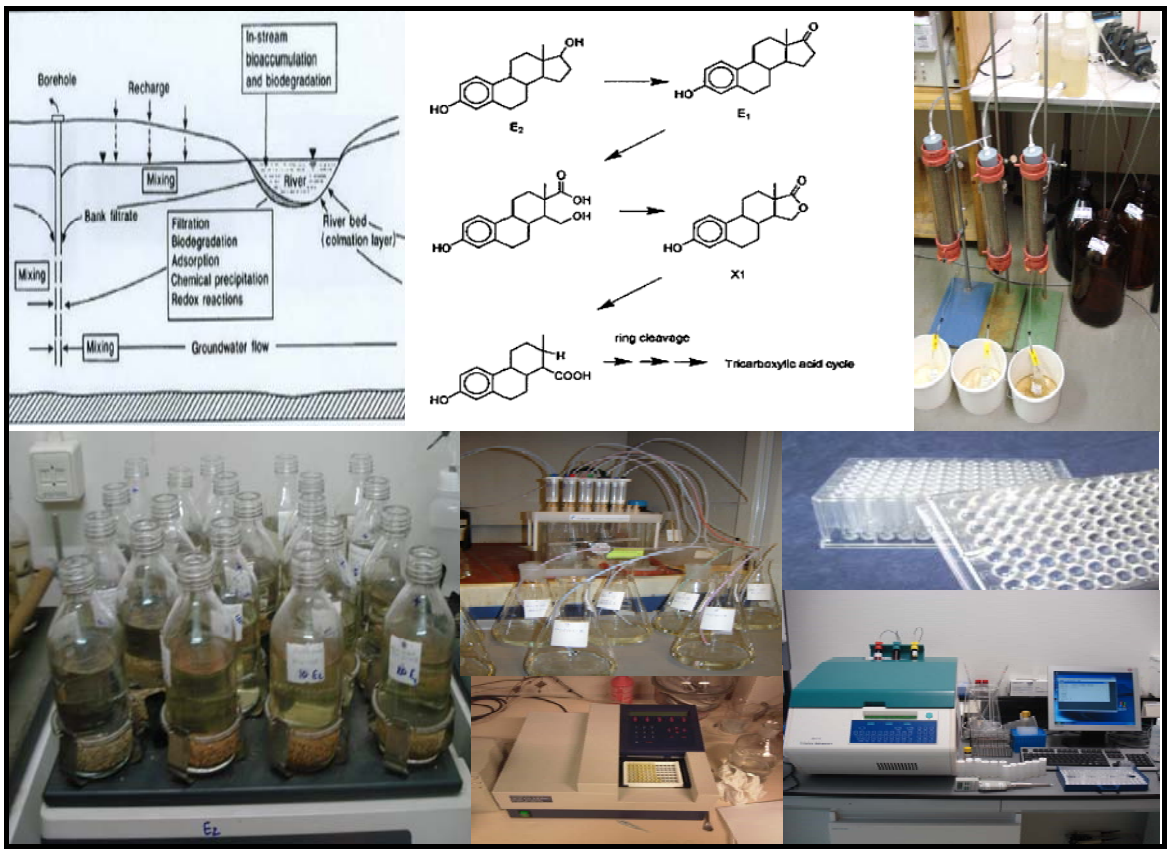


# Endocrine Disrupting Compounds Removal During Riverbank Filtration

Sharada Devkota

MSc Thesis MWI - 2008 - 28
May 2008

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# Endocrine Disrupting Compounds Removal During Riverbank Filtration

Master of Science Thesis
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This research is done for the partial fulfilment of requirements for the Master of Science degree at the UNESCO-IHE Institute for Water Education, Delft, the Netherlands

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The findings, interpretations and conclusions expressed in this study do neither necessarily reflect the views of the UNESCO-IHE Institute for Water Education, nor of the individual members of the MSc committee, nor of their respective employers

# Abstract

Riverbank filtration (RBF) is the natural process of water treatment. It is a low cost and robust technology and relevant to both developed and developing countries. It is well known technology in USA and Europe where it has been utilized for decades. In RBF process filtration, biodegradation, adsorption and dilution are the main mechanisms.

Nowadays, large varieties of Endocrine Disrupting Compounds (EDCs) are present in raw water resources where the raw water is strongly influenced by wastewater effluent. Among many EDCs, estrogens such as estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) showed the most estrogenic activity, than alkylphenols (APs) and their ethoxylates (APEOs). RBF is a promising technology for pre-treatment of raw water prior to conventional water treatment which reduces the cost of chemicals. Therefore, this study was focused on analysis of estrogen removal during RBF. Better understanding of EDCs removal helps in rational design of RBF systems.

Laboratory-scale batch and soil column experiments were conducted to study the removal of estrogens during RBF. Mixture of Delft canal water (DCW) and secondary effluent (SE) in 1:1 ratio was used as influent. Silica sand of size 0.8-1.25 mm diameter was used as a filter media. Mainly estrogens compounds were quantified by using enzyme linked immunosorbent assay (ELISA). Solid phase extraction (SPE) was applied for sample pre-treatment. Similarly, fluorescence excitation-emission matrix (F-EEM) was used for kinetic fate study.

Abiotic batch experiment was conducted to determine the optimum concentration of sodium azide ($NaN_{3)}$ to inactivate the micro-organisms and it was found that 2 mM $NaN_3$ was appropriate without any interference with ripened layer of the sand.

Batch adsorption experiments were conducted to establish isotherms of E2 and EE2. It was found that, in ripened sand removal of E2 was 99.9 % isotherms was not obtained but in the case of EE2 adsorption capacity and intensity was 0.28 µg/g and 0.67. Similarly, in clean sand adsorption capacity of E2 and EE2 was 0.20 µg/g and 0.21 µg/g and corresponding intensity was 0.95 and 0.63.

Batch study for determination of factors affecting estrogen removal showed that the maximum removal was 99.9 % and 95.8 % for E2 and EE2 respectively. Adsorption and biodegradation played the main role of removal. Biotic and oxic conditions and organic material content in soil aquifer were main favorable factors for removal.

EE2 removal in soil column with tap water was 25.9 % to 64.1 %, with mixture of DCW & SE (abiotic) was 46.0 % to 66.7 % and with mixture of DCW & SE was 49.0 % to 87.0 % with variation in flow rate was 0.4 ± 0.15 m/d. Removal efficiency depended on flow rate, organic material and microbial activity.

***Keywords*****:** *Riverbank Filtration, Endocrine Disrupting Compounds, Estrogens, Removal Efficiency, Biodegradation, Adsorption*

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# Table of contents

# List of tables

# List of figures

# List of acronyms and abbreviations

| | |
|---|---|
| APs | Alkylphenols |
| APEOs | Alkylphenol polyethoxylates |
| ATP | Adenosine triphosphate |
| ACTW | Autoclaved tap water |
| ACDW | Autoclaved demi water |
| AC (DCW + SE) | Autoclaved delft canal water and secondary effluent |
| DCW | Delft canal water |
| DOC | Dissolved organic carbon |
| EDCs | Endocrine disrupting compounds |
| EU | European Union |
| E1 | Estrone |
| E2 | 17β-estradiol |
| EE2 | 17α-ethinylestradiol |
| EC | Electrical Conductivity |
| EEM | Excitation-emission matrix |
| ELISA | Enzyme-linked immunosorbent assay |
| F-EEM | Fluorescence excitation-emission matrix |
| FOG | Fat, oil, and grease |
| FS | Fresh sand |
| GAC | Granular activated carbon |
| GUL | Glucuronide |
| HLR | Hydraulic loading rate |
| HPSEC | High performance size exclusion chromatography |
| HRT | Hydraulic residence time |
| MATLAB | MATrix LABoratory |
| MF | Microfiltration |
| MQ | Milli - Q water |
| NOM | Natural organic matter |
| PAHs | Polyaromatic hydrocarbons |
| PCBs | Polychlorinated biphenlys |
| PAC | Powdered activated carbon |
| RBF | Riverbank filtration |
| RS | Ripened sand |
| SC1 | Soil column 1 |
| SC2 | Soil column 2 |
| SC3 | Soil column 3 |
| SPE | Solid phase extraction |
| SUL | Sulfate |
| SAT | Soil aquifer treatment |
| SRT | Solid retention time |
| SE | Secondary effluent |
| SC | Soil column |
| THMs | Trihalomethanes |
| TOC | Total organic carbon |

| | |
|---|---|
| TAC | Tricarboxylic acid |
| UF | Ultrafiltration |
| UV | Ultraviolet |
| UVA | Ultraviolet absorbance |

# 1. INTRODUCTION

## 1.1. Background

Increasing rate of endocrine disrupting compounds (EDCs) especially estrogens in surface water source and mechanism of removal during riverbank filtration (RBF) is the issue of concern. Population in the world is increasing rapidly specially in urban areas. Small cities are growing at faster rate and converted into the mega cities. The current urban population of 2.8 billion will increase to 3.8 billion in 2015 and to 4.5 billion in 2025 (WHO/UNICEF, 2001). Hence, water demand on those cities is increasing rapidly. Consequently, wastewater production and water resource pollution is increasing rapidly. One reason of water resource pollution is the disposal of insufficiently treated or untreated municipal wastewater and other reason is influence of agriculture runoff.

Nowadays, water companies have no alternative source for the production of potable drinking water. Therefore, they are attracted to reuse treated domestic wastewater for ground water recharge to augment the drinking water production which enhances the capabilities of existing water treatment infrastructures.

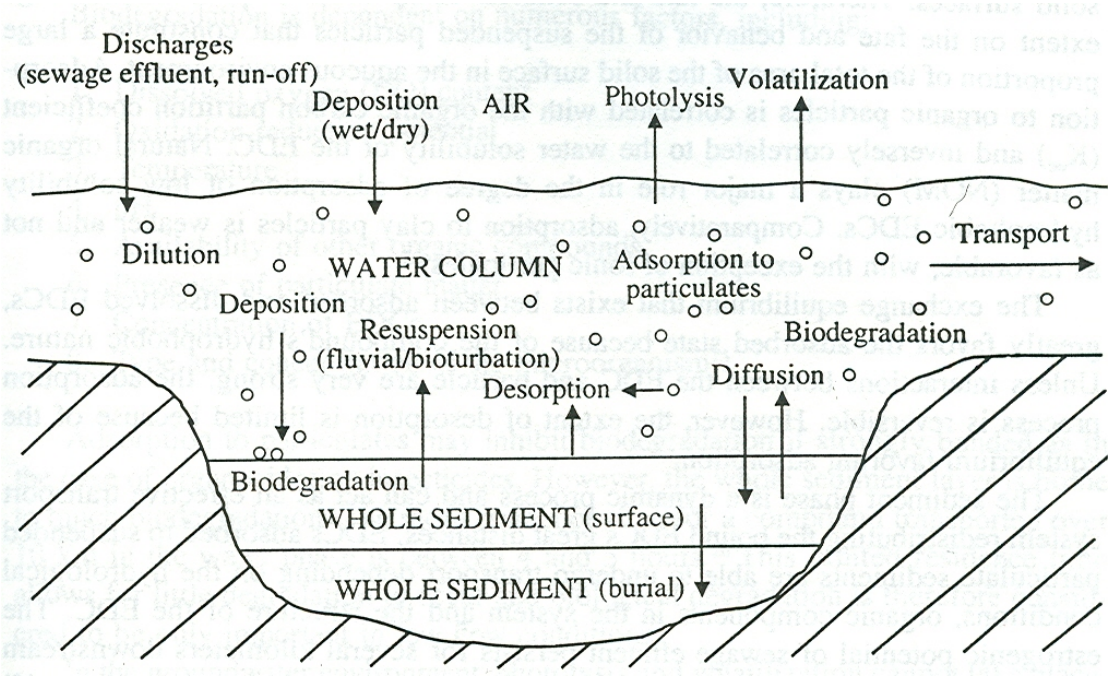
EDCs are focus of the current environmental concern; they can cause adverse health effect in an intact organism, or its progeny, subsequent to endocrine function. EDCs are present in wastewater effluents, agricultural runoff, and feminization of male aquatic species in water bodies. Following are the effect of EDCs present in the surface water:

- harmful effects have been observed on reproduction, growth and development in certain species of wildlife and
- Increases in some human reproductive disorders and some cancers which could be related to disturbance of the endocrine system (Birkett and Lister, 2003).

Although, there is no substantial evidence, it is believed that EDCs exposure could be harmful to humans and could be a reason for some of the increases in human disorders such as breast cancer, uterine cancer, testicular cancer, prostate cancer or thyroid cancer. Estrogen compounds are EDCs, which is especially available in polluted surface water. Generally, estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are detected on the surface water resources and secondary as well as tertiary treated municipal wastewater effluent. However, certain treatment process have been shown for better removal of these compounds which is soil aquifer treatment (SAT) (Mansell *et al.*, 2004).

Furthermore, most of EDCs are often concentrated in suspended solids or sediment than in the aqueous phase, and the intrusion of EDCs into ground water is minimized by its characteristics so the ground water is less influenced by the EDCs compared to surface waters. However, sediments can be dynamic under seasonal variations and enabling bound EDCs for long distances into an aquifer. Therefore, the degradation kinetics of bound EDCs was investigated and found that it is dependent on numerous factors: redox

conditions, temperature, dissolved as well as suspended organic matter, travel distance and pH. Figure 1-1 shows the sources and behaviour of EDCs in the receiving aquatic environment.




*Source: (Gomes and Lester, 2003)*

Figure 1-1. Sources and behaviour of EDCs in the receiving aquatic environment

RBF technology provides the sustainable treatment and pre-treatment service both in develop and developing world (Amy, 2006). In this technology alluvial sediments is effective filter and remove physical, chemical and biological contaminants (McClain, 2004). It can provide the opportunity for a water treatment system avoiding the use of chemicals. Finally, riverbank filtration is the low cost and robust solution of drinking water treatment. By this method, many specific compounds such as estrogen compound can also be removed effectively. Hence, this technology has been practiced more than 150 years.

## 1.2. Problem of statement

Population in the world is increase rapidly. Consequently, water demand and number of utilities are also increasing at a faster rate in developing countries. Hence, water utilities are currently obtaining drinking water from the source under the influence of agriculture run-off, industrial discharge from point sources or discharge from municipal wastewater treatment facilities. So, increasing water demand and lack of alternative sources utilities are attracted to reuse of treated domestic wastewater for groundwater recharge. They argue the drinking water supply with the portable reuse of this water. However, there is a concern that organic pollutants such as estrogen compounds might remain on those source.

EDCs are considered to be any compound natural or anthropogenic, which interfere with endocrine system function (Iguchi T *et al.*, 2001; Silva E *et al.*, 2002). Among these compounds, the natural estrogens estrone and 17β-estradiol and synthetic estrogens 17α-ethinylestradiol is the endogenous EDCs observed in aquatic environments and possess estrogenic potency 10,000 to 100,000 times higher than exogenous EDCs such as organochlorine aromatic compounds (Gomes and Lester, 2003). Hence, presence of estrogenic EDCs in drinking water sources is the issue of concern.

Furthermore, estrogenic EDCs can remain in wastewater effluents which pollute the surface water source. Consequently, it can adversely affect on drinking water quality. Hence, this indicates the possibility of development of cancer in humans (Iguchi T *et al.*, 2001) and cause a raised concern over the exposure of humans to increased levels of hormones in drinking water.

The major concern for water utilities is the source of drinking water. Therefore, there is a possibility for residual of EDCs in drinking water sources that could cause impacts on human health. Hence, there is a need to understand the biodegradability, sorption and transport of estrogen compounds (E1, E2 and EE2) during RBF. Therefore, understanding the removal of those estrogens during RBF may help to provide a provision against the estrogens contamination and the development of a model will help to predict estrogen compounds removal.

Finally, many studies have shown that RBF is a promising technology for pre-treatment of raw water prior to conventional water treatment by removing natural as well as anthropogenic contaminants such as EDCs and reducing the cost of chemicals. However, the performance of RBF in removing those contaminants depends on several factors including hydraulic conductivity, temperature and travel time. But, there is a lack of fundamental understanding of degradation of bulk organic matter and trace organic compounds under different redox conditions during RBF.

## 1.3. Goal and objectives

The main goal of this MSc study is to evaluate the fate of estrogen compounds during the riverbank filtration.

The specific objectives to achieve this goal are:

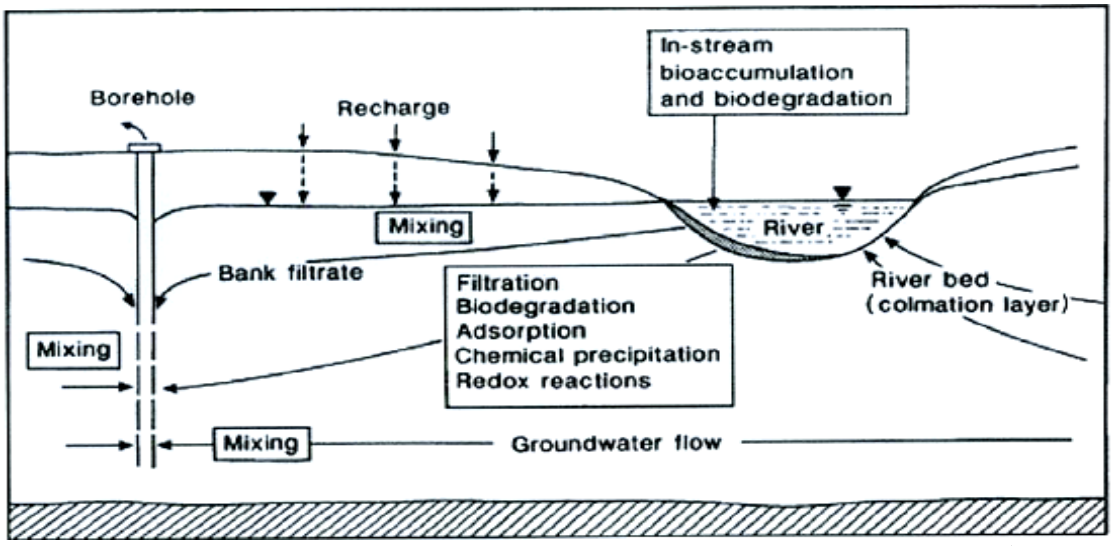
(i) To investigate the mechanism of removal of estrogen compounds (E1, E2 and EE2) during RBF under the biotic and abiotic conditions.
(ii) To compare the removal of E1, E2 and EE2 during different redox condition (oxic and anoxic)
(iii) To determine the kinetics of removal of E2 and EE2 during RBF by conducting soil column and batch studies

(iv) To determine the relative performance of biodegradation and adsorption process for the removal of three different types of estrogens compounds namely as E1, E2 and EE2.

# 2. LITERATURE REVIEW

## 2.1. Riverbank filtration system (RBF)

RBF is a process for producing drinking water. It acquires advantage of existing geologic formations adjacent to the water body to filter drinking water. River water is brought to flow through riverbed soils to pumping wells located on the banks of the river. During the passage of this water through the riverbed and aquifer, dissolved and suspended contaminants as well as pathogens are removed due to a combination of physical, chemical and biological processes (Amy, 2006; Huisman, 1989). Mixing, biodegradation and sorption are the main beneficial attenuation processes. These processes take place within two main zones: the biologically active colmation layer, where intensive degradation and adsorption processes occur within a short residence time; and along the main flow path between the river and abstraction borehole where degradation rates and sorption capacities are lower and mixing processes are greater (Hiscock and Grischek, 2002). Figure 2-1 shows the different activities occur in RBF.




*Source:(Amy, 2006)*

Figure 2-1. Schematic diagram of process affecting water quality during RBF

## 2.2. Factors affecting RBF

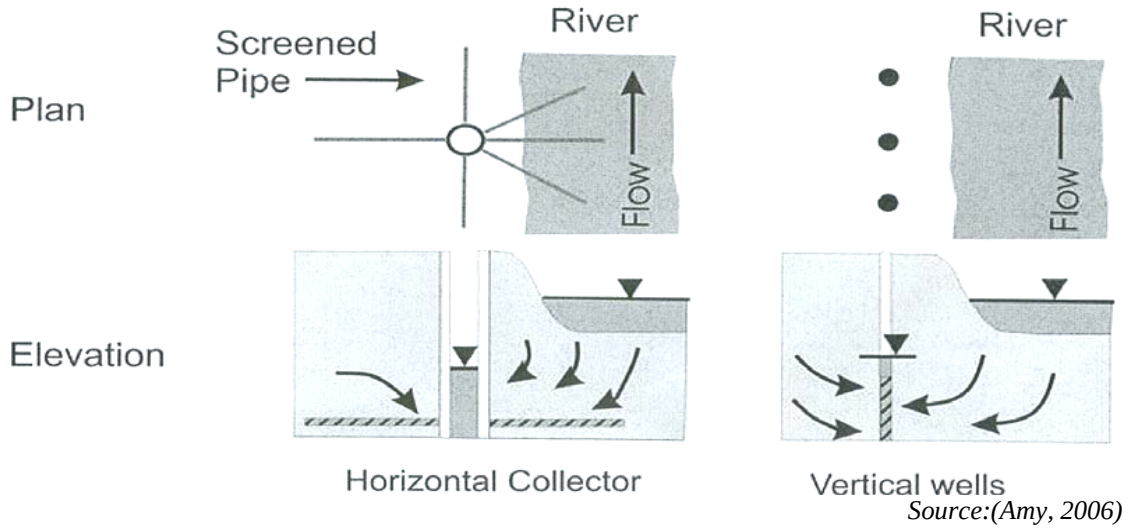
Main factors that can affect the quality and quantity of filtered water during RBF process are mentioned as below:

**a) Raw water quality**

Available raw water quality directly affects the quantity and quality of filtrate water through the riverbank. Heavily polluted river water requires higher travel length, presence of organic matter and more residence time.

**b) Well types and distance from the river**

In the RBF process filtration, biodegradation, adsorption, chemical precipitation and redox reactions occur between zone of river and production well. In the case of horizontal well, well is directed towards the river hence traveling distance is less. Consequently, those mechanisms does not complete adequately due to the residence time. However, in the case of vertical well residence time inside the aquifer is enough for those mechanisms. Consequently, vertical well is used for qualitative production and horizontal well is used for quantitative production. Figure 1-1 shows the illustration of the two types of bank filtration wells.




*Source:(Amy, 2006)*

Figure 2-2. Schematic representations of horizontal and vertical wells

**c) Variation of water level in rivers**

The direction of natural water flow depends on the relative water levels of ground water and river water. During low flow period, rivers can have water levels below the ground water table; in this case, water flows towards the river and percolates into the flowing wave of the river. In the case of high flows, however, river water infiltrates vice versa into the aquifer. Thus, the flow direction of the groundwater is variable under natural conditions even without any anthropogenic extraction of groundwater.

**d) Local ground water table**

Mixing with ground water (dilution) is one of the processes that improve the water quality during RBF system operation. The efficiency of this process depends on the elevation of ground water table. Table 2-1 shows the results of a study made on RBF sites in Reston, Virginia to investigate the effect of dilution process on water quality improvement in riverbank filtrate.

Table 2-1. Water quality improvement during riverbank filtration

| Parameters | % total removal | % removal due to dilution | % removal due to subsurface filtration |
|---|---|---|---|
| Turbidity | 87 | 10 | 77 |
| DOC | 63 | 29 | 34 |

*Source: (Partinoudi et al., 2003)*

**e) Site hydrogeology**

The local hydrogeology of a site affects the quality of surface water. In alluvial aquifers with coarse-grained materials, a significant amount of dissolved chemicals can be stored when the river water level is high. However, the water and associated chemicals drain back to the river when the floods in the river recede. The sediments present on the banks and riverbed affect the infiltration of water in to the aquifer. The bank material plays an important role during flood seasons. If the sediments on the banks and beds are fine-grained and thick, they can retard the flow of river water into the aquifer. Coarse-grained sediments at the river-aquifer interface may not retard the migration of dissolved chemicals and pathogens to the same degree as thick, low permeability sediments.

**f) Schmutzdecke and riverbed clogging**

A layer/bio-film formed at the bed and bank of the river is known as "Schmutzdecke". In RBF water flows through a bio-film formed at the river bed/bank - water interface. In general, it consists of numerous forms of life including algae, plankton, diatoms, protozoa, and bacteria. The schmutzdecke layer is a place where inert suspended particles can be mechanically strained, organic matter and nitrogenous compounds broken down, and microorganisms entrapped (Huisman, 1989). Riverbed clogging is one of the issues of research on the performance of RBF system. Clogging reduces aquifer recharge rates and may therefore be perceived as a problem, which retards the quantitative production.

## 2.3. Removal of trace organic matter during RBF

In industrialized and urban areas, surface water resources are exposed to anthropogenic environmental impacts and contaminated with organic trace compounds. River bank filtration is the natural process of water treatment, which is well-established technique in Europe. It is a multi-barrier system. During this process physical, chemical and biological changes occur hence improve the water quality significantly.

During the removal of organic and trace organic micro-pollutants through soil aquifer biotic and abiotic condition could occur. Abiotic process like sorption, hydrolysis or chemical degradation can reduce the amount of certain organic substances. In this removal process organic substances are fully hydrolyzed to $CO_2$, $H_2O$ and mineral salt.

RBF is an excellent option for removing the anthropogenic organic micro pollutants in reduced condition. Some pollutants are more degradable under anaerobic condition than under aerobic condition and vice versa. Removal of micro-pollutants through RBF depends on residence time of infiltration, redox environment of aquifer, temperature of water and organic material present in aquifer (Grunheid et al., 2005).

## 2.4. Endocrine disrupting compounds

The endocrine system is a set of glands. They produce hormones, which help to guide the development, growth, reproduction, and behavior of animals and humans. Some hormones are also released from parts of the body that are not glands, such as the stomach, intestines or nerve cells, and act closer to where they are released (IPCS, 2002). Gland synthesis hormones, which are then transported via blood stream to target organs where, receptor remain. Generally, hormones molecules are short lived in body due to metabolic clearance mechanism. But, due to the presence of the exogenous substance bioaccumulation is occurs instead of metabolic clearance mechanism. This bioaccumulation process is known as the endocrine disruption and compound formed due to this bioaccumulation is known as endocrine disrupting compound (Birkett and Lister, 2003).

Endocrine disrupting compounds (EDCs) is defined as an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects. Many human-made compounds have been implicated in disrupting endocrine function in animals and humans life worldwide, including agricultural and industrial chemicals (Birkett and Lister, 2003). Another definition of endocrine disrupting compound or an endocrine disrupter is an exogenous agent that interferes with the synthesis, production, transport, binding action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and behavior (Birkett and Lister, 2003).

Chemicals that exhibit an endocrine disrupting property is known as endocrine disrupting substance (Birkett and Lister, 2003) e.g. alkylphenols (APs), alkylphenol polyethoxylates (APEOs), polyaromatic hydrocarbons (PAHs), polychlorinated biphenlys (PCBs), phthalates, bisphenol-A, polybrominated flame retardants, dioxins, furans, herbicides, pesticides, and steroid hormones (e.g., estrogens) etc. European Union (EU) identify and reported that overall 118 endocrine disrupters; out of these 12 have been assigned for priority for depth study. These are carbon disulfide, o-phenylphenol, tetrabrominated diphenyl ether, 4-chloro-3-metahylphenol, 4-octylphenol, estrone, ethinylestradiol, and estradiol etc. which are highly obtained in surface water source and can affect adversely in human health. Mostly endocrine disrupter belongs to the pesticide category and metals (cadmium, mercury). Following are the specific group of EDCs:

- Steroid compounds (e.g., estrogens)
- Surfactants (e.g., nonylphenol and its ethoxylates)
- Pesticides, herbicides, fungicides
- Polyaromatic compounds (e.g., PAHs, PCBs, brominated flame retardants)
- Organic oxygen compounds (phthalates, bisphenol A)

The potential presence of EDCs in river water is a topic of increasing interest and the potential transport of EDCs into riverbank-filtered water presents a major concern (Ray et al., 2002). EDCs are present in industrial, domestic wastewater, agricultural runoff, and feminization of male aquatic species in water bodies. Those are sources of surface water pollution. The presence of EDCs in the environment raise concerns because:

harmful effects have been observed on reproduction, growth and development in certain species of wildlife there are increases in some human reproductive disorders and some cancers which could be related to disturbance of the endocrine system

Although, there is no substantial evidence, it is believed that EDCs exposure could be harmful to humans and could be a reason for some of the increases in human disorders such as breast cancer, uterine cancer, testicular cancer, prostate cancer or thyroid cancer. EDCs can occur mostly by drinking water, and chemicals used in consumer products (Birkett and Lister, 2003). In conclusion, RBF is a one of the cheapest and natural water treatment technique which can remove almost all pollutant such natural as well as anthropogenic.

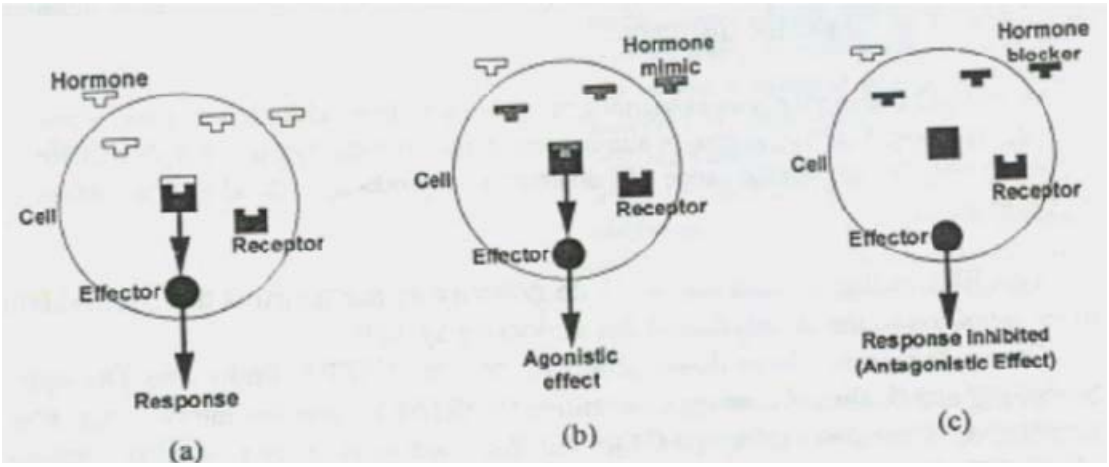
## 2.5. Concept of estrogen compounds

Estrogens are defined as a "family steroid hormones that regulate and maintain female sexual development and reproductive function". However, some scientists have broadened the definition of estrogens to include any compound that stimulate tissue growth by:

- Promoting cell production in the female sex organs
- Promoting hypertrophy or increase a cell's size, such as occurs in female breast and male muscle tissue during youth
- Initiating synthesis of specific proteins

Only sex steroid hormones (Natural hormones produced via biological processes) should classified as estrogens while other substances mimicking their estrogenic effect are classified as environmental estrogens.

Many EDCs compete with estradiol for estrogen receptor, which exert a feminizing effect on endocrine system (get natural response). The substances that mimic feminizing effect are known as estrogenic. An antiestrogenic substances such as tamoxifen, inhibits the biological action of estrogens by binding to and thus inactivating the estrogen receptor of target tissue. At the same time agonistic and antagonistic effect, occur on the cells. Detail of this process as shown in Figure 2-3.




*Source: (Birkett and Lister, 2003).*

Figure 2-3. Endocrine disrupting processes

Compounds release from human and livestock in the form of waste product. Natural steroidal estrogen hormones are most potent endocrine disrupter. It is available mostly in wastewater or source of surface water fed by the wastewater and agriculture runoff. Estrogen compounds available in the surface water are in nanograms per litter level e.g. E1, E2, EE2 and estriol (E3). E1 and E2 are naturally occurring; however, EE2 is synthetic compounds from contraceptive sources. Estrogen has property of endocrine disrupting chemicals (EDCs). Human and animal waste-bone steroidal hormone called natural steroidal estrogen. It is belonging to the endogenous steroidal EDCs and synthetic chemical such as organochlorine aromatic compounds belong to exogenous EDCs. Endogenous compound has estrogenic potency 10,000 to 100,000 times higher than exogenous EDCs (Birkett and Lister, 2003).

Natural steroidal estrogens (also known as the C18 steroidal group) share the same tetra cyclic molecular framework, which is composed of the four rings; a phenol, two cyclo hexanes and a cyclo-pentane. The difference in compound within the C18 group lies in the configuration of the D-ring at positions C16 and C17 as shown in the Figure 2-4. For example, estrone has a carbonyl group on C17, estradiol has hydroxyl group on C17 and estriol has two alcohol groups on C16 and C17. The C17 hydroxyl group of estradiol can either point downward of the molecular plain forming the 17α- compound or upward forming the E2 compound. E2 is using industrially for the synthesis of ethinylestradiol (EE2) the commonly used active ingredient for oral contraceptive pills. Free estrogens also known as un-conjugated estrogens are moderately hydrophobic and poorly soluble in water. Conjugated estrogens are formed by esterification of free estrogens by glucuronide (GLU) and sulfate (SUL) group at the position of C17. Conjugated estrogens do not posses biological activity and dissolve in aqueous solution at much larger quantity than their un-conjugated counterparts. This is due to the substitution of the original hydroxyl group (Khanal et al., 2006)

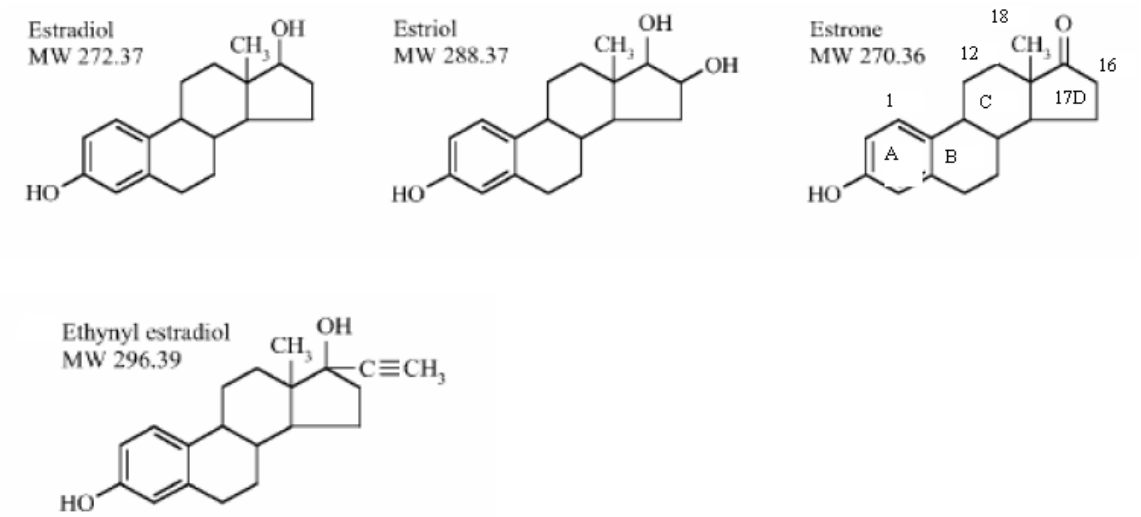



Figure 2-4. The chemical structures of the different estrogens compound

*Source:(López de Alda et al., 2001)*

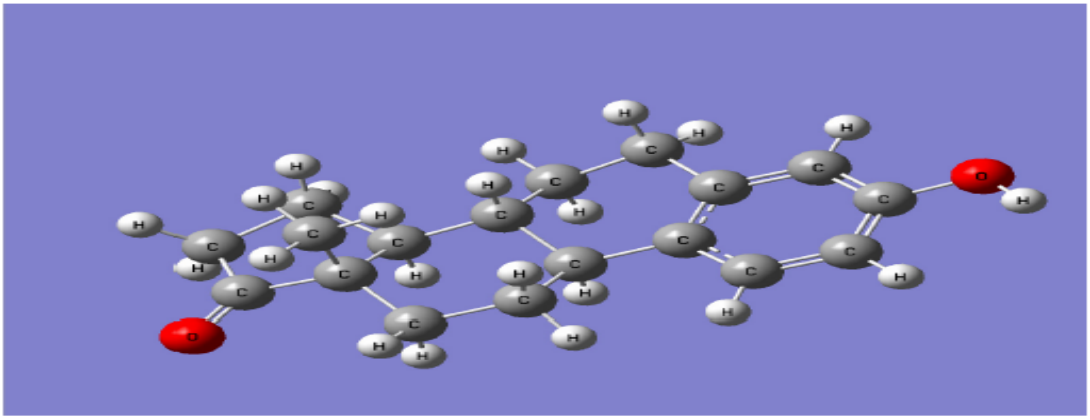


Figure 2-5. The structure of estrogen molecules

*Source:(López de Alda et al., 2001)*

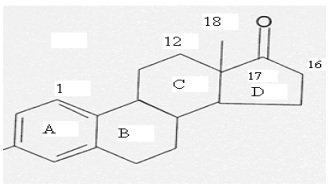
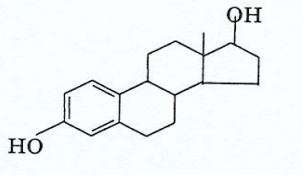
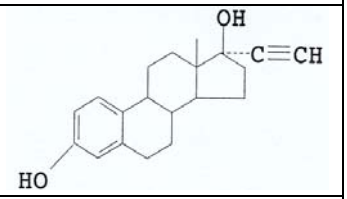
Many EDCs (Conjugated estrogens/synthetic) have a high sorption potential and low water solubility and volatility. The water solubility of steroid estrogens range from 0.3 to approximately 13 mg/L. Natural steroid (free estrogens) has the highest solubility. The synthetic steroids have the highest octanol-water partitioning coefficients (logKow) and can be expected to adsorb to solids. Hence, it can be removed by RBF.

### 2.5.1. Description about estrone, 17β-estradiol and 17α-ethinylestradiol

Natural steroidal estrogens have same molecular framework, which is composed of the four rings; a phenol, two cyclo hexanes and a cyclo-pentane. The difference in compound lies in the only in D-ring configuration. During RBF, redox conditions significantly influence the degradation kinetics of organic compounds and trace compounds (Grunheid et al., 2005). In this process, E2 was transformed to E1 under

different redox conditions but the transformation rate varied according to each electron acceptor such as organic matter and properties of the estrogens compound (Czajka and Londry, 2006). Detail property of the estrogens compound is shown in the Table 2-2 below:

Table 2-2. Physiochemical properties of steroid estrogens

| **Properties of estrogens** | **Estrone (E1)** | **17β-Estradiol (E2)** | **17α-Ethinylestradiol (EE2)** |
|---|---|---|---|
| Structure of steroidal hormones |  |  |  |
| It consists of four ring a phenol two cyclohexanes and a cyclopentane | √ | √ | √ |
| Chemical formula | $C_{18}H_{22}O_2$ | $C_{18}H_{24}O_2$ | $C_{20}H_{24}O_2$ |
| Vapor pressure (KPa) | 0.1 – 0.2 | 1 | 1 - 2 |
| Solubility in water at 20ºC | 0.8 – 12.4 | 5.4 – 13.3 | 3.4 – 4 |
| Octanol-water partitioning coefficients (Log $k_{ow}$) | 3.43 | 3.43 | 4.15 |
| Molecular weight g/mol | 270.37 | 272.38 | 296.4 |
| Natural estradiol estrogen | √ | √ | (synthetic) |
| carbonyl group | $C_{17}$ | $C_{17}$ | $C_{17}$ |
| Form of estrogen | un-conjugated (E1) | conjugated [E1-17-GUL,E1-17-SUL] | conjugated [E2-17-GUL, E2-17-SUL] |
| Removal mechanism | sorption and subsequent biodegradation | sorption and subsequent biodegradation | sorption and subsequent biodegradation |
| Type of compound | moderately hydrophobic and moderately soluble in water | moderately hydrophobic and moderately soluble in water | hydrophobic and poorly soluble in water |
| Acronyms | Estrone (E1) | 17β-Estradiol (E2) | 17α-Ethinylestradiol (EE2) |
| Lies on plane | horizontal plane of molecular structure | point downward of the molecular plane | Point upward the molecular plane |

*Source:(Birkett and Lister, 2003)*

## 2.6. Removal mechanism of estrogen compounds

### 2.6.1. Removal of free estrogens from the aqueous phase

**a) Volatilization from the liquid phase into the gas phase**

The loss of natural estrogens through volatilization could be judged best by their Henry's law constants. As indicated in Table 2-3 below; free natural estrogens have low vapor pressures. Hence, they are likely to have very small Henry's law constants (Hc). Thus natural estrogens are not easily volatilized under normal temperature and pressure conditions, and their loss from the aqueous phase through volatilization is likely to be quite limited.

Table 2-3. Vapour pressures of estrogens under normal temperature and pressure

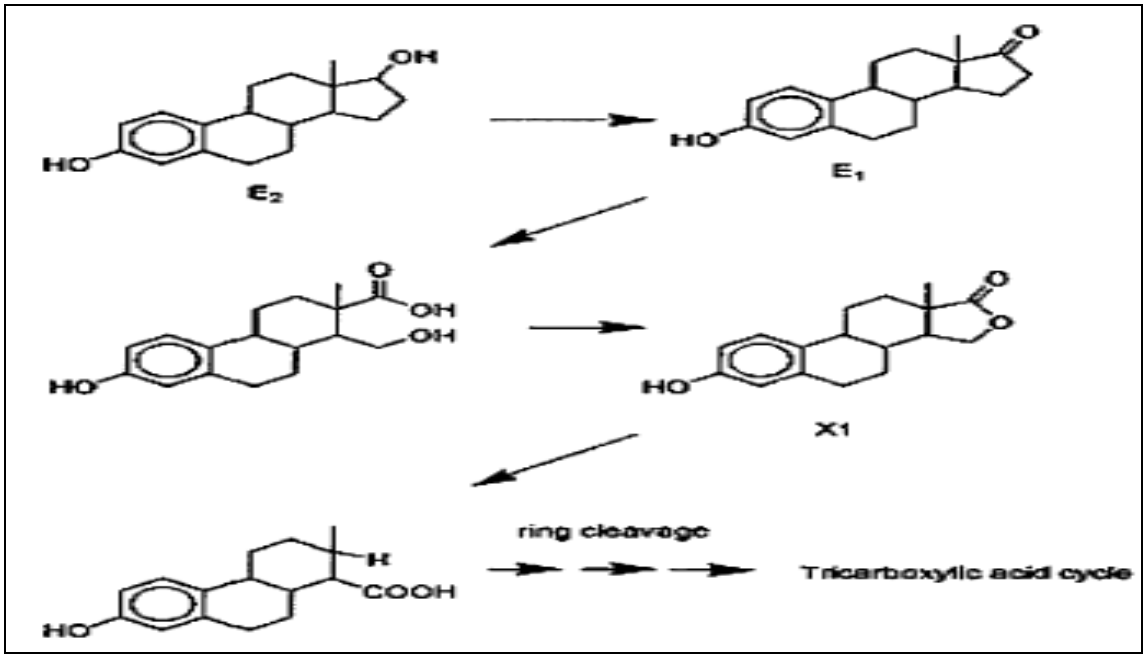
| Estrogen hormone | Vapor pressure($VP^{\Psi}$) (KPa) |
|---|---|
| Estone | $3\times10^{-8}$ |
| 17β-Estradiol | $3\times10^{-8}$ |
| 17α-Estradiol | $3\times10^{-8}$ |
| Estriol | $9\times10^{-13}$ |

*Source: (Birkett and Lister, 2003)*

**b) Biotic and abiotic degradation**

The biodegradation of EDCs from water, sediments, and soils is expected to be occurred as a result of a combination of physical sorption and binding to biota. Published studies (Amy, 2006) showed that natural estrogens estrone (E1) and 17β-estradiol (E2) in sewage treatment plants or batch experiment using municipal sludge were largely degraded biologically (Andersen et al., 2003; Khanal et al., 2006; Shappell and N.W., 2006; Suzuki and Maruyama, 2006). The removal mechanism of the estrogens during soil infiltration was adsorption to the soil and subsequent additional attenuation by biodegradation (Mansell et al., 2004; Ying et al., 2003). Column study indicated that degradation of free estrogens was achieved mainly through a biotic route, whereas under abiotic conditions, the estrogen level fairly remained constant at an initial estrogen level.

The removal of E2 should be considered more than just degradation of its compound because E1 which is intermediate compound from E2 oxidation still posses estrogenic activity at 0.1-0.2 of E2 equivalent. Thus, 14C-labeled E2 compound was used to demonstrate its fate during mineralization, and bacteria presented in the activated sludge from wastewater treatment plant were capable to mineralize 70 to 80 % of E2 to carbon dioxide in 24 h (Layton et al., 2000). The degradation pathway of E2 and E1 with microbial enzyme and industrial catalyst is illustrated in Figure 2-6. E2 was oxidized from the cyclo-pentane ring D at C17 into E1 during enzymatic degradation and then further degraded into metabolite X1 and finally to carbon dioxide through a tricarboxylic acid (TCA) cycle.




*Source: (Khanal et al., 2006)*

Figure 2-6. Pathway of estrogens (E2 and E1) degradation

**c) Sorption onto solids**

Natural estrogen compounds are mainly removed from the aqueous phase by adsorption onto associated solid phases, such as sludge in wastewater treatment or soil in the case of land application (RBF). Free estrogens are non-volatile and moderately hydrophobic nature. So, have high octanol water coefficient ($K_{ow}$). $K_{ow}$ is concentration ratio at equilibrium of organic compounds partitioned between octanol and water. Therefore, it can easily adsorb into the riverbank sediment during infiltration. Adsorption capacity coefficient ($K_f$) values of different kinds of soil is varies from 4 (sand) to as high as 667 (LaDelle silt loam). It is apparent that $K_f$ is strongly correlated to the organic content of the sorbent, as pure sand, silt, and clay contain very low organic matter; whereas LaDelle silt loam, which contains the highest organic matter, was a strong adsorbent of free estrogens. Furthermore, $K_f$ values are also governed by the specific surface area of the adsorbent. $K_f$ values are somewhat correlated to clay and silt contents of the soil, since fine-textured soils usually have a higher organic matter content than those with coarse textures (Khanal *et al.*, 2006).

Adsorption of estrogen on to adsorbent media is given by

$$q = \frac{X}{M} = \frac{(C_o - C_e)v}{M}$$

Where,

q = Equilibrium estrogen concentration in sediment (µg/g) sand

Ce = Equilibrium estrogen concentration in water phase (µg/L)

Co = Initial concentration of estrogen (µg/L)

V = working volume (L)

M = weight of absorbent (g)

Freundlich isotherm is normally used for adsorption modeling by plotting equilibrium estrogen concentration in media (q) against the equilibrium estrogen concentration (Ce) in water phase.

$q = K\ Ce^{1/n}$

Where,

K = sorption coefficient

1/n = sorption constant

The equation of the trend line was noted, and gradient 1/n was started. The gradient value signifies whether sorption of the compound is limited (nonlinear) (1/n < 1) or limitless (linear) (1/n > 1) (Sharma, 2007).

### 2.6.2. Removal of free estrogen compounds from solid phase

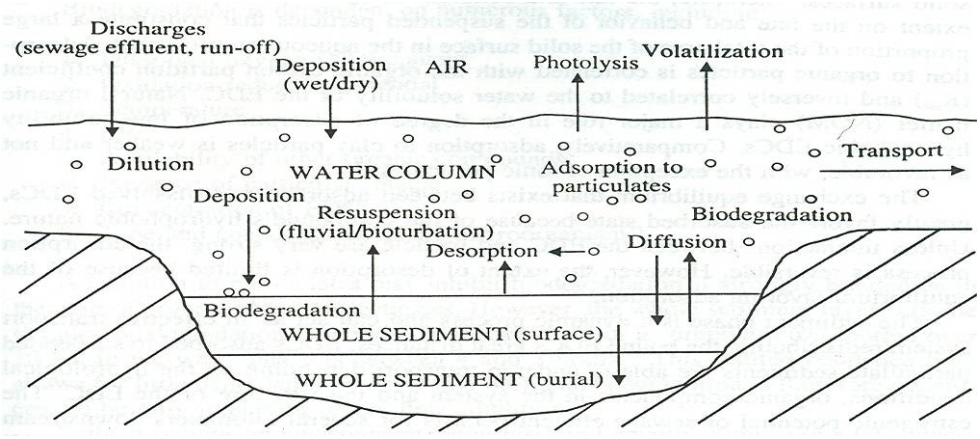
Estrogenic compounds with octanol-water partition coefficient ($K_{ow}$) of 2.6 - 4.0 are readily adsorbed on to solids, including soil, river sediment etc. The removal of free estrogens from the solid phase occurs mainly through biodegradation by soil microbes, which is fast and complete; biodegradation by soil microbes is rather slow and incomplete. The half-lives of E2 in a loam soil with 3.2 % organic matter and a silt loam soil with 2.9 % organic matter were 61 and 72 hour, respectively, at room temperature. The mineralization efficiency measured in terms of carbon dioxide released did not exceed 15 % in both loam and silt soils.

Many study shows that the aerobic biodegradation of estrogen compounds in the solid phase occurs at a much faster rate than the anaerobic route. For example, in deeper horizons, the oxygen content is variable and depends particularly on the degree of water-saturated subsoil, microbial activity can rapidly deplete oxygen, leading to anaerobic condition. Biodegradation is expected to proceed much faster in aerobic surface horizons. Accumulation of estrogen compounds in the deeper layer of soils might be possible because of low estrogen biodegradation due to oxygen limiting conditions. However, no published research particularly elucidates the estrogen degradation profile at different soil depths (Khanal *et al.*, 2006).

## 2.7. Fate of estrogen compounds in the environment

### 2.7.1. Fate of estrogen compound in receiving waters (natural system)

Estrogenic effects are reduced after discharging the sewage effluent into the receiving water because of dilution, degradation and sorption processes. In many developed countries, the percentage of sewage effluent in receiving water can be 50 % to 90 %. Regarding the discharge of EDCs to surface waters, partitioning to the solid phase (suspended solid or whole sediment) and biota will decrease their presence in the water column. Seasonal variation can affect the presence of EDCs in surface waters by microbial activity, temperature and rainfall. Figure 2-7 illustrates several fate processes associated with receiving surface water.




Source:(Gomes and Lester, 2003)

Figure 2-7. Sources of EDCs and several fate processes

The intrusion of EDCs to ground water source is by percolation or effluent recharge. The hydrophobic and low solubility nature of most EDCs will decrease percolation through to underlying ground water due to sorption to solid organic matter. For EDCs introduced via effluent recharge, behavior and transport are depending on dispersion, sorption and biodegradation.

**a) Partitioning and transport**

Sediment constitutes an important compartment of EDCs partitioning into the suspended or whole sediment phase. The main processes that determine the fate of sediment-bound EDCs are sorption degradation and transport. Most EDCs favor sorption to surfaces. Therefore, the fate and behavior of EDCs are dependent on the large proportion of the total area of the solid surface in the aqueous environment. Adsorption to organic particle is correlated with organic carbon partition coefficient $K_{oc}$ and inversely correlated to the water solubility of EDCs. Natural organic matter play an major role in degree of adsorption of low solubility hydrophobic EDCs. Comparatively, adsorption to clay particle is weaker. The exchange equilibrium that exist between adsorbed and dissolved EDCs. Adsorbed state is greatly favor then dissolve state because of the hydrophobic nature of the compounds. In the sediment there is high concentration of organic matter and have large surface area. So, adsorption is high then dissolves. Hence, partitioning and adsorption is more then transport in the river sediment (Gomes and Lester, 2003).

**b) Transformation process**

Photolysis, exposure to ultraviolet (UV) sunlight, may degrade certain EDCs to simpler compounds by volatilization and hydrolysis. That action depends on the EDCs sorption potential, temperature and pH of water. Adsorption to particles of NOM can catalyze hydrolysis. Biodegradation of estrogen on the environment depends on the following factor:

- Dissolve oxygen content
- Oxidation- reduction potential
- Temperature
- pH

- Availability of other organic compounds
- Presence of particulate matter
- Composition of EDCs
- Type and concentration of organic matter

Adsorption and bio degradation process is side by side process .This process is depends on the residence time and flow condition. Generally low flow condition more biodegradation.

In the groundwater environment, photosynthetic and volatilization cannot takes place. Degradation process is also not as evident with the depleted oxygen content, resulting in a decreased prevalence of microorganism. Percolation through the soil strata to the ground water is consider to be the main method of removing undesirable organic compounds as a result of sorption process. Hence, groundwater is more pure source of drinking water treatment than surface water. In ground water, age of EDCs is high. Hence, have low degradative resistance. Estrogenic effect can be reduced through dilution, degradation, and sorption processes. Partitioning to the solid phase is the dominant process for the majority of EDCs as a result their hydrophobic character and low solubility. In the case aerobic sediment layer have high estrogen removal capacity then in anaerobic sediment layer (Birkett and Lister, 2003).

### 2.7.2. Fate of estrogen compounds in an engineered system

**a) Fate of estrogen compounds during the biological wastewater treatment**
**Preliminary treatment**
Preliminary treatment involves the screening of raw sewage through bar screens to remove large objects, including rags, plastic bottles and bags, branches, etc. A large amount of organic material remains in dissolved and in suspensions from. So, it is poorly removed during preliminary treatment. Thus, essentially no free estrogen removal takes place during preliminary treatment.

**Primary treatment**
Primary treatment mainly removes organic solids by gravity. Natural estrogens are removed from the water phase by partitioning onto fat, oil, and grease (FOG) and/or onto primary sludge. Slid-bound estrogens are then removed through solid-liquid separation, such as flotation or sedimentation.

**Secondary treatment**
Natural estrogens are removed from the aqueous phase by adsorption onto bio-film and are further degraded by microbes within the bio-film. The activated sludge process has been reported to have better estrogen removal efficiency than trickling filters. In this method nitrification takes place and removes the organic matter.

**Tertiary treatment**
In this method, wastewater passes through sand filtration or activated carbon and membrane filtration. This method is generally use for recycling of wastewater in small scale.

**b) Fate of estrogens during advanced treatment**
**Activated carbon**
E2 was quickly adsorbed onto granular activated carbon (GAC) as the adsorption reached equilibrium within 3 hours in a series of bench-scale adsorbent evaluation tests. The removal efficiency of GAC was found to decrease with the decrease in initial concentration of E2.When the initial concentration of E2 was decreased from100 to 1 ng/l, the removal efficiency decreased from 81 to 49 %. Powdered activated carbon (PAC) was found to remove over 90 % of E2 at initial concentrations ranging from 27 to 135 ng/L, and the efficiency was further improved by increasing the retention time. However, the PAC-based system requires a continuous supply of media, which makes the application suitable only for temporary or seasonal use (Caxey *et al.*, 2003).

**Membrane bioreactor (MBR)**
MBR is able to maintain an extremely long solid retention time (SRT) and diverse microbial community, which facilitates the degradation of estrogen compounds. Microfiltration (MF) and ultrafiltration (UF) membranes have pore size 100 to 10 000 times larger than the estrogen molecules, which have a molecular mass ranging from 270 to 288 Da. Therefore, the removal of estrogens in the membrane system is achieved by sorption on suspended and colloidal particles and biological degradation. Liu *et al.*, (2005) employed cross-flow UF membranes to determine the partition coefficient of EDCs, including estrogen compounds in molecular and colloidal forms. The authors reported the overall removal efficiency is 82 % for estrogens (E2, E1 and EE2).

**Chemical oxidation and other advanced oxidation processes**
- Chemical oxidation :

The use of chemical oxidants has been found to be highly efficient for estrogen removal from the aqueous phase in several bench-scale studies. The time for oxidation of E2 into E1 was reduced from 48 hours to 10 min and 2 hours, respectively, when ozone and chlorine were employed. Ozone dosages of 10-50 mg/L provided excellent removal of estrogen compounds. Westerhoff *et al.*, (2005) reported that the implementation of ozone was more efficient in oxidizing compounds with phenolic rings. An ozone dosage of 5 m/L successfully reduced the initial concentration of 3.ng/L E2 and 13 ng/L E1 to below detection limits of 1 ng/L.

- Photodegradation:

In this method E2 occurred with direct exposure to UV lamps above 30 W, and that of E1 required direct exposure above 12 W. The degradation of estrogens at the initial concentration of 3-20 mg/L was followed first-order kinetics. The rate was higher with an increase in pH from 7.0 to 8.0, but it was lowest at a pH around 5.0. E1 required a higher energy input for degradation than E2 (Khanal et al., 2006).

# 3. MATERIALS AND METHODS

This chapter explains the methods and experimental procedures followed for data collection and analysis in order to attain the objectives of the research. Data collection was carried out by literature review on riverbank filtration and by conducting laboratory-scale soil columns and batch experiments. Finally, the collected data were analyzed and interpreted.

## 3.1. Experimental setup and procedure

### 3.1.1. Abiotic experiment

Laboratory-scale batch experimental setup was used to investigate the sodium azide concentration which inactivates the microbial activity. Appropriate concentration of sodium azide was required to determine the removal efficacy of estrogen under abiotic condition. Ripened sand was used for this study and oxic condition was maintained. Mixture of Delft canal water (DCW) and secondary effluent (SE) from Hoek van Holland wastewater treatment plant, The Netherlands was used as influent. Three different concentrations (2 mM, 10 mM, and 20 mM) of sodium azide were used for identification of the appropriate concentration. Table 3-1 and Figure 3-1 shows the details of reactors condition and schematic diagram of this experiment.

Table 3-1. The conditions of batch reactors for abiotic experiments

| Sodium azide concentration | Influent | redox condition | No of reactor | Filter media |
|---|---|---|---|---|
| 2 mM | DCW + SE (1:1 ratio) | Aerobic | 2 | Ripened sand |
| 10 mM | DCW + SE (1:1 ratio) | Aerobic | 2 | Ripened sand |
| 20 mM | DCW + SE (1:1 ratio) | Aerobic | 2 | Ripened sand |
| Control | DCW + SE (1:1 ratio) | Aerobic | 2 | Ripened sand |

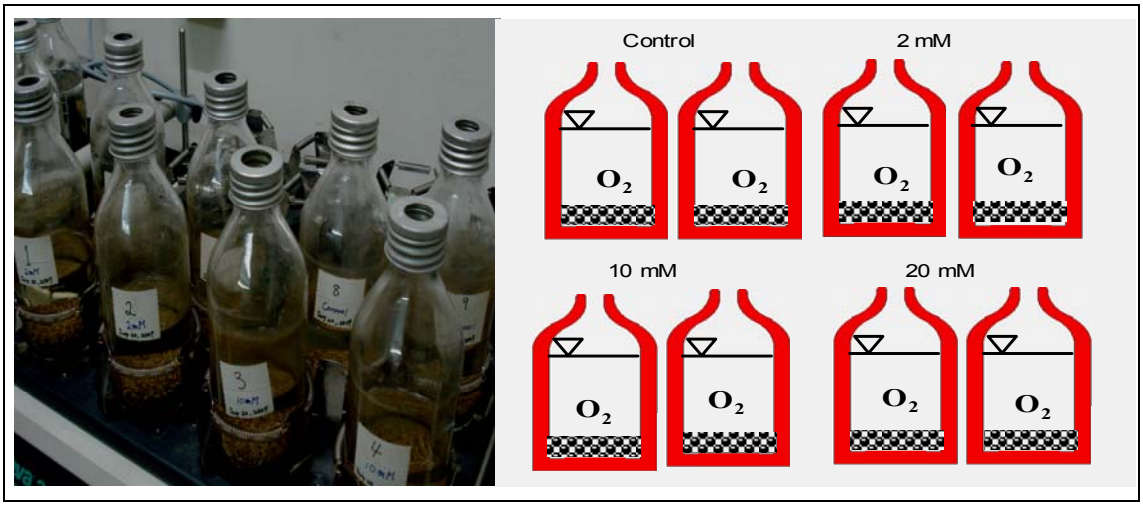



Figure 3-1. Photograph and schematic diagrams of abiotic batch experimental setup

The detailed procedure for abiotic experiment is given below:

(i) First of all, clean sand was prepared after washing with 0.1 N $H_2SO_4$ followed by 0.1 N NaOH solution before using as media in order to remove background materials. Dry clean sand was prepared after drying on oven over 24 hour at temperature 120$^oC$.
(ii) 100 g of clean dry sand of size 0.8 - 1.25 mm diameter was kept in each reactor of volume 500 mL and filled with secondary effluent up to 400 mL.
(iii) Reactors were kept on shaker and the rotational speed was adjusted at 100 RPM
(iv) Influent on reactors was renewed every five days, over the period of more then 70 days until sand was ripened
(v) DOC and $UV_{254}$ were measured during the ripening period.
(vi) After ripening the batch reactors, abiotic experiment was started
(vii) Sodium azide of three different concentrations (2 mM, 10 mM, and 20 mM) were spiked in duplicate reactors. Mixture of DCW and SE (1:1) was used as influent during experiment
(viii) One set of reactor was maintained as the control condition.
(ix) Effluent DOC were measured up to 25 days at an interval of 5 days
(x) Influent from the reactors were renewed after 25 days and same concentration of sodium azide was spiked then DOC were measured up to 45 days
(xi) ATP on sand was measured (sonication was used for extraction) and followed by HPC
(xii) EEM measurement was carried out after 70 days of experiment

### 3.1.2. Adsorption isotherms of E2 and EE2

Adsorption capacity of adsorbent was measured by conducting lab-scale batch experiment using silica sand of size 0.8 to 1.25 mm diameter and estrogen compounds specially 17β-Estradiol (E2) and 17α-Ethinylestradiol (EE2) used as adsorbate. ELISA test results were used for analysis. According to the ELISA test guide manual, EE2 interferes around 50 % with E1 and E2 during the reaction. Hence, separate identical setups were used for E2 and EE2 isotherm to get the accurate result.

**a) 17β-Estradiol (E2)**
Adsorption isotherm of 17β-Estradiol (E2) was determined by laboratory scale batch experiment. Silica sand of size 0.8 to 1.25 mm was used as a media. Experiment was divided into biotic and abiotic. Ripened sand was used for biotic and clean sand was used for abiotic condition. Mixture of DCW and SE (1:1) was used as an influent. In total 13 reactors were used for the experiment. Out of 13 reactors 5 reactors were used for isotherms test under abiotic condition, 5 reactors, were used for isotherms test under biotic condition and two reactors were used for control of the experiment. Details of the experimental setup and condition of reactors are shown in Table 3-2 and Figure 3-2. Similarly, detail procedure of the experiment is explained below:

Table 3-2. Experimental conditions for absorption isotherm of E2

| S.N | Reactors condition | E2 spiked conc.(µg/L) |
|---|---|---|
| 1 | Autoclaved demi-water (ACDW) with no sand (Abiotic) | 100 |
| 2 | Autoclaved DCW and SE no sand (Abiotic) | 100 |
| 3 | DCW and SE with no sand (Biotic) | 100 |
| 4 | Mixture of DCW and SE (1:1) with 50 g ripened sand of dry weight 39.42 g (Biotic) | 0 |
| 5 | | 20 |
| 6 | | 40 |
| 7 | | 80 |
| 8 | | 100 |
| 9 | Autoclaved demi-water with 39.42 g fresh sand (Abiotic) | 0 |
| 10 | | 20 |
| 11 | | 40 |
| 12 | | 80 |
| 13 | | 100 |

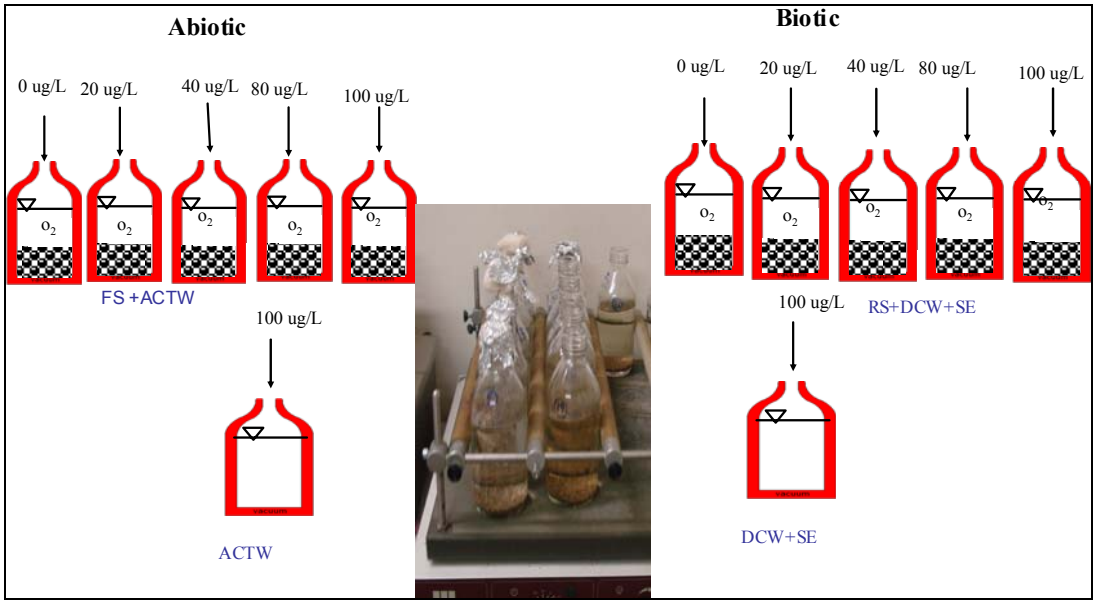



Figure 3-2. Experimental setup for adsorption isotherms of E2

(i) Clean sand was prepared in the same way as in case of abiotic experiment in section 3.1.1.

(ii) Ripened sand was prepared according to the process explained in section 4.2.1.

(iii) 50 g of ripened sand (39.42 g dry weight) was kept in five biotic reactors of volume 500 mL and filled with mixture of DCW and SE (1:1) up to 400 mL

(iv) Clean sand and demi-water were autoclaved for abiotic reactors. Then, 39.42 g of clean sand was kept in five reactors of volume 500 ml and filled with autoclaved demi-water (ACDW) up to 400 mL.

(v) Five different concentrations (0 µg/L, 20µg/L, 40 µg/L, 80 µg/L and 100 µg/L) of E2 were spiked in five biotic and five abiotic reactors. On the other hand, three reactors were used as control of the experiment with no sand and 100 µg/L

of E2 was spiked in each. Autoclaved demi-water (ACDW), AC(DCW+SE) and DCW was used as influent in each reactors.

(vi) Intermediate samples were taken at 0 hour, 24 hours, 48 hours and 120 hours for F-EEM analysis.

(vii) ELISA was carried out for measurement of equilibrium concentration of E2 in water phase after 5 days. Solid phase extraction was used for sample pretreatment.

(viii) Initial and final DOC concentrations were also measured.

**b) 17α-Ethinylestradiol (EE2)**

Adsorption isotherm of 17α-Ethinylestradiol (EE2) was determined by conducting laboratory-scale batch experiment. Experimental setup was exactly similar to that of E2 isotherm experiment. Mixture of DCW and SE (1:1) was used as an influent for biotic experiment. Similarly, ACDW was used for abiotic experiment. Ripened sand was used for biotic and autoclaved clean sand was used for abiotic as absorbent. Two reactors were used for control of the experiment. Schematic and photographic view of experimental setup and different conditions of experimental setup are shown in Table 3-3 and Figure 3-3 and detail procedure of experiment is explained below:

Table 3-3. Experimental conditions of absorption isotherm of EE2

| S.N | Reactors condition | EE2 spiked conc.(µg/L) |
|---|---|---|
| 1 | Autoclaved demi-water (ACDW) with no sand (Abiotic) | 100 |
| 2 | Autoclaved DCW and SE no sand (Abiotic) | 100 |
| 3 | DCW and SE with no sand (Biotic) | 100 |
| 4 | Mixture of DCW and SE (1:1 ratio) with 50 g ripened sand of dry weight 39.42 g (Biotic) | 0 |
| 5 | | 20 |
| 6 | | 40 |
| 7 | | 80 |
| 8 | | 100 |
| 9 | Autoclaved demi-water with 39.42 g fresh sand (Abiotic) | 0 |
| 10 | | 20 |
| 11 | | 40 |
| 12 | | 80 |
| 13 | | 100 |

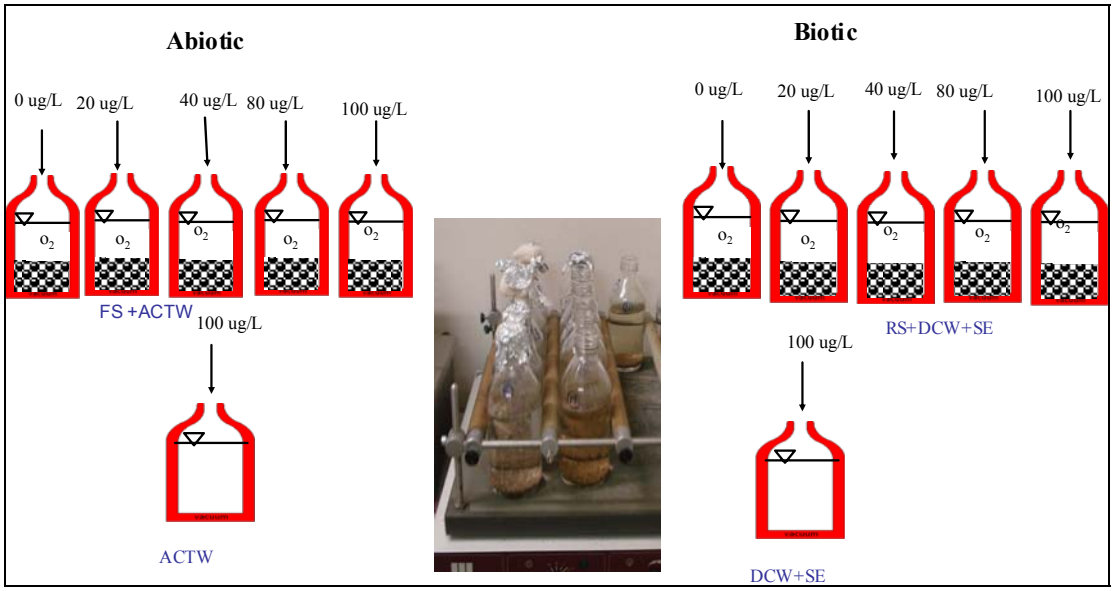



Figure 3-3. Experimental setup for adsorption isotherm of EE2

(i) Clean sand was prepared in same way as in abiotic experiment in section 3.1.1.
(ii) Ripened sand was prepared according to the process explained in section 4.2.1
(iii) Batch reactors for abiotic isotherms test and biotic isotherms test and controls were prepared as for E2 isotherms test mentioned in 3.1.2 (a)
(iv) Five different concentrations (0 µg/L, 20 µg/L, 40 µg/L, 80 µg/L and 100 µg/L) of EE2 were spiked in each set of biotic and abiotic reactors.
(v) 100 µg/L of EE2 was spiked in each three control reactors with influent ACDW, AC(DCW+SE) and DCW.
(vi) Intermediate samples were taken at 0 hour, 24 hours, 48 hours and 120 hours for F-EEM analysis.
(vii) ELISA was carried out for the measurement of equilibrium concentration of EE2 in water phase after 5 days. Solid phase extraction was used for sample pretreatment.
(viii) Initial and final DOC concentrations were measured.

### 3.1.3. Determination of factors affecting E2 and EE2 removal

Laboratory-scale based batch experiments were conducted for identification of factors governing E2 and EE2 removal. ELISA is the well known method to determine the estrogen concentration in aqueous phase. According to the ELISA test guide manual EE2 has high interference nature with E1 and E2 during the reaction. Manual mentions that interference was around 50 %. Hence, batch experimental setup was divided into two identical setups for determination of factors which affect the removal of estrogen during RBF. One setup was used for determination of E2 and E1 others were used for determination of EE2.

**a) Investigation of factors affecting E2 and E1 removal**

A laboratory-scale batch experiment was conducted for the determination of the factors affecting the removal efficiency of 17β-Estradiol (E2) and Estrone (E1) during RBF. Total 24 batch reactors were used for the experiment. 10 batch reactors were used to investigate the role of adsorption and biodegradation (biotic). Similarly, another 14 reactors were used to investigate the adsorption (abiotic). Summary of reactors condition and schematic diagram is shown Figure 3-4 and Table 3-4. Detail procedure of experiment is explained below:

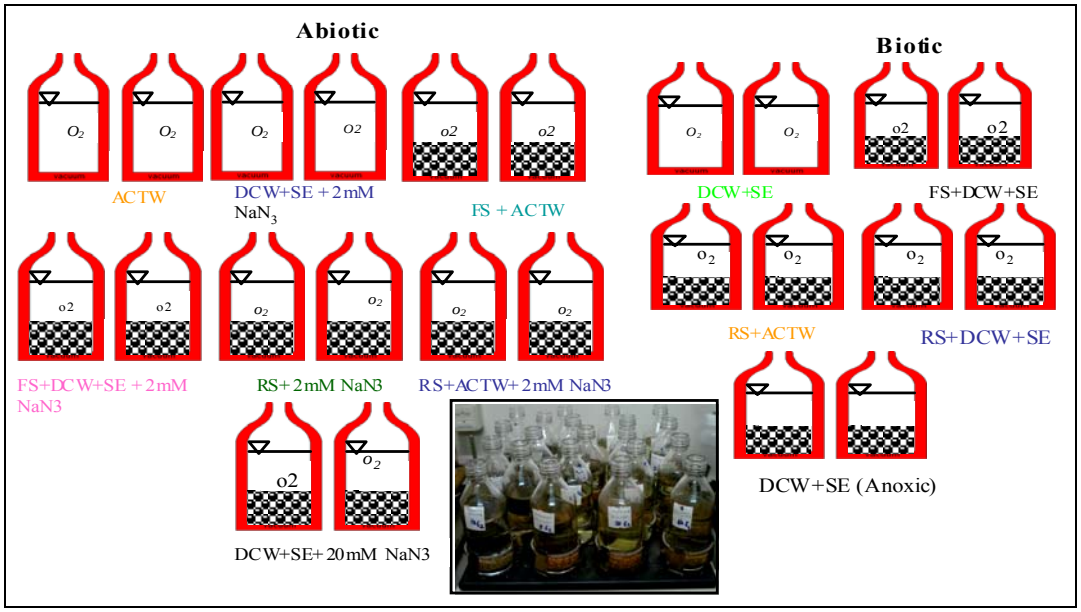



Figure 3-4. Experimental setup to determine the factors affecting E2 and E1 removal

Table 3-4. Reactors condition to determine the factors affecting E2 and E1 removal

| Reactors condition | No of reactors | Removal process conditions |
|---|---|---|
| Autoclaved tap water (ACTW) (no sand) | 2 | Oxic – Abiotic (adsorption) |
| Mixture of DCW and SE (1:1) (no sand) + 2 mM sodium azide | 2 | Oxic – Aiotic (adsorption) |
| 39.420 g of fresh sand + ACTW | 2 | Oxic– Abiotic (adsorption) |
| Mixture of DCW and SE (1:1) (no sand) | 2 | Oxic– Biotic (adsorption + biodegradation) |
| 39.42 g of fresh sand + Mixture of DCW and SE (1:1) | 2 | Oxic– Biotic (adsorption + biodegradation) |
| 39.42 g fresh sand + DCW + SE + 2 mM sodium azide | 2 | Oxic– Abiotic (adsorption) |
| 50 g ripened sand + Mixture of DCW and SE(1:1) + 2 mM sodium azide | 2 | Oxic - Abiotic |
| 50 g of ripened sand + ACTW | 2 | Oxic - Abiotic |
| 50 g of ripened sand + ACTW | 2 | Oxic – Biotic |
| 50 g of ripened sand + Mixture of DCW and SE (1:1) | 2 | Oxic – Biotic (adsorption + biodegradation) |
| 50 g of ripened sand + Mixture of DCW and SE(1:1) + 20 mM of sodium azide | | Oxic - Abiotic |
| Mixture of DCW and SE (1:1) | | Anoxic + Biotic |

(i) Sand was washed with 0.1 N $H_2SO_4$ solution followed by 0.1 N NaOH before using as media in order to remove background materials. Dry clean sand was prepared after drying on oven over 24 hour at temperature 120$^oC$.
(ii) 100 g of clean dry sand of size 0.8 - 1.25 mm diameter was kept in each reactor of volume 500 mL and filled with secondary effluent up to 400 mL.
(iii) Reactors were kept in a shaker and the rotational speed was adjusted at 100 RPM.
(iv) Influent on reactors was renewed every five days, over the period of more than 70 days until sand was ripened.
(v) DOC and UV $_{254}$ were measured during the ripening period. Detail of batch reactor ripening process was explained in section 4.2.1.
(vi) After ripening pre-stabilization of batch reactor were started in the case of DOC removal. 50 g of ripened sand (39.42 g dry weight) was kept in 10 batch reactors of volume 500 mL. Out of them 6 batch reactors were filled with mixture of DCW and SE (1:1) and 4 batch reactors were filled with MQ water up to 400 mL.
(vii) Abiotic condition was maintained by using 2 mM of sodium azide and anoxic condition was maintained by flushing the nitrogen in anoxic chamber.
(viii) Influent was renewed once in 5 days and DOC of influent and effluent was measured until 20 days before experiment was started.
(ix) Fresh sand was prepared by burning at 550$^oC$ for 2 hours in burning chamber.
(x) Clean sand and demi-water were autoclaved for abiotic condition of experiment. Then, 39.42 g of clean sand was kept in five reactors of volume 500 mL and filled with ACTW up to 400 mL. Detail description of reactor condition is explained in Figure 3-4.
(xi) 300 µg/L of E2 was spiked in each duplicate reactor
(xii) Intermediate samples were taken (after 1.5 hours, 3.5 hours, 24 hours, 43 hours, 96 hours and 120 hours) from those reactors which contained DCW + SE + E2 with no sand, ACTW + E2 with no sand and DCW + SE + E2 in biotic condition.
(xiii) Fluorescence measurement was carried out for intermediate samples.
(xiv) Influent and effluent DOC were measured during experiment.
(xv) ATP on sand was measured (sonication was used for extraction) and followed by HPC.
(xvi) ELISA test was carried out. SPE was used for sample pretreatment.

**b) Investigation on factors affecting EE2 removal**

Laboratory-scale batch experiment was conducted to determine the factors affecting 17α-Ethinylestradiol (EE2) removal during RBF. Experimental setup and other conditions of batch reactors were exactly same that for E2 experiments. Details of batch experimental setup and condition of batch reactors are shown in Figure 3-5 and Table 3-5. Similarly detail experimental procedure is explained below:

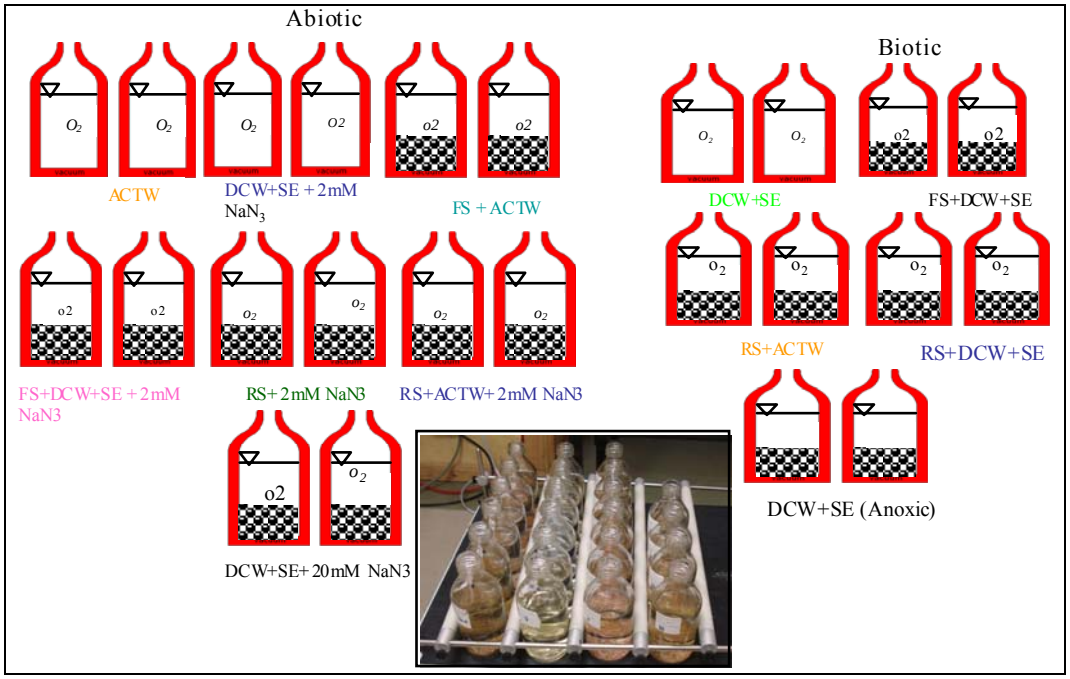



Figure 3-5. Experimental setup to determine the factors affecting EE2 removal

Table 3-5. Reactors condition to determine the factors affecting EE2 removal

| Reactors condition | No of Reactors | Removal process conditions |
|---|---|---|
| Autoclaved tap water (ACTW) with no sand | 2 | Oxic– Abiotic (adsorption) |
| Mixture of DCW and SE (1:1) (no sand) + 2 mM sodium azide | 2 | Oxic – Aiotic (adsorption) |
| 39.420 g of fresh sand + Autoclaved tap water | 2 | Oxic– Abiotic (adsorption) |
| Mixture of DCW and SE (1:1) with no sand | 2 | Oxic– Biotic (adsorption + biodegradation) |
| 39.42 g of fresh sand + Mixture of DCW and SE (1:1) | 2 | Oxic– Biotic (adsorption + biodegradation) |
| 39.42 g fresh sand + DCW + SE + 2 mM sodium azide | 2 | Oxic– Abiotic (adsorption) |
| 50 g ripened sand + Mixture of DCW and SE(1:1) + 2 mM sodium azide | 2 | Oxic - Abiotic |
| 50 g of ripened sand + ACTW | 2 | Oxic - Abiotic |
| 50 g of ripened sand + ACTW | 2 | Oxic – Biotic |
| 50 g of ripened sand + Mixture of DCW and SE (1:1) | 2 | Oxic – Biotic (adsorption + biodegradation) |
| 50 g of ripened sand + Mixture of DCW and SE (1:1) + 20 mM of sodium azide | 2 | Oxic - Abiotic |
| Mixture of DCW and SE (1:1) | 2 | Anoxic + Biotic |

(i) Sand was ripened according to the section 3.1.3 (a).
(ii) Preconditioning of batch reactors was carried out in similar way as explained in the case of E2 experiment.
(iii) Fresh sand and autoclaved demi-water was prepared by same way as for E2 experiment.
(iv) 300 µg/L EE2 was spiked in each of the duplicate reactors. Intermediate samples were taken (after 1.5 hours, 3.5 hours, 24 hours, 43 hours, 96 hours and 120

hours) from those reactors which contained DCW + SE + EE2 with no sand, ACTW + EE2 with no sand and DCW + SE + EE2 + ripened sand.

(v) Fluorescence measurement was carried out for intermediate samples.

(vi) Influent and effluent DOC were measured during experiment.

(vii) ATP on sand was measured (sonication was used for extraction) and followed by HPC.

(viii) ELISA test was carried out. Solid phase extraction was used for sample pretreatment

### 3.1.4. Soil columns study

Removal efficiency of estrogen compounds during the river bank filtration was determined by conducting laboratory-scale soil column experiments. Soil columns were constructed with borosilicate glass pipe of an internal diameter of 5 cm and length of 30 cm. One end of the pipe was closed with rubber stopper and other end was closed with O-ring on the plastic plunger on outer adjusting tube as shown in Figure 3-6. These soil columns were used to determine the removal of estrogen compound particularly 17α-ethinylestradiol (EE2). Each column was packed with silica sand of size 0.8 to 1.25 mm diameter. Experiment was conducted in dark to simulate the process in river bank filtration with HLR: 0.4 m/d and EBCT: 18 hours. Detail procedures are explained below:

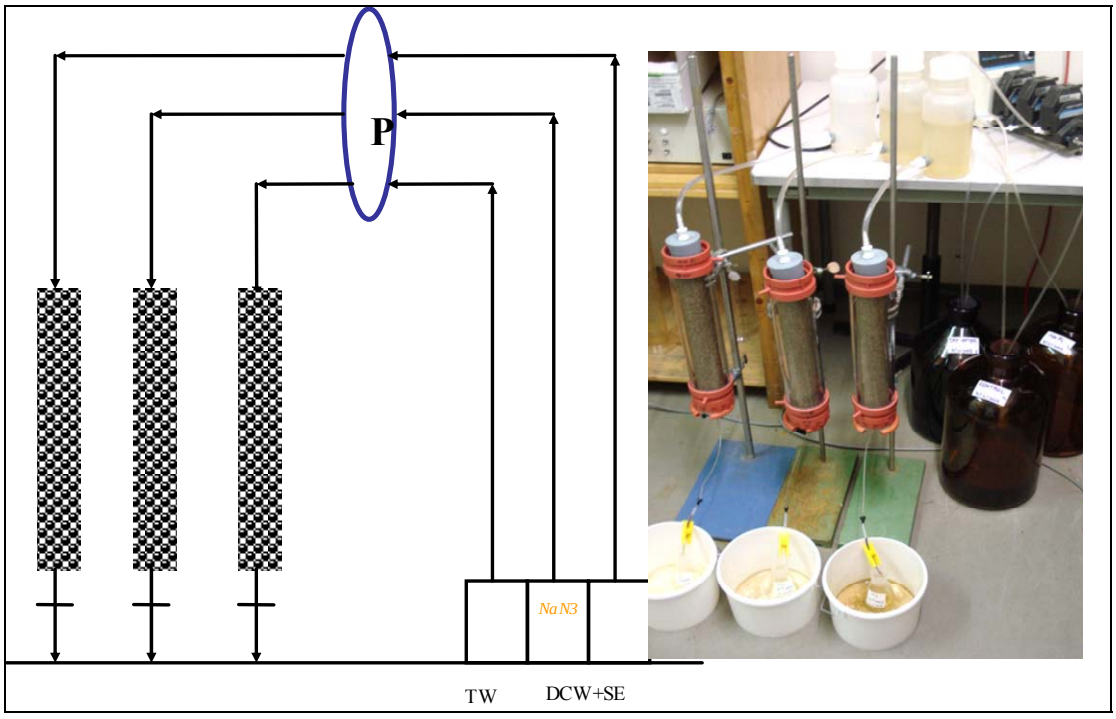



Figure 3-6. Schematic diagram of the soil column experimental setup

(i) Sand was washed with 0.1 N $H_2SO_4$ solution followed by 0.1 N NaOH solution before using as media in order to remove background materials

(ii) Soil columns were packed with clean sand and ripening was started.
(iii) Secondary effluent (SE) from Hoek van Holland wastewater treatment plant was used as an influent and flow rate was adjusted 0.4 m/d or 18 hours EBCT.
(iv) Influent on soil columns were renewed within 24 hours, over the period of more than 70 days until sand was ripened.
(v) DOC and $UV_{254}$ were measured during the ripening period. Detail of sand ripening process is explained in section 4.2.2.
(vi) Oxic conditions were maintained through out the experiment.
(vii) After ripening, columns were pre-conditioned for the experiment by using tap water in SC1 and mixture of secondary effluent and Delft canal water(1:1) in SC2 and SC3
(viii) 2 mM sodium azide was used to inactivate the microbial activities in SC2.
(ix) After 20 days of pre-conditioning, experiments were started with same influent as in pre-conditioning periods.
(x) 1000 µg/L of EE2 was spiked in each influent tank. Samples were taken at 24 hours interval until 15 days .ELISA test was carried out solid phase extraction was used for sample pre-treatment. Similarly, fluorescence measurement was carried out.
(xi) SC1 and SC3 were used to investigate the role of adsorption and biodegradation of estrogen compounds. But, SC2 was used to investigate the role of adsorption only.

## 3.2. Analytical methods and equipments

A brief description of the methods and equipments used to measure the parameters of concern for this research work is presented in the following sub sections.

### 3.2.1. Enzyme-linked immunosorbent assay (ELISA)

Quantification of estrogenic compounds (E1, E2 and EE2) was carried out by using biological technique known as ELISA (Enzyme-linked immunosorbent assay) and F-EEM technique:

**a) The principle of EDCs measurement.**

E1, E2, and EE2 ELISA test kits specifically detect estrogenic hormone E1, E2, and EE2 respectively. The analysis is based on a competitive reaction where enzyme labeled standard E1 (E2 or EE2) competes with free E1 (E2 or EE2) in the sample for binding to a specific monoclonal antibody immobilized to the surface of a microplate. The amount of labeled E1 (E2 or EE2) bound to the antibody is determined by addition of a non-colored substrate which is converted into a colored product. The color intensity is measured at 450 nm and allows to measure the concentration of E1 (E2 or EE2) in the sample. The assay is calibrated using a standard solution of E1 (E2 or EE2) supplied with the kit(Japan EnviroChemicals, 2007).

**b) Procedure**

**Sample pre-treatment**

- Filtration of raw water samples (500 mL) through glass fiber filters (1 µm pore diameter).
- Concentration and cleanup (extraction) of filtered samples using solid phase extraction technique to a final 1 ml sample volume.

**ELISA test**

- 100 µL of conjugate solution, 100 µL of E1 (E2 or EE2) standards and 100 µL of sample (prepared as 10 % methanol) were transferred into each well of the uncoated microplate. Then 100 µL of the mixture prepared in the above step was dispensed into each coated well of the microplate and incubated for 60 minutes at room temperature
- Then the content of each well of the microplate was decanted and washed three times with wash solution of 300 µL. The plate was firmly tapped out to remove solution from the microplate well.
- 100 µL of color solution was added to each well of the microplate and incubated for 30 minutes at room temperature. This changed the solution to blue color.
- Then 100 µL of stop solution was added to terminate the reaction. This turned the blue colored solution to yellow.
- Finally absorbance at 450 nm of both the standard solutions and the samples were determined using a spectrophotometer (microplate reader) within 15 minutes time.
- Then standard calibration curve was prepared form the readings of the standard solutions
- Sample concentration was determined from the calibration line for a particular absorbance.

### 3.2.2. Dissolved organic carbon

**Sample preparation**

Samples were first filtered with 0.45 µm HV durapore membrane filter (Millipore) using a 30 mL syringe and filter head. However, to avoid leaching of DOC from the filter itself, the filter was washed twice with 30 mL Milli-Q water and then flushed a bit with the sample itself before filtering the samples. The samples were prepared for analysis in an injection vials (glass vials) of 40 mL volume.

Hydrochloric acid:- 0.1 N HCL solution was used for pH control of the sample.

Milli-Q:- Two Milli-Q water gallon was used for washing the machines those gallon were filled every day with fresh Milli-Q water

**Measurement**

DOC of the samples was measured using TOC - $V_{cpn}$ total organic carbon analyzer ('Shimadzu Corporation' analytical and measuring instruments deviation Kayuto, Japan) shown in Figure 3-7. During each analysis, TOC analyzer measured duplicates

and an average final value of DOC and TOC were displayed on the computer. Before measurement of the samples, three Milli-Q water samples were measured in order to clean the system and a DOC concentration of about 0.05 mg/L would imply that the machine is ready for analysis of the samples. Similarly, one tap water vial was measured for control. It takes about 15 minutes to analyze of each sample.


Figure 3-7. Total organic carbon analyzer (Shimadzu corporation, Japan)

### 3.2.3. Fluorescence excitation - emission matrix

**Sample preparation**

Samples were first filtered with 0.45 µm HV durapore membrane filter (Millipore) which was pre-rinsed with Milli-Q water and sample water. DOC of the samples was then measured using TOC - Vcpn total organic carbon analyzer ('Shimadzu Corporation' analytical and measuring instruments deviation kayuto, Japan) or Model 700 total organic carbon analyzer (O.I Corporation, USA). Based on the measured DOC level, samples were diluted to about 1 mg/L of DOC with 0.01M KCl solution which was pre-adjusted to a pH of 2.8 using 0.1 M HCl solution. pH of the samples were again measured and adjusted 2.8 by using 0.1 M HCl solution.

**Measurement**

A spectrofluorometer was used to measure and record the fluorescence of a sample. The Fluorescence excitation-emission matrix (F-EEM) of the influent and effluent samples from the soil column and batch reactors were analyzed by using Fluoro Max-3 spectrofluorometer (HORIBA Jobin Yvon Inc., USA) shown in Figure 3-8. The entire excitation and emission matrix (EEMs) were obtained by measuring the emission spectra in the range of 290 – 500 nm at 2 nm intervals, with an excitation range of 240 to 450 nm at 10 nm intervals. EEMs of each sample were subtracted with an EEM of 0.01 M KCl solution set as a blank F-EEM. The machine required 15 minutes to analyze single sample.

F-EEM plots were prepared by using MATLAB software. Ready made MATLAB code was used. According to the code, three xls data sheet were required for one F-EEM plot these are: water.xls, blank.xls and sample xls. Dilution factor was changed according to the sample dilution. No of contour and scale were changed for attractive image.


Figure 3-8. Fluoro Max-3 for fluorescence measurement

### 3.2.4. UV absorbance (UVA254)

UV absorbance measurement at a wavelength of 254 nm was carried out by Perkin Elmer UV/VIS Spectrophotometer using 1 cm cell of type QS suitable for wavelength below 300 nm. The samples were first filtered in a similar way as for the DOC measurement. Care was taken not to have stain on the surface and air bubbles inside the cell wall. Cuvette was flushed thoroughly with Milli-Q water between measurements of different samples and was rinsed with each sample before being filled. The spectrophotometer used for absorbance measurements of various parameters is shown in Figure 3-9

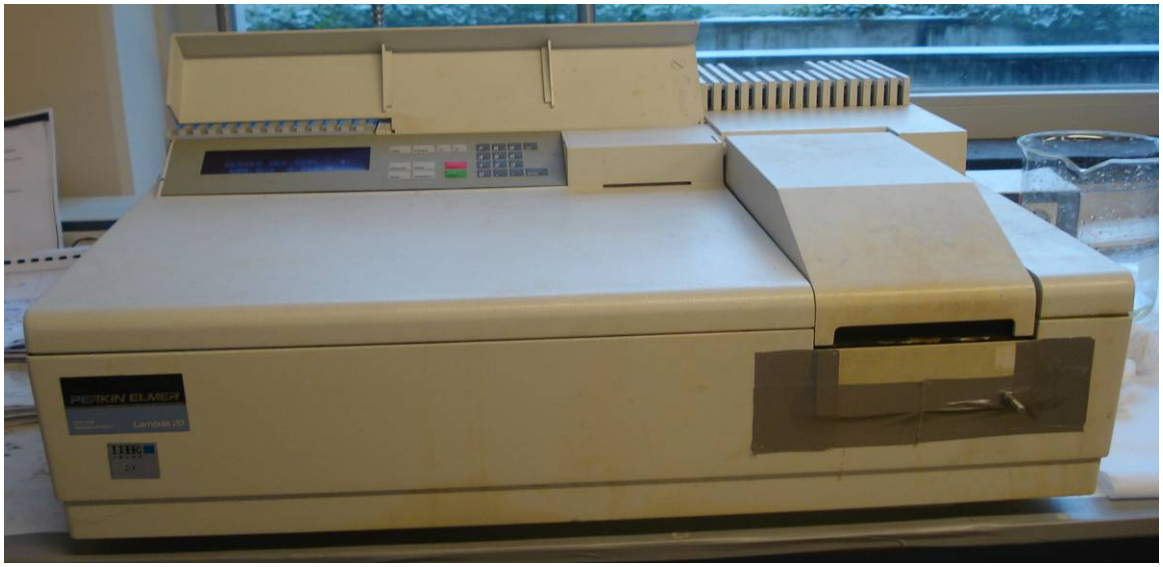



Figure 3-9. Perkin Elmer UV/VIS spectrophotometer

### 3.2.5. UV Scanner between 200 - 400 nm

UV absorbance measurement at a wavelength of 200-400 nm was carried out by using UV-2501 PC, UV-VIS recording Spectrophotometer (SHIMADZU) using 1 cm cell of type UV suitable for this wavelength. The samples were first filtered in a similar way as for the DOC measurement. Care was taken not to have stain on the surface and air bubbles inside of the cell wall. Cuvette was flushed thoroughly with Milli-Q water between samples and was rinsed with each sample before being filled. The spectrophotometer used for absorbance measurements as well as scanning of UV absorbance of various samples water is shown in Figure 3-10.

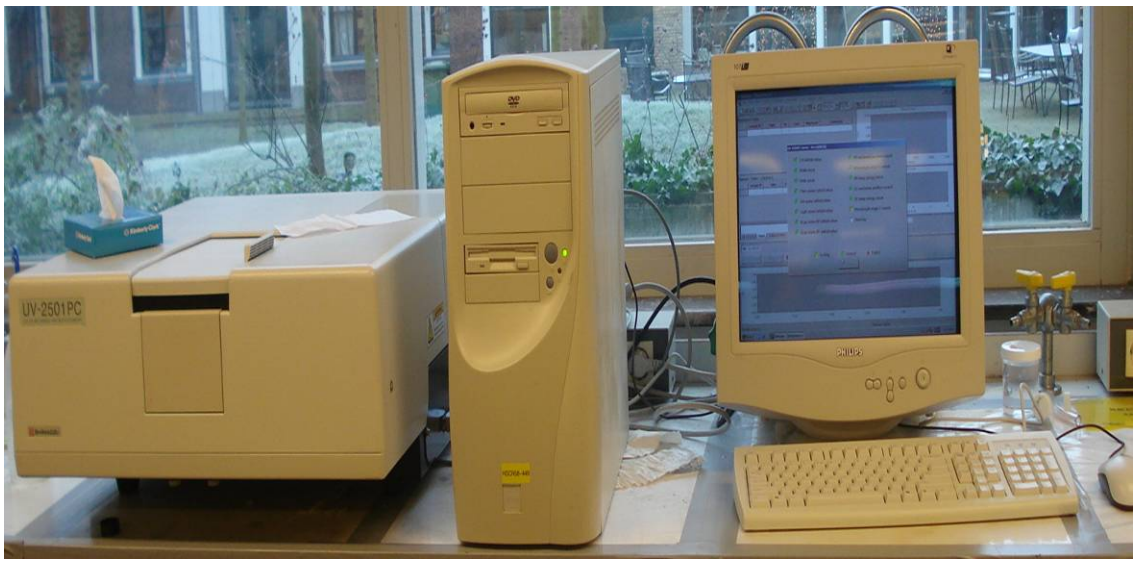



Figure 3-10. Shimazdu UV-VIS recording spectrophotometer for UV scans between 200 to 400 nm

## 3.2.6. Size Exclusion Chromatography, HPSEC - UVA/DOC/Fluorescence

**Sample preparation**
Samples were first filtered with 0.45 µm cellulose acetate membrane filter in the same way as for DOC analysis. Ionic strength (conductivity) and pH of the sample was adjusted to the same level as the mobile phase before injection (EC ≈ 4.9 to 5.1 mS/cm). 4 mL of sample was then injected to the machine: 2 mL being used for flushing the coil (injection loop) and 2 ml for HPSEC-UVA/DOC analysis.

**Measurement**
The HP-SEC measurement was performed with HPLC (Shimadzu LC600) coupled with a UV-Vis detector (Shimadzu SPD-10Avp) and an on-line DOC detector (modified Ionic Sievers Turbo TOC analyzer) as shown in Figure 3-11. The system used a TSK HW-50S column (column size: 2 cm x 25 cm, particle size: 35 µm Toyopearl HW resin) and the flow rate was 1 mL/min. The mobile phase (eluent) was prepared with Milli-Q water buffered with phosphate (0.0024 M $NaH_2PO_4$ + 0.0016 M $Na_2HPO_4$, pH 6.8) and 0.025 M $Na_2SO_4$, producing 0.1 M of an ionic strength. The inorganic carbon was converted to $CO_2$ and removed through inorganic carbon remover (ICR). The machine required about 2 hours to analyze single sample.


Figure 3-11. HPLC – SEC - DOC/UV analyser

## 3.2.7. ATP

ATP analysis is the method to determine the active biomass. According to this method active microbial mass was measured. High energy sonication at a power input of 40 W was used for measurement.

**Ultrasonic treatment**

Three gram of wet sand was taken and 50 $cm^3$ of autoclave tap water (pH 8.4 ± 0.2) was added in screw-capped borosilicate flask. Sonication was applied for 2 minutes. 5 $cm^3$ of the obtained suspension was collected and kept on ice for examination.

**Measurement**

The measurement of ATP is based on the production of light in the luciferine-luciferase assay. ATP was released from the suspended cells with nucleotide-releasing buffer (NRB, Celsis). The intensity of emitted light was measured in a luminometer (Celsis Advance $^{TM}$) calibrated with solution of free ATP with Autoclaved tap water as shown in Figure 3-12.

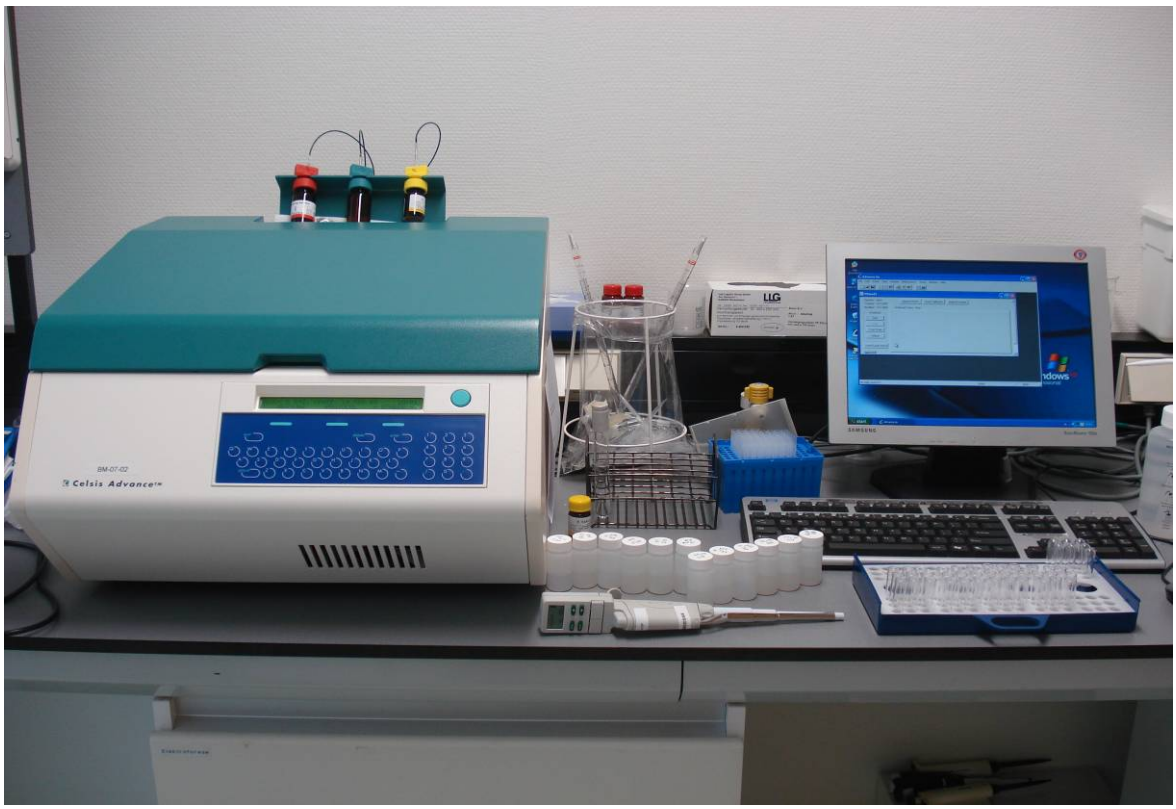



Figure 3-12. ATP analyser

# 4. RESULTS AND DISCUSSION

This chapter primarily presents the results obtained after conducting the laboratory-scale batch and soil column experiments under various process conditions and secondly, discusses the efficiency of estrogens removal through RBF system.

## 4.1. Influent characterization

Two sources of water were selected as influent for batch and soil column experiments: Delft canal water and wastewater secondary effluent. Canal water was collected from Oude Delft, on the back side of UNESCO-IHE and wastewater secondary effluent from Hoek van Holland wastewater treatment plant located in South Holland on the North Sea coast. Wastewater effluent was mixed with canal water (1:1 ratio) before application to the batch reactors and soil columns. This was done to investigate the impact of wastewater effluent on surface water during RBF. In many developed countries, the percentage of wastewater effluent in receiving water can be 50 % in normal condition. In periods of low flow and the highest demand, this percentage can be up to 90 % (Birkett and Lester, 2003).

The characteristics of influents applied to the soil column and batch reactors are presented in Table 4-1, Figure 4-1, Figure 4-2 and Figure 4-3. Influents were stored in dark at 4°C immediately after collection. Pre-filtration of the samples were done with 45 µm sieve to avoid settling of larger size materials to prevent the columns from clogging.

Table 4-1. Influent water quality parameters

| Parameter | Unit | Delft canal water | Secondary effluent |
|---|---|---|---|
| pH | - | 7.2 | 7.82 |
| Temperature | ºC | 6.6 | 12.5 |
| $O_2$ | mg/L | 7.7 | 1.7 |
| EC | µc/cm | 1257 | 1000 |
| DOC | mg/L | 17.42 ±2.02 | 12.31 ± 4.77 |
| $UV_{254}$ | $cm^{-1}$ | 0.541 ± 0.05 | 0.441 ± 0.05 |
| SUVA | L/mg-m | 3.21 ± 0.16 | 3.18 ± 0.15 |
| $NH_4$-N | mg/L | 0.154 | 0.045 |
| $NO_3$-N | mg/L | 2.23 | 1.81 |
| $PO_4$-P | mg/L | 1.1 | 0.33 |
| $SO4^{2-}$ | mg/L | 74.5 | 97.7 |

Table 4-1 shows the major water quality parameters of the influent water. Some of those parameters were measured immediately after taking the water from the source. So

oxygen content in secondary effluent (SE) was found too low. Rest of the parameters in Delft canal water (DCW) was higher than in SE except $SO_4^{2-}$, pH and temperature.

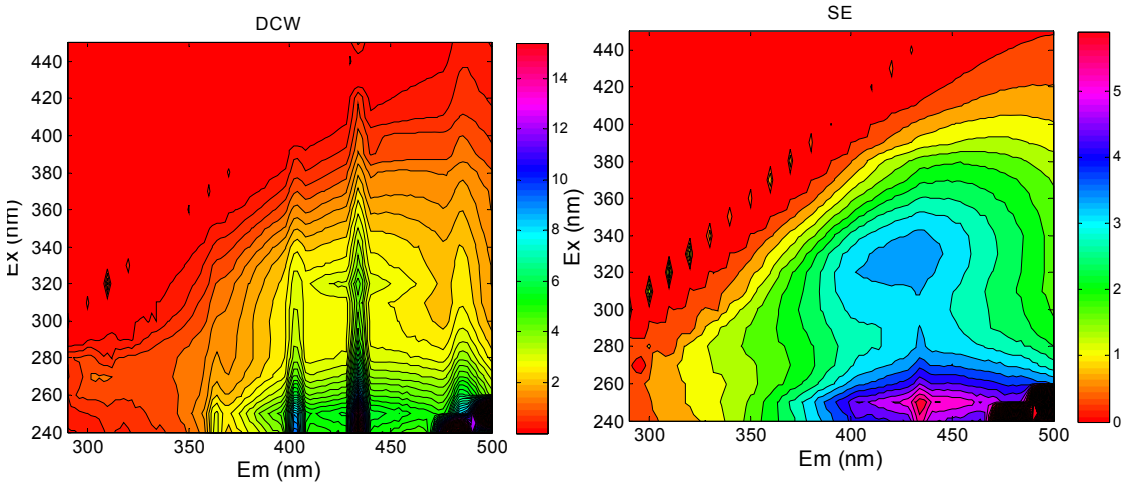



Figure 4-1. F-EEM spectra of DCW and SE

Figure 4-1, F-EEM spectra shows the protein peaks in DCW and SE. The protein peak in DCW was higher than that of SE. But, spectra showed that humic and fulvic substances were the major components. Table 4-2.

Table 4-2. F-EEM detected intensity level of protein and humic peak in influent water

| Sample | Intensity level of protein peak | Intensity level of humic peak |
|---|---|---|
| DCW | 1.23 | 15.42 |
| SE | 0.2 | 6 |

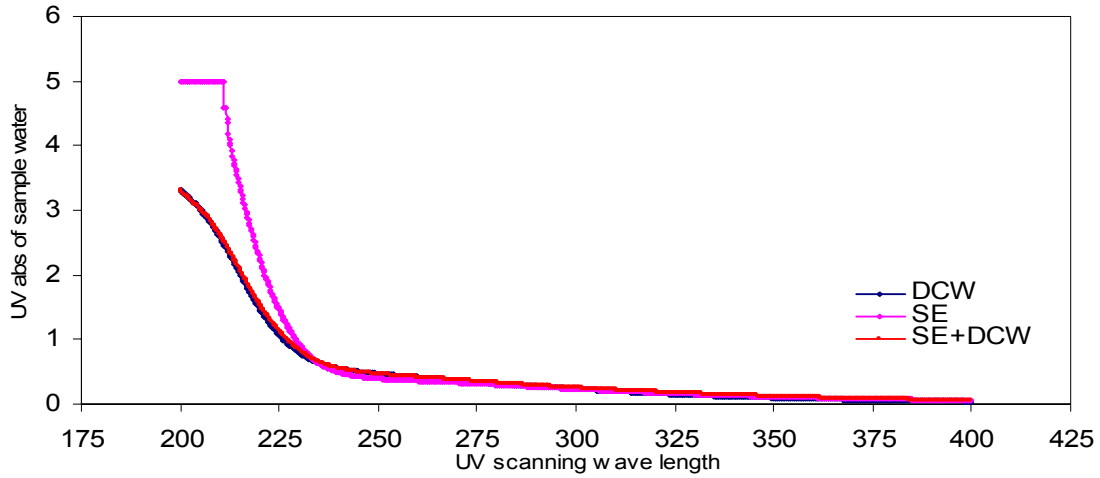



Figure 4-2. UV scans of influents between 200 - 400 nm

Figure 4-2 shows the UV scans in DCW, SE. and mixture of DCW and SE (1:1 ratio). Maximum UV appeared in SE than DCW as well as mixture of DCW and SE (1:1 ratio). Maximum value was observed at $UV_{210}$ nm in all cases. Then it continuously decreased

when wavelength was increased. Scan graph in mixture of DCW and SE (1:1 ratio) showed less UV absorbance than SE.

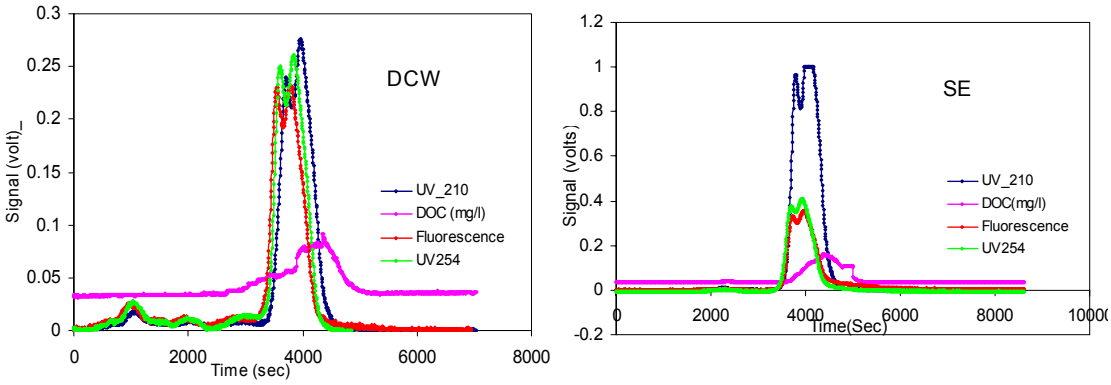



Figure 4-3. HPSEC – UVA/DOC/Fluorescence of the DCW and SE

Figure 4-3 shows that the UVA/DOC/Fluorescence in DCW and SE. All parameters were higher in SE than DCW. The signal of $UVA_{210}$ in DCW was 0.255 mV and in SE it was 1.0 mV. Likewise, $UVA_{254}$ in DCW was 0.23 mV and in SE it was 0.39 mV. Signals from different detectors could be used to differentiate the bulk organic matter in different samples. Finally, organic materials in DCW were higher than in SE except protein.

## 4.2. Ripening Process

It is a process in which a thin layer of bio-films of microorganism was formed on the surface of sand. Secondary effluent was used as influent water over two and half months until steady state was reached with respect to DOC removal. In this study batch reactors and soil columns were used for ripening.

### 4.2.1. Batch reactors ripening process

For batch experiments total 20 reactors (volume 500 mL) were prepared as follows: 100 g of clean dry sand of size 0.8 - 1.25 mm diameter was kept in each reactor and filled with secondary effluent up to 400 mL. Reactors were kept in a shaker with rotational speed of 100 rpm. The biological acclimation (ripening) process was continued for more than 2 months till the batch reactors stabilized with respect to DOC removal. During the ripening period, influent and effluent DOC and $UV_{254}$ were continuously monitored. The influent on the reactors was renewed every 5 days. Figure 4-4 shows the variation in influent and effluent DOC concentrations during the ripening process while Figure 4-5 shows the amount of DOC degradation in percentage during the ripening process. Figure 4-6 represents a normalized plot of the data which shows the extent of DOC removal during the process. The average influent DOC concentration during the ripening period was 12.31 ± 4.77 and the effluent DOC was 11.05 ± 4.47mg/L.

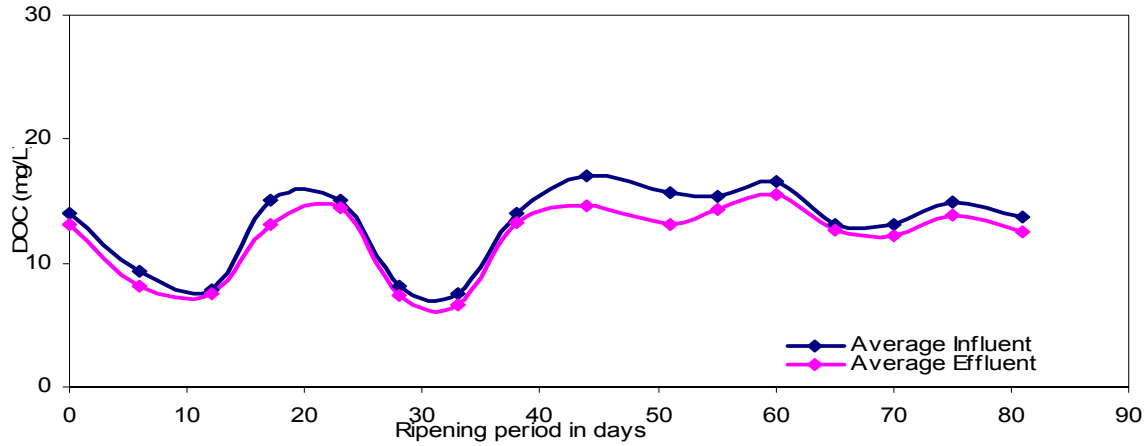



Figure 4-4. Variation of DOC concentration in influent and effluent during ripening of batch reactors (Influent: secondary effluent; media size: 0.8 – 1.25 mm, oxic condition)

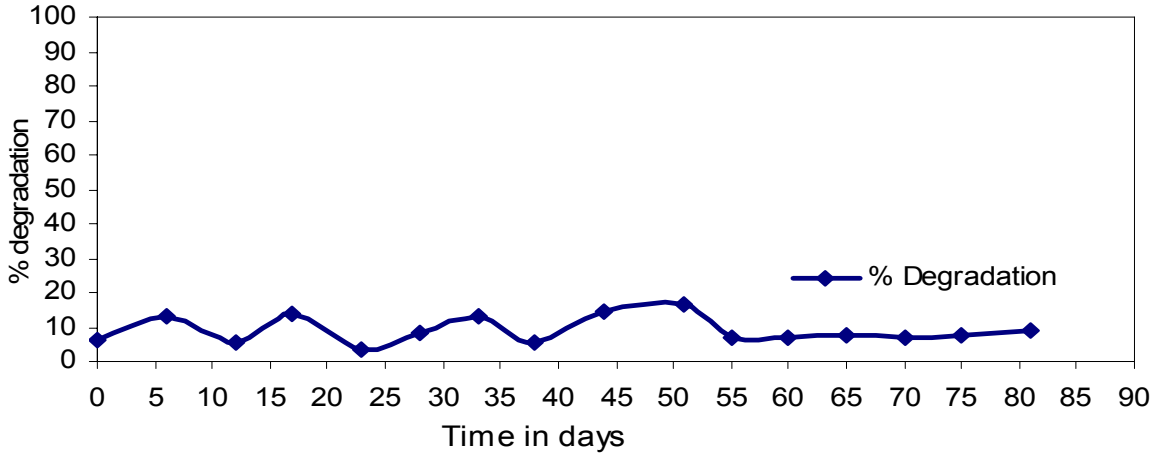



Figure 4-5. Change in DOC degradation during the ripening of batch reactors (Influent: secondary effluent; media size: 0.8 – 1.25 mm, oxic condition)

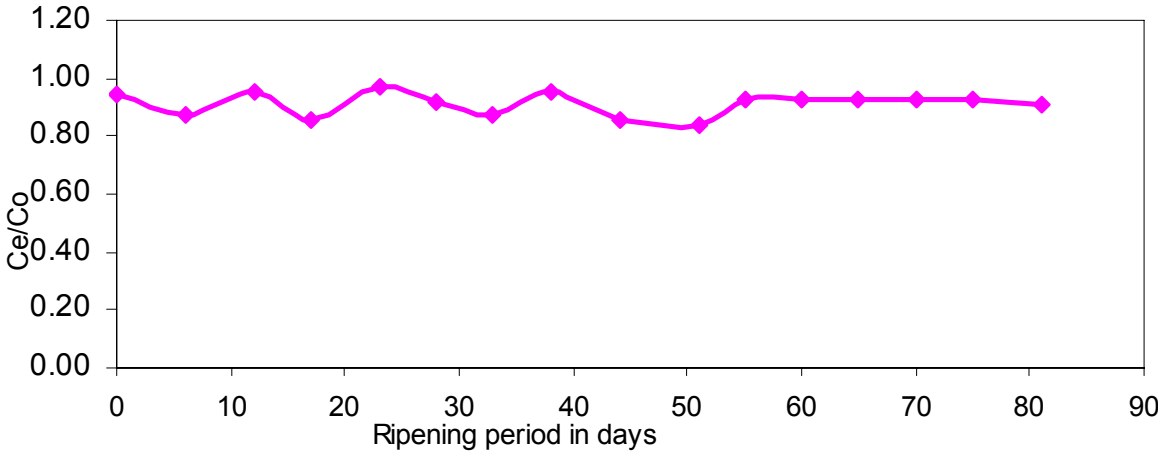



Figure 4-6. DOC removal during ripening period of batch reactors (Influent: secondary effluent; media size: 0.8 – 1.25 mm, oxic condition)

### 4.2.2. Soil column ripening process

For soil column experiments, three borosilicate glass columns of internal diameter of 5 cm and length of 30 cm packed with clean sand of size 0.8 - 1.25 mm diameter were used. Secondary effluent was used as influent with flow rate 0.4 m/d. Ripening process was continued for more than two months till the soil columns were stabilized with respect to DOC removal. During the process, influent and effluent DOC were continuously monitored. The small intermediate plastic bottle was used in order to prevent DOC degradation of the influent before entering the column by reducing residence time in the tank. For the constant flow rate, constant head was needed. So, constant head was maintained by this plastic bottle. Influent tank was fed daily with the amount of water needed to operate the column; the rest of the influent being stored in the fridge. Figure 4-7 shows the variation in influent and effluent DOC concentrations during the ripening process while Figure 4-8 shows the amount of DOC degradation in percentage during the ripening process. Figure 4-9 represents a normalized plot of the data which shows the extent of DOC removal during the process. The average influent DOC concentration during the ripening period was 12.47 ± 4.69 mg/L and the effluent DOC was 11.65 ± 4.54, 11.74 ± 4.55 and 12.54 ± 4.04 mg/L for SC1, SC2 and SC3 respectively. On average a DOC removal of about 12 % was obtained for three columns at the end of ripening period.

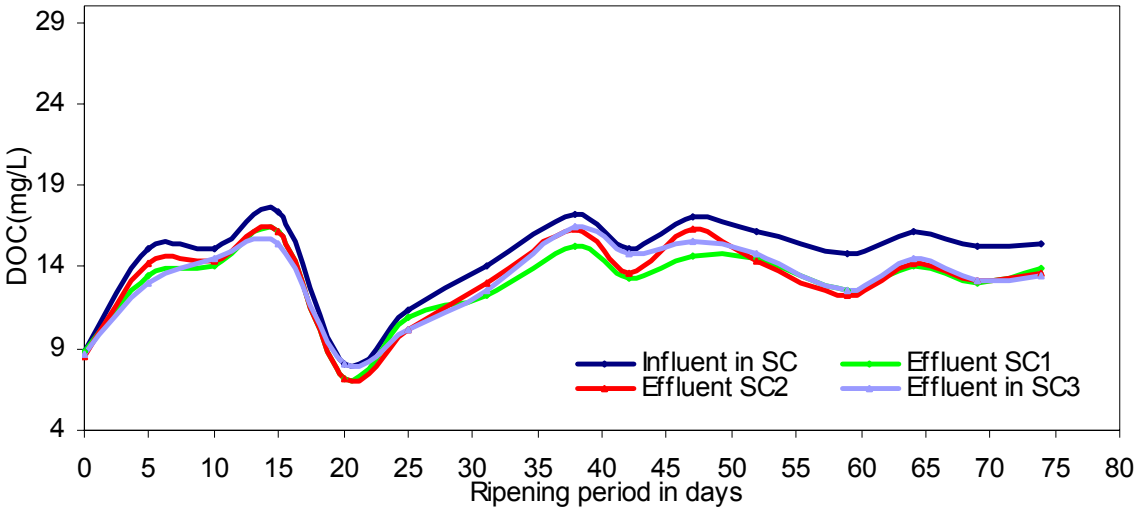



Figure 4-7. Change in DOC concentration of influent and effluent during ripening of soil columns (Influent: secondary effluent; HLR = 0.4 m/day, column depth = 30 cm, media size: 0.8 – 1.25 mm, oxic condition)

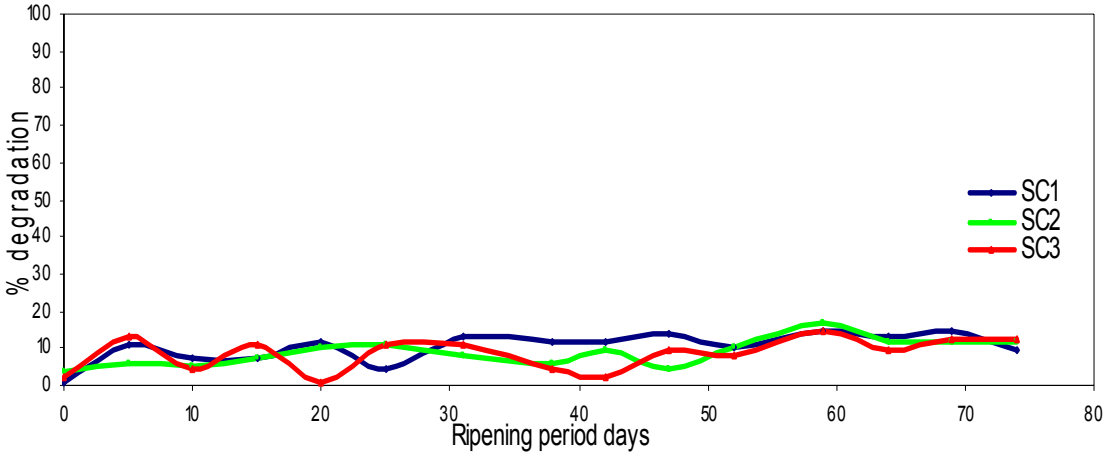



Figure 4-8. Change in DOC degradation in soil columns during the ripening period (Influent: SE, HLR = 0.4 m/day, column depth = 30 cm, media size: 0.8 – 1.25 mm, oxic condition)

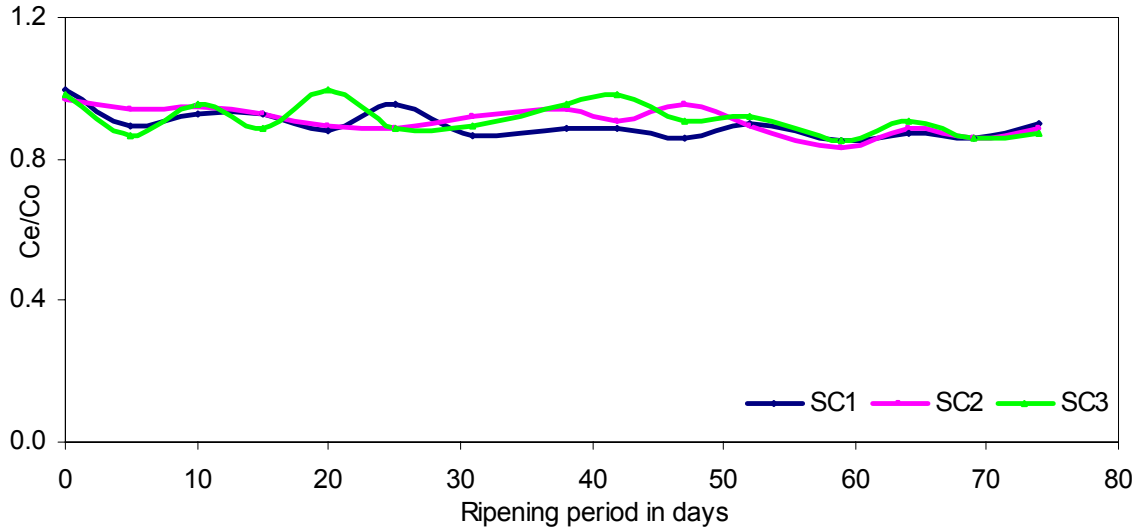



Figure 4-9. DOC removal during the ripening of soil columns (Influent: secondary effluent; HLR = 0.4 m/day, column depth = 30 cm, media size: 0.8 – 1.25 mm, oxic condition)

## 4.3. E2 and EE2 detection by using F-EEM

Concentration of estrogen compounds is generally determined by ELISA technique. This technique is more expensive, time consuming, and requires a high degree of analytical knowledge. F-EEM detection has been also used to determine the relative concentrations of estrogen compounds in µg/L level (Yoon *et al.*, 2003).

According to this technique fluorescence of E2 and EE2 was detected at excitation wave length of 280 nm and emission wavelength of 310 nm. Fluorescence peaks were observed on F-EEM. Figure 4-10 shows E2 and EE2 peaks in MQ water.

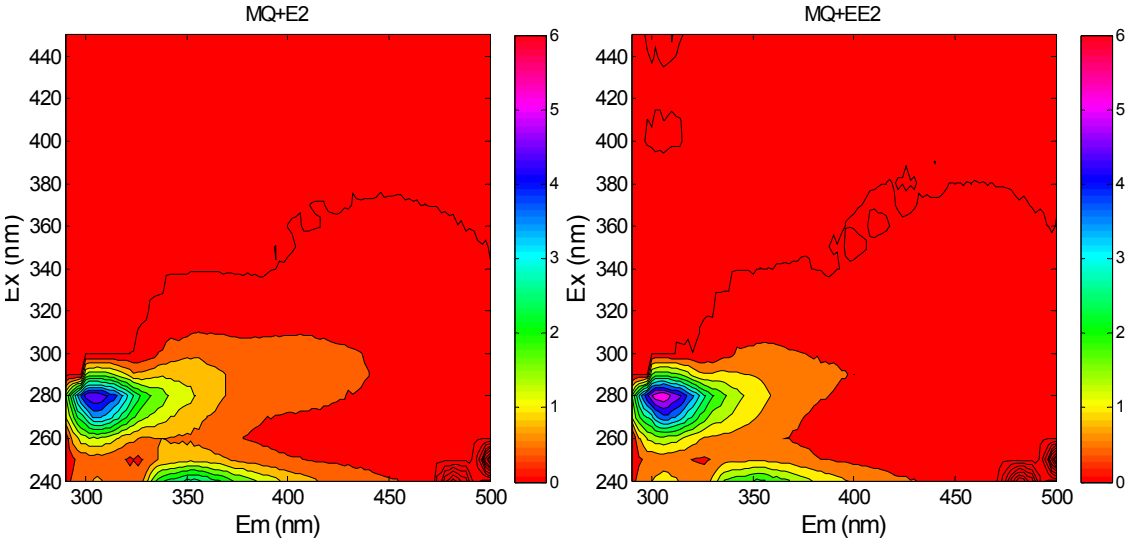



Figure 4-10. F-EEM spectra of E2 and EE2 standards with MQ water of concentration 100 µg/L

On the F-EEM plot estrogen peak appeared at intensity level of excitation wavelength of 280 nm and emission wavelength of 310 nm

### 4.3.1. 17β-Estradiol

Fluorescence detected E2 peak at excitation wavelength of 280 nm and emission wavelength of 310 nm. F-EEM was used to determine the relative concentration of estrogen in µg/L level. So, for the application of this technique calibration curve were prepared by using MQ water and sample water with E2. The entire F-EEM plot for calibration curve determination is given in Appendix D1.

**a) Calibration curves for known concentration of E2 in MQ water**

Calibration curves of E2 were prepared by using fluorescence detected intensity level (Ex – 280 nm and Em – 310 nm). Standard solutions were prepared with known concentrations (5 - 100 µg/L) of E2 in MQ water. Fluoro Max-3 fluorescence measurement instrument was used for E2 detection. Duplicate calibration curves were prepared and degree of accuracy was achieved. Figure 4-11 shows the calibration curves of E2.with $R^2$ = 0.9957 and $R^2$ = 0.9144. Using F-EEM, the fate of E2 can be determined in sample with µg/L level. However, further more works are needed to measure E2, accurately.

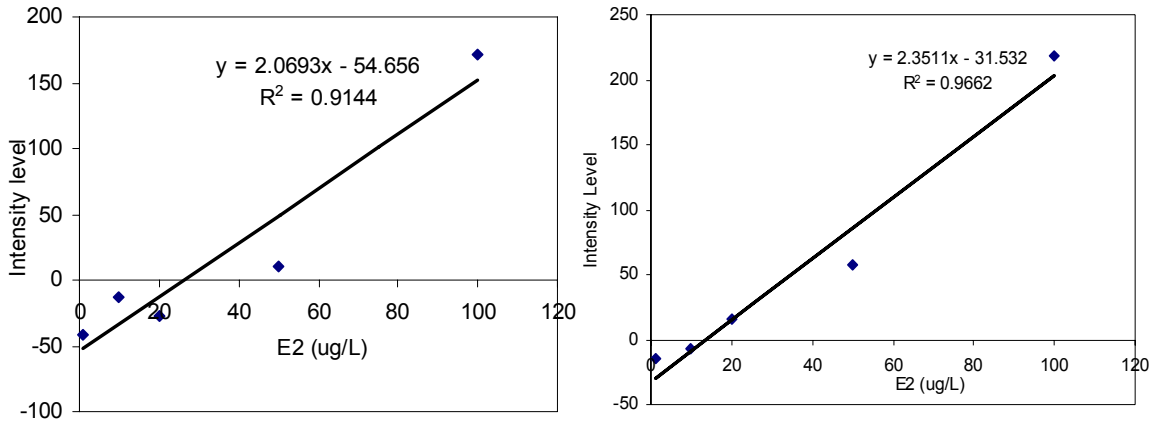



Figure 4-11. Calibration curve of E2 at Ex – 280 nm and Em – 310 nm with MQ

**b) Calibration curve for known concentration of E2 in mixture of DCW and SE**

Calibration curve of E2 in MQ water with known concentration was appropriate. But, in this research mixture of DCW and SE (1:1) was used as influent. Hence, calibration curve of E2 with known concentration in mixture of DCW and SE (1:1) was also plotted. Intensity levels of F-EEM spectra were used for calibration. Solid phase extraction (SPE) was used for sample pre-treatment. MQ water with known concentration was used as a control. Detail of the process and F-EEM spectra are shown in Figure 4-12.

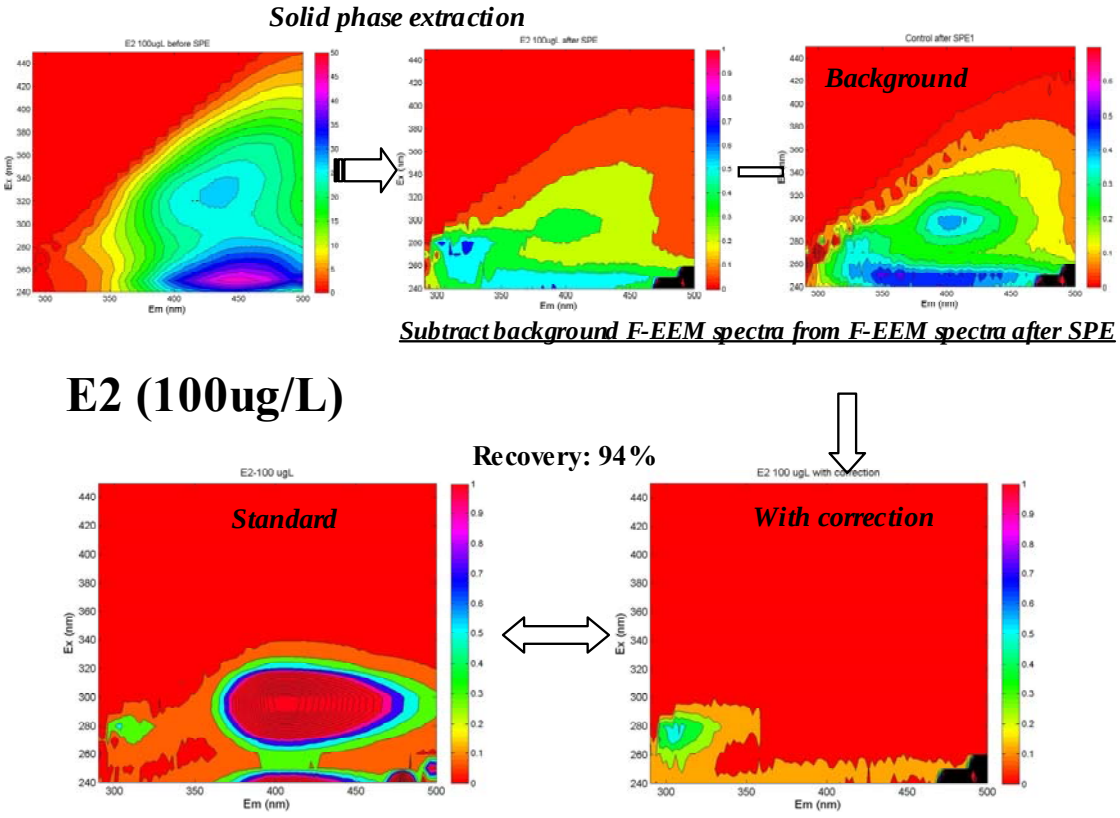



Figure 4-12. F-EEM spectra of E2 for the process of sample correction

Figure 4-12 shows that 100 µg/L of E2 was spiked in a mixture of DCW and SE (1:1) and F-EEM spectra were observed with SPE and without SPE. Similarly, F-EEM spectra of background were measured and background F-EEM spectra was subtracted from F-EEM after SPE. MQ water with known concentration was used as control. The results were then compared with control and the accuracy was found to be 94 %.

This study showed that it is possible to determine E2 concentration in sample using F-EEM, but certain correction factor was required to deduct (spectra of background water). Hence, by applying this method, calibration curve of E2 in mixture of DCW and SE was plotted. Detailed method is explained below:

- Mixture of DCW and SE (1:1 ratio) was used as sample water.
- Three different concentrations (50 µg/L, 100 µg/L and 200 µg/L) of E2 were spiked
- Sample water without E2 was prepared for correction.
- SPE was applied for sample pre-treatment.
- 10 % methanol was used for dilution
- Methanol water was used as blank
- Intensity level of spiked samples were measured at Ex – 280 nm and Em – 310 nm
- Intensity level of background (mixture of DCW and SE (1:1 ratio) water (no E2) was measured.
- Intensity level of background water was deducted from the intensity level of spiked samples
- After deduction, remaining value of intensity level at Ex – 280 nm and Em – 310 nm were used to plot the calibration curve as shown in Figure 4-13.

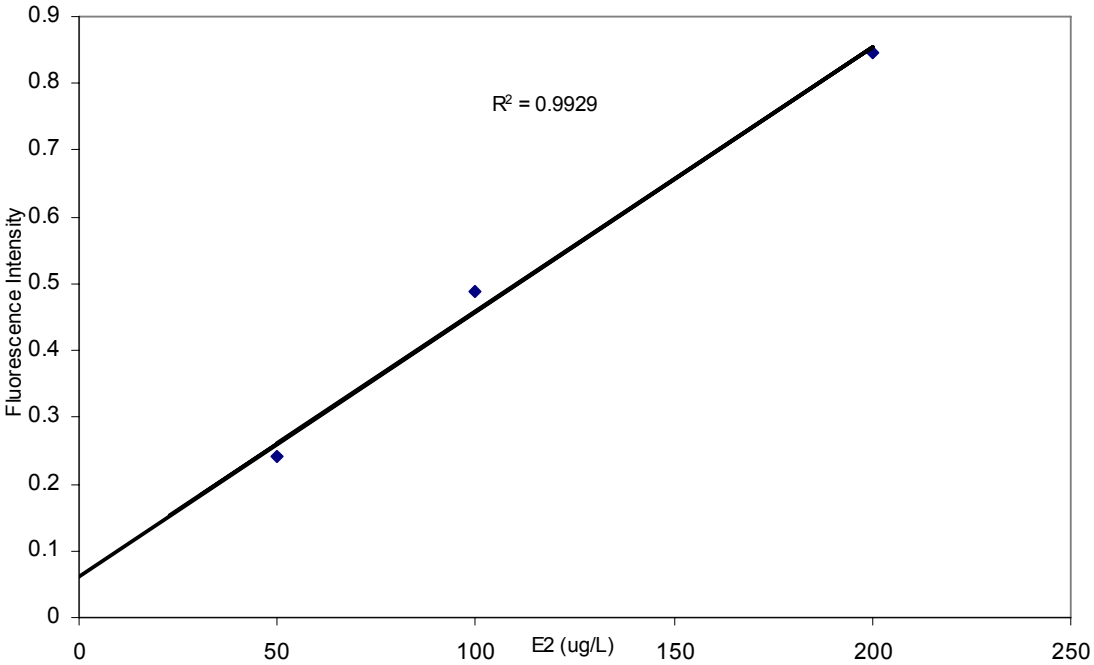



Figure 4-13. The calibration curve for E2 measurement using F-EEM after applying the correction factor

## 4.3.2. 17α-Ethinylestradiol (EE2):

F-EEM plots in Figure 4-10 shows that fluoresance peak appears at wave length Ex – 280 nm and Em – 310 nm. So, F-EEM was used to determine the concentration of EE2 in µg/L level and calibration curve was plotted. Mixture of DCW and SE (1:1) as well as MQ water was used for sample preparation. Known concentration of EE2 in MQ water and mixture of DCW and SE (1:1) was spiked and F-EEM intensity level was measured. Detail procedure is explained below and the entire F-EEM plots are given in Appendix D1.

**a) Calibration curves for known concentration of EE2 in MQ**

Calibration curve of EE2 was prepared by using fluorescence detected intensity level. Standard solutions (5 - 100 µg/L) were prepared with MQ water. For the degree of accuracy, two sets of F-EEM graphs were prepared. Intensity level at Ex – 280 nm and Em – 310 nm was used for calibration. Figure 4-14 showed that $R^2$ = 0.9957 and $R^2$ = 0.9351. Hence, it can be concluded that F-EEM technique was appropriate for determination of EE2 concentration in MQ water.

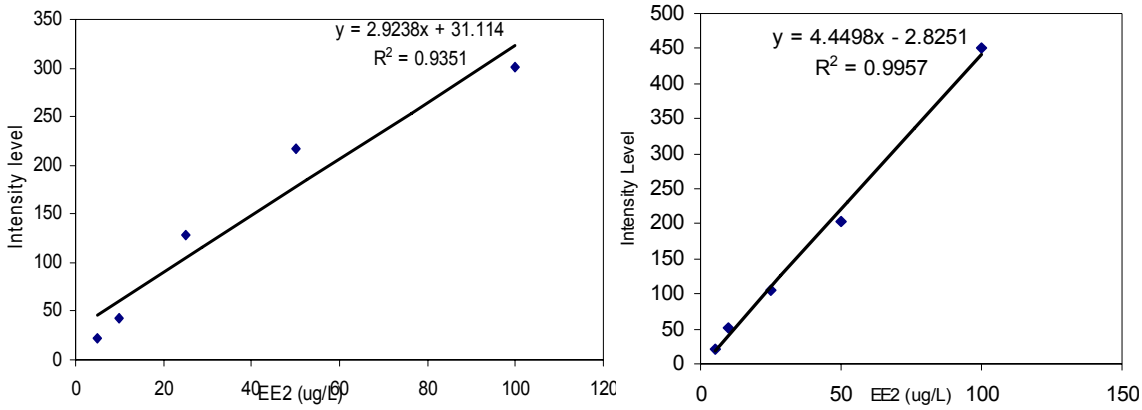



Figure 4-14. Calibration curve of EE2 at Ex – 280 nm and Em – 310 nm with MQ

**b) Calibration curve for known concentration of EE2 in mixture of DCW and SE**

Calibration curve of EE2 in mixture of DCW and SE (1:1 ratio) with known concentration (50 µg/L, 100 µg/L and 200 µg/L) was plotted. SPE was used for sample pre-treatment. MQ water with known concentration was used as a control to determine if there is intensity difference. Detail procedure for calibration curve of EE2 is exactly similar to the E2. Detail of the process and F-EEM spectra were shown in Appendix D1 – III. In this case, accuracy of the corrected sample was found to be 85 %. Final calibration curve of EE2 after subtracting background spectra is shown in Figure 4-15

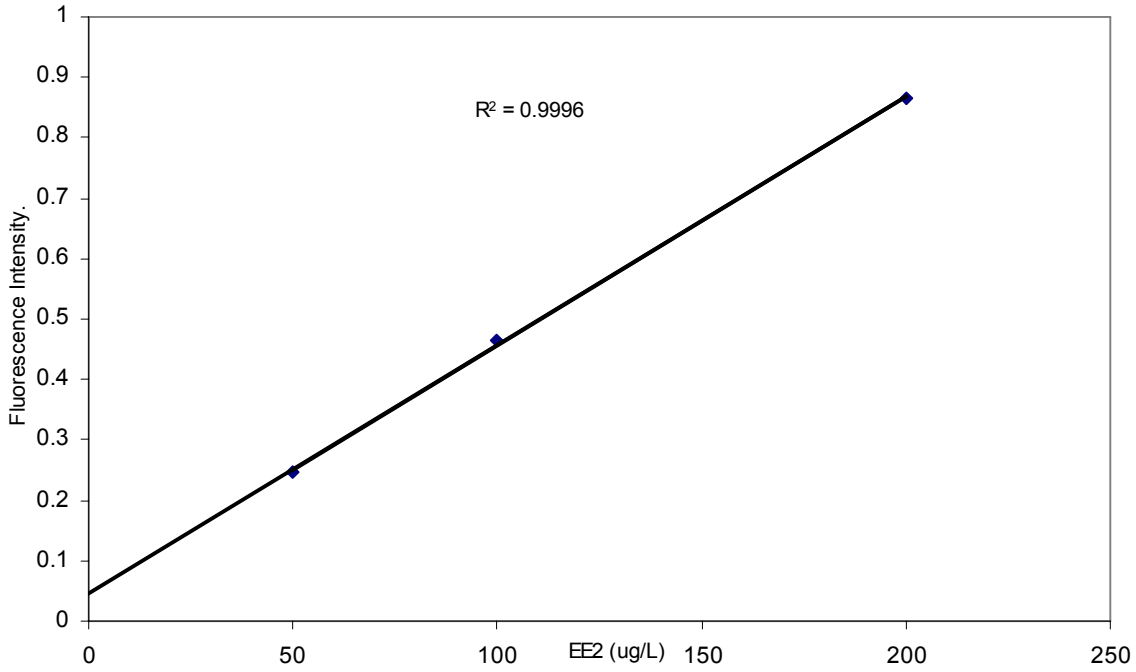



Figure 4-15. Calibration curve for EE2 measurement using F-EEM after applying the correction factor

## 4.4. Abiotic batch experiment using sodium azide

In this research the experiments were conducted under abiotic and biotic conditions to compare the removal efficiency of estrogen between adsorption and biodegradation.

Laboratory-scale abiotic experiment was conducted to investigate the appropriate concentration of sodium azide which minimizes the microbial activity during the experiment. For that, batch reactors were deployed as follows: 100 g of clean dry sand was kept in eight batch reactors of volume 500 mL and filled with wastewater secondary effluent up to 400 mL. Then, ripening process was continued till the batch reactors were stabilized with respect to DOC removal. After stabilization, three different concentrations of sodium azide (2 mM, 10 mM and 20 mM) were spiked in duplicates and DOC removal was observed until 70 days. DOC removal during the study period is presented in Figure 4-16. F-EEM spectra were measured after 5 days and 70 days of the experiment (Figure 4-17).

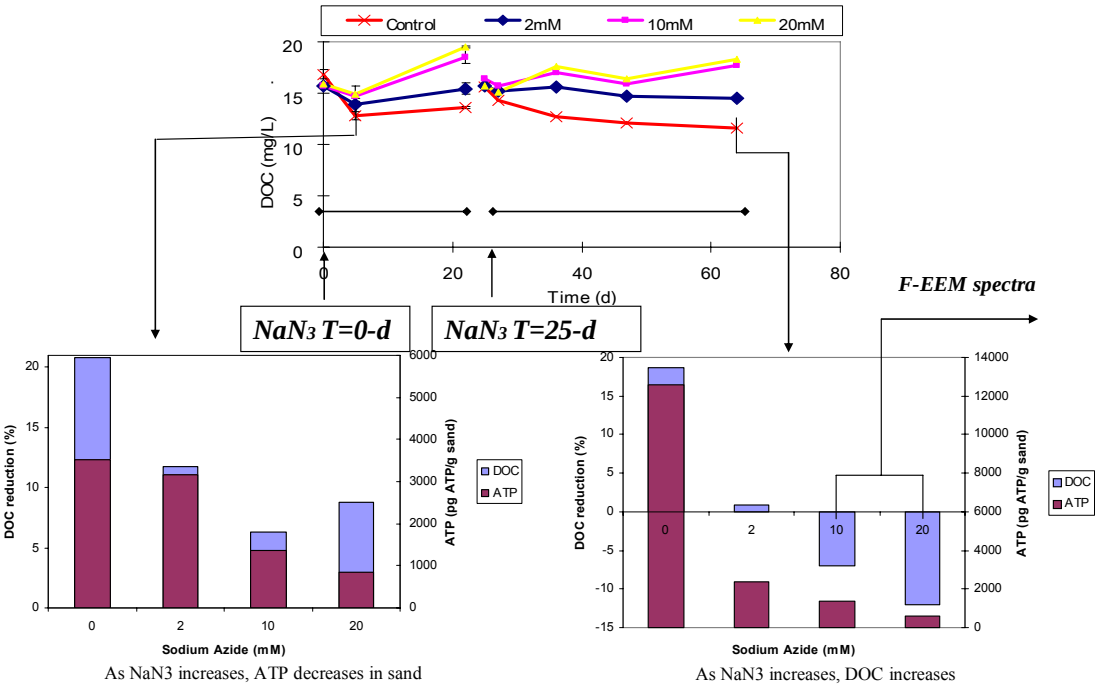



Figure 4-16. DOC removal in different concentration of the sodium azide

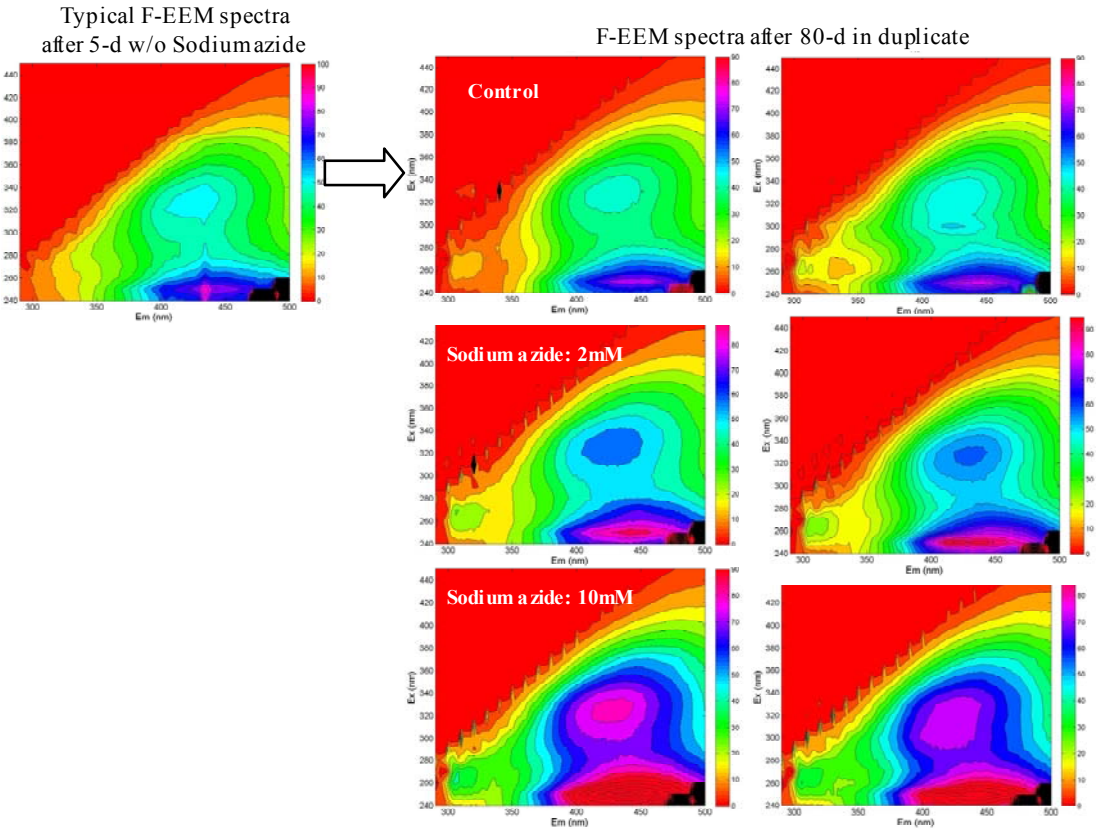



Figure 4-17. F-EEM spectra after 5 days and 70 days of the abiotic experiment with sodium azide on ripened sand

Table 4-3. F-EEM detected intensity level after 70 days of abiotic experiment in different reactors

| Conc.of $NaN_3$ | Intensity Level | | |
|---|---|---|---|
| | Protein Peak | Primary humic peak | Secondary humic peak |
| 2 mM | 29.92 | 95.32 | 60.97 |
| | 26.11 | 94.24 | 59.37 |
| 10 mM | 39.74 | 123.04 | 82.41 |
| | 39.74 | 110.99 | 74.72 |
| Control | 14.79 | 73.106 | 46.7 |
| | 19.18 | 76.2 | 49.01 |

In first phase, the experiment was started with spiked known concentration of sodium azide and DOC was measured up to 25 days. Result of DOC removal rate is shown in Figure 4-16 (a) & (b). Similarly, in second phase, influent on the reactor was replaced and same concentrations of sodium azide were spiked and DOC was measured up to 45 days. Result of DOC removal rate is shown in Figure 4-16 (a) & (c). In both phases, DOC removal in case of 2 mM sodium azide was remaining constant. But, it increased in case of 10 mM and 20 mM as shown in Figure 4-16. The corresponding values of DOC are presented in Appendix A3.

Figure 4-17 shows the F-EEM spectra at beginning and end of experiment. Similarly, Table 4-3 shows the corresponding intensity level after 70 days of the experiment. This shows that protein peaks increased with increase in sodium azide concentration. Additionally humic and fulvic peaks also increased.

According to the F-EEM spectra and DOC value, high concentration of sodium azide interfered with bio-film. It means 2 mM of sodium azide was appropriate to inactivate the microorganism without any interference on the bio-film around the sand surface. ATP appeared even in case of sample with 20 mM of $NaN_3$ which was the lowest value. Hence, according to the result of ATP measurement 20 mM of $NaN_3$ was appropriate for inactivation of microorganism. But, high concentration of $NaN_3$ interfered with ripened layered of sand and detached the bio-film. However, both 2 mM and 20 mM of $NaN_3$ were used in this study.

## 4.5. Adsorption isotherms of E2 and EE2

Adsorption isotherms of 17β-Estradiol (E2) and 17α-Ethinylestradiol (EE2) was determined by conducting a laboratory-scale batch experiments. Batch reactors were prepared with various influents and different conditions. Experiment was conducted in dark to simulate the process that occurs in RBF. The different arrangements of batch reactors for this study are presented under section 3.1.2. F-EEM and ELISA were used for analysis of the results. Intermediate samples were taken for F-EEM analysis. But, final estrogen concentrations were identified by ELISA test. Solid phase extraction was used for sample pre-treatment. Adsorption isotherms of estrogens were analyzed by using Freundlich sorption model. Only ELISA test results were used for this model.

In this experiment, isotherms of estrogen were determined in the case of adsorption and biodegradation as well as adsorption alone. Hence, this experiment was divided into biotic and abiotic part. In abiotic part, it is expected that only adsorption is occurring. But in biotic part, adsorption and biodegradation both are occurring. Each part of the experiment is explained below:

### 4.5.1. 17β-Estradiol (E2)

In this experiment, isotherm of 17β-Estradiol (E2) was determined by Freundlich model. The experiment was carried out by using fixed amount of media and different initial concentrations of adsorbate. Effluents concentration of E2 was determined by using ELISA. F-EEM spectra were measured to determine the fate of E2 during the experiment.

**ELISA.**
ELISA test was conducted according to the instruction manual. Solid phase extraction was applied for sample pre-treatment. ELISA test result of 17β-Estradiol (E2) is presented in Appendix C1 – I.

In this experiment, all the reactors were under oxic condition. Ripened sand was used for biotic and clean sand was used for abiotic reactors. Similarly, mixture of DCW and SE effluent (1:1) was used for biotic reactors and ACDW was used for abiotic reactors. Five different concentrations of E2 were spiked in each reactor and the effluent concentration of the estrogens was measured after five days Figure 4-18 shows equilibrium concentrations of E2 (µg/L) against adsorbed amount of E2 per gram of sand (µg/g) under abiotic condition.

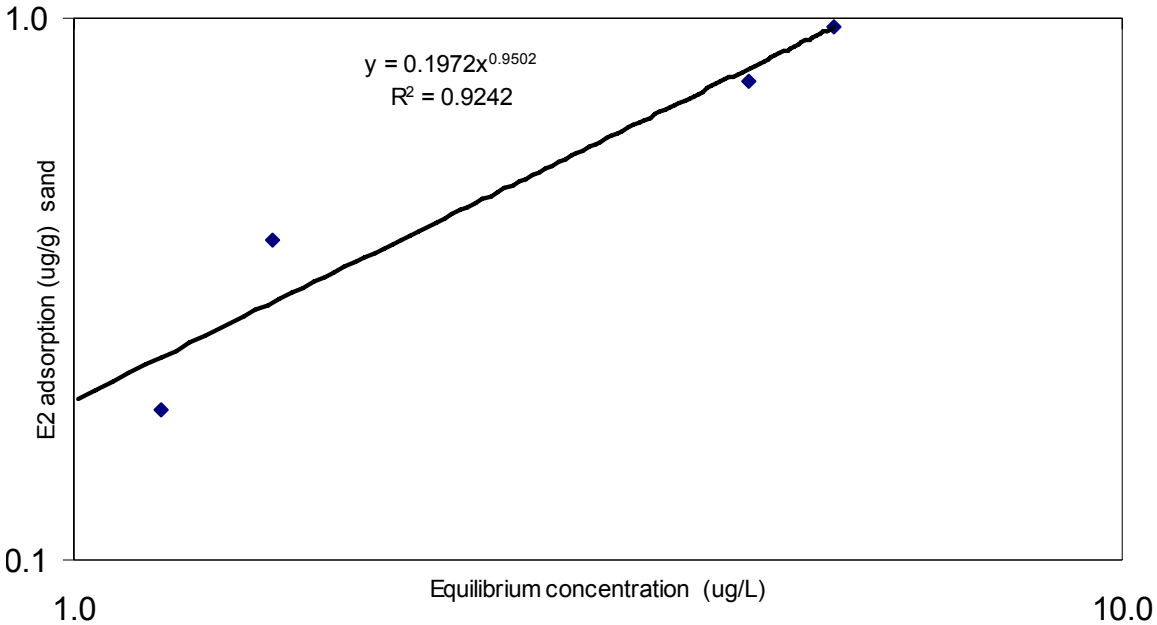



Figure 4-18. Freundlich isotherm of 17β-Estradiol (E2) in abiotic reactors (clean sand)

Figure 4-18 shows the adsorption capacity and intensity E2 in abiotic reactors. In clean sand only adsorption should have occurring and found that the adsorption capacity of sand (k) = 0.20 µg/g and adsorption intensity of sand (1/n) = 0.92. In this case, the adsorbate removal per unit weight of adsorbent is not changing significantly. But in the case of biotic reactors adsorption as well as biodegradation was taken place and found that 99.96 % removal. In this case E2 compound was removed efficiently and isotherms was not obtained. From the above graph, value of 1/n is less than one. Hence, isotherm of E2 was favorable in abioitc condition. Therefore, adsorption and biodegradation was the main mechanism of E2 removal. F-EEM detected intensity levels of E2 in case of biotic and abiotic condition are discussed as below:

**F-EEM**

F-EEM detected intensity level at wavelength of Ex – 280 nm and Em – 310 nm showed that the decreasing rate of E2 in biotic and abiotic condition. Sorption and biodegradation of 17β-Estradiol in biotic and abiotic reactors showed the different rate and different amount. In abiotic reactors only sorption was occurring but in biotic reactors sorption and biodegradation was occurring. The results and discussion for both conditions are given below.

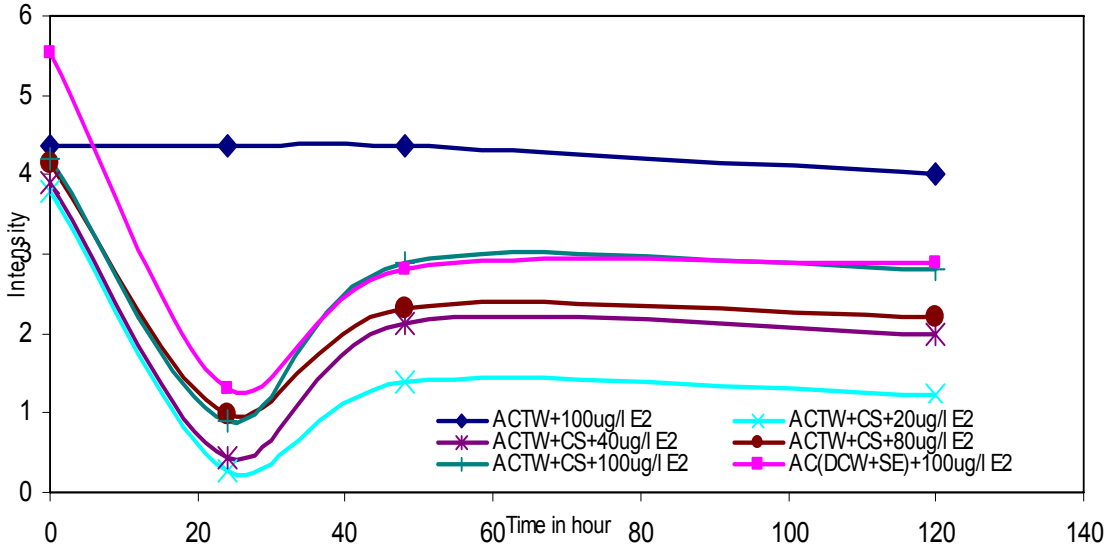



Figure 4-19. FEEM detected intensity level of 17β-Estradiol (E2) in abiotic reactors (with clean sand)

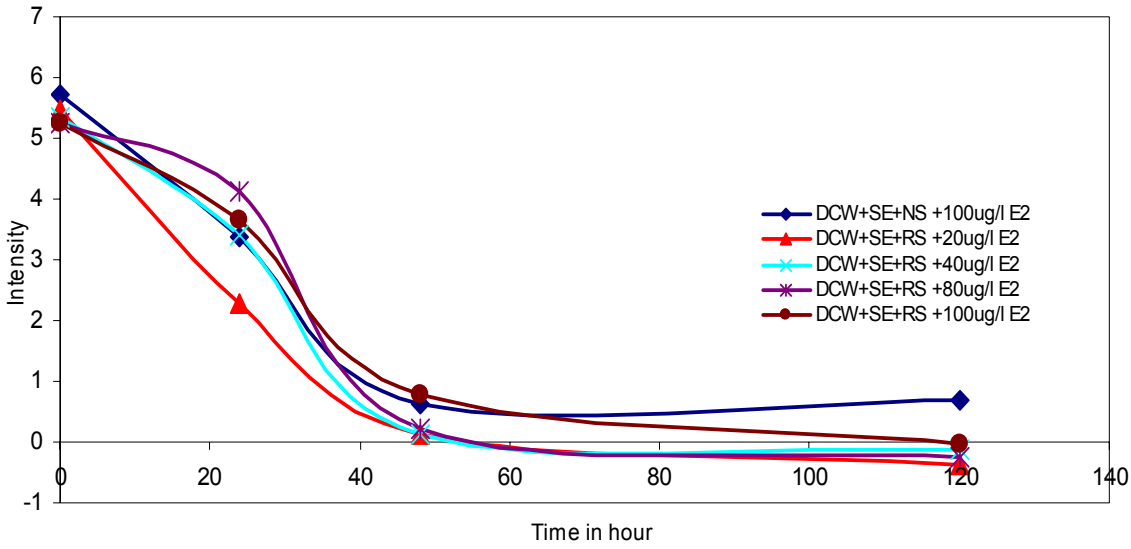



Figure 4-20. FEEM detected intensity level of 17β-Estradiol (E2) in biotic reactors (with ripened sand)

Figure 4-19 shows the removal rate of E2 in abiotic condition. Florescence detected intensity level of E2 showed that only sorption should have occurred because intensity level in 100 µg/L of E2 with no sand remains constant during the experiment. But, in rest of the reactors intensity level of the fluorescence were significantly reduced up to 24 hour but from 24 to 48 hours it increased and remained constant. It was likely that progressive increase in sorption rate from 0 hour and saturation of sorbent binding site at 24 hours may reflect the duration of maximum sorption. However, decrease in amount of sorption between 24 to 48 hours suggested to represent E2 desorption back into the aqueous phase and after 48 hours concentration remain constant. So, that should be the sorption capacity of sand. Similar type of result is mentioned in Lai *et al.*, 2000.

Figure 4-20 shows the removal rate of E2 in biotic reactors. Florescence detected intensity level of E2 showed that sorption and biodegradation should have occurred because F-EEM detected intensity level was rapidly decreased up to 48 hours and bit slower decrease between 48 to 60 hours and remained constant. In the literature, for comparable conditions sorption kinetic of estrogen compounds were identified in three stages. An initial rapid sorption (4.0 – 9.4 µg/g/h) between 0 and 0.5 hour, followed by a period of 1 hour slower sorption (1.5 – 2.9 µg/g/h) prior to steady decrease in sorption (0.07 – 0.37 µg/g/h) (Lai *et al.*, 2000). In this experiment, this may also have occurred with the sorption of 17β-Estradiol (E2). However, the temporal resolution of the data was not fine enough to clearly demonstrate this effect.

On the other hands, in the case of ripen sand sorption and desorption processes was not occurred. In the literature, sorption of E2 in the sediment exhibited correlation with DOC content (Lai *et al.*, 2000). In biotic reactors DOC content was enough. Hence, removal of E2 was not limited. Influent and effluent DOC are shown in Appendix A4.

Finally, in abiotic reactors approximately 90 % E2 was removed after 24 hours of the experiment and between 24 to 48 hours removal of E2 was negative then remained

constant, the constant state might be the adsorption isotherms of E2.(see Figure 4-18). But, in the case of biotic reactors removals were drastically reduced up to 48 hours and remain constant after 60 hours. Study showed that maximum 99 % of E2 was removed in biotic reactors. F-EEM spectra showed that organic materials play the major role in removal of estrogen. In conclusion, adsorption and biodegradation was the major mechanism for E2 removal.

### 4.5.2. 17α-Ethinylestradiol (EE2)

Adsorption isotherms of 17α-Ethinylestradiol (EE2) was determined by laboratory scale batch experiment in oxic (Biotic – Abiotic) condition. Experiment was carried out by using equal mass of absorbent and different concentration of adsorbate. F-EEM spectra were measured to determine the fate of EE2 during the experiment.

**ELISA**
All the method, process and condition were exactly same as in the case of 17β-Estradiol (E2). Experimental setup was divided into abiotic and biotic part for determination of the isotherms. The detail experimental setup and procedure was explained in section 3.1.2. Isotherm of EE2 was determined by Freundlich model.

ELISA test was carried out for the determination of effluent concentration of EE2. Solid phase extraction was applied for sample pre-treatment. F-EEM spectra were measured to determine the fate of EE2 during the experiment. ELISA test results of EE2 are shown in Appendix C1 – II.

Equilibrium concentrations of the EE2 in reactors were determined by using ELISA. Five different concentrations of EE2 were spiked in each reactor then the effluent concentrations of the estrogens were measured and the graphs between equilibrium concentrations (µg/L) against adsorbed amount of estrogen per gram of sand (µg/g) were showed in (Figure 4-21 and Figure 4-22).

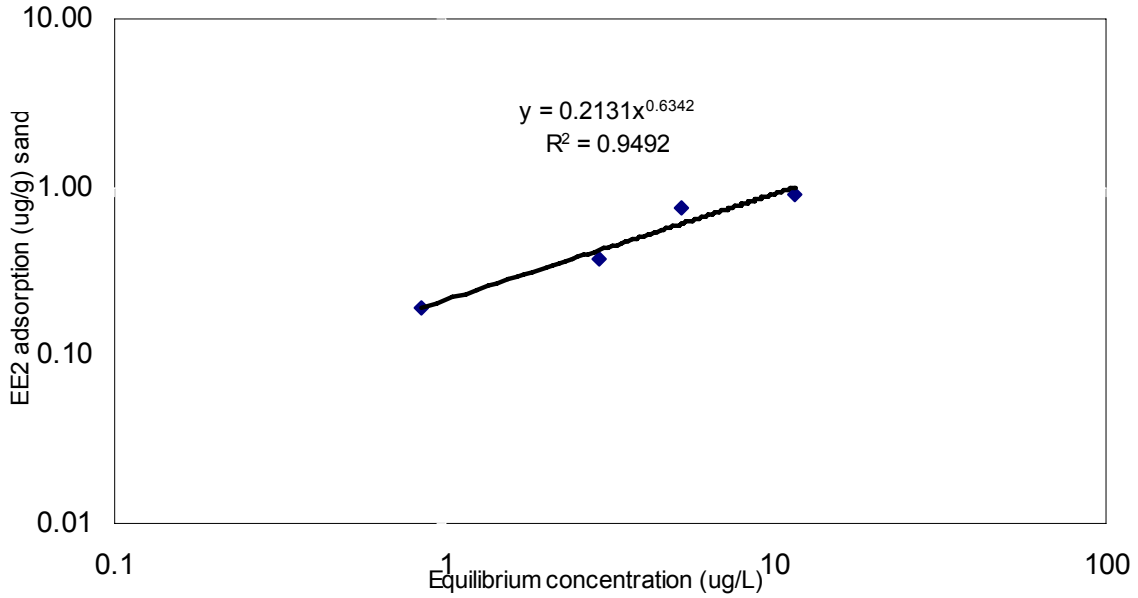



Figure 4-21. Freundlich isotherm of 17α-Ethinylestradiol (EE2) in abiotic reactors (clean sand with oxic condition)

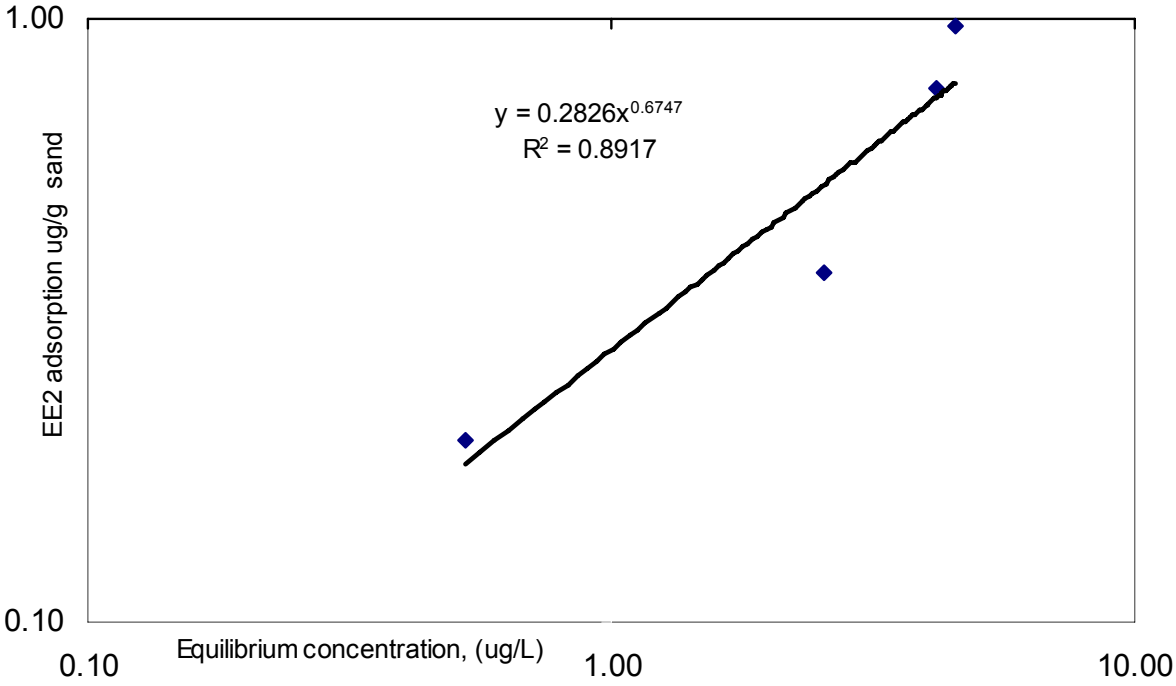



Figure 4-22. Freundlich isotherm of 17α-Ethinylestradiol (EE2) in biotic reactors (ripened sand with oxic condition)

Figure 4-21 shows that in abiotic reactors only adsorption was occurred and found that the adsorption capacity of sand was (k) = 0.21 µg/g and adsorption intensity of sand was (1/n) = 0.63. Similarly, Figure 4-22 shows that in biotic reactors adsorption as well as biodegradation were taken place and found that the adsorption capacity of sand (k) = 0.22 µg/g and adsorption intensity of sand (1/n) = 0.68. From graphs removal capacity of EE2 in biotic condition was slightly higher than abiotic condition. In both cases

adsorption intensity was less than one. Hence isotherms were favorable. In conclusion, adsorption and biodegradation was the main removal mechanism of EE2. F-EEM detected intensity levels of EE2 in case of biotic and abiotic conditions are discussed as below:

**F-EEM**

ELISA test results, from isotherms experiment showed that sorption and biodegradation was the main removal mechanism for EE2. It depends on the organic materials present in the reactors as well as microbial activities. In abiotic reactors sorption should have occurred on clean sand surface but in biotic reactors sorption and biodegradation should have occurred on ripened layer of sand. Trend of removal in biotic and abiotic reactors are describe below:

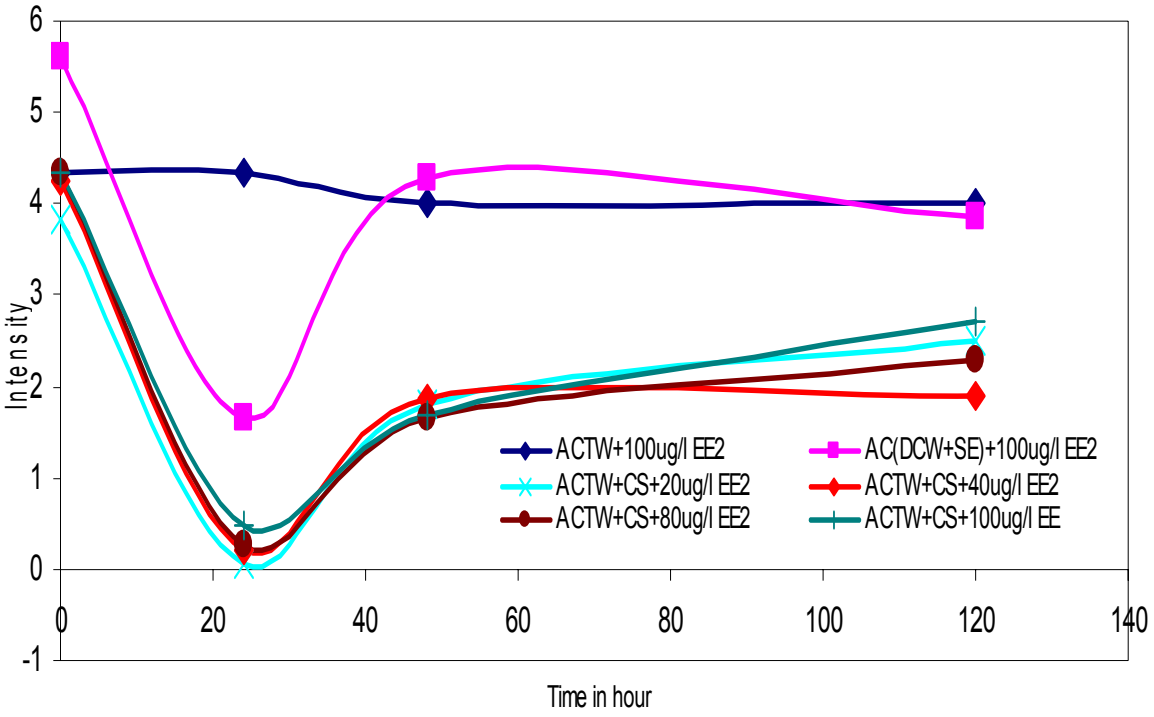



Figure 4-23. Intensity level of 17α-Ethinylestradiol (EE2) during the experiment in abiotic (oxic) reactors with ACTW as well as clean sand

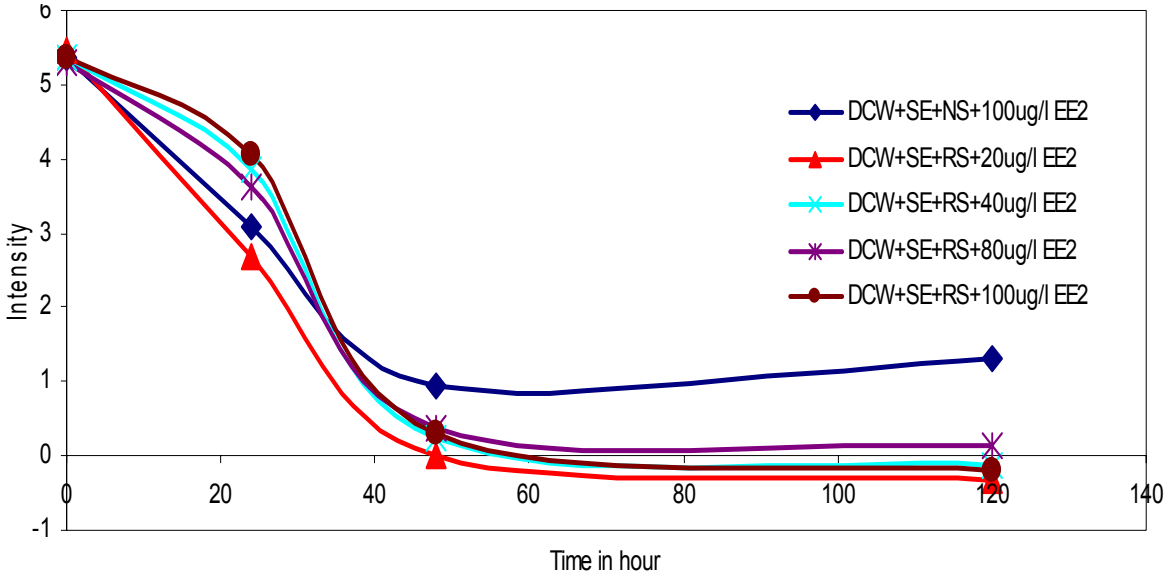



Figure 4-24. Intensity level of 17α-Ethinylestradiol (EE2) during the experiment in biotic (oxic) reactors with mixture DCW and SE (1:1) as well as ripened sand

Figure 4-23 shows the florescence detected intensity level of EE2 on abiotic reactors during the experiment. Result showed that only sorption occurred in abiotic reactors. Hence, fluctuation of F-EEM detected intensity level was caused by sorption and desorption process of EE2. Detail is explained in section 4.5.1.

On the other hand, Figure 4-24 shows that in biotic reactors sorption as well bio-degradation should have occurred. As described in section 4.5.1 sorption kinetic of estrogen compounds were identified in three stages. Those stages may also have occurred in the case of EE2. However, the temporal resolution of the data was not fine enough to clearly demonstrate this effect.

On the other hand, in the literature sorption of EE2 into the sediments exhibited correlation with DOC content. In biotic reactors DOC content was sufficient which shown in Appendix A5. Hence, F-EEM detected intensity level is not fluctuating. Consequently, EE2 removal was higher in biotic reactors than in abiotic reactors because it should be the presence of organic compounds as well as microbial activities.

Finally, in abiotic reactors approximately 90 % of EE2 was removed up to 24 hours of the experiment and between 24 to 48 hours removal of EE2 was negative and remained constant. But, in the case of biotic reactors removal was drastically reduced up to 48 hours and remained constant after 60 hours. Study showed that maximum 97 % of EE2 was reduced in biotic reactors with ripened sand. In conclusion, adsorption and biodegradation played the major role of removal of EE2. However, adsorption was the dominant in removal. All the F-EEM plots and intensity level are shown in Appendix D2 – II.

**Conclusion of isotherms test**

Isotherms experiment was conducted to determine the adsorption capacity for estrogen in different type of soil aquifer. Summary of the result is presented below:

Table 4-4. Adsorption capacity and intensity of estrogen in ripened sand (biotic) and clean sand (abiotic)

| | E2 | EE2 | |
|---|---|---|---|
| Isotherms Parameter | Clean sand (Abiotic) | Clean sand (Abiotic) | Ripened sand (Biotic) |
| K (µg/g) | 0.20 | 0.21 | 0.28 |
| 1/n | 0.95 | 0.63 | 0.67 |

Table 4-4 shows the adsorption capacity of E2 and EE2 in different condition. It showed EE2 has bit higher adsorption capacity in clean sand. On the other hand, removal of E2 compound in ripen sand was 99.9 %. Hence isotherm was not obtained. But, isotherm of EE2 was obtained with adsorption capacity K = 0.28 µg/g and intensity 1/n = 0.67. Result showed that adsorption and biodegradation was the major removal mechanism in both E2 and EE2. However, removal of E2 was more efficient than EE2. Main factors affecting the removal of estrogen were microbial activities, organic materials present in influent and ripened layer over the sand.

## 4.6. Determination of factors affecting E2 and EE2 removal

Batch reactors were deployed with various influents under different redox conditions to simulate the processes occurring during RBF and to study EDCs (steroid estrogens) removal during percolation of water through porous media. The different arrangements of batch reactors for this study are presented under section 3.1.3 in Table 3.4 and Table 3-5. Adsorption and biodegradation are the two mechanisms responsible for removal of EDCs during the RBF. Compounds that are being removed primarily via adsorption have the potential to remobilize (Birkett and Lester, 2003). If the compounds are being degraded, then they might be transformed to a non-estrogenic form and no longer be capable of causing endocrine disruption.

Mainly ELISA was used for analysis of EDCs removal. But, F-EEM was also used to observe the trend of removal. ELISA test guide manual mention that EE2 has high interference with E2 and E1. So, separate batch experimental setup was used for E2 and EE2 study.

In this set of experiment E2 and EE2, with concentration 300 µg/L were spiked in each reactor. Several methods were considered to inactivate microbial activity such as autoclaved demi-water, autoclaved fresh sand with demi-water and sodium azide (2 mM & 20 mM). Then samples were taken after 5 days and analyzed initial and residual E2 and EE2 concentrations as well as transformation of E2 to E1 and adsorption of E1 were analyzed using ELISA. Intermediate samples were taken for kinetic removal study and analyzed by using F-EEM.

## 4.6.1. 17β-Estradiol (E2) and Estrone (E1)

One set experiment was used to determine the factor which affected the removal of 17β-Estradiol (E2) and Estrone (E1) during the RBF. Different conditions of batch reactors were deployed with respect to the condition occur during RBF. Detail of the batch reactors are shown in section 3.1.3 and in table 3.4.

**a) Investigation on factors affecting E2 and E1 removal by ELISA**

Experiment was conducted according to the condition mentioned in section 3.1.3. 300 µg/L of E2 was spiked in each reactor but not E1. In this experiment E2 was converted into E1 and adsorptions as well as bio-degradation were taking place in different reactors. The results obtained from the experiment are shown in Table 4-5 and Figure 4-2 below:

Table 4-5. Percentage removal of E2 and remaining amount of E1

| Reactors condition | Spiked conc (µg/L) | Measured effl. conc (µg/L) | | Average effl conc (µg/L) | | Standard deviation | | % removal | Avg. % removal |
|---|---|---|---|---|---|---|---|---|---|
| | E2 | E1 | E2 | E1 | E2 | E1 | E2 | E2 | E2 |
| Autoclaved tap water | 300 | | 261.63 | | | | | | |
| | | | 246.63 | | | | | | |
| DCW+SE (2 mM $NaN_3$) | 300 | 1.51 | 5.24 | 1.51 | 4.72 | 0.01 | 0.74 | 98.25 | 98.43 |
| | | 1.5 | 4.19 | | | | | 98.6 | |
| Fresh sand + ACTW | 300 | 6.36 | 5.64 | 5.1 | 7.91 | 1.78 | 3.21 | 98.12 | 97.37 |
| | | 3.84 | 10.18 | | | | | 96.61 | |
| DCW+SE | 300 | 0.07 | ND | 0.09 | ND | 0.03 | ND | 100 | 100 |
| | | 0.11 | ND | | | | | 100 | |
| Fresh sand + DCW+SE | 300 | 0.13 | ND | 0.17 | ND | 0.05 | ND | 100 | 100 |
| | | 0.2 | ND | | | | | 100 | |
| Fresh sand + DCW+SE (2 mM $NaN_3$) | 300 | 15.7 | 3.04 | 8.53 | 3.66 | 10.1 | 0.87 | 98.99 | 98.79 |
| | | 1.36 | 4.27 | | | | | 98.58 | |
| Ripened sand + DCW+SE (2 mM $NaN_3$) | 300 | 1.33 | 0.87 | 2.32 | 1.22 | 1.39 | 0.49 | 99.71 | 99.6 |
| | | 3.3 | 1.57 | | | | | 99.48 | |
| Ripened sand + ACTW (2 mM $NaN_3$) | 300 | 8.18 | 2.4 | 8.13 | 2.46 | 0.08 | 0.49 | 99.2 | 99.18 |
| | | 8.07 | 2.52 | | | | | 99.16 | |
| Ripened sand + ACTW | 300 | 10.5 | 1.23 | 10.45 | 2.49 | 0.07 | 1.77 | 99.59 | 99.17 |
| | | 10.4 | 3.74 | | | | | 98.75 | |
| Ripened sand +DCW+SE | 300 | 0.53 | ND | 0.53 | ND | 0 | ND | 100 | 100 |
| | | 0.53 | ND | | | | | 100 | |
| Ripened sand +DCW+SE+(20 mM $NaN_3$) | 300 | 1.31 | 0.83 | 1.5 | 0.95 | 0.26 | 0.16 | 99.72 | 99.69 |
| | | 1.68 | 1.06 | | | | | 99.65 | |
| Ripened sand +DCW+SE (Anoxic) | 300 | 1.31 | 1.6 | 1.5 | 4.77 | 0.26 | 4.48 | 99.47 | 98.41 |
| | | 1.68 | 7.94 | | | | | 97.35 | |

The removal of E2 in batch reactors under different conditions ranges from 97.37 and 99 9 %. Result showed that different factors that should have affected the removal of E2 during the RBF. These are:

i. Organic material present in influent
ii. Suspended solid in the influent
iii. Bio-film associated on the ripened layer of sand
iv. Microbial activities
v. Different redox conditions

In Table 4-5, mixture of DCW and SE effluent with no sand has high capacity on estrogen removal due to the presence of organic materials, with high microbial activities as well as favorable redox condition. But, ACTW showed the low removal due to the absence of the entire factors except favorable redox condition. According to the results obtained in Table 4-5, all the responsible factors for removal of estrogen were analyzed. Figure 4-25 shows the comparative analysis of E2 removal in biotic and abiotic condition.

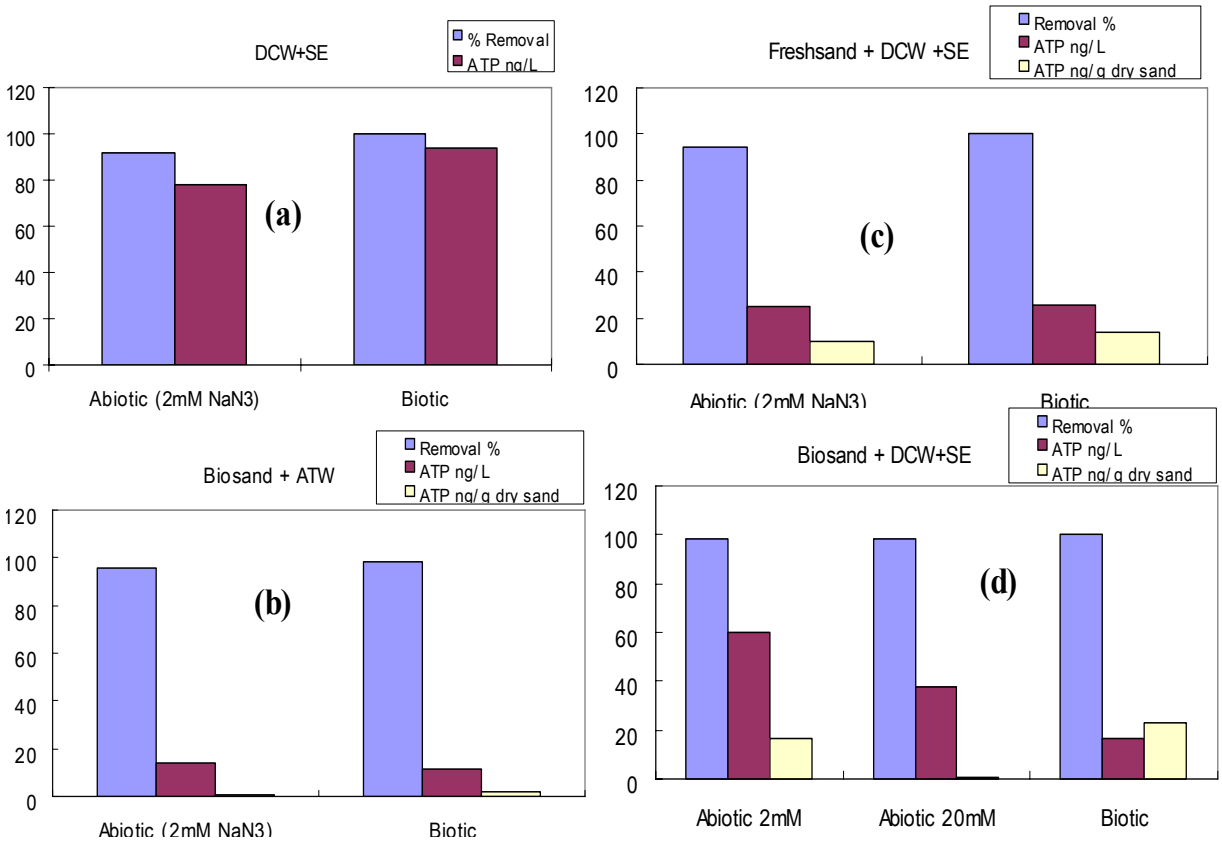



Figure 4-25. Comparative analysis of the microbial activities in different conditions and its impact on E2 removal

Figure 4-25 (a) shows that the ATP in mixture of DCW and SE without $NaN_3$ was higher than with $NaN_3$. It means microbial activity was higher in biotic condition. Hence, removal of E2 was higher under biotic than abiotic condition.

Figure 4-25 (b) shows that ATP in water phase as well as in sand phase in bio-sand and ACTW without sodium azide were higher than with sodium azide. It is due to the fact

that, microbial activities were higher in biotic condition. Hence, removal of E2 was higher in biotic condition than in abiotic condition.
Figure 4-25 (c) shows that ATP in water as well as sand phase was higher in biotic condition with ripened sand and a mixture of DCW and SE (no $NaN_3$) than in abiotic condition (2 mM $NaN_3$). Consequently, removal of E2 was higher in biotic condition than abiotic condition due to microbial activity.
Figure 4-25 (d) shows that mixture of DCW and SE with RS was used as media. 2 mM and 20 mM of $NaN_3$ were used for abiotic condition. It shows that water phase ATP was the highest in 2 mM $NaN_3$ and sand phase ATP was the highest in ripen sand (no $NaN_3$). But, it was the lowest in 20 mM of NaN3. Hence, removal of E2 was the highest in ripened sand with biotic condition. But, almost equal removal in abiotic conditions because adsorption was the dominant in removal.

Finally, maximum ATP in water phase was in the mixture of DCW and SE (biotic) and minimum in bio-sand with ACTW (abiotic). Similarly, ATP in sand phase was maximum in bio-sand with mixture of DCW and SE (biotic) and minimum in bio-sand with ACTW (abiotic). As a whole the highest ATP was in bio-sand with mixture of DCW and SE (biotic) and the lowest ATP in bio-sand with ACTW (2 mM $NaN_3$). Consequently, removal of E2 compound was the highest in bio-sand with mixture of DCW and SE (biotic) and the lowest in bio-sand with ACTW (2 mM $NaN_3$). In conclusion, no significant difference was found in removal of E2 in biotic and abiotic condition because adsorption was the dominant in removal. However, adsorption and biodegradation was the main mechanism for estrogen removal.

Similarly, other responsible factors for estrogen removal are shown in Figure 4-26. All the details are explained below:

Figure 4-26 (a) shows the four main responsible environments for estrogen removal. Those are mixture of DCW and SE (no sand), fresh sand with mixture of DCW and SE, bio-sand with ACTW and bio-sand with mixture of DCW and SE. Result showed that removal was bit less in ACTW with ripened sand but in other cases removal was almost equal. It means adsorption was dominant in E2 removal. However, adsorption and biodegradation were responsible removal mechanisms.

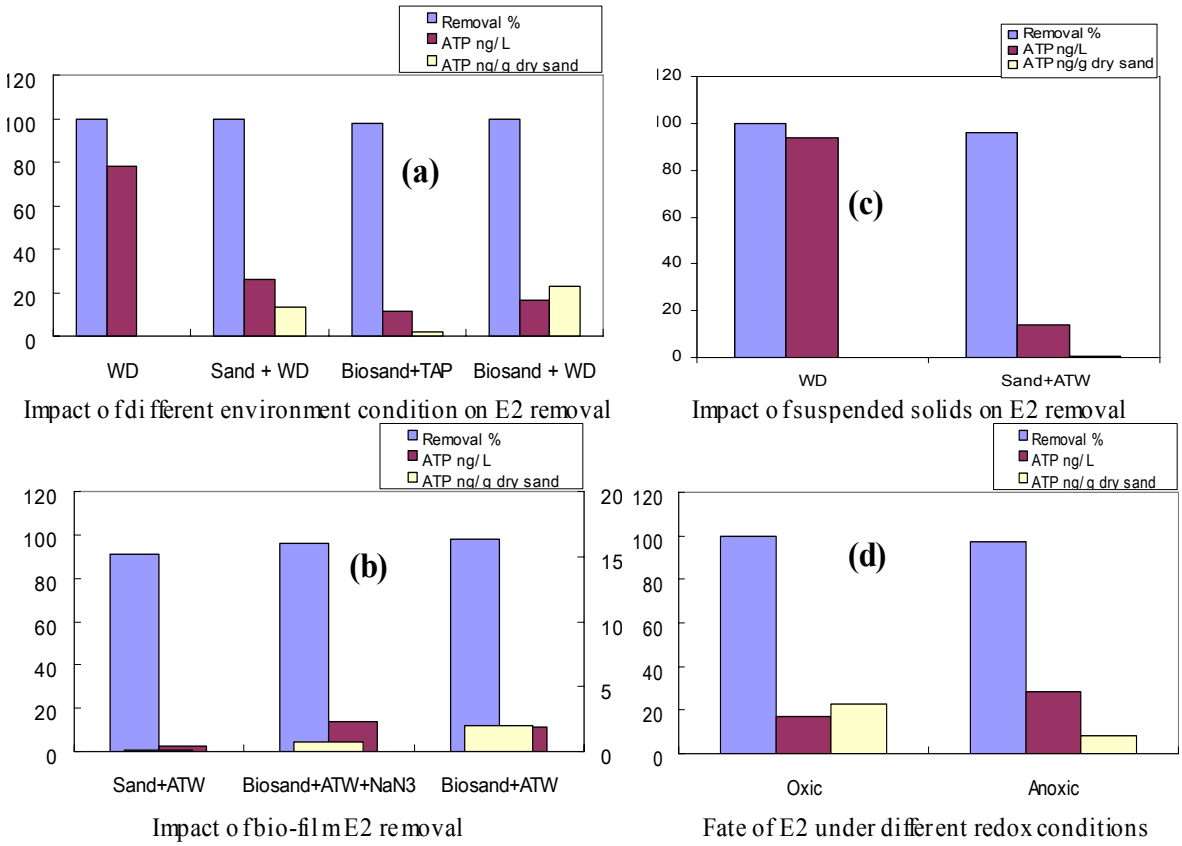



Figure 4-26. Impact of different environmental condition on E2 removal

Figure 4-26 (b) shows that the removal of E2 was bit higher in bio-sand with ACTW (no $NaN_3$) than with $NaN_3$ but lower removal in fresh sand with ACTW. This shows the impact of bio-film on removal. In bio-film there was organic material as well as microbial activity which help to remove the estrogen compound.

Figure 4-26 (c) shows the removal of E2 was higher in mixture of DCW and SE (no sand) but lower removal was found in sand with ACTW. Hence, this should be the impact of suspended solid in the influent.

Figure 4-26 (d) shows the higher removal of E2 in oxic condition than in anoxic because microbial activities were higher in oxic condition. However, adsorption was the dominant in removal. Hence, removal was almost equal in oxic and anoxic condition.

In conclusion, different environmental conditions played the major role on estrogen removal such as: bio-film associated with sand (soil organic matter), presence of suspended solid in influent and associate redox condition. Due to the fact that, removal of E2 in ripened sand with mixture of DCW and SE with biotic oxic condition was the highest and it was found the lowest in bio-sand with autoclaved tap water in abiotic condition. Due to adsorption and biodegradation the removal efficiency of estrogens was increased. However, adsorption was dominant in removal. Hence, removal of E2 in

each case was almost equal during the experiment. Detail of the ELISA test results are shown in Appendix C2 – I.

**b) Investigation on factors affecting E2 removal by F- EEM**

F-EEM detected intensity level was used to determine the relative concentration of E2 in µg/L level. Intermediate samples were taken from reactors with only MQ water, mixture of DCW and SE and mixture of DCW and SE with bio-sand and then F –EEM plots was prepared. The intensity of peaks (280 - 310 nm) was responsible for estrogen Figure 4-27 and Table 4-6 shows the F-EEM plot and responsible estrogen intensity level of the E2. Detail description of the F-EEM plots are shown below:

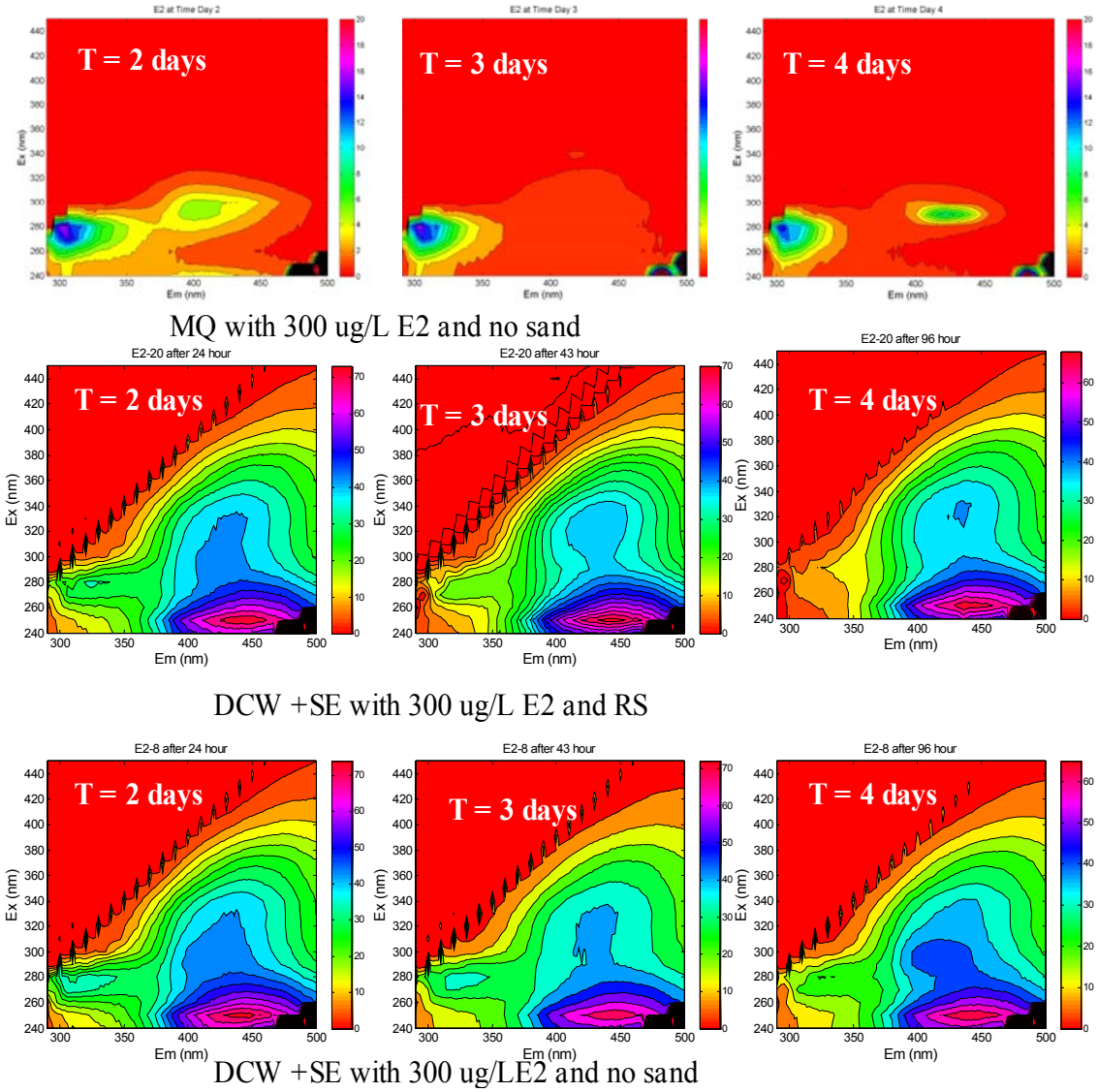



Figure 4-27. Fate of E2 during the experiment and effect of different condition

Figure 4-27 shows the fate of E2 in different reactors during the experiment. No significant change in estrogen peaks was found in control reactor: MQ with E2 (no sand) during the experiment. But, in the case of DCW and SE with no sand and with bio-sand E2 peaks changed as time elapsed as shown in Table 4-6.

Table 4-6. Intensity level E2 peak at Ex – 280 nm and Em – 310 nm

| Reactors condition (300 ug/L E2) | Intensity level | | | |
|---|---|---|---|---|
| | T = 24 - h | T = 48 - h | T = 96 - h | T = 120 - h |
| DCW + SE | 32.6 | 30.1 | 17.47 | 15.1 |
| DCW + SE + RS | 25.7 | 12.63 | 5.45 | 4.96 |

From above table, intensity level shows that the removal of E2 in mixture of DCW and SE (1:1) without sand was higher than the mixture of DCW and SE (1:1) with ripened sand due to the high percentage of organic materials and microbial activities. F-EEM plot shows the clear difference on the fate process at intensity level of 280 - 310 nm.

In conclusion, bio-film layer around sand, microbial activities and the organic material present in influent played the vital role in removal. Hence, adsorption and biodegradation was the main mechanism of E2 removal.

### 4.6.2. 17α-Ethinylestradiol (EE2):

This experiment was carried out to investigate the factors responsible for EE2 removal as well as to determine the main mechanism of removal during the RBF Detailed experimental set up and condition are explained in section 3.1.3. ELISA and F-EEM plots were used for sample analysis.

**a) Investigation on factors affecting EE2 removal by ELISA**

ELISA was used to determine the EE2 concentration in the effluent. Solid phase extraction was used for sample pre-treatment. Table 4-7 shows the remaining concentration of EE2 and percentage removal.

Table 4-7. Percentage removal of EE2 in different reactor conditions

| Reactor conditions | spiking conc (µg/L) | Actual conc (µg/L) | Avg.Eff conc (µg/L) | Standard deviation | % removal | % Ave removal |
|---|---|---|---|---|---|---|
| ACTW with no sand | 300 | 282.75 | | | | |
| | 300 | 282.75 | | | | |
| DCW+SE (no sand) + 2mM $NaN_3$ | 300 | 47.26 | | | 84.25 | |
| | 300 | 23.14 | 35.20 | 17.06 | 92.29 | 88.27 |
| 39.420 g of fresh sand + ACTW | 300 | 51.61 | | | 82.80 | |
| | 300 | 47.78 | 49.69 | 2.71 | 84.07 | 83.44 |
| DCW+SE(1:1) no sand | 300 | 14.64 | | | 95.12 | |
| | 300 | 17.22 | 15.93 | 1.82 | 94.26 | 94.69 |
| 39.42 g of fresh sand + DCW+SE(1:1) | 300 | 44.32 | | | 85.23 | |
| | 300 | 29.53 | 36.93 | 10.46 | 90.16 | 87.69 |
| 39.42 g fresh sand + DCW+SE+2mM $NaN_3$ | 300 | 37.63 | | | 87.46 | |
| | 300 | 23.95 | 30.79 | 9.67 | 92.02 | 89.74 |
| 50 g ripened sand + DCW+SE (1:1)+2mM $NaN_3$ | 300 | 19.82 | | | 93.39 | |
| | 300 | 27.55 | 23.68 | 5.47 | 90.82 | 92.11 |
| 50 g of ripen sand + ACTW | 300 | 45.31 | | | 84.90 | |
| | 300 | 33.14 | 39.23 | 8.60 | 88.95 | 86.92 |
| 50 g of ripened sand + ACTW | 300 | 28.82 | | | 90.39 | |
| | 300 | 30.45 | 29.63 | 1.15 | 89.85 | 90.12 |
| 50 g of ripened sand + DCW+SE(1:1) | 300 | 12.61 | | | 95.80 | |
| | 300 | 5.57 | 9.09 | 4.98 | 98.14 | 96.97 |
| 50 g of ripened sand + DCW + SE + 20 mM $NaN_3$ | 300 | 20.14 | | | 93.29 | |
| | 300 | 19.66 | 19.90 | 0.34 | 93.45 | 93.37 |

Table 4-7 shows that the removal of EE2 under different conditions. Removal ranged from 82.8 and 98.14 %. Biotic (oxic) reactor showed better performance than abiotic. Detail of ELISA test results are shown in Appendix C2- II and comparative analysis of EE2 removal under biotic and abiotic conditions are shown in Figure 4-28.

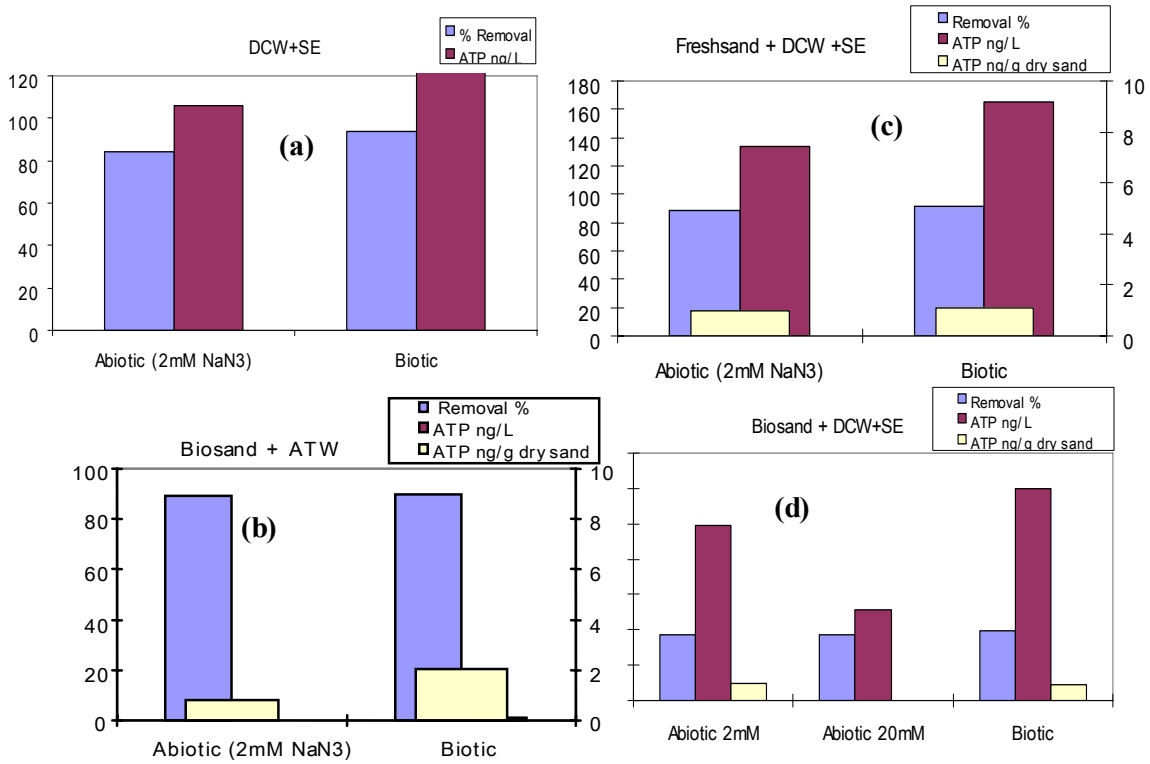



Figure 4-28. Comparative analysis of the microbial activities in different condition and its impact on EE2 removal

Figure 4-28 (a) shows the removal of EE2 in the mixture of DCW and SE (no sand) with 2 mM of $NaN_3$ and without $NaN_3$ for biotic and abiotic condition. ATP was measured and it was found higher in biotic condition than abiotic. Consequently, EE2 removal was higher in biotic than in abiotic due to the microbial activity.

Figure 4-28 (b) shows that the comparative analysis of bio-sand and ACTW with 2 mM of $NaN_3$ and without $NaN_3$ for EE2 removal. No ATP was found in water phase but ATP in sand phase was higher in biotic condition. Hence, removal was higher due to the microbial activity.

Figure 4-28 (c) shows EE2 removal and ATP in case of fresh sand (FS) and a mixture of DCW and SE with 2 mM $NaN_3$ and without $NaN_3$. It was found that ATP was higher in biotic condition than abiotic because microbial activity was higher in biotic reactor. Consequently, removal of EE2 was higher in biotic reactor than in abiotic reactor.

Figure 4-28 (d) shows the effect of EE2 removal in the case of ripened sand with a mixture of DCW and SE in biotic and abiotic (2 mM and 20 mM of $NaN_3$). It was found that ATP in water and sand phase was the highest in biotic reactor but the lowest in 20 mM $NaN_3$. However, there was no significant difference in removal. But slightly higher removal was observed in biotic reactors.

Finally, Figure 4-28 showed that ATP in water phase was maximum in mixture of DCW and SE (biotic) but minimum in bio-sand with ACDW with 2 mM of $NaN_3$. Similarly,

ATP in sand phase was maximum in bio-sand with mixture of DCW and SE (biotic) but minimum in bio-sand with ACTW (abiotic). Overall highest ATP was found in bio-sand with mixture of DCW and SE (biotic) but the lowest ATP in bio-sand ACTW with 2 mM of sodium azide. Consequently, the removal of EE2 compound was the highest in bio-sand with mixture of DCW and SE (biotic) and the lowest in bio-sand ACTW with 2 mM of $NaN_3$. Similarly, impact of different environmental conditions played a major role on estrogen removal (see Figure 4-29)

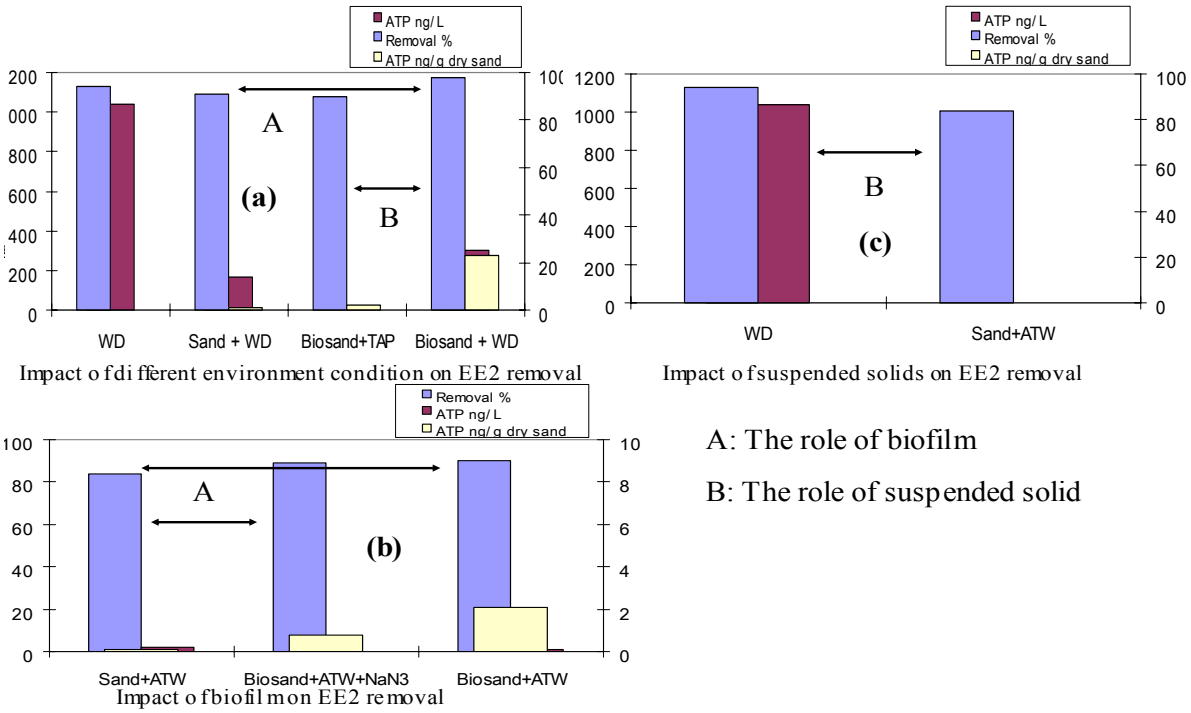



Figure 4-29. Impact of different environmental condition on EE2 removal

Figure 4-29 (a) shows the comparative analysis of the removal of EE2 in four different conditions. These are: mixture of DCW and SE, FS with a mixture of DCW and SE, bio-sand with ACTW and bio-sand with a mixture of DCW and SE. Result showed that removal was lower in ripened sand with ACTW than bio-sand with a mixture of DCW and SE. Similarly, FS with mixture of DCW and SE showed less removal than ripened sand with a mixture of DCW and SE. This is due to the microbial activities and presence of organic materials. Hence, different environmental conditions played the vital role on EE2 removal during the RBF.

Figure 4-29 (b) shows that the comparative analysis of the EE2 removal during RBF. Higher removal was found in bio-sand with ACTW than FS with ACTW and RS with ACTW (2 mM $NaN_3$). This is due to the impact of bio-film associated with sand (soil organic matter)

Figure 4-29 (c) shows the comparison of EE2 removal in sand with ACTW and a mixture of DCW and SE. Higher removal was found in a mixture of DCW and SE. It was because of the impact of suspended matters.

Finally, Figure 4-29 shows the impact of different environmental conditions on estrogen removal such as, bio-film associated with sand (soil organic matter), presence of suspended solid in influent and associated redox condition play the major role in estrogen removal. Due to the fact that, removal of EE2 in ripened sand with mixture of DCW and SE with biotic under oxic condition was the highest and in bio-sand with autoclave tap water in abiotic condition was the lowest.

**b) Investigation on factors affecting EE2 removal by F- EEM**

F-EEM detected intensity level was used to determine the relative concentration of EE2 in µg/L level. Intermediate samples were taken on those reactors which contained only MQ, mixture of DCW and SE and mixture of DCW and SE with bio-sand. Estrogen peak appeared at excitation wavelength of 280 nm and emission wave length of 310 nm. Figure 4-30 and Figure 4-31 shows the fate of estrogen compound during the experiment and Table 4-8 shows the corresponding intensity.

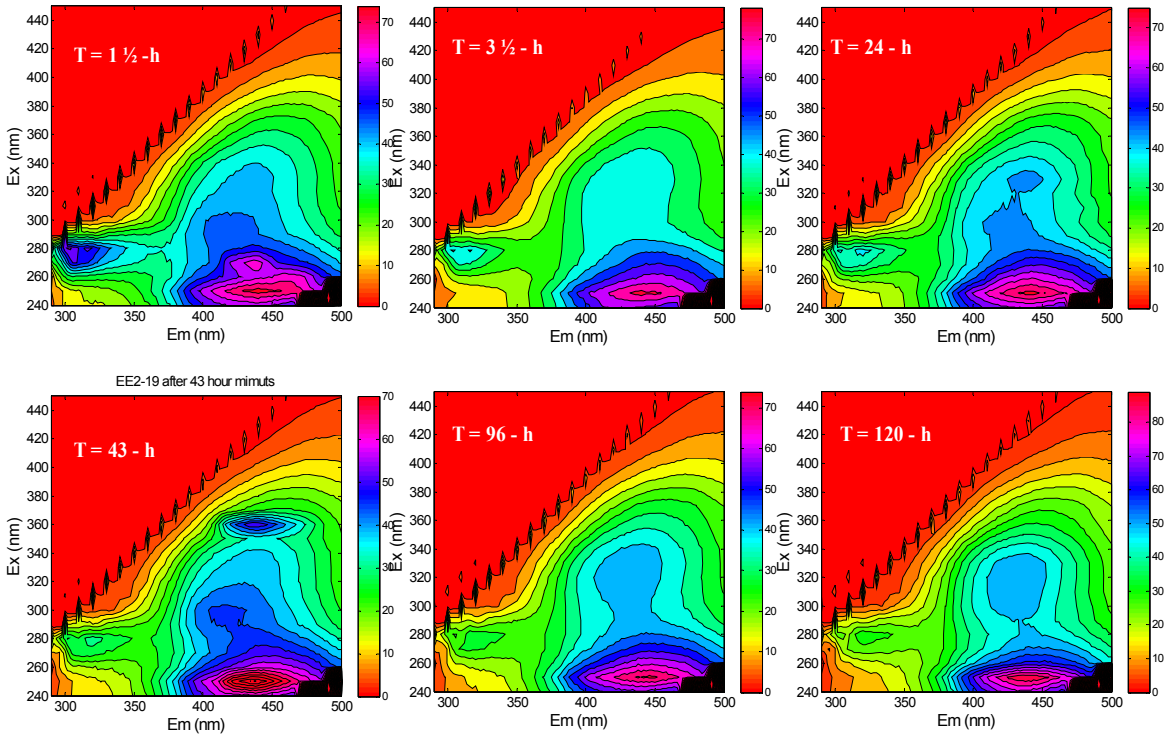



Figure 4-30. Fate of EE2 in DCW and SE with ripen sand during experiment.

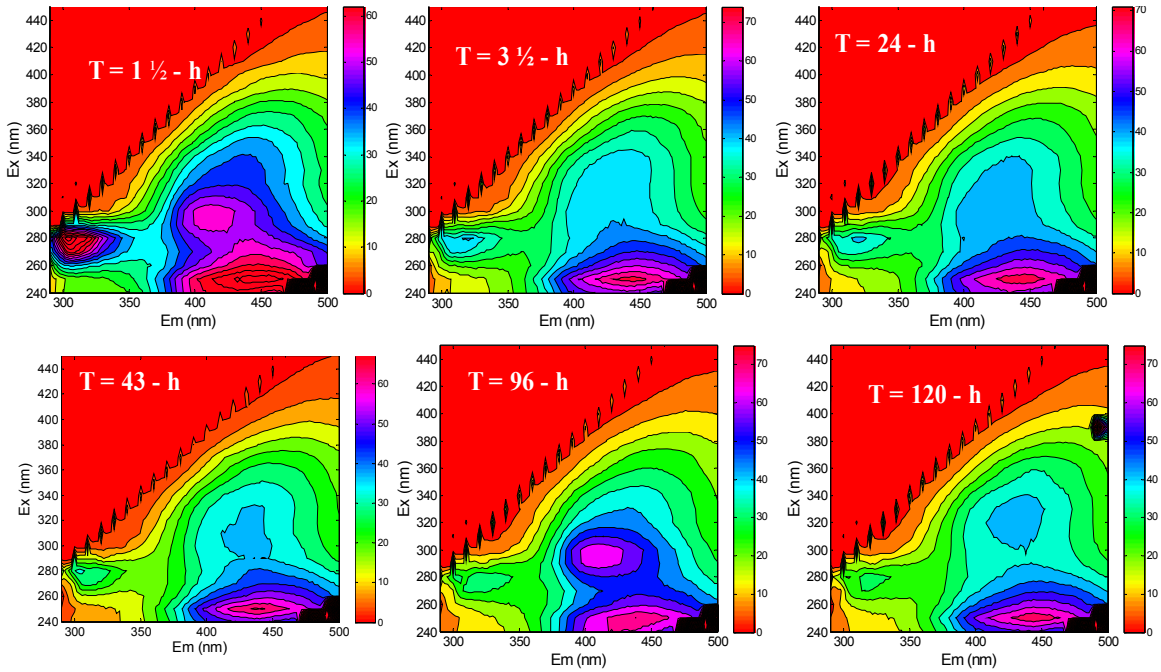



Figure 4-31. Fate of EE2 in DCW and SE with no sand during experiment

Table 4-8. Intensity level EE2 peak at Ex – 280 nm and Em – 310 nm

| Reactors condition | Intensity level | | | | |
|---|---|---|---|---|---|
| | T = 3 1/2 - h | T = 24 - h | T = 48 - h | T = 96 - h | T = 120 - h |
| DCW + SE | 76.5 | 38.8 | 34.76 | 26.87 | 25.6 |
| DCW + SE + RS | 49.16 | 36.16 | 33.49 | 24.52 | 23.34 |

Results show that 17α-Ethinylestradiol (EE2) concentration decreased with time. Figure 4-30 shows the fate of EE2 in DCW and SE with ripened sand. Similarly, Figure 4-31 shows the fate of EE2 in DCW and SE with no sand. Likewise Table 4-8 shows the corresponding intensity during the experiment. The results showed that EE2 removal in DCW and SE with ripened sand was higher than in DCW and SE with no sand. Both reactors were at exactly same conditions except ripened sand. This shows the impact of bio-film layers around the sand on removal efficiency.

## 4.7. Soil column study

Soil columns study for EE2 removal was conducted after ripening of the columns. Oxic biotic and abiotic conditions were maintained. In this study small glass column of diameter 5 cm and length 30 cm was used. Three columns were filled with clean sand of size 0.8 to 1.25 mm diameter as a filter media. SE was used for ripening. Experiment was conducted in dark room to simulate the process occurring in RBF. During the experiment influent used in SC-1 was tap water. But, SC-2 and SC-3 used mixture of DCW and SE (1:1). SC-2 was maintained at abiotic condition by using 2 mM of $NaN_3$. Samples were taken at least once a day. Flow through columns with above-mentioned influents was used to study the EDCs (steroid estrogens) removal during water percolation through porous media.

17α-Ethinylestradiol (EE2), with concentration of 1000 µg/L was spiked in each column. Samples were analyzed by using ELISA and F-EEM. Only selected samples were used for ELISA but the entire sample in soil column experiment was analyzed by using F-EEM technique. Detailed ELISA results are presented in Appendix C3 and F-EEM results are included in Appendix D3.

**a) Determination of estrogen by using ELISA**

ELISA was used to determine the EE2 concentration in soil column effluent. Solid phase extraction was applied for sample pre-treatment. Test results are shown in Table 4-9.

Table 4-9. EE2 removal in flow through soil columns during the experiment

| Time hours/days | Soil columns | Spiked conc (ug/l). | Remaining Conc (ug/l). | % removed | Remarks |
|---|---|---|---|---|---|
| T = 24 hours | SC1 | 1000 | 740.8 | 25.92 | Influents of soil columns were SC1 = Tap water SC2-DCW+SE(Abiotic), and SC3 = DCW+SE |
| | SC2 | 1000 | 539.93 | 46.01 | |
| | SC3 | 1000 | 509.40 | 49.06 | |
| T = 48 hours | SC1 | 1000 | 667.25 | 33.28 | |
| | SC2 | 1000 | 453.61 | 54.64 | |
| | SC3 | 1000 | 241.35 | 75.86 | |
| T = 8 $^{th}$ day | SC1 | 1000 | 467.04 | 53.30 | |
| | SC2 | 1000 | 348.41 | 65.16 | |
| | SC3 | 1000 | 306.71 | 69.33 | |
| T = 9 $^{th}$ day | SC1 | 1000 | 508.05 | 49.20 | |
| | SC2 | 1000 | 394.39 | 60.56 | |
| | SC3 | 1000 | 375.33 | 62.47 | |
| T = 14 $^{th}$ day | SC1 | 1000 | 359.01 | 64.10 | |
| | SC2 | 1000 | 332.69 | 66.73 | |
| | SC3 | 1000 | 129.90 | 87.01 | |

Table 4-9 shows the removal of EE2 in three different conditions of the soil columns at different time interval of the experiment. Result of removal in SC1 ranged from 25.92 % to 64.1 %, in SC2 it ranged from 46.01 % to 66.73 % and in SC3 it ranged from 48.06 to 87.01 %. In conclusion, SC1 showed the lowest removal capacity and SC3 showed that the highest removal capacity. Flow rate of this experiment was too low. Hence, it was difficult to adjust accurately and it varied in the range 0.4 ± 0.148 m/day. Consequently, removal of estrogen should have also varied. However, ELISA test result was not fine enough to demonstrate the effect of fluctuations in flow rate because only selected samples were taken. F-EEM detected intensity levels were measured for all samples. These intensity levels showed the effect of flow rate (see Table 4-10).

ELISA test result showed that SC1 has less removal capacity due to the absence of organic material in the influent. But SC3 has highest removal capacity due to the high percentage of organic material as well as microbial activities. SC2 showed bit less removal than SC3 due to the absence of microbial activities. Therefore, it was concluded that adsorption and biodegradation was the main mechanism of EE2 removal. In conclusion, removal of estrogen compounds during RBF was mainly

depending up on the flow rate, organic material present in aquifer, microbial activity in the aquifer and travel distance.

**b) Estrogen detection by using F-EEM**

F-EEM was used to compute the removal efficacy of estrogen compounds when water passes through the different conditions of soil aquifer. F-EEM detected intensity level showed the remaining EE2 concentration. Following are the F-EEM plots and corresponding intensity level of EE2 spectra in flow through columns

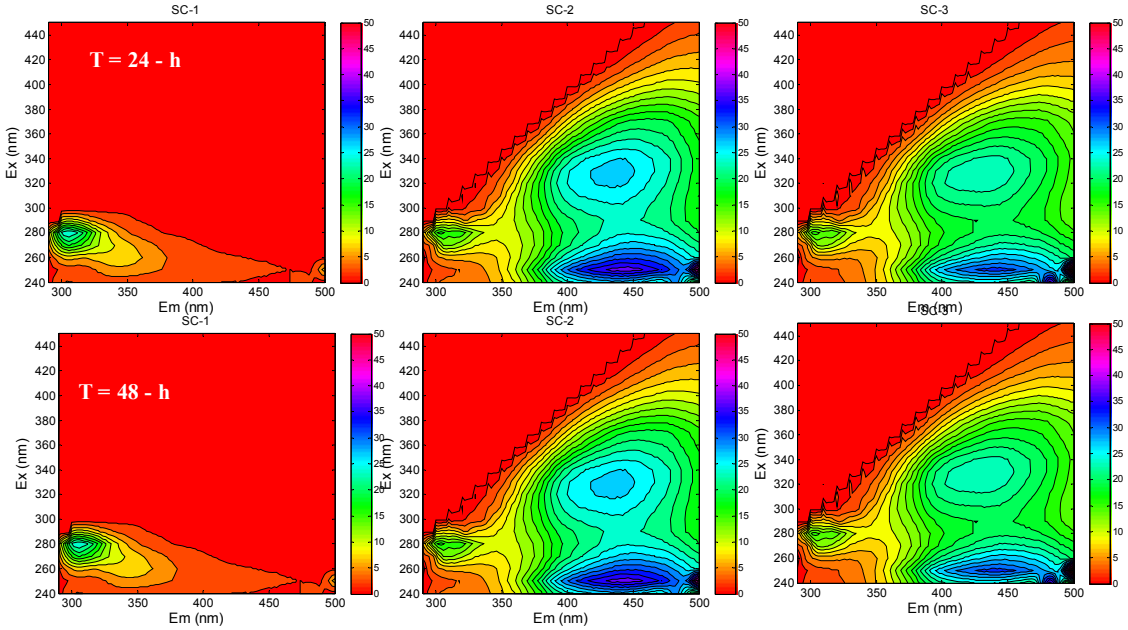



Figure 4-32. Concentration of EE2 in soil columns effluent with flow rate 0.4±0.148 m/day

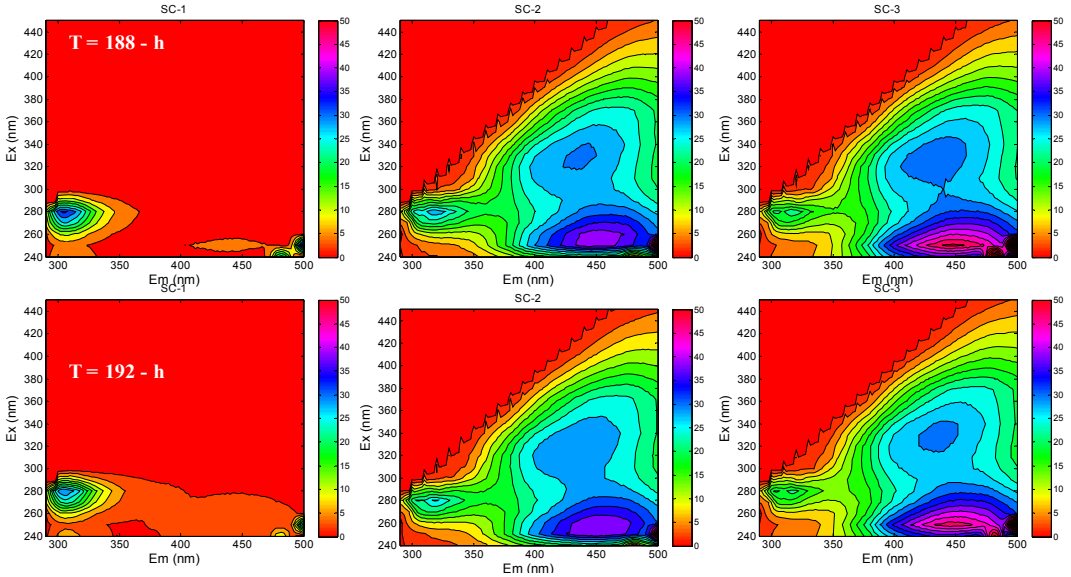



Figure 4-33. Concentration of EE2 in soil columns effluent with flow rate 0.4 ±0.148 m/day

Table 4-10. Intensity level of EE2 in different soil column effluent during the experiment

| Soil columns | Sampling time during experiment | Intensity level | Soil column | Sampling time during experiment | Intensity level |
|---|---|---|---|---|---|
| SC1 | | 25.96 | SC1 | | 36.59 |
| SC2 | | 18.28 | SC2 | | 26.58 |
| SC3 | T = 24 hours | 17.3 | SC3 | T = 8 $^{th}$days | 22.71 |
| SC1 | | 27.07 | SC1 | | 31.5 |
| SC2 | | 17.58 | SC2 | | 25.16 |
| SC3 | T = 30 hours | 16.37 | SC3 | T = 9 $^{th}$ days | 22.16 |
| SC1 | | 25.96 | SC1 | | 30.96 |
| SC2 | | 18.28 | SC2 | | 21.15 |
| SC3 | T = 48 hours | 17.31 | SC3 | T = 10 $^{th}$ days | 22.26 |
| SC1 | | 36.7 | SC1 | | 31.65 |
| SC2 | | 22.54 | SC2 | | 21.17 |
| SC3 | T = 60 hours | 25.9 | SC3 | T = 11 $^{th}$ days | 21.56 |
| SC1 | | 35.47 | SC1 | | 21.87 |
| SC2 | | 22.68 | SC2 | | 22.36 |
| SC3 | T = 4 $^{th}$ days | 24.48 | SC3 | T = 13 $^{th}$ days | 24.04 |
| SC1 | | 34.04 | SC1 | | 35.92 |
| SC2 | | 24.82 | SC2 | | 22.66 |
| SC3 | T = 6 $^{th}$ days | 24.36 | SC3 | T = 14 $^{th}$ days | 18.96 |
| SC1 | | 35.95 | SC1 | | 34.96 |
| SC2 | | 24.35 | SC2 | | 16.86 |
| SC3 | T = 7 $^{th}$ days | 25.03 | SC3 | T = 15 $^{th}$ days | 22.47 |

Figure 4-32 and Figure 4-33 show the F-EEM plot of the soil columns effluent during the experiment and Table 4-10 shows the corresponding intensity level of the estrogen peaks. Results showed that the highest removal of EE2 was in SC3 with mixture of DCW and SE (biotic) and the lowest removal was in SC1 with tap water biotic condition. Bio-film associated with sand (soil organic matter), presence of dissolved organic carbon in influent, flow rate and associated redox condition played the major role in estrogen removal. Due to this fact, removal of EE2 in ripened sand with mixture of DCW and SE with biotic oxic condition was the highest and removal in bio-sand with tap water in biotic condition was the lowest. Biodegradation increased the removal efficiency of estrogens. However, adsorption was dominant in removal, therefore removal of EE2 in SC2 and SC3 was almost equal. Literature mentioned that, in flow through column removal of estrogen compounds were constant in same flow rate. If the flow rate was increased removal decreased and vice versa (Lai *et al.*, 2000). In this study flow rate was too low and it was difficult to adjust and it was varying in the range 0.4 ± 0.15 m/day. Consequently, estrogen detected intensity level also varied as shown in Table 4-10. Experiment showed that flow rate is the most dominant factor on estrogen removal in RBF.

In conclusion, removal of estrogen compounds during the RBF depended up on the flow rate, organic material on the soil sediments, dissolved organic carbon in influent and microbial activities. Hence, adsorption and biodegradation were the two main mechanism of estrogen removal during RBF.

## 4.8. Practical implications of the study

The result obtained from this study on EDCs removal during the RBF could have following practical implications:

- Estrogen removal efficiency up to 99 % for E2 and 97 % for EE2 was obtained from an experiment simulating surface water 50 % impacted by wastewater. These experimental results imply that RBF could be a potential pre-treatment method with regard to EDCs removal from wastewater impacted source.

- Study found that RBF can remove the estrogen compounds. Removal of E2 ranged from 97 % to 99 % and EE2 ranged from 82 % to 95 %. The removal efficiency depends up on the background water quality and process conditions applied (oxic/anoxic, biotic/abiotic).

- Result showed that removal was high in organic-rich reactors. Similarly, higher removal was found in oxic reactors than in anoxic reactors. From these results, it is expected that organic-rich oxic soil aquifer could be favorable of EDCs removal. Maximum five days travel time was enough for estrogen removal.

# 5. CONCLUSIONS AND RECOMMENDATIONS

## 5.1. Conclusions

Based on the experimental results, the following conclusion can be drawn:

- Abiotic batch experiment using sodium azide showed that, 2 mM sodium azide was appropriate for inactivation of the micro-organisms without any interference with the ripened layer of sand.

- Fluorescence detected estrogen peaks, especially E2 and EE2 at an excitation wavelength of 280 nm and emission wave length of 310 nm. It was found that F-EEM can be used for quick detection of estrogen compounds.

- Batch adsorption experiment was conducted in ripened sand (biotic) and clean sand (abiotic) to establish isotherms of E2 and EE2. It was found that, in ripened sand removal of E2 was 99.9 % isotherm was not obtained but in the case of EE2 adsorption capacity and intensity were 0.28 µg/g and 0.67. Similarly, in clean sand adsorption capacity of E2 and EE2 was 0.20 µg/g and 0.21 µg/g and corresponding intensity was 0.95 and 0.63.

- Kinetic fate study of E2 and EE2 in abiotic reactors showed approximately 90 % removal from 0 to 24 hours (maximum sorption taken place). Similarly, between 24 to 48 hours removal was negative, estrogens were back to the water (desorption was taking place). Finally, removal was constant at 97.5% and 94.9 % of E2 and EE2 respectively. In conclusion E2 has higher adsorption capacity than EE2. Adsorption was depending up on the organic material contained in soil aquifer.

- Kinetic fate studies of E2 and EE2 in biotic reactors showed that the rapid removal from 0 to 48 hours, slow removal 48 to 60 hours. After 60 hours, in the case of EE2 removal was remaining constant at 95.1 %. But E2 was continuously removed up to 99 %.

- There was maximum removal of E2 and EE2 in bio-sand with mixture of DCW and SE (1:1 ratio) and minimum removal in bio-sand with ACTW (2 mM of $NaN_3$). Hence, presence of organic material in the influent and microbial activities played the major role in the removal of estrogens.

- Batch study showed that under similar conditions estrogen removal in anoxic reactors was 98 % and in oxic reactor was 99.9 %. This implies oxic condition was favorable for removal.

- Soil column study showed that EE2 removal in soil column with tap water was 25 to 64 %, with mixture of DCW & SE (abiotic) was 46 to 66 % and with mixture of DCW & SE was 49 to 87 %.
- Batch and soil column experiments under different redox conditions revealed that adsorption and biodegradation are the two main mechanisms responding for removal of estrogens during RBF.

- Removal of estrogens through the RBF were affected the bio-film associated with sand (soil organic matter), presence of dissolved organic carbon in influent, flow rate, travel distance and microbial activity.

- RBF was appeared to be an appropriate technology for the organic micro-pollutant removal specially E1, E2 and EE2.

## 5.2. Recommendations

The following is recommended for better understanding of EDCs removing during RBF:

- EDCs removal during RBF should be further investigated by conducting batch and soil column experiments under anoxic conditions.

- EDCs removal during RBF should be further investigated by conducting long soil column experiments at different flow rates.

- Measurement of E2 and EE2 by using F-EEM technique should be further investigated.

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# APPENDICES

## Appendix A – Measurement of DOC

A1 – DOC in batch reactors during ripening
A2 – DOC in soil columns during ripening
A3 – DOC of the samples during the abiotic experiment
A4 – DOC of the samples during adsorption isotherm experiment of E2
A5 – DOC of the samples during adsorption isotherm experiment of EE2
A6 – DOC during the determination of factors affecting E2 and E1 removal at pre-stabilization periods
A7 –DOC during the determination of factors affecting EE2 removal at pre-stabilization periods

## Appendix B – Measurement of UV absorbance (UVA)

B1 – UVA during the ripening of the batch reactors
B2 – UVA during the ripening of the soil columns

## Appendix C – Measurement of estrogens by ELISA

**C1 – Adsorption isotherms of E2 and EE2**

C1 – I – 17β-Estradiol (E2) (First test)
C1 – I – 17β-Estradiol (E2) (Second test)
C1 – I – Summary of ELISA test result of 17β-Estradiol (E2)
C1 – II – 17α-Ethinylestradiol (EE2) (First test)
C1 – II – 17α-Ethinylestradiol (EE2) (Second test)
C1 – II – 17α-Ethinylestradiol (EE2) (Third test)
C1 – II – Summary of ELISA test result of 17α-Ethinylestradiol (EE2)

**C2 – Determination of factor affecting E2 and EE2 removal**

C2 – I – 17β-Estradiol (E2)
C2 – II – 17α-Ethinylestradiol (EE2)
C2 – III –Estrone (E1) (First test)
C2 – III –Estrone (E1) (Second test)

**C3 – Soil columns study**

C3 – I – 17α-Ethinylestradiol (EE2) (First test)
C3– I – 17α-Ethinylestradiol (EE2) (Second test)
C3 – I – 17α-Ethinylestradiol (EE2) (Third test)

# Appendix D – Measurement of estrogens by F-EEM

**D1 – F-EEM plot for calibration curves of estrogen with MQ and mixture of DCW and SE (1:1)**

D1 – I – 17β-Estradiol (E2)
D1 – II – 17α-Ethinylestradiol (EE2)
D1 – III – Sample correction process for calibration curve of EE2 with known concentration in mixture of DCW and SE

**D2 – F-EEM plot and intensity level of adsorption isotherm experiment of E2 and EE2**

D2 – I – Intensity level of E2 during experiment
D2 – I – F-EEM plot of E2 during experiment
D2 – II – Intensity level of EE2 during experiment
D2 – II – F-EEM plot of EE2 during experiment

D3 – **F-EEM plot of soil columns study**

# Appendix A – Measurement of DOC

## A1 - DOC in batch reactors during ripening

| Time in days | DOC (mg/l) | | | | | | Averae Influent | Average Effluent |
|---|---|---|---|---|---|---|---|---|
| | Reactor 1 | | Reactor 2 | | Reactor 3 | | | |
| | Influent | Effluent | Influent | Effluent | Influent | Effluent | | |
| 0 | 14.04 | 13.12 | 14.01 | 13.10 | 14.02 | 13.22 | 14.02 | 13.146 |
| 6 | 9.564 | 7.79 | 9.122 | 8.404 | 9.33 | 8.182 | 9.33 | 8.125 |
| 12 | 8.189 | 7.29 | 7.598 | 7.466 | 7.932 | 7.714 | 7.906 | 7.49 |
| 17 | 15.34 | 12.39 | 14.97 | 13.51 | 15.08 | 13.27 | 15.13 | 13.05 |
| 23 | 14.8 | 14.327 | 15.09 | 14.492 | 15.21 | 14.723 | 15.03 | 14.514 |
| 28 | 8.21 | 7.726 | 8.065 | 7.379 | 7.998 | 7.233 | 8.091 | 7.447 |
| 33 | 7.678 | 6.79 | 7.41 | 6.38 | 7.549 | 6.57 | 7.545 | 6.58 |
| 38 | 13.93 | 13.28 | 14.38 | 13.39 | 13.95 | 13.26 | 14.08 | 13.31 |
| 44 | 16.49 | 14.61 | 17.16 | 14.81 | 17.59 | 14.53 | 17.08 | 14.65 |
| 51 | 15.81 | 13.13 | 15.35 | 13.2 | 15.79 | 12.95 | 15.65 | 13.09 |
| 55 | 15.17 | 14.13 | 15.55 | 14.59 | 15.52 | 14.21 | 15.41 | 14.31 |
| 60 | 17.17 | 16.1 | 16.32 | 15.4 | 16.43 | 15.1 | 16.64 | 15.53 |
| 65 | 13.33 | 12.2 | 13.04 | 12.17 | 13.07 | 12.25 | 13.14 | 12.69 |
| 70 | 14.16 | 12.12 | 12.7 | 12.07 | 12.4 | 12.34 | 13.08 | 12.17 |
| 75 | 14.66 | 13.76 | 15.02 | 13.96 | 15.24 | 13.84 | 14.97 | 13.85 |
| 81 | 13.7 | 12.35 | 13.4 | 12.5 | 13.94 | 12.51 | 13.68 | 12.45 |

## A2 – DOC in soil columns during ripening

| Time in days | DOC (mg/l) | | | |
|---|---|---|---|---|
| | Influent in SC | Effluent in SC1 | Effluent in SC2 | Effluent in SC3 |
| 0 | 8.809 | 8.749 | 8.5 | 8.64 |
| 5 | 15.07 | 13.41 | 14.21 | 13.07 |
| 10 | 15.15 | 14.01 | 14.38 | 14.45 |
| 15 | 17.43 | 16.2 | 16.18 | 15.47 |
| 20 | 8.05 | 7.107 | 7.198 | 8.015 |
| 25 | 11.36 | 10.87 | 10.09 | 10.1 |
| 31 | 14.06 | 12.22 | 12.95 | 12.55 |
| 38 | 17.26 | 15.29 | 16.29 | 16.45 |
| 42 | 15.07 | 13.36 | 13.65 | 14.79 |
| 47 | 17.09 | 14.67 | 16.29 | 15.5 |
| 52 | 16.11 | 14.5 | 14.41 | 14.85 |
| 59 | 14.77 | 12.6 | 12.27 | 12.58 |
| 64 | 16.11 | 14.01 | 14.24 | 14.58 |
| 69 | 15.28 | 13.09 | 13.16 | 13.13 |
| 74 | 15.45 | 13.93 | 13.69 | 13.46 |

## A3 – DOC of the samples during the abiotic experiment

| Experimental Phase | Time (days) | $NaN_3$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2 mM | | 10 mM | | 20 mM | | Control | |
| | | DOC in mg/L | | | | | | | |
| First Phase | 0 | 15.4 | 16 | 15.9 | 15.6 | 16 | 15.9 | 9.3 | 16.9 |
| | 5 | 13.6 | 14.2 | 14.9 | 14.6 | 14.6 | 15.3 | 13.3 | 12.3 |
| | 22 | 15.5 | 15.4 | 18.1 | 18.9 | 18.9 | 20.1 | 13.7 | 13.5 |
| | % | -0.6 | 3.8 | -13.8 | -21.2 | -18.1 | -26.4 | -47.3 | 20.1 |
| Second Phase | 0 | 15.8 | 15.6 | 16.1 | 16.7 | 16.2 | 15.3 | 15.8 | 15.4 |
| | 2 | 14.7 | 15.8 | 15.1 | 16.4 | 15.1 | 15.1 | 14.1 | 14.6 |
| | 9 | 15.53 | 15.61 | 17.22 | 16.77 | 17.84 | 17.42 | 12.76 | 12.63 |
| | 11 | 14.63 | 14.68 | 15.79 | 16.05 | 16.28 | 16.56 | 12.23 | 12.04 |
| | % | **7.4** | **5.9** | **1.9** | **3.9** | **-0.5** | **-8.2** | **22.6** | **21.8** |

## A4 –DOC of the samples during adsorption isotherm experiment of E2

| Reactor conditions | Influent DOC conc (mg/L) | Effluent DOC conc (mg/L) |
|---|---|---|
| ACDW + 100 µg/L of E2 | 1.49 | 1.49 |
| AC(DCW + SE) + 100u g/L of E2 | 17.2 | 14.23 |
| ACDW + clean sand (39.42 g) no E2 | 1.052 | 1.052 |
| ACDW + clean sand(39.42 g) + 20 µg/L E2 | 1.99 | 1.068 |
| ACDW + clean sand(39.42 g) + 40 µg/L E2 | 2.28 | 1.114 |
| ACDW + clean sand(39.42 g) + 80 µg/L E2 | 2.87 | 1.109 |
| ACDW + clean sand (39.42 g) + 100 µg/L E2 | 3.16 | 1.13 |
| DCW + SE + 100 µg/L E2 no sand | 15.74 | 15.19 |
| DCW + SE + 50 g ripened sand + 0 µg/L E2 | 15.74 | 14.07 |
| DCW + SE + 50 g ripened sand + 20 µg/L E2 | 16.04 | 13.67 |
| DCW + SE + 50 g ripened sand + 40 µg/L E2 | 16.34 | 13.29 |
| DCW + SE + 50 g ripened sand + 80 µg/L E2 | 16.94 | 13.26 |
| DCW + SE + 50 g ripened sand + 100 µg/L E2 | 17.24 | 13.71 |

## A5 – DOC of the samples during adsorption isotherm experiment of EE2

| Reactor conditions | Influent DOC conc (mg/L) | Effluent DOC conc (mg/L) |
|---|---|---|
| ACDW + 100 µg/L of EE2 | 1.87 | 1.782 |
| AC(DCW + SE) + 100 µg/Lof EE2 | 16.95 | 14.22 |
| ACDW + clean sand (39.42 g) no EE2 | 0.8 | 0.9766 |
| ACDW + clean sand (39.42 g) + 20µg/L EE2 | 1.12 | 1.185 |
| ACDW + clean sand (39.42 g) + 40µg/L EE2 | 1.42 | 1.045 |
| ACDW + clean sand (39.42 g) + 80µg/L EE2 | 2.05 | 1.046 |
| ACDW + clean sand (39.42 g) + 100 µg/L EE2 | 2.37 | 1.084 |
| DCW + SE + 100 µg/L EE2 no sand | 17.23 | 16.34 |
| DCW + SE + 50 g ripened sand + 0 µg/L EE2 | 15.74 | 13.69 |
| DCW + SE + 50 g ripened sand + 20 µg/L EE2 | 16.03 | 13.59 |
| DCW + SE + 50 g ripened sand + 40 µg/L EE2 | 16.47 | 13.96 |
| DCW + SE + 50 g ripened sand + 80 µg/L EE2 | 16.91 | 14.51 |
| DCW + SE + 50 g ripened sand + 100 µg/L EE2 | 17.2 | 13.86 |

## A6 – DOC during the determination of factors affecting E2 and E1 removal at pre-stabilization periods

### A6 – I abiotic condition

| Time in days | DOC (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Reactor 13 | | Reactor 14 | | Reactor 15 | | Reactor 16 | |
| | Inf | Eff | Inf | Eff | Inf | Eff | Inf | Eff |
| 0 | 15.08 | 15.19 | 15 | 14.91 | 0.699 | 1.213 | 0.876 | 0.894 |
| 5 | 14.86 | 14.05 | 15.3 | 13.84 | 0.475 | 0.828 | 0.339 | 0.545 |
| 10 | 14.38 | 14.41 | 13.69 | 14.43 | 0.727 | 1.057 | 0.398 | 0.932 |
| Characteristics of batch reactors | Acclimated sand + DCW + SE (Sodium azide) | | | | Acclimated sand + MQ (Sodium azide) | | | |

### A6 – II under biotic condition

| Time in days | DOC (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Reactor 17 | | Reactor 18 | | Reactor 19 | | Reactor 20 | |
| | Inf | Eff | Inf | Eff | Inf | Eff | Inf | Eff |
| 0 | 0.296 | 0.307 | 0.449 | 0.227 | 14.93 | 13.86 | 15.43 | 14.44 |
| 5 | 0.235 | 0.349 | 0.41 | 0.307 | 14.83 | 13.28 | 15.19 | 12.56 |
| 10 | 0.157 | 0.549 | 0.294 | 0.413 | 14.95 | 13.39 | 14.31 | 13.77 |
| Characteristics of batch reactors | Acclimated sand+MQ | | | | Acclimated sand + DCW + SE | | | |

### A6 – III biotic (anoxic) condition

| Time in days | DOC (mg/L) | | | |
|---|---|---|---|---|
| | Reactor 23 | | Reactor 24 | |
| | Inf. | Eff. | Inf. | Eff. |
| 0 | 18.09 | 15.1 | 16.2 | 15.16 |
| 5 | 14.39 | 13.09 | 15.39 | 13.1 |
| 10 | 15.94 | 13.44 | 13.33 | 13.41 |
| Characteristics of batch reactors | Acclimated sand + DCW + SE (Anoxic) | | | |

## A7 – DOC during the determination of factors affecting EE2 removal at pre-stabilization periods

### A7 – I abiotic condition

| Time in days | DOC (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Reactor 14 | | Reactor 15 | | Reactor 16 | | Reactor 17 | |
| | Inf | Eff | Inf | Eff | Inf | Eff | Inf | Eff |
| 0 | 15.47 | 15.4 | 15.11 | 14.59 | 0.67 | 1.11 | 0.45 | 0.82 |
| 5 | 15.02 | 13.5 | 15.7 | 13.64 | 0.778 | 0.64 | 0.54 | 0.49 |
| 10 | 13.73 | 14.5 | 13.5 | 15.97 | 0.312 | 1.09 | 0.22 | 0.6 |
| Characteristics of batch reactors | Acclimated sand + DCW + SE (Sodium azide) | | | | Acclimated sand.+.MQ.(Sodium azide) | | | |

### A7 – II under biotic condition

| Time in days | DOC (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Reactor 17 | | Reactor 18 | | Reactor 19 | | Reactor 20 | |
| | Inf. | Eff | Inf | Eff | Inf | Eff | Inf | Eff |
| 0 | 0.449 | 0.66 | 0.607 | 0.398 | 15.26 | 14.8 | 15.9 | 14.5 |
| 5 | 0.345 | 0.23 | 0.454 | 0.485 | 14.77 | 12.2 | 15.1 | 14 |
| 10 | 0.836 | 1.01 | 0.297 | 0.367 | 13.72 | 13.7 | 14.3 | 13.4 |
| Characteristics of batch reactors | Acclimated sand+MQ | | | | Acclimated sand.+.DCW.+.SE | | | |

### A7 – III biotic (anoxic) condition

| Time in days | DOC (mg/L) | | | |
|---|---|---|---|---|
| | Reactor 23 | | Reactor 24 | |
| | Inf | Eff | Inf | Eff |
| 0 | 16.07 | 14.3 | 15.74 | 15.33 |
| 5 | 14.99 | 12.4 | 15.26 | 12.93 |
| 10 | 14.43 | 13.7 | 13.7 | 13.55 |
| Characteristics of batch reactors | Acclimated sand + DCW + SE (Anoxic) | | | |

# Appendix B – Measurement of UV absorbance (UVA)

## B1 – UVA during the ripening of the batch reactors

| Time in days | | DOC(mg/l) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Reactor 1 | | Reactor 2 | | Reactor 3 | |
| Influent | Effluent | Influent | Effluent | Influent | Effluent | Influent | Effluent |
| 0 | 0 | | | | | | |
| 6 | 6 | | 0.276 | | 0.285 | | 0.296 |
| 12 | 12 | 0.293 | 0.277 | 0.292 | 0.292 | 0.290 | 0.276 |
| 17 | 17 | 0.542 | 0.487 | 0.537 | 0.490 | 0.552 | 0.495 |
| 23 | 23 | 0.545 | 0.526 | 0.55 | 0.526 | 0.56 | 0.524 |
| 28 | 28 | 0.225 | 0.206 | 0.232 | 0.204 | 0.233 | 0.209 |
| 33 | 33 | 0.233 | 0.189 | 0.234 | 0.191 | 0.226 | 0.209 |
| 38 | 38 | 0.456 | 0.522 | 0.439 | 0.523 | 0.455 | 0.521 |
| 44 | 44 | 0.403 | 0.465 | 0.414 | 0.435 | 0.413 | 0.427 |
| 51 | 51 | 0.467 | 0.445 | 0.457 | 0.447 | 0.457 | 0.434 |
| 55 | 55 | 0.538 | 0.482 | 0.522 | 0.491 | 0.509 | 0.48 |
| 60 | 60 | 0.52 | 0.482 | 0.516 | 0.49 | 0.51 | 0.891 |

## B2 – UVA during the ripening of the soil columns

| Time in days | UVA($cm^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Inf .SC1 | Inf .SC2 | Inf .SC3 | Eff.SC1 | Eff.SC2 | EffSC3 |
| 0 | 0.290 | 0.293 | 0.292 | 0.283 | 0.273 | 0.289 |
| 5 | 0.50 | 0.489 | 0.494 | 0.450 | 0.467 | 0.434 |
| 10 | 0.544 | 0.537 | 0.538 | 0.530 | 0.530 | 0.501 |
| 15 | 0.292 | 0.290 | .286 | 0.282 | 0.284 | 0.279 |
| 20 | 0.224 | 0.226 | 0.222 | 0.248 | 0.239 | 0.238 |
| 25 | 0.340 | 0.331 | 0.342 | 0.284 | 0.285 | 0.294 |
| 31 | 0.392 | 0.391 | 0.397 | 0.375 | 0.392 | 0.383 |
| 38 | 0.486 | 0.489 | 0.489 | 0.479 | 0.468 | 0.474 |
| 42 | 0.526 | 0.5 | 0.489 | 0.548 | 0.449 | 0.489 |
| 47 | 0.481 | 0.509 | 0.525 | 0.477 | 0.502 | 0.409 |

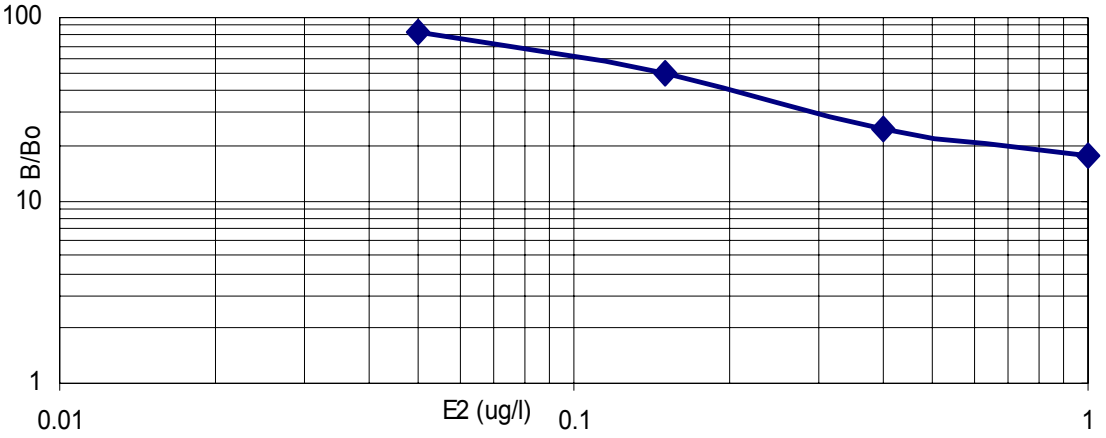
# Appendix C – Measurement of estrogens by ELISA

## C1 – Adsorption isotherm of E2 and EE2

### C1 – I – 17β-Estradiol (E2) (First test)

| E2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.818 | 0.897 | 0.8575 | 100 |
| 0.05 | 0.742 | 0.704 | 0.723 | 84.32 |
| 0.15 | 0.421 | 0.424 | 0.4225 | 49.28 |
| 0.4 | 0.209 | 0.214 | 0.2115 | 24.67 |
| 1 | 0.143 | 0.157 | 0.15 | 17.49 |

*B/Bo = Standard E2 absorbance / absorbance at E2 = 0 µg/L



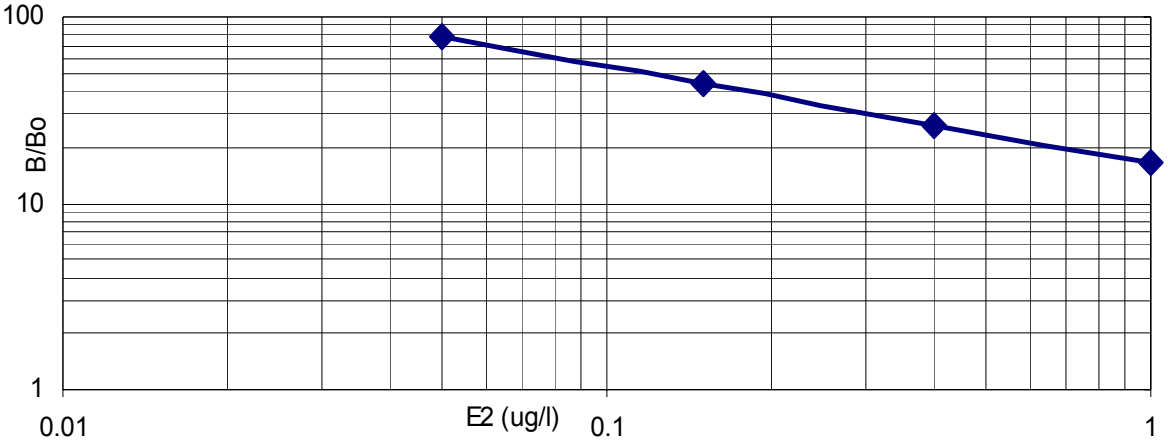

Result of 17β-Estradiol (E2) in different reactors

| Abs | | Avg abs | B/Bo(%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) | Reactor conditions |
|---|---|---|---|---|---|---|
| 0.589 | 0.587 | 0.588 | 68.58 | 0.08 | 7.63 | AC(DCW + SE) + 100 µg/L (no sand) |
| 0.464 | 0.475 | 0.4695 | 54.76 | 0.12 | 1.21 | ACTW + 20 µg/L (39.42 g clean sand) |
| 0.408 | 0.419 | 0.4135 | 48.23 | 0.15 | 1.55 | ACTW + 40 µg/L (39.42 g clean sand) |
| 0.202 | 0.206 | 0.204 | 23.79 | 0.44 | 4.39 | ACTW + 80 µg/L (39.42 g clean sand) |
| 0.174 | 0.206 | 0.19 | 22.16 | 0.53 | 5.31 | ACTW + 100 µg/L (39.42 g clean sand) |
| 1.003 | 0.947 | 0.975 | 113.72 | ND | ND | DCW + SE + 0 µg/L (50 g ripened sand) |

## C1 – I – 17β-Estradiol (E2) (Second test)

| E2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.892 | 0.878 | 0.885 | 100 |
| 0.05 | 0.728 | 0.653 | 0.6905 | 78.02 |
| 0.15 | 0.392 | 0.387 | 0.3895 | 44.01 |
| 0.4 | 0.232 | 0.231 | 0.2315 | 26.16 |
| 1 | 0.143 | 0.146 | 0.1445 | 16.33 |

*B/Bo = Standard E2 absorbance / absorbance at E2 = 0 µg/L




Result of 17β-Estradiol (E2) in different reactors

| Absorbance | | Avg. abs | B/Bo (%) | Conc. (µg/L) w/ dilution | Act. conc (µg/L) | Reactor conditions |
|---|---|---|---|---|---|---|
| 0.155 | 0.154 | 0.1545 | 17.46 | 0.88 | 87.82 | ACTW + 100 µg/L (no sand) |
| 0.706 | 0.353 | 0.5295 | 39.89 | 0.18 | 0.18 | (DCW + SE) + 100 µg/L (no sand) |
| 0.845 | 0.845 | 0.845 | 95.48 | 0.03 | 0.033 | DCW + SE + 20 µg/L (50 g ripened sand) |
| 0.59 | 0.65 | 0.62 | 70.06 | 0.06 | 0.06 | DCW + SE + 40 µg/L (50 g ripened sand) |
| 0.736 | 0.785 | 0.7605 | 85.93 | 0.04 | 0.04 | DCW + SE + 80 µg/L (50 g ripened sand) |
| 0.679 | 0.682 | 0.6805 | 76.89 | 0.05 | 0.05 | DCW + SE+ 100 µg/L (50 g ripened sand) |
| 0.899 | 0.802 | 0.89 | 100.5 | ND | 0.00 | ACTW + 0 µg/L (39.42 g clean sand) |

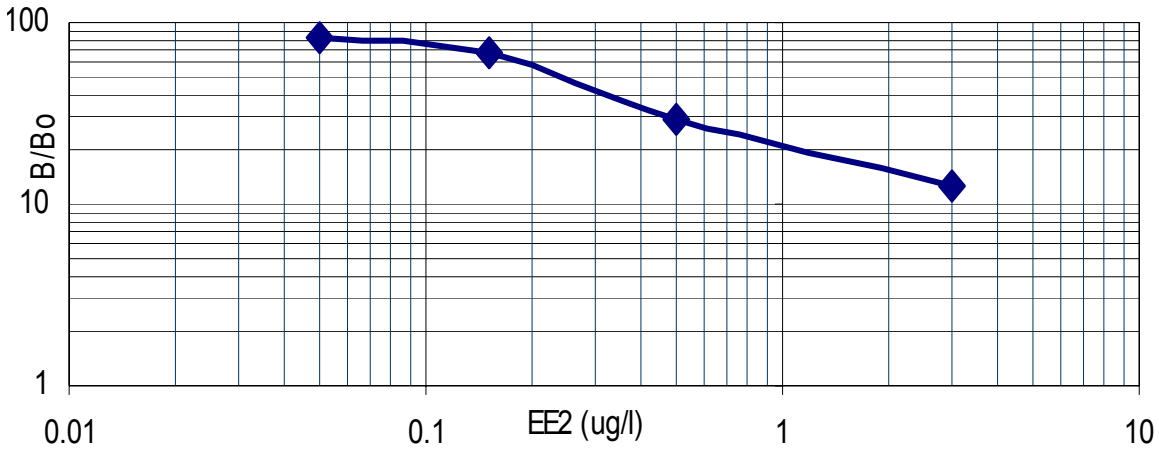
## C1 – I – Summary of ELISA test result of 17β-Estradiol (E2)

| Reactors conditions | Spike cont(µg/l). | Remained Cont (µg/L) | % removed |
|---|---|---|---|
| ACTW+100ug/l(no sand) | 100 | 87.82 | 0 |
| AC(DCW+SE)+100ug/l(no sand) | 100 | 0.76 | 99.24 |
| ACTW+0ug/l(39.42g clean sand) | 0 | 0.00 | 0 |
| ACTW+20ug/l(39.42g clean sand) | 20 | 1.21 | 93.95 |
| ACTW+40ug/l(39.42g clean sand) | 40 | 1.55 | 96.13 |
| ACTW+80ug/l(39.42g clean sand) | 80 | 4.39 | 94.51 |
| ACTW+100ug/l(39.42g clean sand) | 100 | 5.31 | 94.69 |
| DCW+SE+100ug/l (no sand) | 100 | 0.18 | 99.82 |
| DCW+SE+0ug/l(50g ripen sand) | 0 | ND | 0 |
| DCW+SE+20ug/l(50g ripen sand) | 20 | 0.03 | 99.9 |
| DCW+SE+40ug/l(50g ripen sand) | 40 | 0.06 | 99.8 |
| DCW+SE+80ug/l(50g ripen sand) | 80 | 0.04 | 100.0 |
| DCW+SE+100ug/l(50g ripen sand) | 100 | 0.05 | 100.0 |

## C1 – II – 17α-Ethinylestradiol (EE2) (First test)

| EE2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 1.021 | 0.948 | 0.9845 | 100 |
| 0.05 | 0.783 | 0.812 | 0.7975 | 81.01 |
| 0.15 | 0.726 | 0.604 | 0.665 | 67.55 |
| 0.5 | 0.331 | 0.254 | 0.2925 | 29.71 |
| 3 | 0.128 | 0.123 | 0.1255 | 12.75 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L



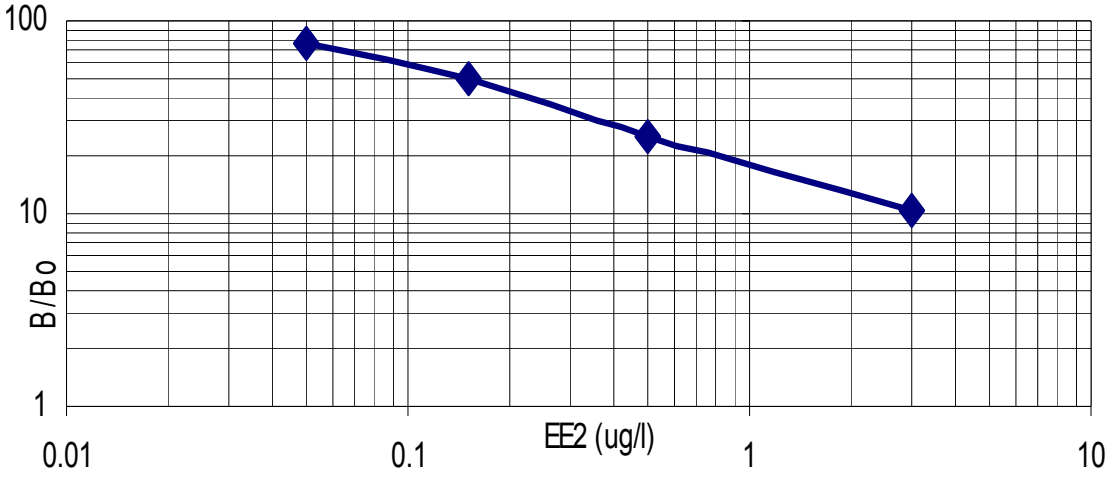
Result of 17α-Ethinylestradiol (EE2) in different reactors

| Absorbance | | Avg. abs. | B/Bo (%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) | % of removal |
|---|---|---|---|---|---|---|
| 0.161 | 0.188 | 0.1745 | 20.08 | 1.15 | 11.46 | ACTW + 100 µg/L (39.42 g clean sand) |
| 0.26 | 0.249 | 0.2545 | 29.29 | 0.52 | 5.15 | ACTW + 80 µg/L (39.42 g clean sand) |
| 0.389 | 0.357 | 0.373 | 42.92 | 0.29 | 2.92 | ACTW + 40 µg/L (39.42 g clean sand) |
| 0.651 | 0.638 | 0.6445 | 74.17 | 0.09 | 0.85 | ACTW + 20 µg/L (39.42 g clean sand) |
| 1.034 | 0.892 | 0.963 | 110.8 | ND | ND | ACTW + 0 µg/L (39.42 g clean sand) |
| 0.689 | 0.633 | 0.661 | 76.06 | 0.07 | 0.73 | AC(DCW + SE) + 100 µg/L (no sand) |
| 1.04 | 0.525 | 0.7825 | 90.05 | 0.03 | 0.03 | DCW + SE+0 µg/L (50 g ripened sand) |
| 0.101 | 0.089 | 0.095 | 10.93 | 4.16 | 4.16 | DCW + SE + 100 µg/L (no sand) |

**C1 – II – 17α-Ethinylestradiol (EE2) (Second test)**

| EE2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.997 | 1.129 | 1.063 | 100 |
| 0.05 | 0.74 | 0.904 | 0.822 | 77.33 |
| 0.15 | 0.518 | 0.535 | 0.5265 | 49.53 |
| 0.5 | 0.257 | 0.27 | 0.2635 | 24.79 |
| 3 | 0.106 | 0.116 | 0.111 | 10.44 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L



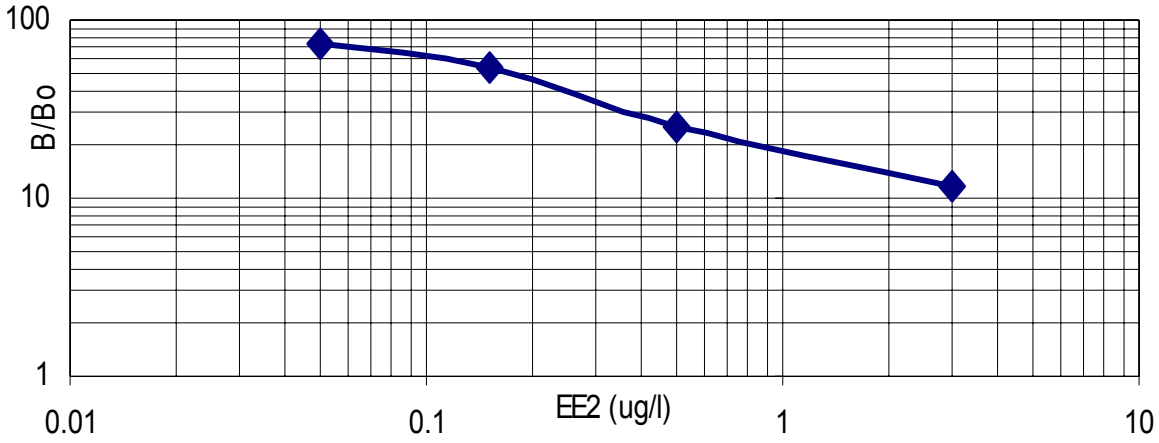
Result of 17α-Ethinylestradiol (EE2) in different reactors

| Abs. | | Avg. abs. | B/Bo (%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) | % of removal |
|---|---|---|---|---|---|---|
| 1.274 | 1.378 | 1.326 | 124.74 | 0.00 | 0.00 | ACTW + 0 µg/L (39.42 g clean sand) |
| 0.251 | 0.281 | 0.266 | 25.02 | 0.49 | 4.92 | DCW + SE + 100 µg/L (50 g ripened sand) |
| 0.375 | 0.41 | 0.3925 | 36.92 | 0.25 | 2.50 | DCW + SE + 80 µg/L ( 50 g ripened sand) |
| 1.1 | 1.022 | 1.061 | 99.81 | 0.00 | 0.00 | DCW + SE + 0 µg/L (50 g ripened sand) |
| 0.244 | 0.253 | 0.2485 | 23.38 | 0.19 | 1.88 | DCW + SE + 100 µg/L (no sand) |

**C1 – II – 17α-Ethinylestradiol (EE2) (Third test)**

| EE2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.827 | 0.885 | 0.856 | 100 |
| 0.05 | 0.642 | 0.637 | 0.6395 | 74.71 |
| 0.15 | 0.469 | 0.451 | 0.46 | 53.74 |
| 0.5 | 0.222 | 0.212 | 0.217 | 25.35 |
| 3 | 0.1 | 0.1 | 0.1 | 11.68 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L




Result of 17α-Ethinylestradiol (EE2) in different reactors

| Absorbance | | Avg abs | B/Bo (%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) | Reactor conditions |
|---|---|---|---|---|---|---|
| 0.166 | 0.144 | 0.155 | 18.11 | 1.09 | 108.85 | ACTW+100µg/L(no sand) |

### C1 – II – Summary of ELISA test result of 17α-Ethinylestradiol (EE2)

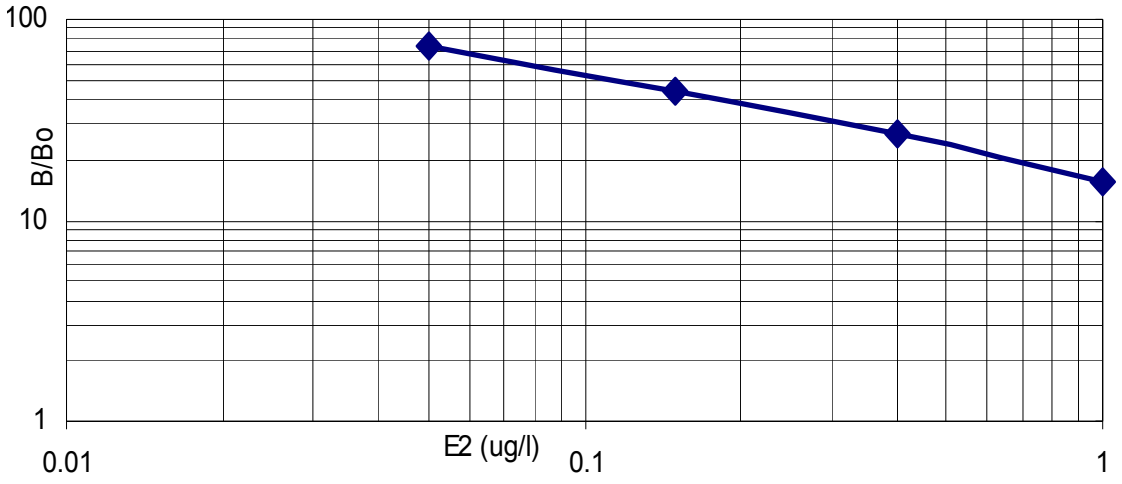
| Reactor conditions | Spike cont (µg/L). | Remained Cont. | % removed |
|---|---|---|---|
| ACTW + 100 µg/L (39.42 g clean sand) | 100 | 11.46 | 88.54 |
| ACTW + 80 µg/L (39.42 g clean sand) | 80 | 5.15 | 93.56 |
| ACTW + 40 µg/L (39.42 g clean sand) | 40 | 2.92 | 92.71 |
| ACTW + 20 µg/L (39.42 g clean sand) | 20 | 0.85 | 95.74 |
| ACTW + 0 µg/L (39.42 g clean sand) | 0 | ND | ND |
| AC(DCW + SE) + 100 µg/L (no sand) | 100 | 0.73 | 99.27 |
| ACTW + 100 µg/L (no sand) | 100 | 108.85 | 0.00 |
| DCW + SE + 100 µg/L (50 g ripened sand) | 100 | 4.55 | 95.45 |
| DCW + SE + 80 µg/L (50 g ripened sand) | 80 | 4.19 | 94.76 |
| DCW + SE + 40µg/L (50 g ripened sand) | 40 | 2.55 | 93.62 |
| DCW + SE + 20 µg/L (50 g ripened sand) | 20 | 0.53 | 97.37 |
| DCW + SE + 0 µg/L ( 50 g ripened sand) | 0 | ND | ND |
| DCW + SE + 100 µg/L (no sand) | 100 | 4.16 | 95.84 |

## C2 – Determination of factors affecting E1, E2 and EE2 removal

### C2 – I – 17β-Estradiol (E2)

| E2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.94 | 0.895 | 0.9175 | 100 |
| 0.05 | 0.65 | 0.704 | 0.677 | 73.79 |
| 0.15 | 0.407 | 0.397 | 0.402 | 43.81 |
| 0.4 | 0.227 | 0.266 | 0.2465 | 26.87 |
| 1 | 0.149 | 0.139 | 0.144 | 15.69 |

*B/Bo = Standard E2 absorbance / absorbance at E2 = 0 µg/L



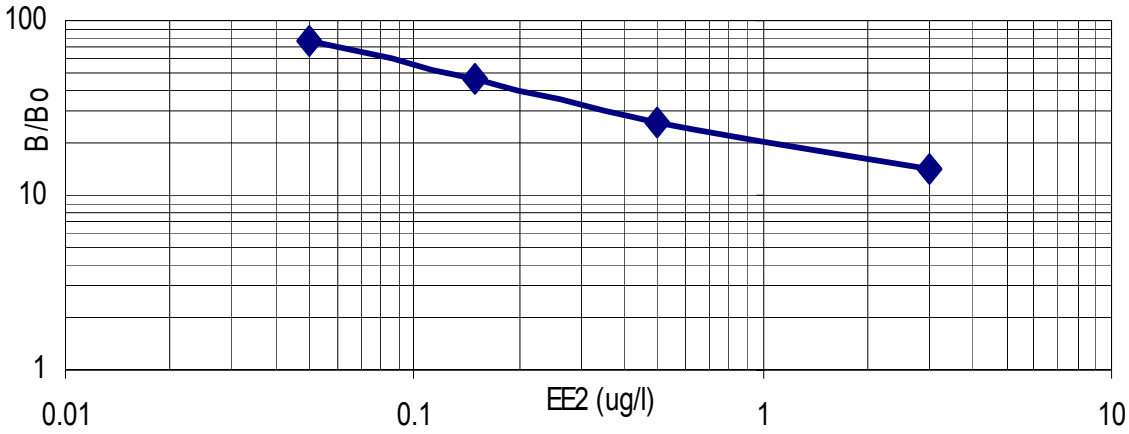
Result of 17β-Estradiol (E2) in different reactors

| Reactors | Absorbance | | Average absorbance | B/Bo(%) | Concentration (µg/L) w/ dilution | Actual concentration (µg/L) |
|---|---|---|---|---|---|---|
| R1 | 0.152 | 0.16 | 0.156 | 17.00 | 0.87 | 261.63 |
| R2 | 0.156 | 0.167 | 0.1615 | 17.60 | 0.82 | 246.63 |
| R3 | 0.289 | 0.32 | 0.3045 | 33.19 | 0.26 | 5.24 |
| R4 | 0.323 | 0.358 | 0.3405 | 37.11 | 0.21 | 4.19 |
| R5 | 0.285 | 0.302 | 0.2935 | 31.99 | 0.28 | 5.64 |
| R6 | 0.186 | 0.242 | 0.214 | 23.32 | 0.51 | 10.18 |
| R7 | 0.951 | 0.826 | 0.8885 | 96.84 | ND | ND |
| R8 | 0.872 | 1.059 | 0.9655 | 105.23 | ND | ND |
| R9 | 0.938 | 0.791 | 0.8645 | 94.22 | ND | ND |
| R10 | 0.901 | 0.887 | 0.894 | 97.44 | ND | ND |
| R11 | 0.418 | 0.381 | 0.3995 | 43.54 | 0.15 | 3.04 |
| R12 | 0.343 | 0.331 | 0.337 | 36.73 | 0.21 | 4.27 |
| R13 | 0.756 | 0.693 | 0.7245 | 78.96 | 0.04 | 0.87 |
| R14 | 0.583 | 0.51 | 0.5465 | 59.56 | 0.08 | 1.57 |
| R15 | 0.452 | 0.441 | 0.4465 | 48.66 | 0.12 | 2.40 |
| R16 | 0.424 | 0.449 | 0.4365 | 47.57 | 0.13 | 2.52 |
| R17 | 0.612 | 0.614 | 0.613 | 66.81 | 0.06 | 1.23 |
| R18 | 0.363 | 0.357 | 0.36 | 39.24 | 0.19 | 3.74 |
| R19 | 1.057 | 1.012 | 1.0345 | 112.75 | ND | ND |
| R20 | 0.965 | 1.011 | 0.988 | 107.68 | ND | ND |
| R21 | 0.708 | 0.774 | 0.741 | 80.76 | 0.04 | 0.83 |
| R22 | 0.649 | 0.67 | 0.6595 | 71.88 | 0.05 | 1.06 |
| R23 | 0.525 | 0.56 | 0.5425 | 59.13 | 0.08 | 1.60 |
| R24 | 0.218 | 0.277 | 0.2475 | 26.98 | 0.40 | 7.94 |

**C2 – II – 17α-Ethinylestradiol (EE2)**

| EE2 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 1.021 | 0.717 | 0.869 | 100 |
| 0.05 | 0.486 | 0.821 | 0.6535 | 75.20 |
| 0.15 | 0.415 | 0.389 | 0.402 | 46.26 |
| 0.5 | 0.213 | 0.241 | 0.227 | 26.12 |
| 3 | 0.112 | 0.13 | 0.121 | 13.92 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L



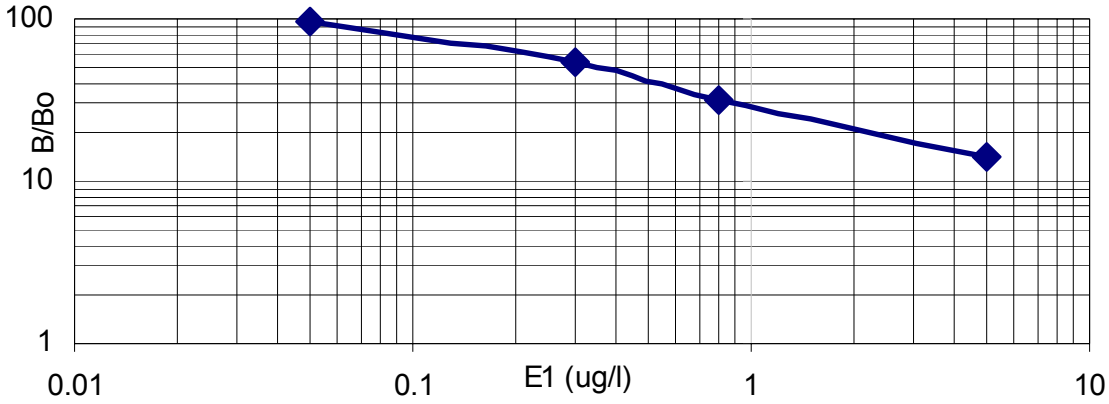

Results of 17α-Ethinylestradiol (EE2) in batch studies in different condition of the reactors:

| Reactors | Absorbance | | Average absorbance | B/Bo(%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) |
|---|---|---|---|---|---|---|
| R1 | 0.124 | 0.123 | 0.1235 | 14.21 | 2.8 | 282.75 |
| R2 | 0.123 | 0.124 | 0.1235 | 14.21 | 2.8 | 282.75 |
| R3 | 0.128 | 0.135 | 0.1315 | 15.13 | 2.36 | 47.26 |
| R4 | 0.16 | 0.178 | 0.169 | 19.45 | 1.16 | 23.14 |
| R5 | 0.129 | 0.126 | 0.1275 | 14.67 | 2.58 | 51.61 |
| R6 | 0.122 | 0.14 | 0.131 | 15.07 | 2.39 | 47.78 |
| R7 | 0.197 | 0.2 | 0.1985 | 22.84 | 0.73 | 14.64 |
| R8 | 0.188 | 0.187 | 0.1875 | 21.58 | 0.86 | 17.22 |
| R9 | 0.119 | 0.15 | 0.1345 | 15.48 | 2.22 | 44.32 |
| R10 | 0.206 | 0.252 | 0.229 | 26.35 | 1.48 | 29.53 |
| R11 | 0.127 | 0.158 | 0.1425 | 16.40 | 1.88 | 37.63 |
| R12 | 0.15 | 0.184 | 0.167 | 19.22 | 1.20 | 23.95 |
| R13 | 0.173 | 0.184 | 0.1785 | 20.54 | 0.99 | 19.82 |
| R14 | 0.149 | 0.169 | 0.159 | 18.30 | 1.38 | 27.55 |
| R15 | 0.122 | 0.145 | 0.1335 | 15.36 | 2.27 | 45.31 |
| R16 | 0.145 | 0.153 | 0.149 | 17.15 | 1.66 | 33.14 |
| R17 | 0.149 | 0.164 | 0.1565 | 18.01 | 1.44 | 28.82 |
| R18 | 0.152 | 0.155 | 0.1535 | 17.66 | 1.52 | 30.45 |
| R19 | 0.162 | 0.166 | 0.164 | 18.87 | 1.26 | 10.09 |
| R20 | 0.217 | 0.22 | 0.2185 | 25.14 | 0.56 | 5.57 |
| R21 | 0.195 | 0.16 | 0.1775 | 20.43 | 1.01 | 20.14 |
| R22 | 0.188 | 0.17 | 0.179 | 20.60 | 0.98 | 19.66 |

## C2 – III –Estrone (E1) (First test)

| E1 standard conc (µg/l) | Absorbance | | Average absorbance | B/Bo(%) |
|---|---|---|---|---|
| 0 | 0.79 | 0.866 | 0.828 | 100 |
| 0.05 | 0.757 | 0.813 | 0.785 | 94.81 |
| 0.3 | 0.459 | 0.449 | 0.454 | 54.83 |
| 0.8 | 0.259 | 0.274 | 0.2665 | 32.19 |
| 5 | 0.12 | 0.114 | 0.117 | 14.13 |

*B/Bo = Standard E1 absorbance / absorbance at E1 = 0 µg/L



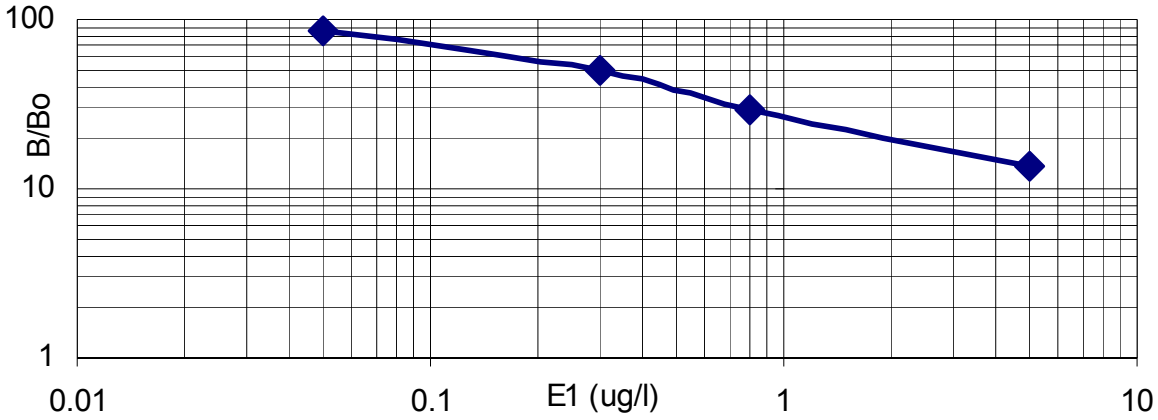

Result of estrone (E1) in different reactors

| Reactors | Absorbance | | Average absorbance | B/Bo (%) | Concentration (µg/L) w/ dilution | Actual concentration (µg/L) |
|---|---|---|---|---|---|---|
| R3 | 0.656 | 0.727 | 0.6915 | 83.51 | 0.08 | 1.51 |
| R5 | 0.456 | 0.424 | 0.44 | 53.14 | 0.32 | 6.36 |
| R6 | 0.501 | 0.54 | 0.5205 | 62.86 | 0.19 | 3.84 |
| R11 | 0.26 | 0.278 | 0.269 | 32.49 | 0.79 | 15.73 |
| R12 | 0.707 | 0.723 | 0.715 | 86.35 | 0.07 | 1.36 |
| R13 | 0.683 | 0.755 | 0.719 | 86.84 | 0.07 | 1.33 |
| R14 | 0.569 | 0.521 | 0.545 | 65.82 | 0.16 | 3.30 |
| R21 | 0.694 | 0.75 | 0.722 | 87.20 | 0.07 | 1.31 |
| R22 | 0.666 | 0.674 | 0.67 | 80.92 | 0.08 | 1.68 |

## C2 – III –Estrone (E1) (Second time test)

| E1 standard conc. (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.792 | 0.803 | 0.7975 | 100 |
| 0.05 | 0.704 | 0.664 | 0.684 | 85.77 |
| 0.3 | 0.408 | 0.398 | 0.403 | 50.53 |
| 0.8 | 0.23 | 0.244 | 0.237 | 29.72 |
| 5 | 0.11 | 0.107 | 0.1085 | 13.61 |

*B/Bo = Standard E1 absorbance / absorbance at E1 = 0 µg/L




Result of estrone (E1) in different reactors

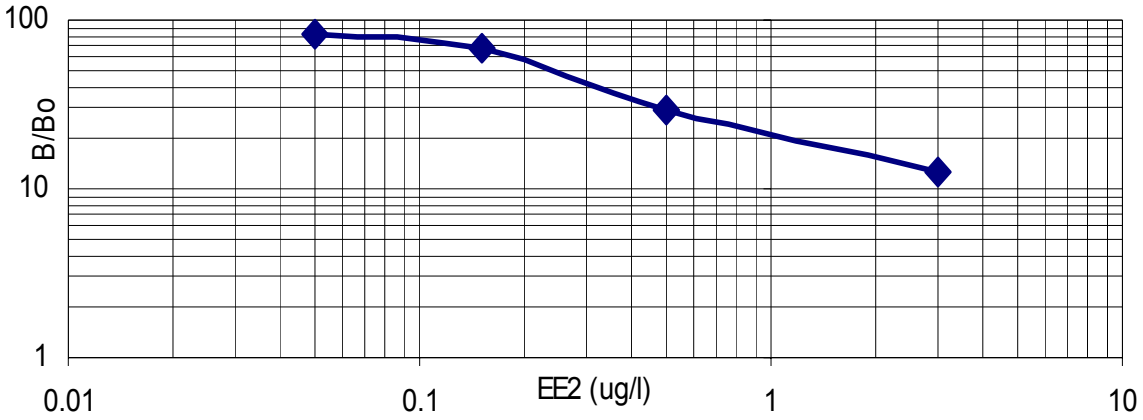
| Reactors | Absorbance | | Average absorbance | B/Bo (%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) |
|---|---|---|---|---|---|---|
| R1 | 0.223 | 0.236 | 0.2295 | 28.78 | 0.86 | 0 |
| R2 | 0.263 | 0.248 | 0.2555 | 32.04 | 0.70 | 0 |
| R7 | 0.632 | 0.602 | 0.617 | 77.37 | 0.07 | 0.07 |
| R8 | 0.54 | 0.535 | 0.5375 | 67.40 | 0.11 | 0.11 |
| R9 | 0.511 | 0.511 | 0.511 | 64.08 | 0.13 | 0.13 |
| R10 | 0.443 | 0.462 | 0.4525 | 56.74 | 0.20 | 0.20 |
| R15 | 0.088 | 0.088 | 0.088 | 11.03 | 8.18 | 8.18 |
| R16 | 0.089 | 0.088 | 0.0885 | 11.10 | 8.07 | 8.07 |
| R17 | 0.08 | 0.078 | 0.079 | 9.91 | 10.54 | 10.54 |
| R18 | 0.081 | 0.078 | 0.0795 | 9.97 | 10.38 | 10.38 |
| R19 | 0.282 | 0.308 | 0.295 | 36.99 | 0.53 | 0.53 |
| R20 | 0.303 | 0.288 | 0.2955 | 37.05 | 0.53 | 0.53 |
| R23 | 0.778 | 0.753 | 0.7655 | 95.99 | ND | ND |
| R24 | 0.201 | 0.188 | 0.1945 | 24.39 | 1.27 | 1.27 |

# C3 – Soil columns study

## C3 – I – 17α-Ethinylestradiol (EE2) (First test)

| EE2 standard conc (µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 1.021 | 0.948 | 0.9845 | 100 |
| 0.05 | 0.783 | 0.812 | 0.7975 | 81.01 |
| 0.15 | 0.726 | 0.604 | 0.665 | 67.55 |
| 0.5 | 0.331 | 0.254 | 0.2925 | 29.71 |
| 3 | 0.128 | 0.123 | 0.1255 | 12.75 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L



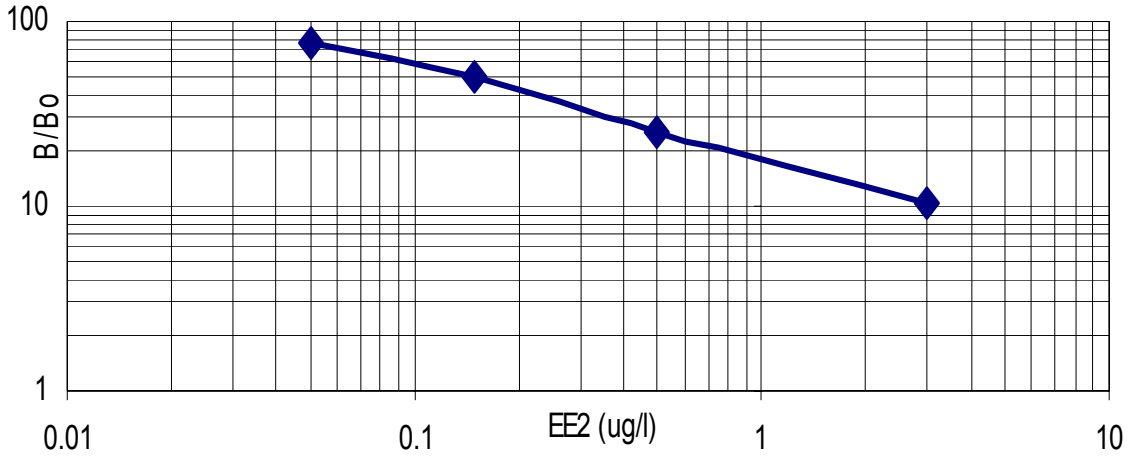

Result of 17α-Ethinylestradiol (EE2) in soil coliumns effluent

| Soil column | Absorbance | | Avg abs | B/Bo (%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) |
|---|---|---|---|---|---|---|
| SC3 | 0.246 | 0.266 | 0.256 | 29.46 | 0.51 | 509.04 |

## C3– I – 17α-Ethinylestradiol (EE2) (Second test)

| EE2 standard conc.(µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 0.997 | 1.129 | 1.063 | 100 |
| 0.05 | 0.74 | 0.904 | 0.822 | 77.33 |
| 0.15 | 0.518 | 0.535 | 0.5265 | 49.53 |
| 0.5 | 0.257 | 0.27 | 0.2635 | 24.79 |
| 3 | 0.106 | 0.116 | 0.111 | 10.44 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L



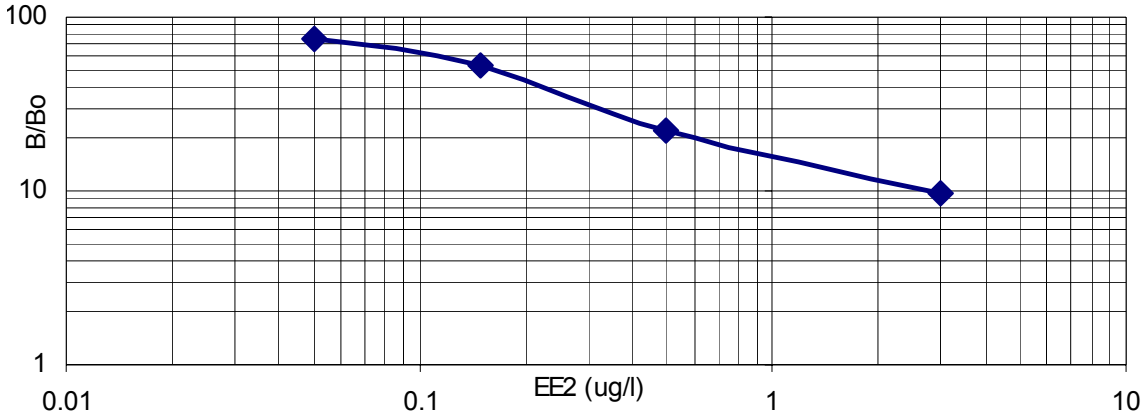

Result of 17α-Ethinylestradiol (EE2) in soil coliumns effluent

| Soil column | Absorbance | | Average absorbance | B/Bo(%) | Conc (ug/l) w/ dilution | Actual conc (ug/L) |
|---|---|---|---|---|---|---|
| SC1- 28 | 0.183 | 0.184 | 0.1835 | 17.26 | 1.06 | 740.80 |
| SC2-28 | 0.218 | 0.238 | 0.228 | 21.45 | 0.67 | 539.93 |
| SC3-29 | 0.385 | 0.416 | 0.4005 | 37.68 | 0.24 | 241.35 |
| SC1-29 | 0.194 | 0.192 | 0.193 | 18.16 | 0.95 | 667.25 |
| SC2-29 | 0.241 | 0.255 | 0.248 | 23.33 | 0.57 | 453.61 |
| SC2 - 5 | 0.4 | 0.419 | 0.4095 | 38.52 | 0.23 | 348.41 |
| SC1- 5 | 0.287 | 0.292 | 0.2895 | 27.23 | 0.42 | 467.04 |
| SC3 - 5 | 0.344 | 0.313 | 0.3285 | 30.90 | 0.34 | 306.71 |
| SC3 - 6 | 0.361 | 0.372 | 0.3665 | 34.48 | 0.28 | 394.39 |
| SC1 - 6 | 0.261 | 0.262 | 0.2615 | 24.60 | 0.51 | 508.05 |
| SC2 - 6 | 0.279 | 0.306 | 0.2925 | 27.52 | 0.42 | 375.33 |
| SC1 - 11 | 0.361 | 0.444 | 0.4025 | 37.86 | 0.24 | 359.01 |
| SC2 - 11 | 0.313 | 0.314 | 0.3135 | 29.49 | 0.37 | 332.69 |

**C3 – I – 17α-Ethinylestradiol (EE2) (Third test)**

| EE2 standard conc.(µg/L) | Absorbance | | Average absorbance | B/Bo (%) |
|---|---|---|---|---|
| 0 | 1.155 | 1.068 | 1.1115 | 100 |
| 0.05 | 0.845 | 0.813 | 0.829 | 74.58 |
| 0.15 | 0.553 | 0.62 | 0.5865 | 52.77 |
| 0.5 | 0.238 | 0.251 | 0.2445 | 22.00 |
| 3 | 0.112 | 0.106 | 0.109 | 9.81 |

*B/Bo = Standard EE2 absorbance / absorbance at EE2 = 0 µg/L



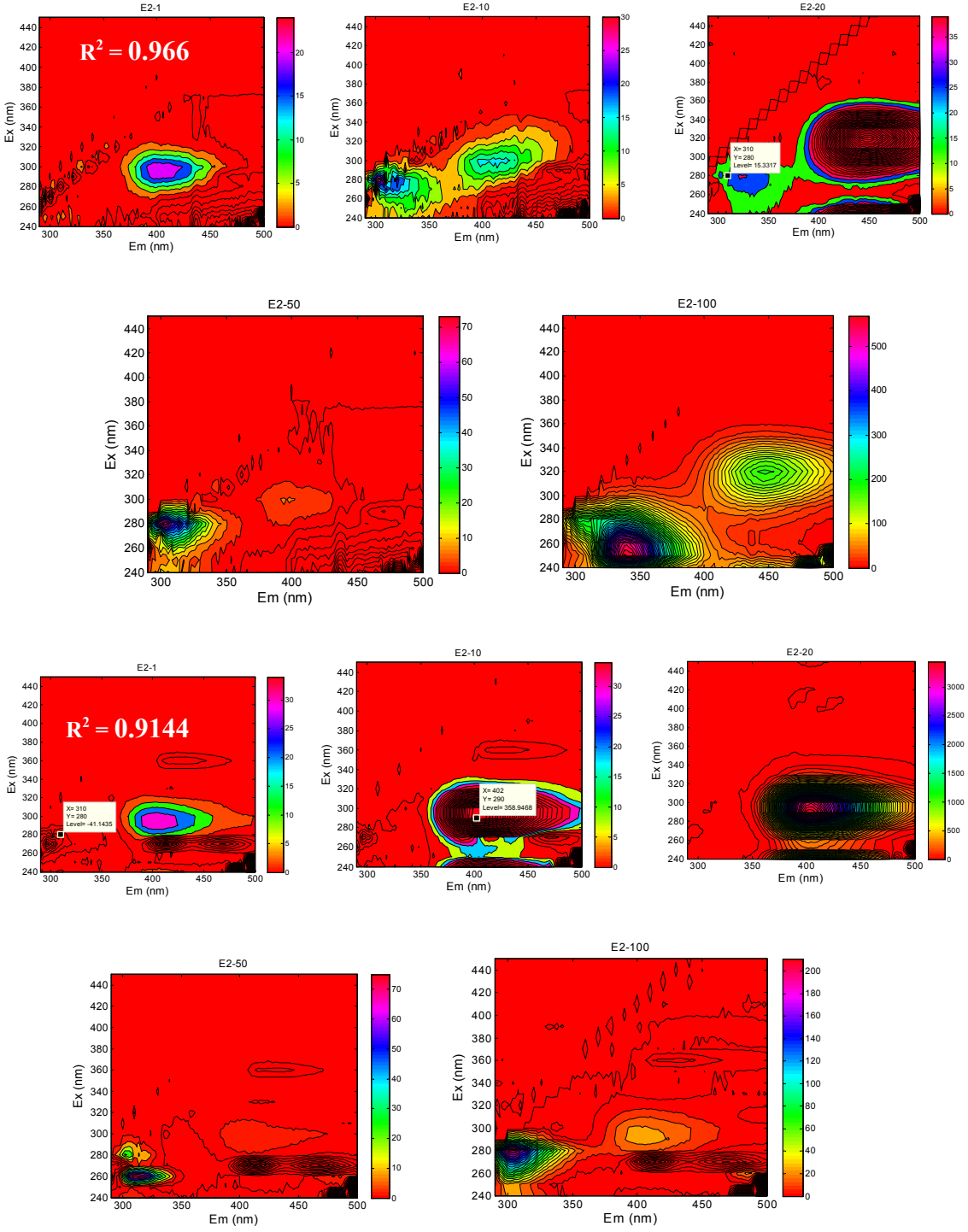

Result of 17α-Ethinylestradiol (EE2) in soil column effluent

| Soil column | Absorbance | | Average absorbance | B/Bo (%) | Conc (µg/L) w/ dilution | Actual conc (µg/L) |
|---|---|---|---|---|---|---|
| SC3-11th | 0.481 | 0.524 | 0.5025 | 45.21 | 0.19 | 129.90 |

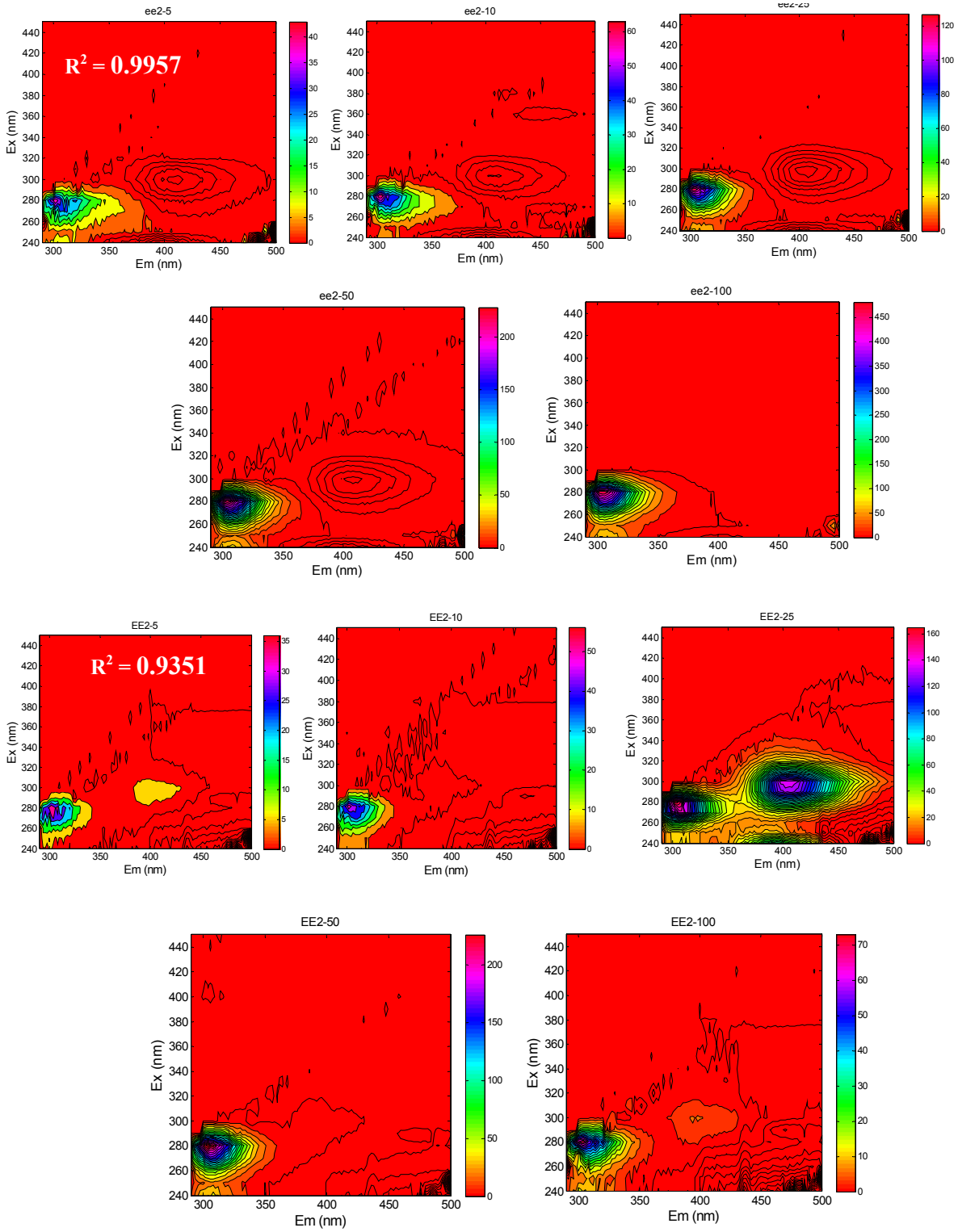
# Appendix D – Measurement of estrogens by F-EEM

## D1 – F-EEM plot for calibration curves of estrogen with MQ water and mixture of DCW and SE (1:1)

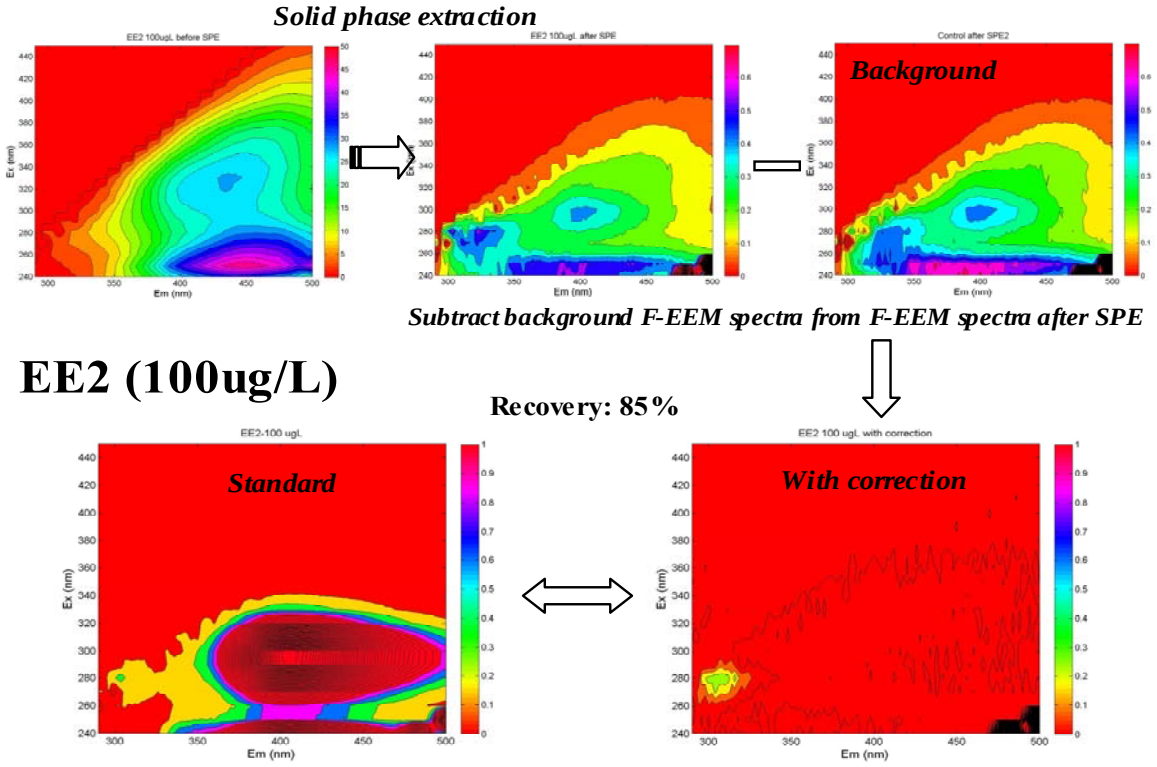
### D1 – I –17β-Estradiol (E2) in MQ water



## D1 – II –17α-Ethinylestradiol (EE2) in MQ



**D1 – III – Sample correction process for calibration curve of EE2 with known concentration in mixture of DCW and SE**

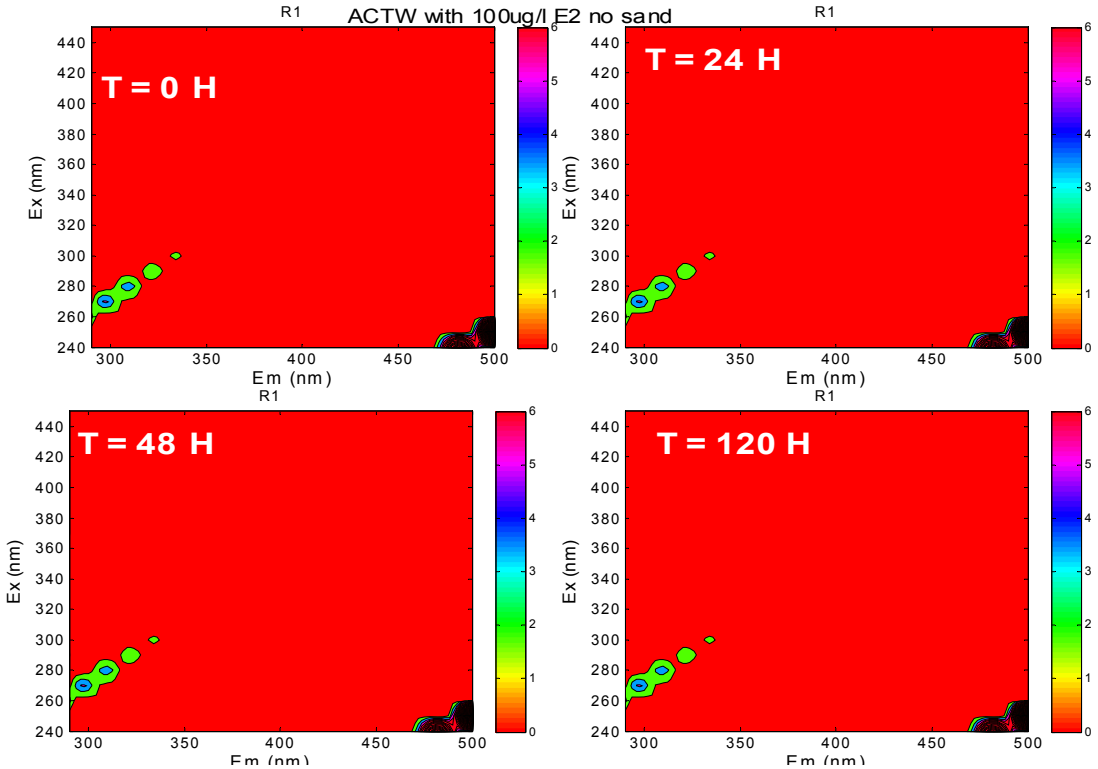


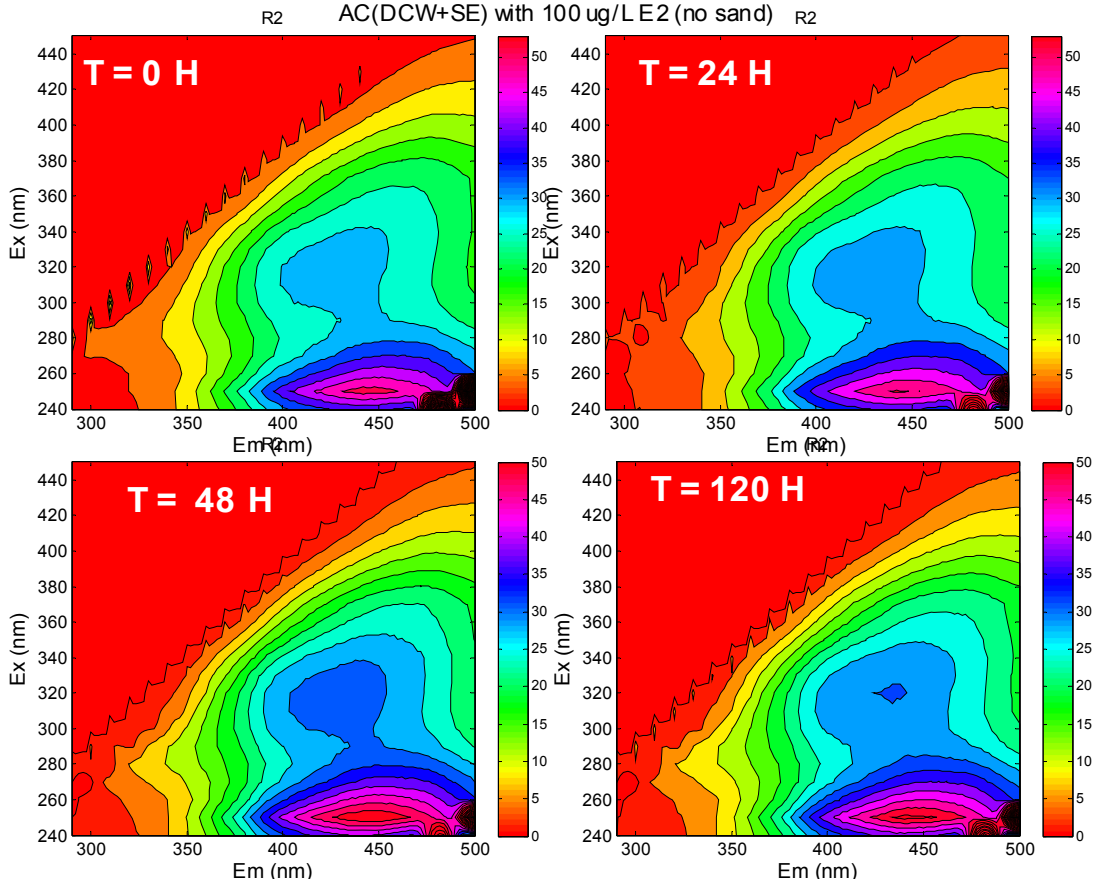

D2 – F-EEM plot and intensity level on adsorption isotherms of E2 and EE2

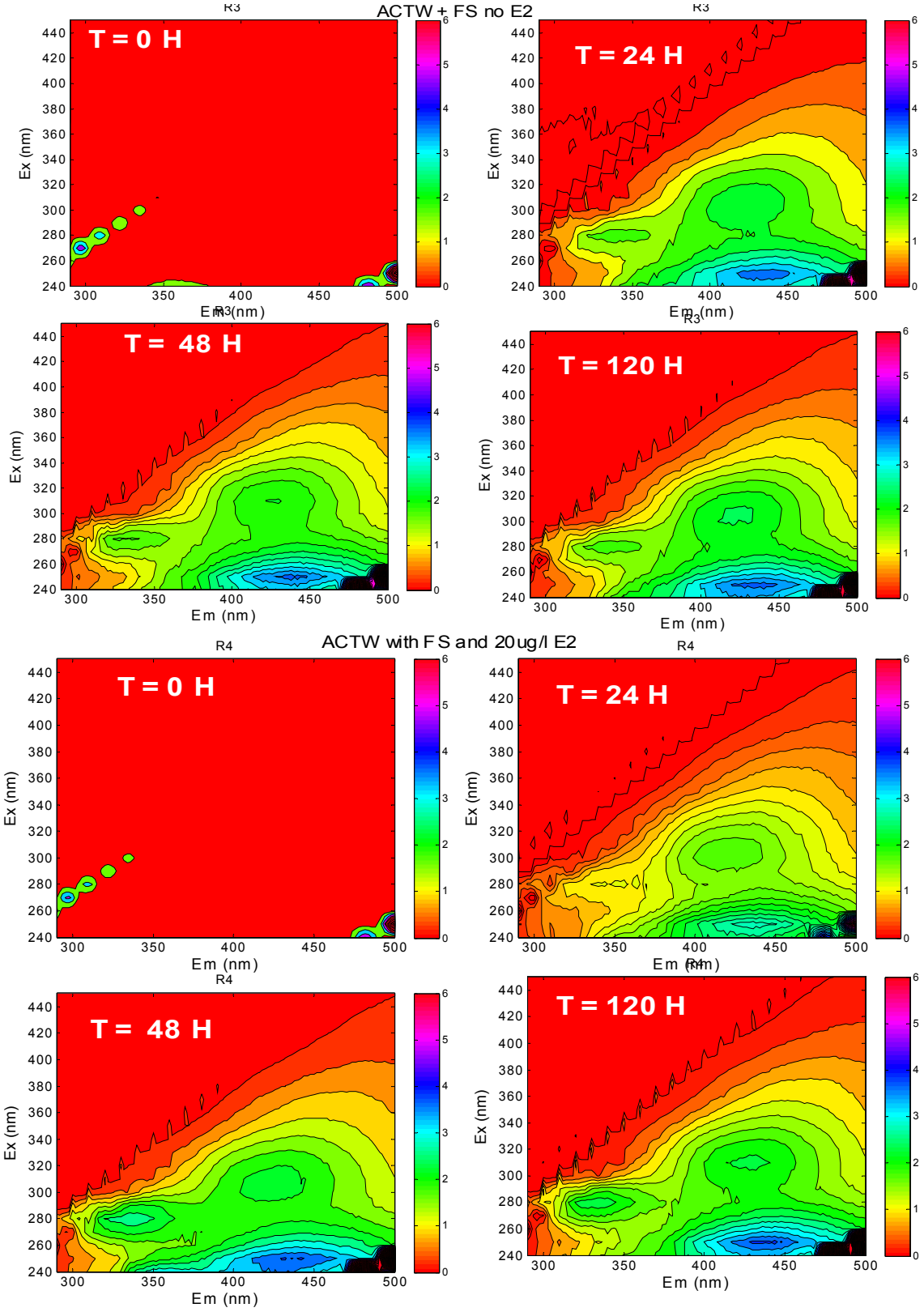
**D2 – I – Intensity level of E2 during experiment**

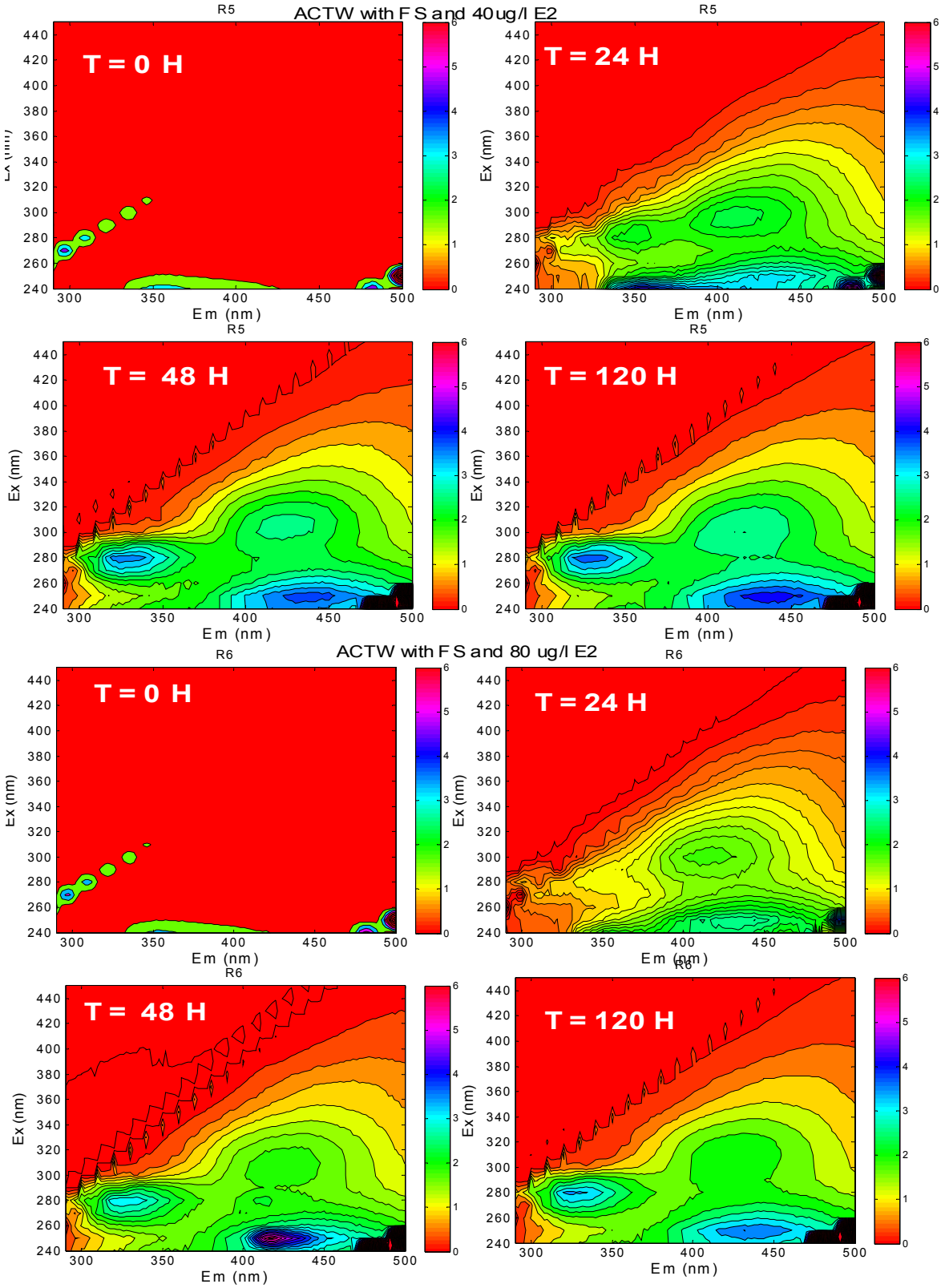
| Reactor conditions | Intensity Level (Ex-280 & Em-310) | | | |
|---|---|---|---|---|
| | 0 hours | 24 hours | 48 hours | 120 hours |
| ACDW + 100µg/L of E2 | 4.37 | 4.37 | 4.37 | 4 |
| AC(DCW + SE) + 100µg/L of E2 | 5.54 | 1.3 | 2.8 | 2.9 |
| ACDW+ clean sand(39.42g) no E2 | 3.5 | 1.3 | 0.87 | 0.82 |
| ACDW+ clean sand(39.42g) + 20µg/L E2 | 3.8 | 0.28 | 1.4 | 1.24 |
| ACDW + clean sand(39.42g) + 40µg/L E2 | 3.91 | 0.43 | 2.14 | 2.3 |
| ACDW+ clean sand(39.42g) + 80µg/L E2 | 4.14 | 0.97 | 2.14 | 2 |
| ACDW + clean sand(39.42g) + 100 µg/L E2 | 4.21 | 0.89 | 2.9 | 2.8 |
| DCW + SE + 100 µg/L E2 no sand | 5.72 | 3.37 | 0.63 | 0.68 |
| DCW + SE + 50g ripened sand + 0 µg/L E2 | 5.29 | 0.9 | 0.21 | -0.14 |
| DCW + SE + 50g ripened sand + 20 µg/L E2 | 5.47 | 2.27 | 0.14 | -0.38 |
| DCW + SE + 50g ripened sand + 40 µg/L E2 | 5.33 | 3.4 | 0.13 | -0.14 |
| DCW + SE + 50g ripened sand + 80 µg/L E2 | 5.24 | 4.12 | 0.22 | -0.26 |
| DCW + SE + 50g ripened sand + 100 µg/L E2 | 5.24 | 3.67 | 0.77 | -0.04 |

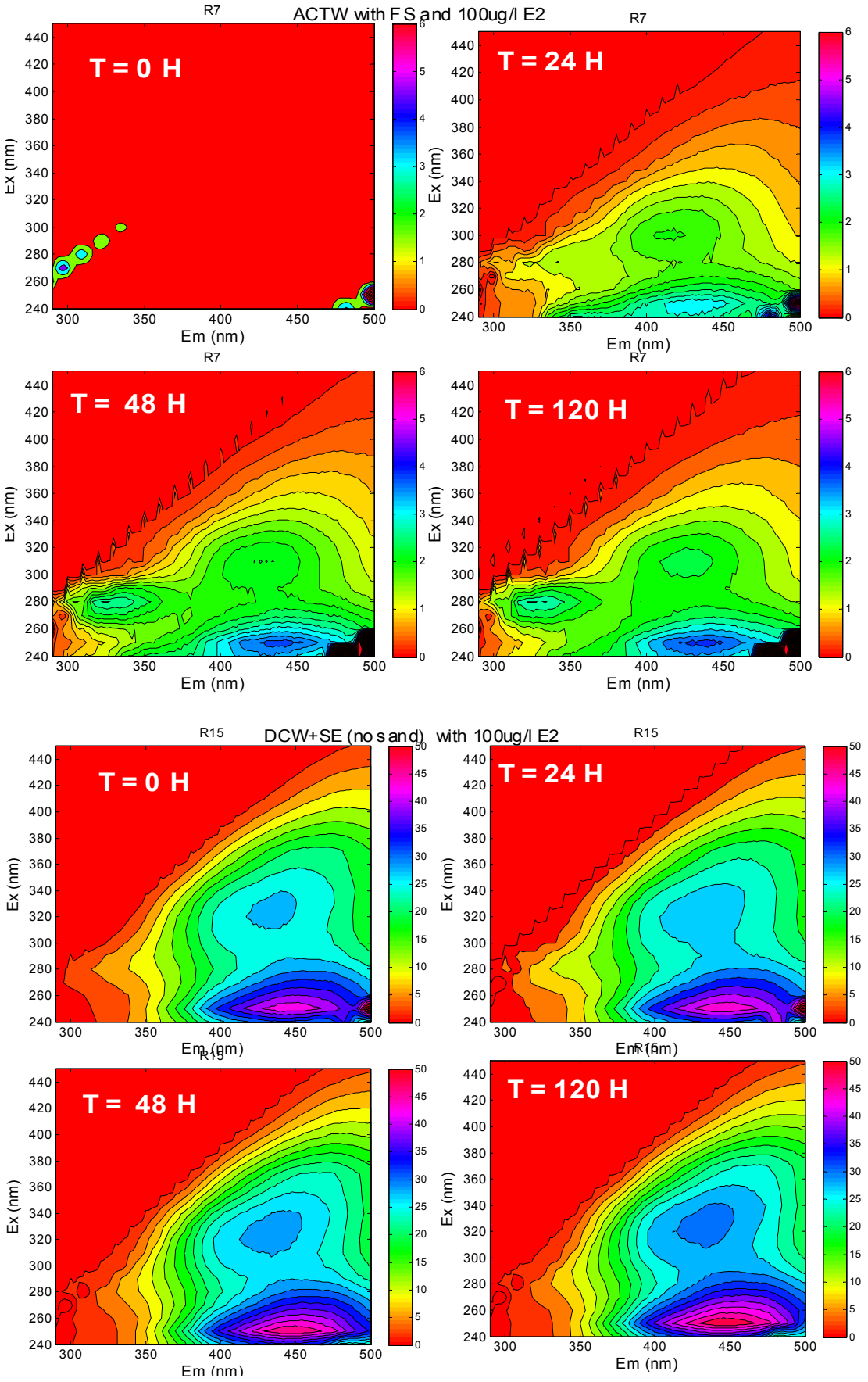
## D2 – I –F-EEM plot of E2 during experiment

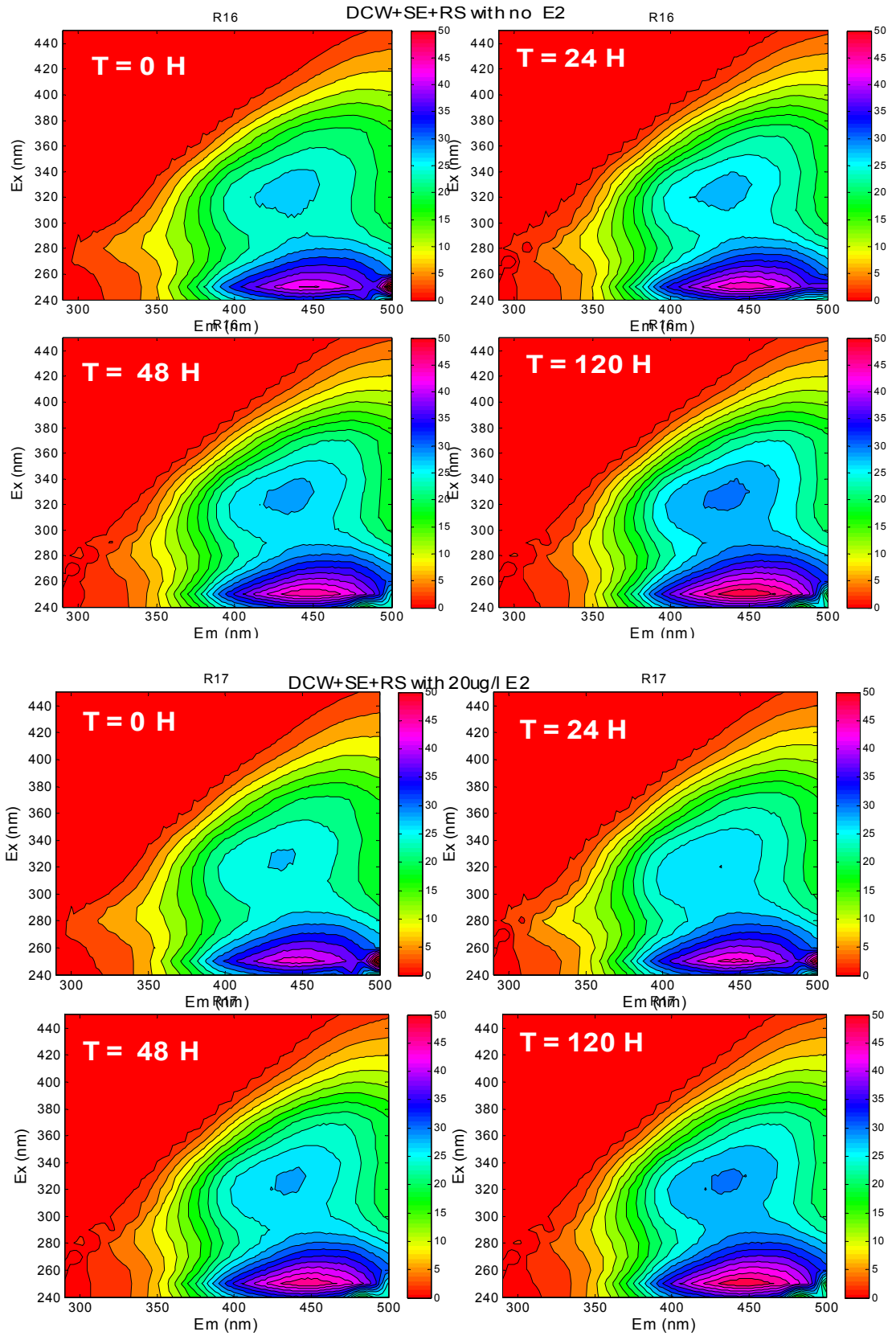


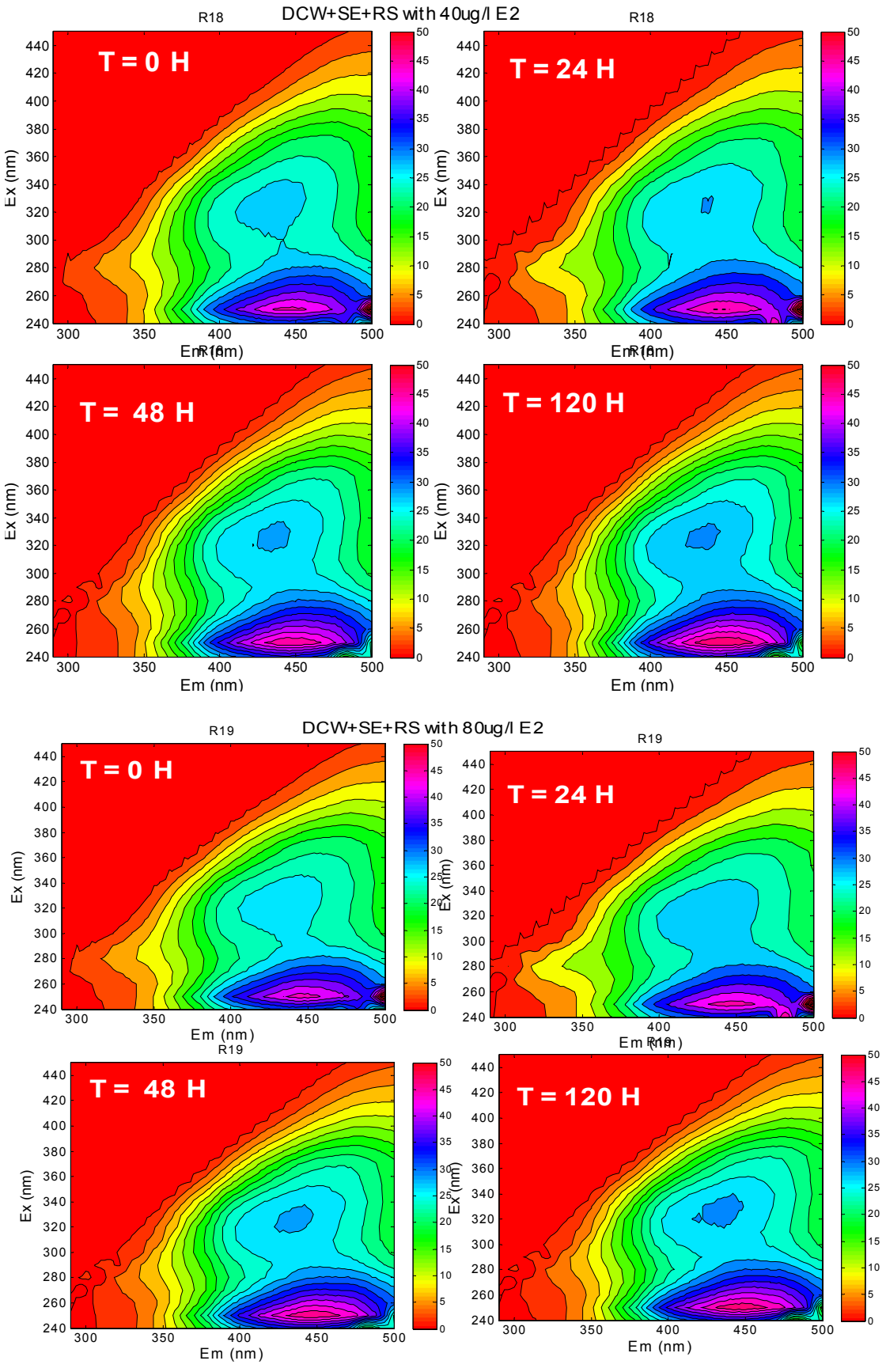



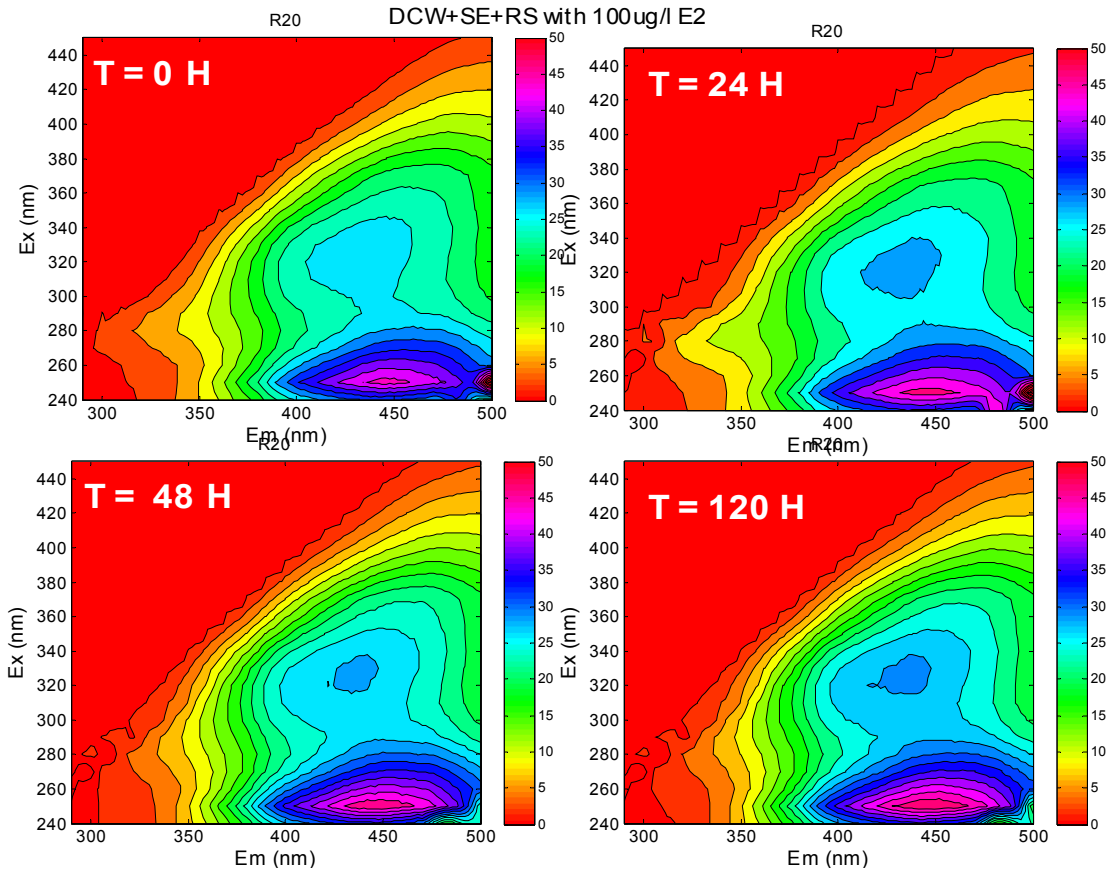

ACTW + FS no E2
R3
T = 0 H
T = 24 H
T = 48 H
T = 120 H
ACTW with FS and 20ug/l E2
R4
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)


ACTW with F S and 40 ug/l E2
R5
T = 0 H
T = 24 H
T = 48 H
T = 120 H
ACTW with F S and 80 ug/l E2
R6
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)


ACTW with F S and 100ug/l E2
R7
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)
DCW+SE (no s and) with 100ug/l E2
R15
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)


DCW+SE+RS with no E2
R16
T = 0 H
T = 24 H
T = 48 H
T = 120 H
DCW+SE+RS with 20ug/l E2
R17
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

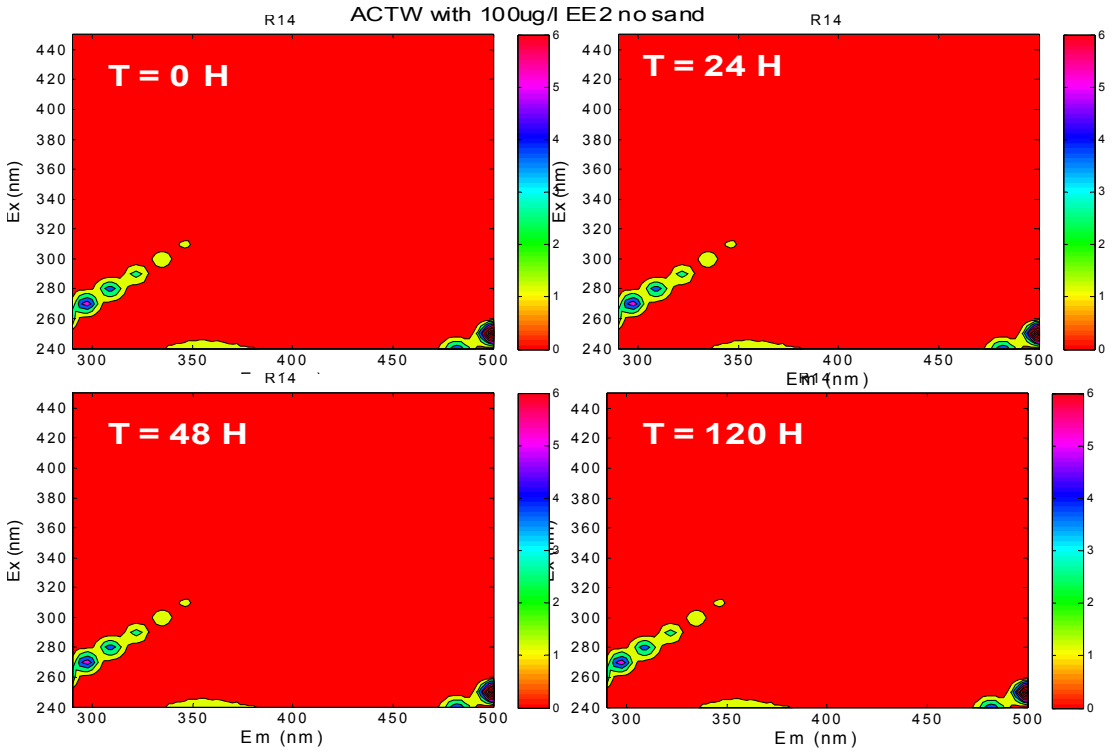

DCW+SE+RS with 40ug/l E2
R18
T = 0 H
T = 24 H
T = 48 H
T = 120 H
DCW+SE+RS with 80ug/l E2
R19
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

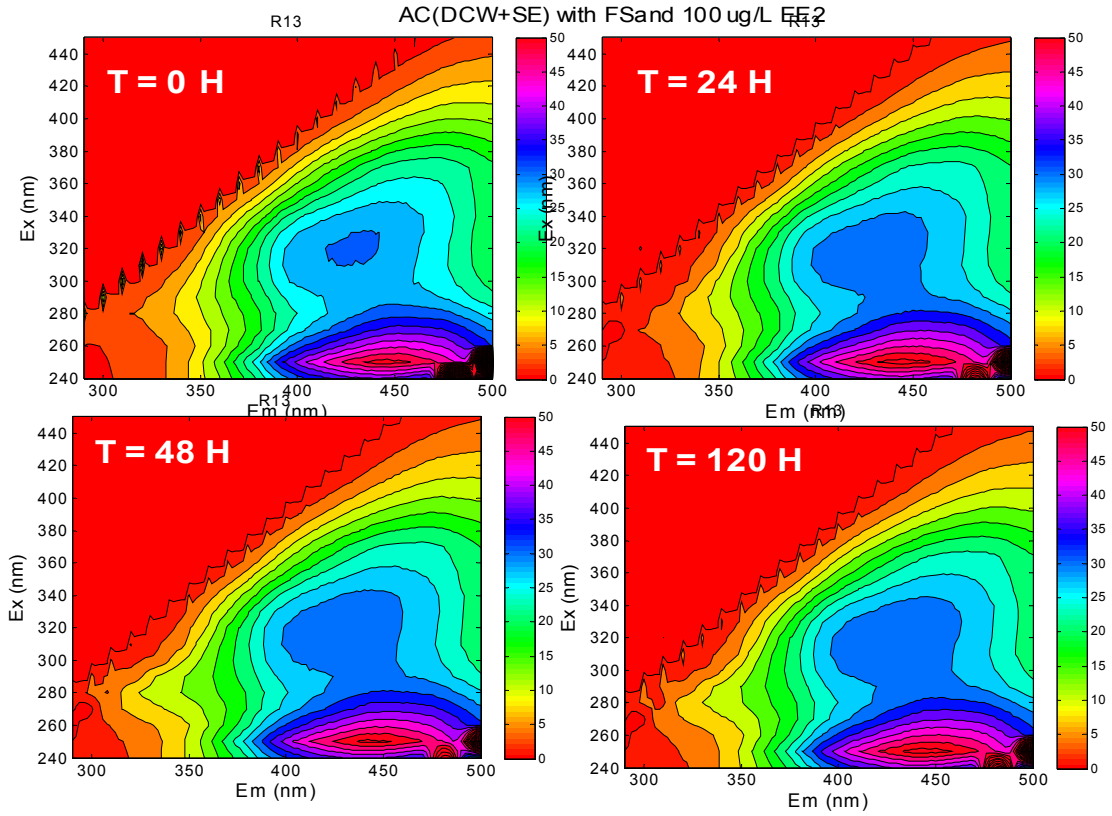


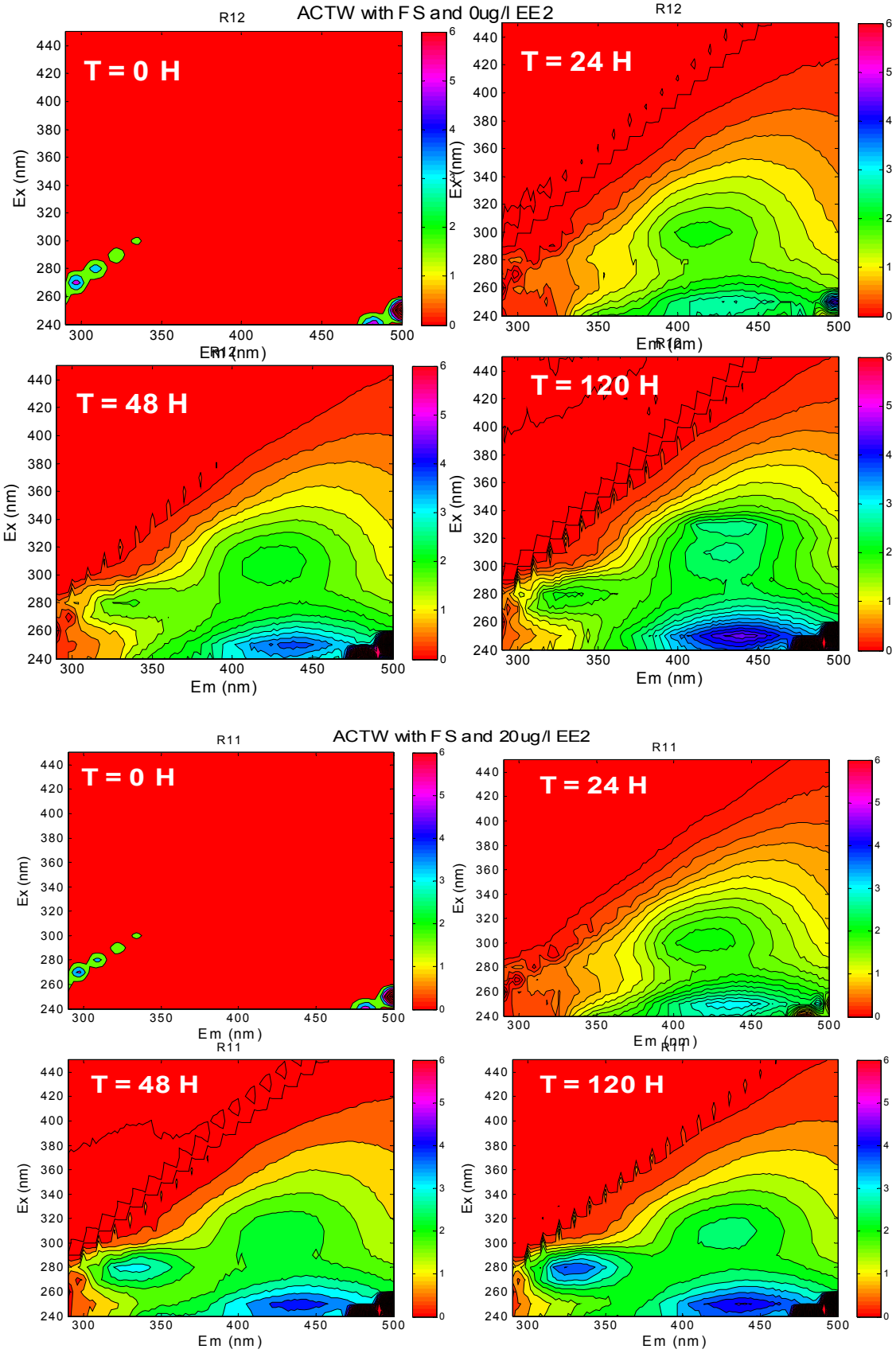

**D2 – II – Intensity level of EE2 during experiment**

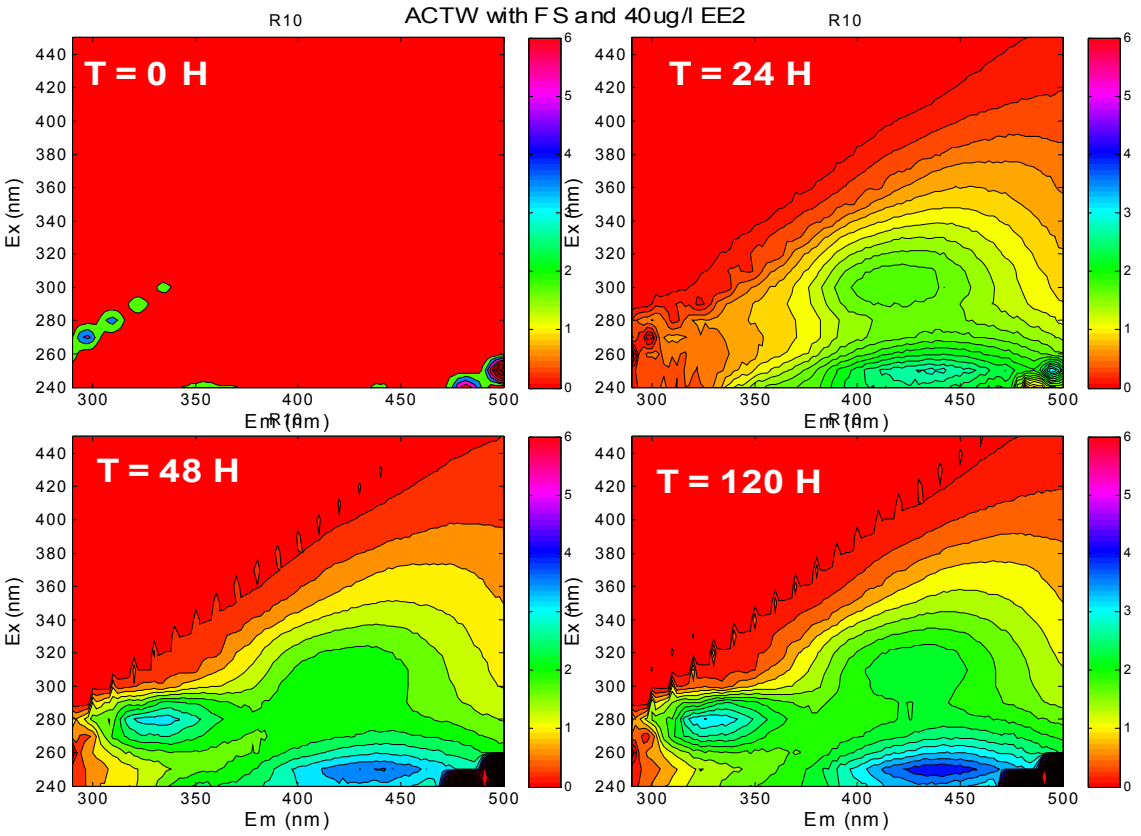
| Reactor conditions | Intensity Level (Ex-280 nm & Em-310 nm) | | | |
|---|---|---|---|---|
| | 0 hour | 24 hours | 48 hours | 120 hours |
| ACDW + 100 µg/L of EE2 | 4.33 | 4.33 | 4.0 | 4.0 |
| AC(DCW + SE) + 100 µg/L of EE2 | 5.61 | 1.66 | 4.27 | 3.85 |
| ACDW+ clean sand (39.42 g) no EE2 | 4.23 | 0.04 | 1.02 | 1.35 |
| ACDW+ clean sand (39.42 g) + 20u g/L EE2 | 3.82 | 0.05 | 1.81 | 2.49 |
| ACDW + clean sand (39.42 g) + 40 µg/L EE2 | 4.25 | 0.21 | 1.87 | 1.9 |
| ACDW+ clean sand (39.42 g) + 80 µg/L EE2 | 4.34 | 0.26 | 1.65 | 2.3 |
| ACDW + clean sand (39.42 g) + 100 µg/L EE2 | 4.34 | 0.48 | 1.7 | 2.7 |
| DCW + SE + 100 µg/L EE2 no sand | 5.36 | 3.07 | 0.94 | 1.3 |
| DCW + SE + 50 g ripened sand + 0 µg/L EE2 | 5.18 | 2.8 | 0.42 | -0.12 |
| DCW + SE + 50 g ripened sand + 20 µg/L EE2 | 5.46 | 2.68 | 0.005 | -0.34 |
| DCW + SE + 50 g ripened sand + 40 µg/L EE2 | 5.36 | 3.87 | 0.25 | -0.13 |
| DCW + SE + 50 g ripened sand + 80 µg/L EE2 | 5.29 | 3.61 | 0.37 | 0.13 |
| DCW + SE + 50 g ripened sand + 100 µg/ EE2 | 5.38 | 4.07 | 0.31 | -0.18 |

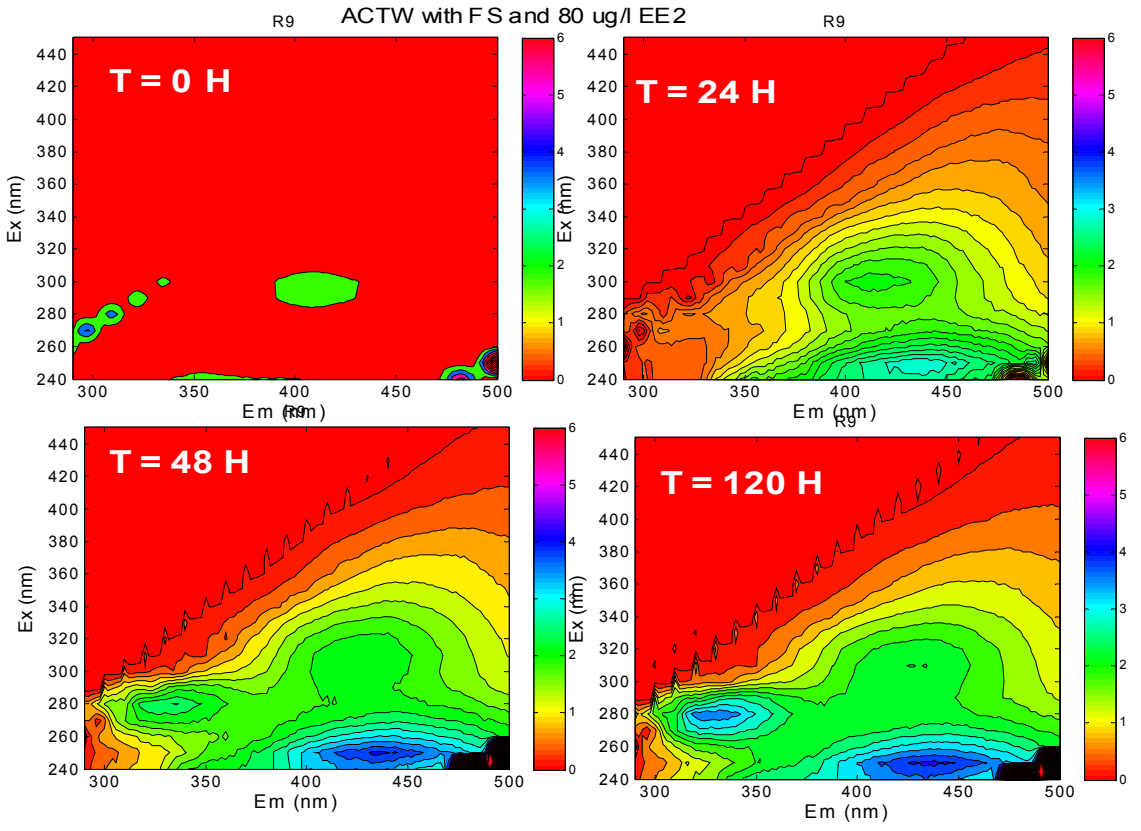
## D2 – II – F-EEM plots of EE2 during experiment

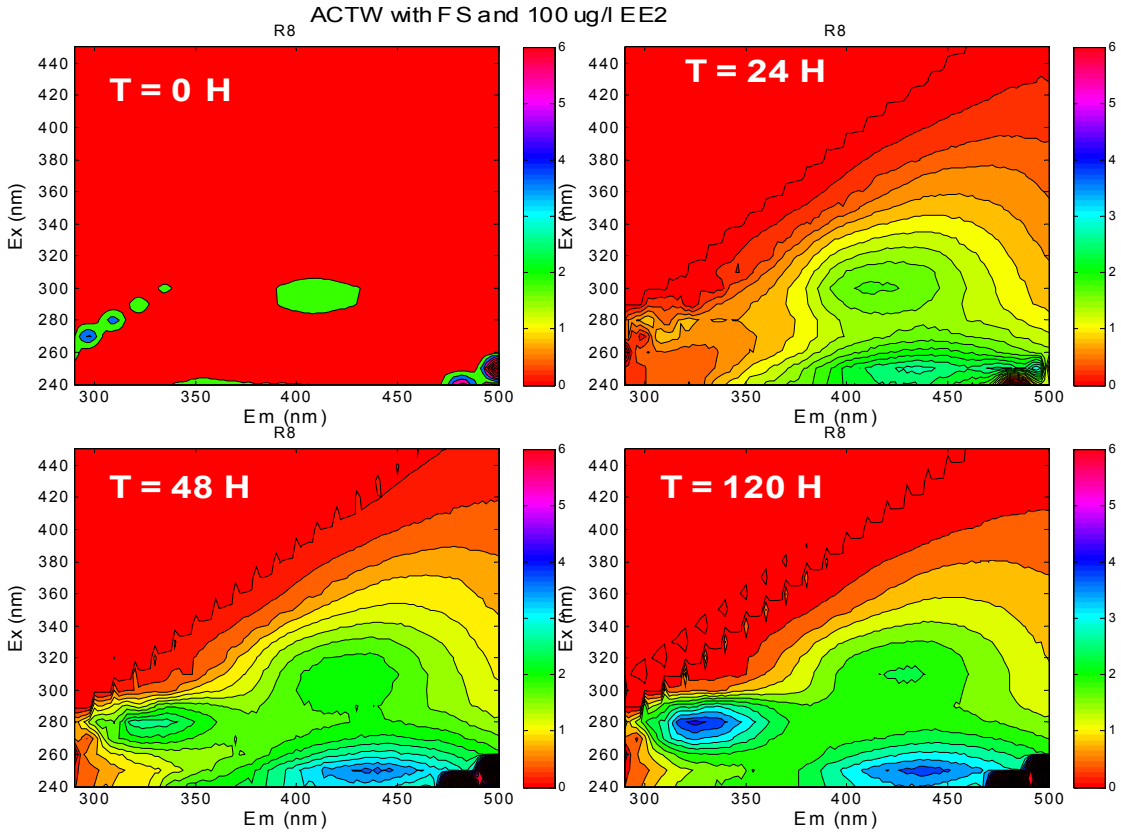


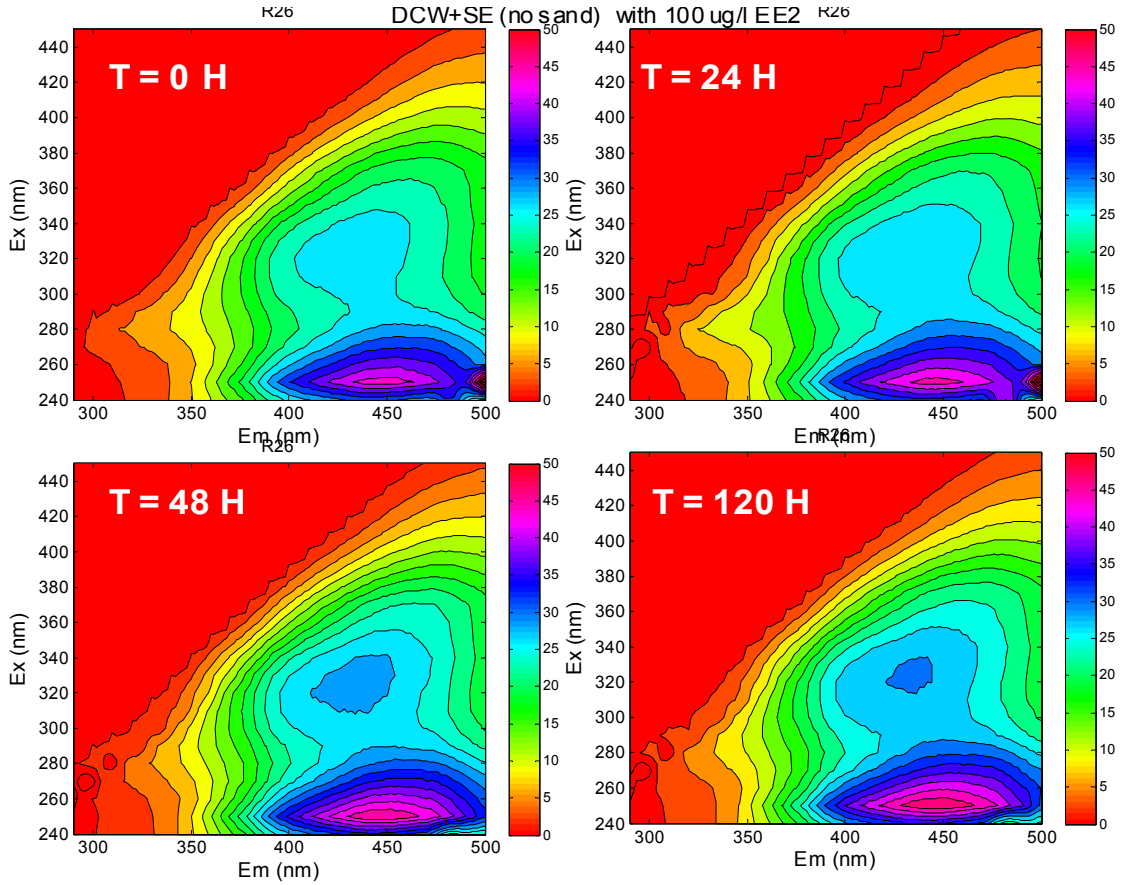



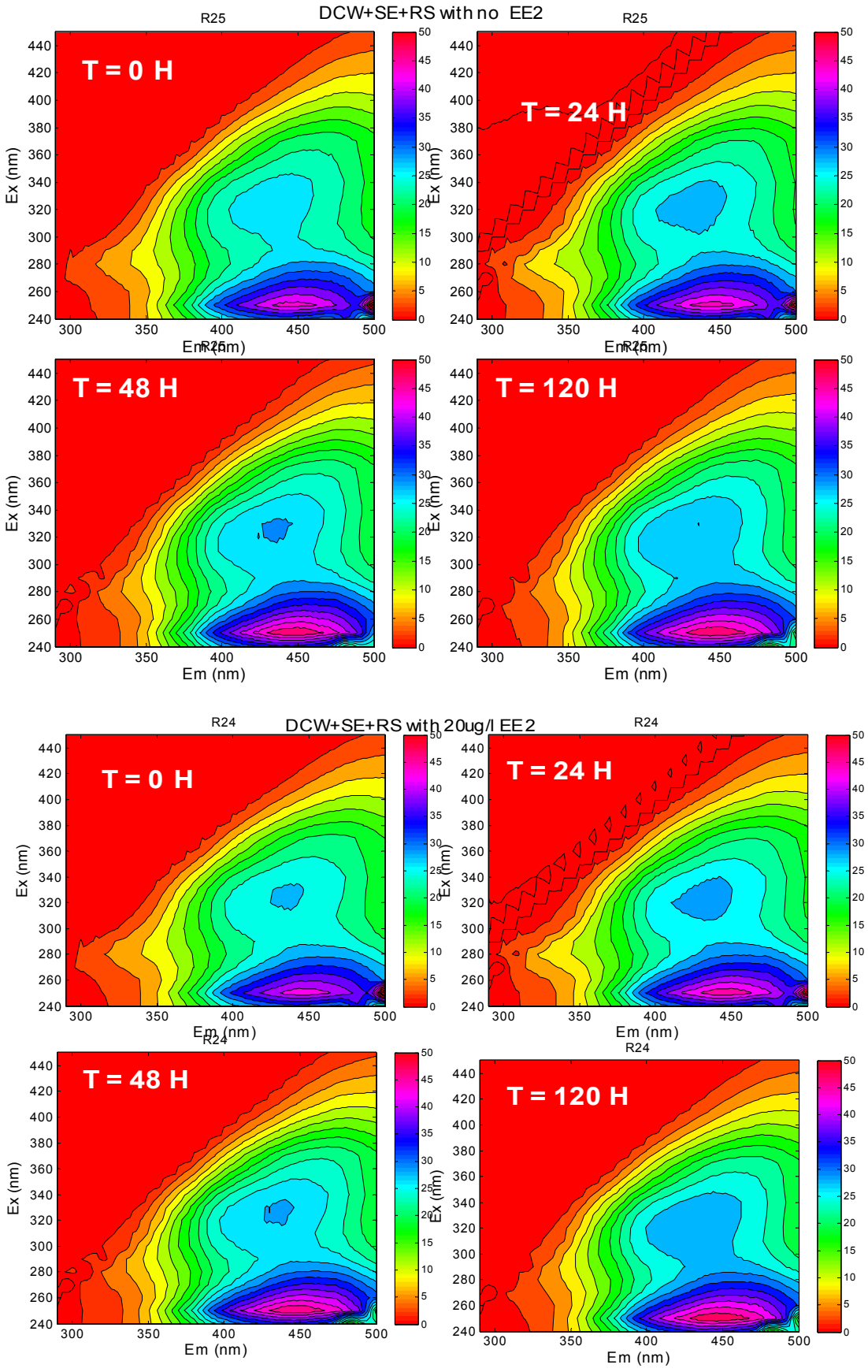

ACTW with FS and 0ug/l EE2
R12
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)
ACTW with FS and 20ug/l EE2
R11
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

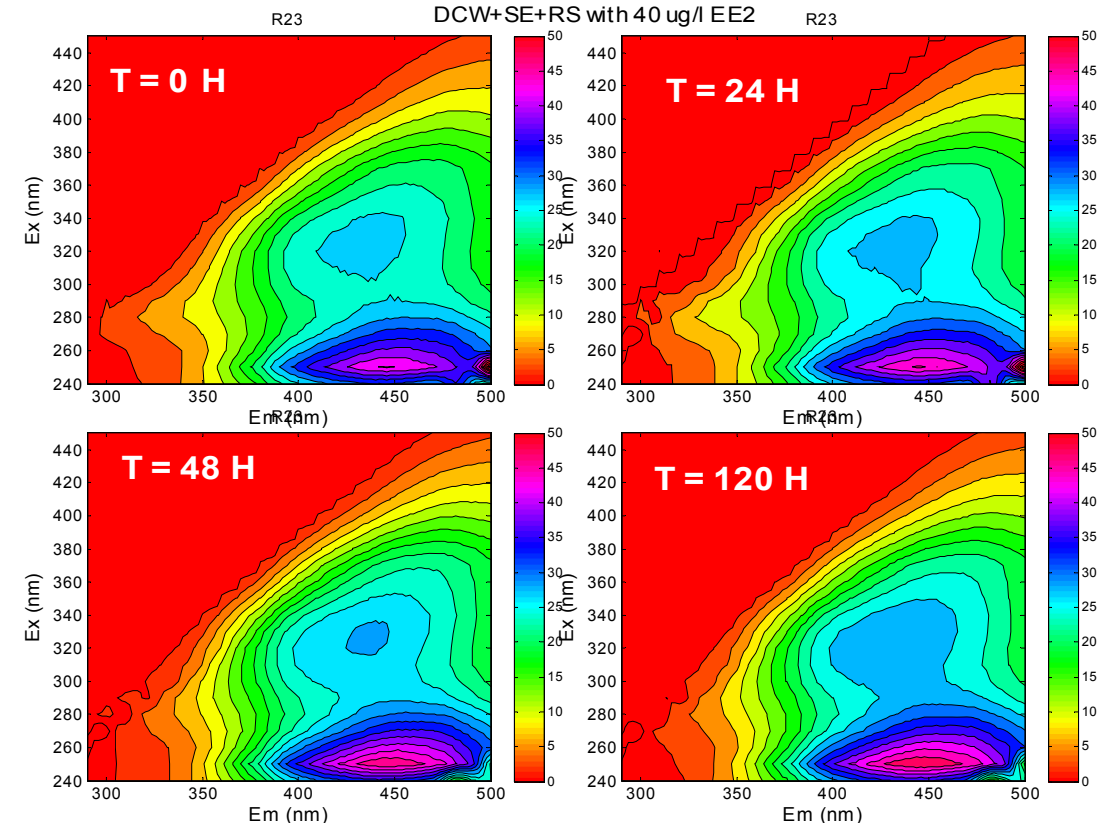

ACTW with F S and 40ug/l EE2
R10
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

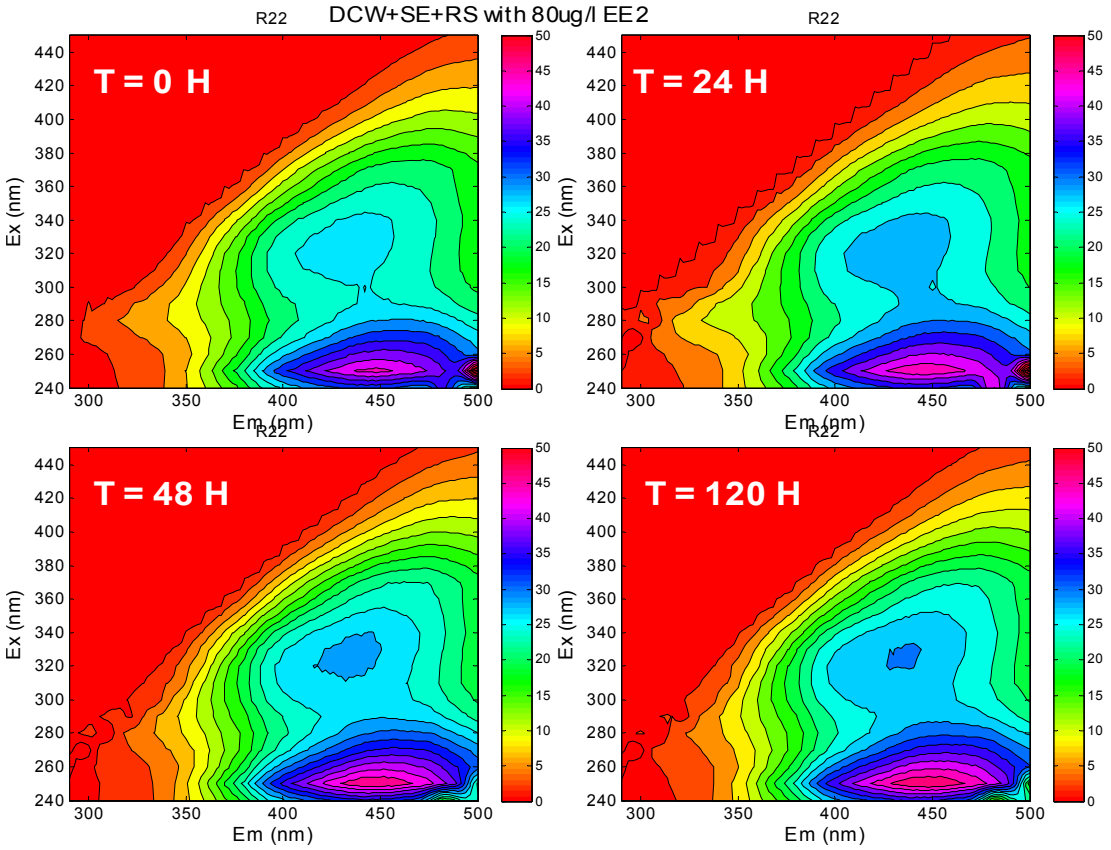

ACTW with F S and 80 ug/l EE2
R9
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

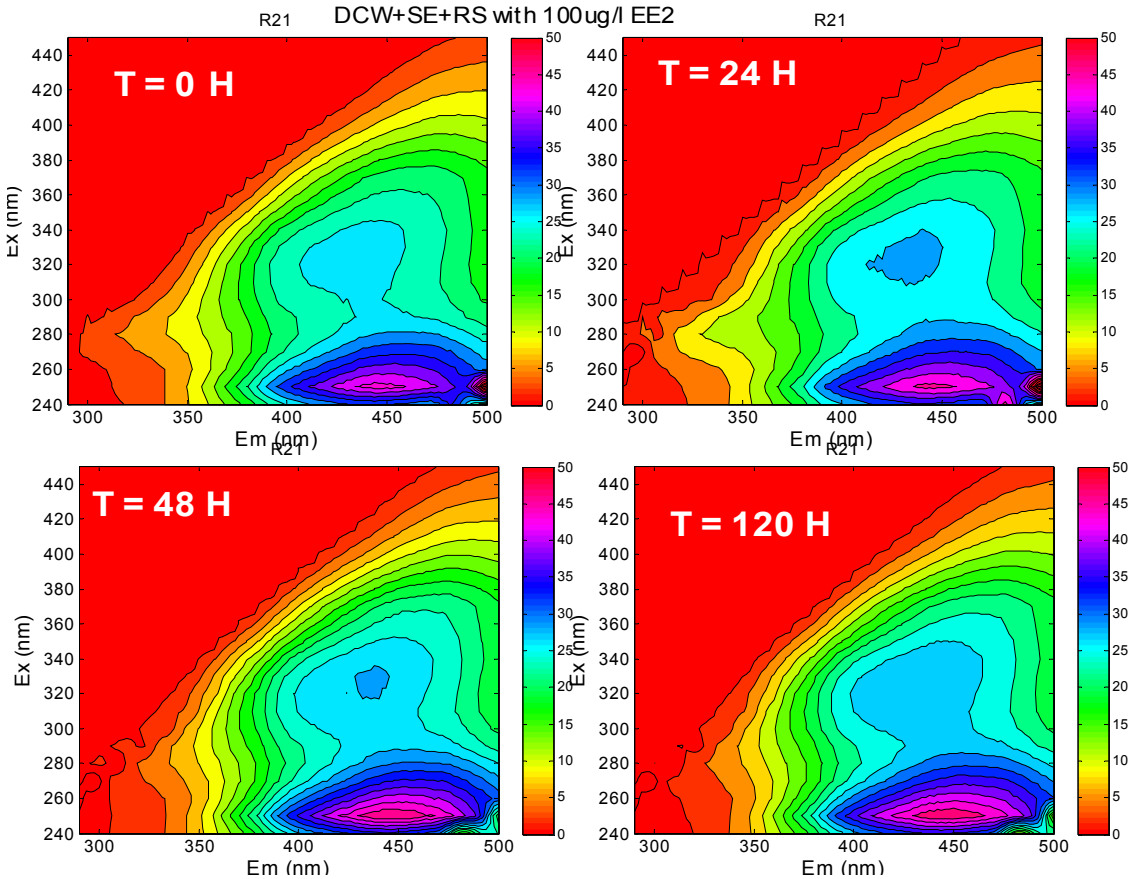

ACTW with F S and 100 ug/l EE2
R8
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)


DCW+SE (no sand) with 100 ug/l EE2
R26
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

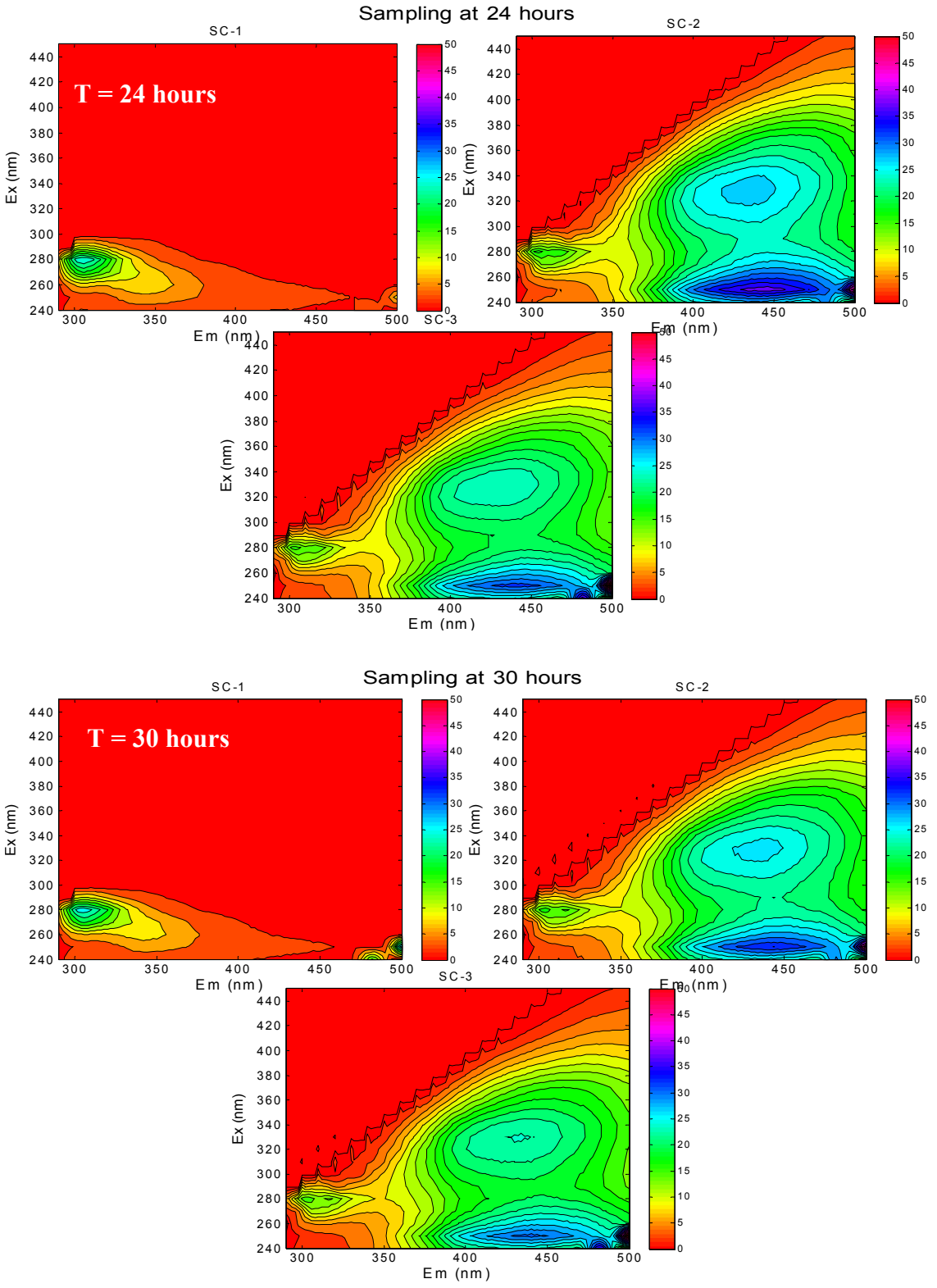

DCW+SE+RS with no EE2
R25
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)
DCW+SE+RS with 20ug/l EE2
R24
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

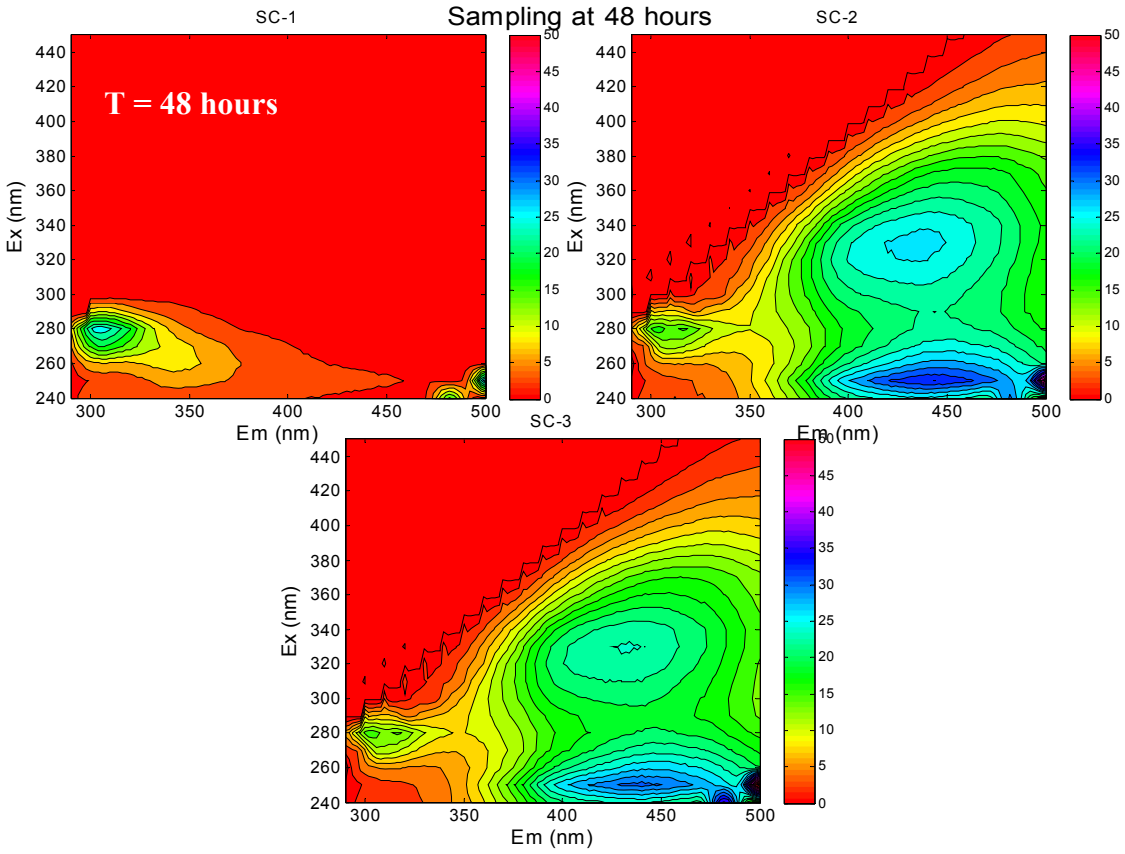

DCW+SE+RS with 40 ug/l EE2
R23
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

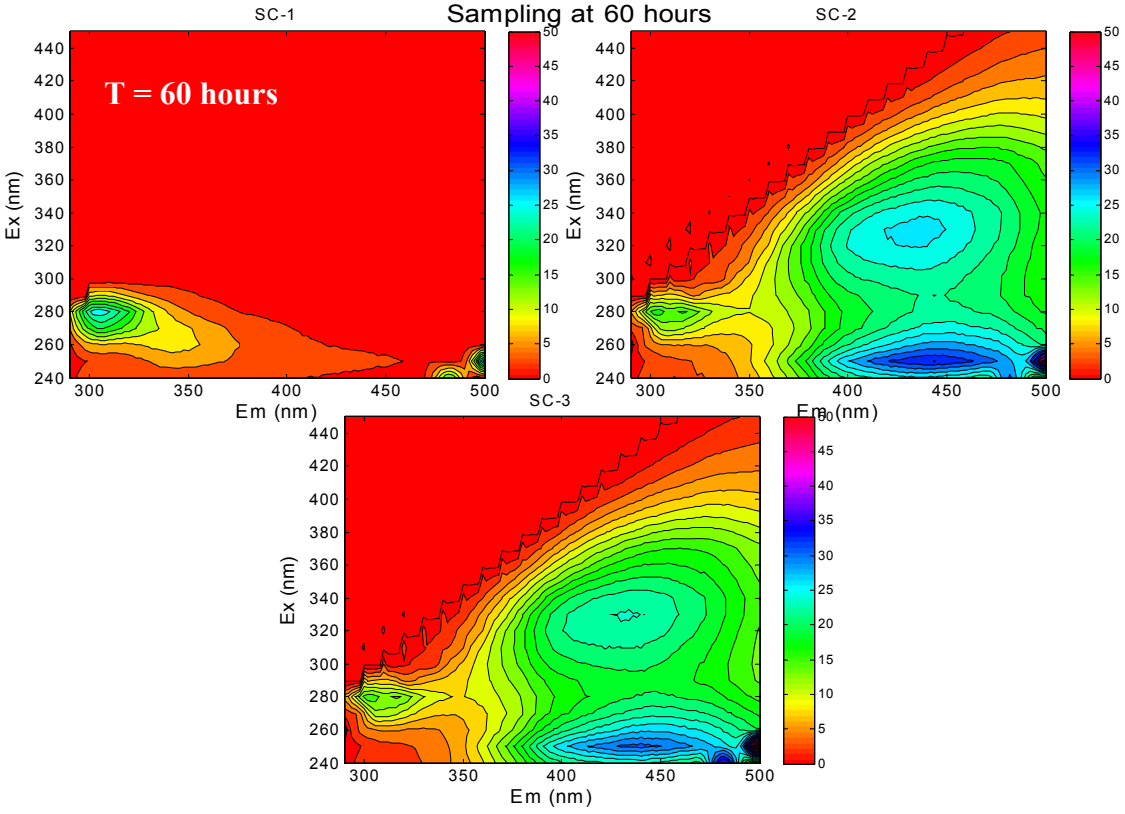

DCW+SE+RS with 80ug/l EE2
R22
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

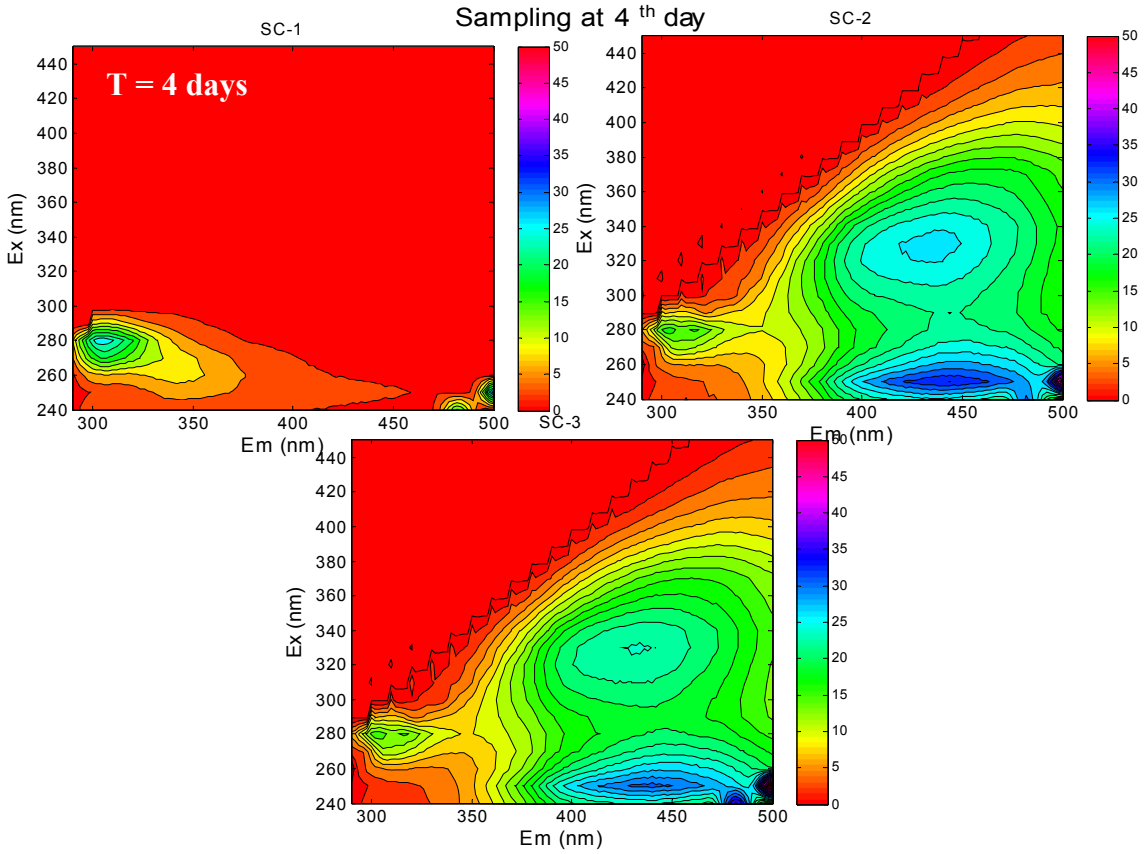

R21
DCW+SE+RS with 100ug/l EE2
T = 0 H
T = 24 H
T = 48 H
T = 120 H
Ex (nm)
Em (nm)

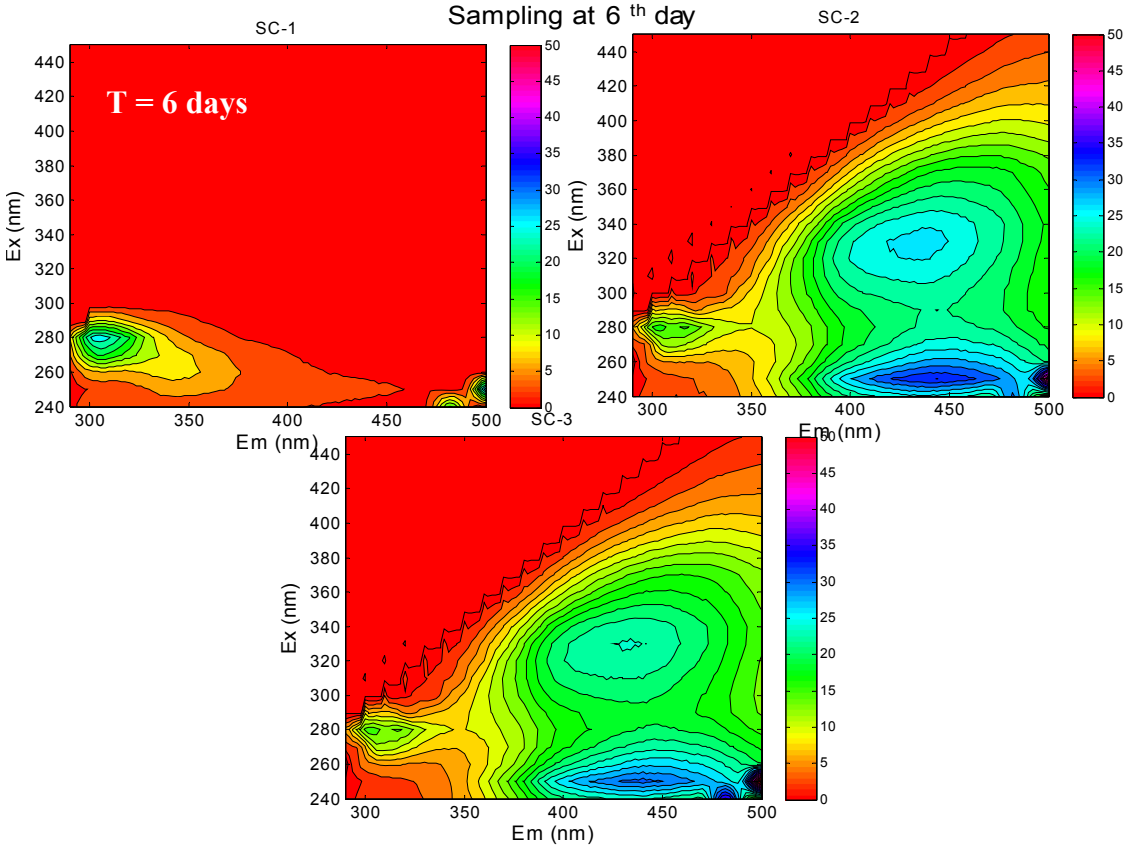
# D3 – F-EEM plots on soil columns study

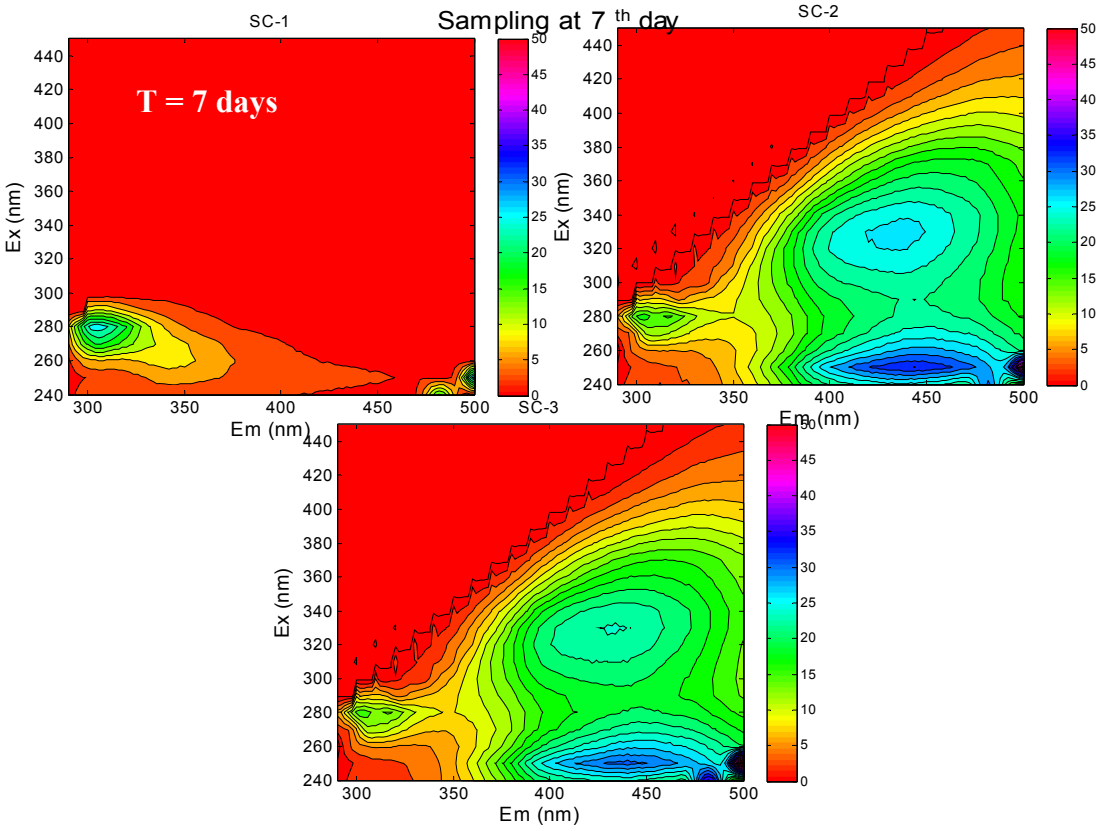


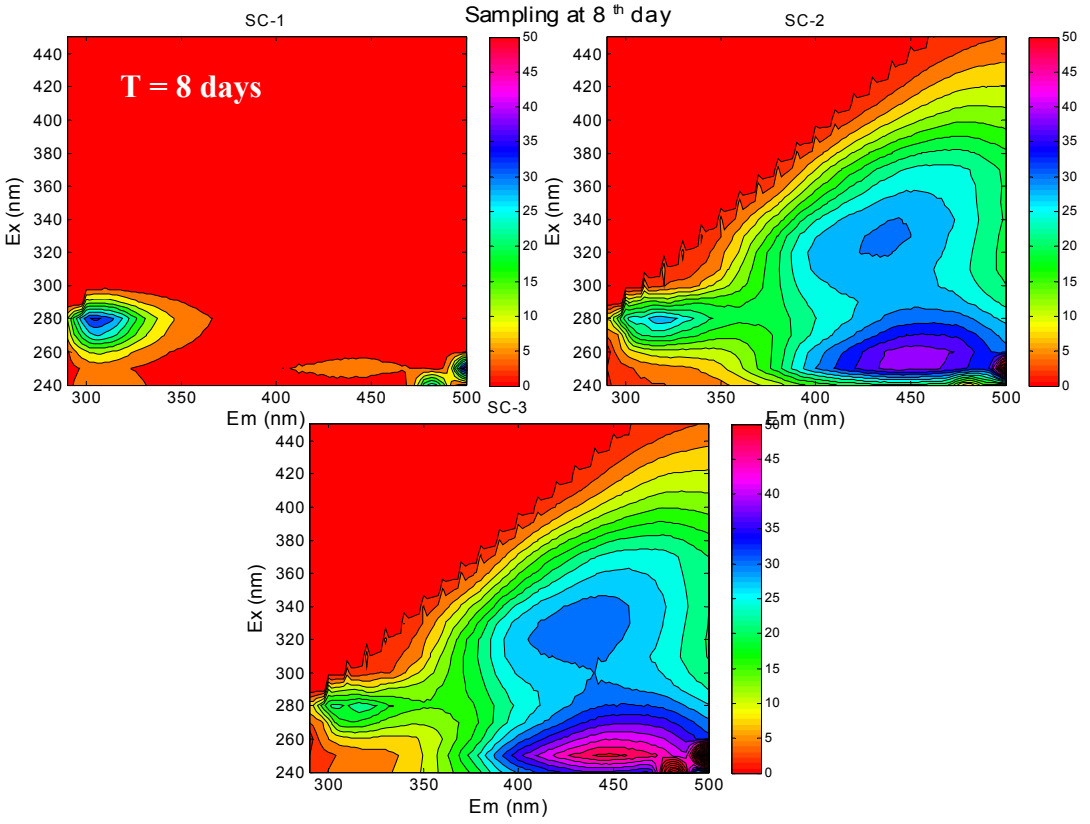

Sampling at 48 hours
SC-1
SC-2
SC-3
T = 48 hours
Ex (nm)
Em (nm)

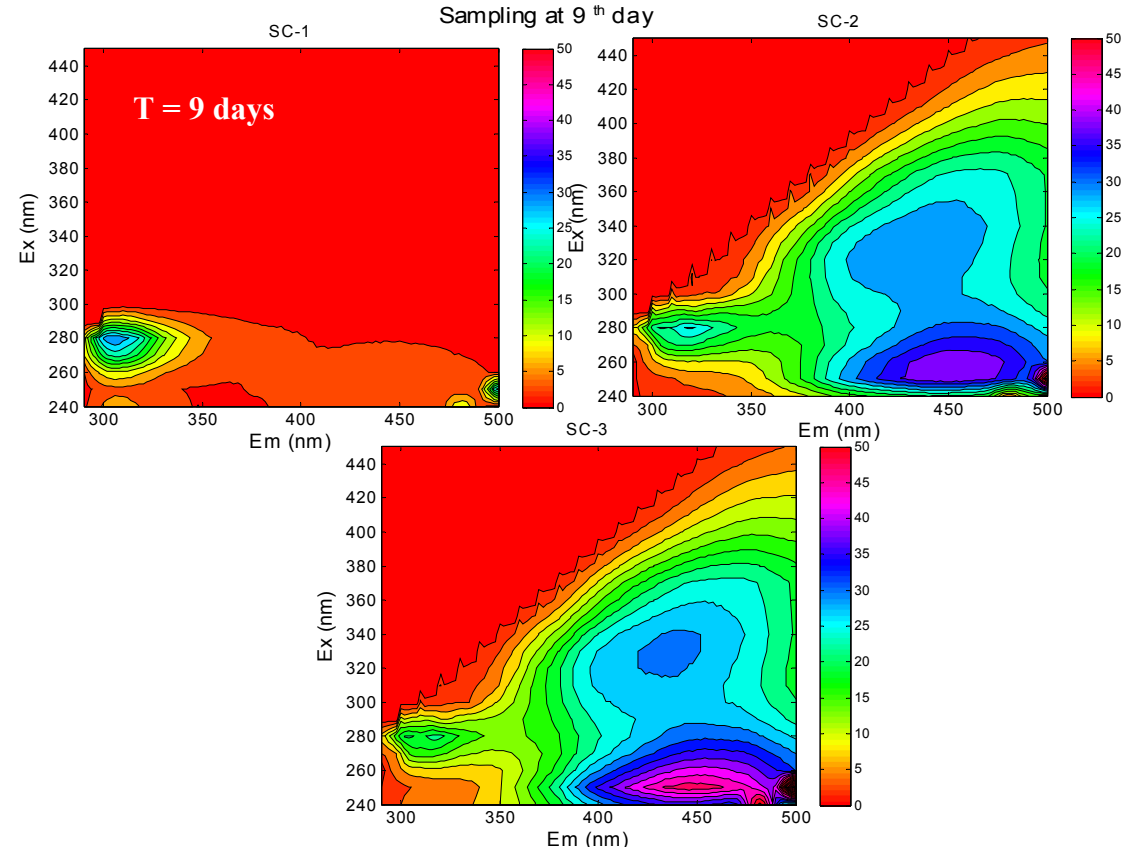

Sampling at 60 hours
SC-1
SC-2
SC-3
T = 60 hours
Ex (nm)
Em (nm)

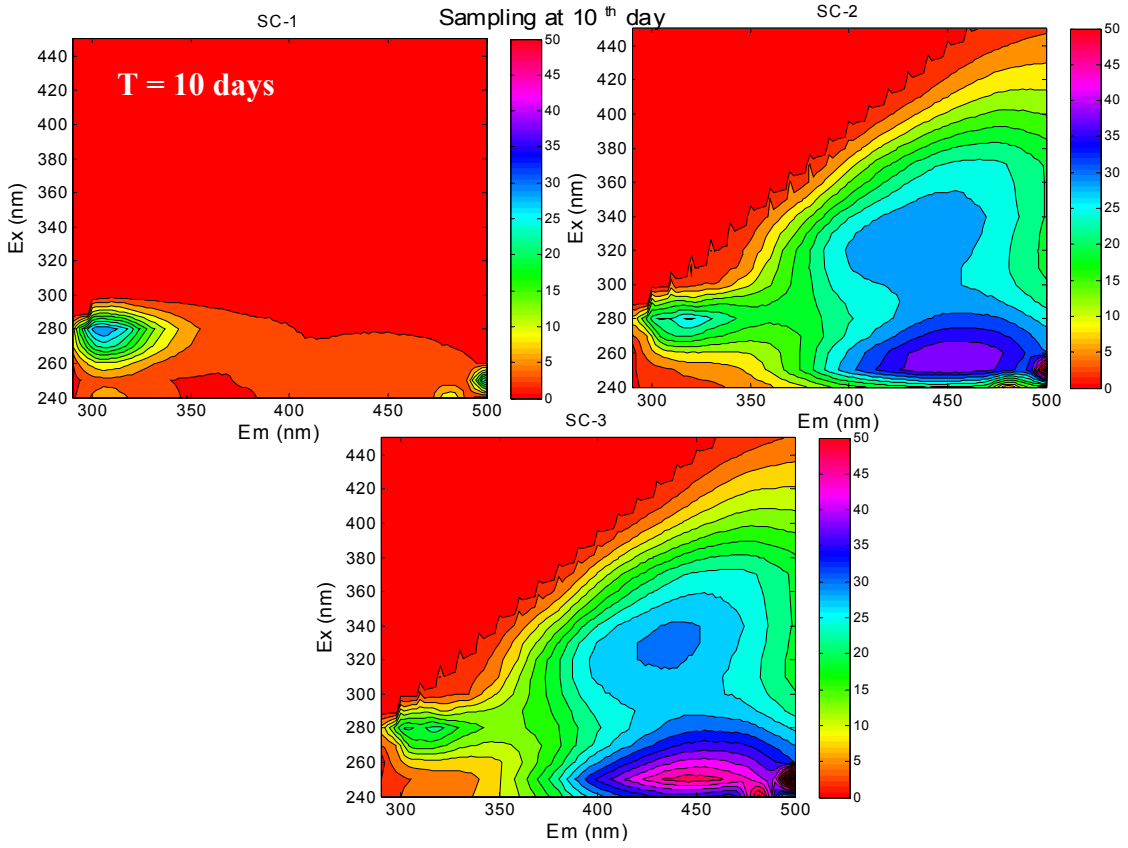

Sampling at 4 th day
SC-1
T = 4 days
SC-2
SC-3
Ex (nm)
Em (nm)

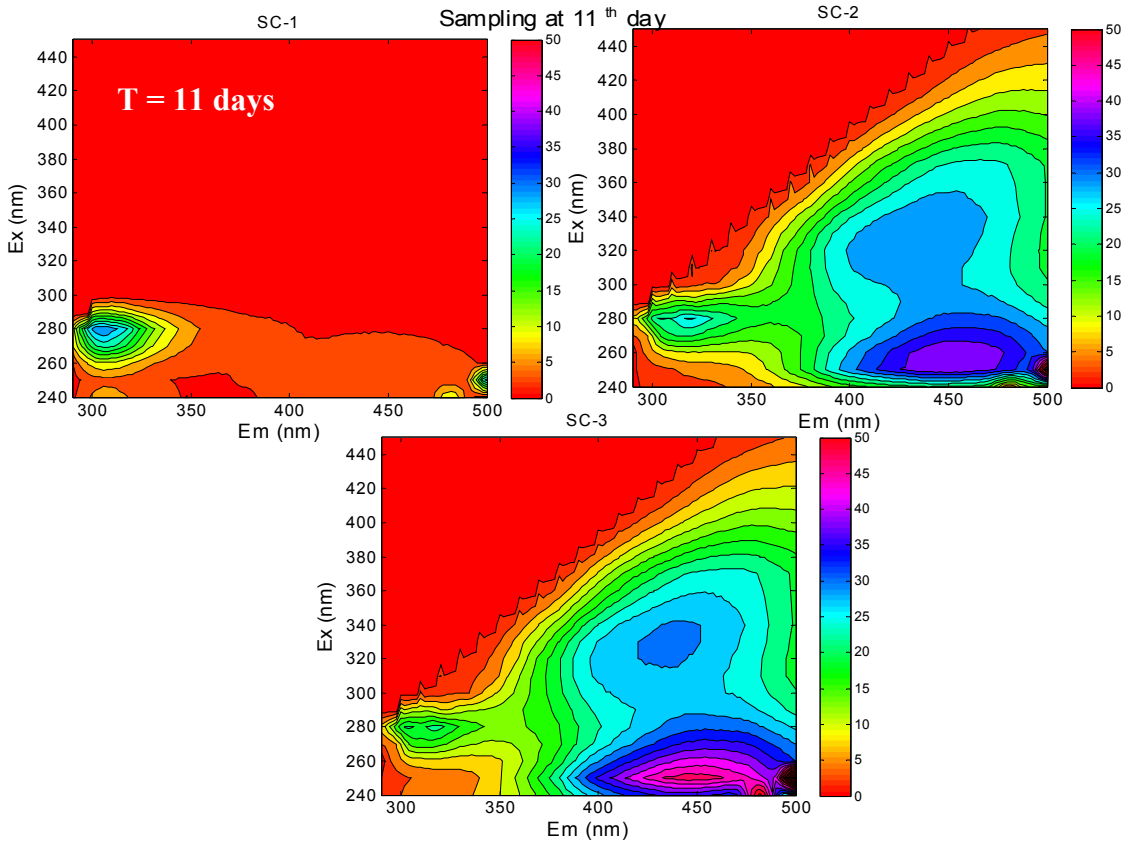

Sampling at 6 th day
SC-1
T = 6 days
SC-2
SC-3
Ex (nm)
Em (nm)

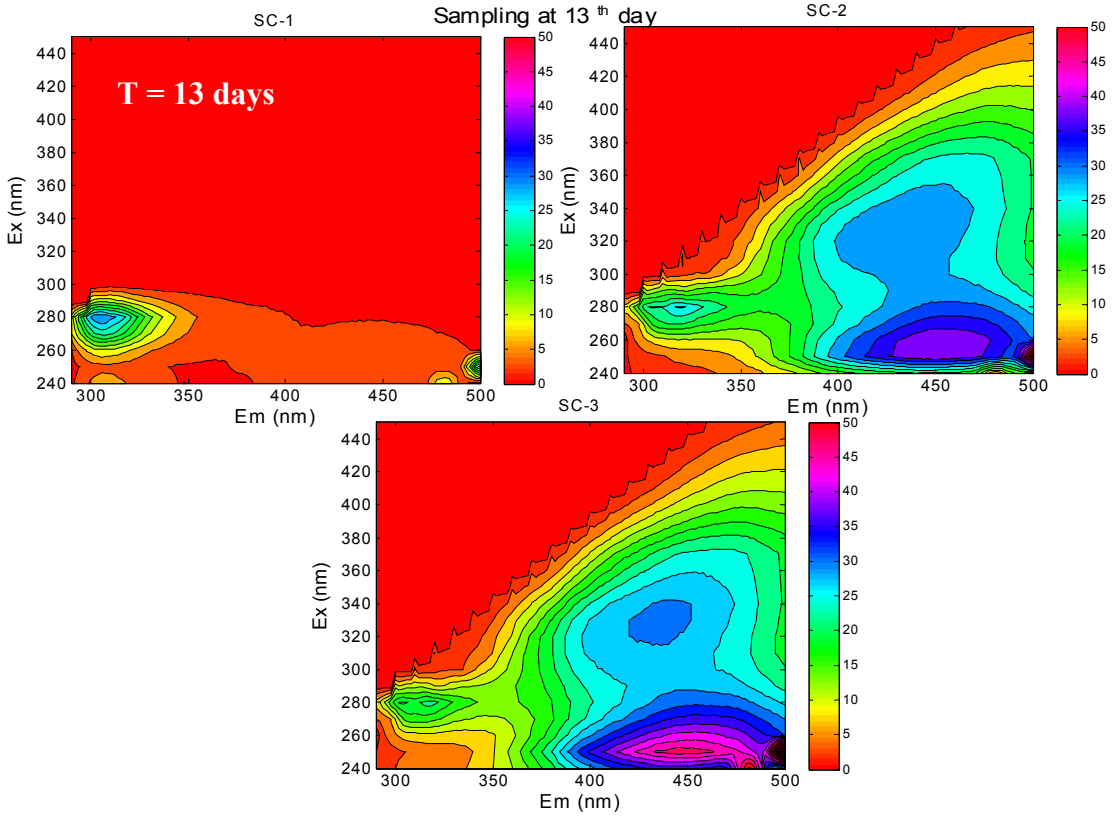

Sampling at 7 th day
SC-1
T = 7 days
SC-2
SC-3
Ex (nm)
Em (nm)

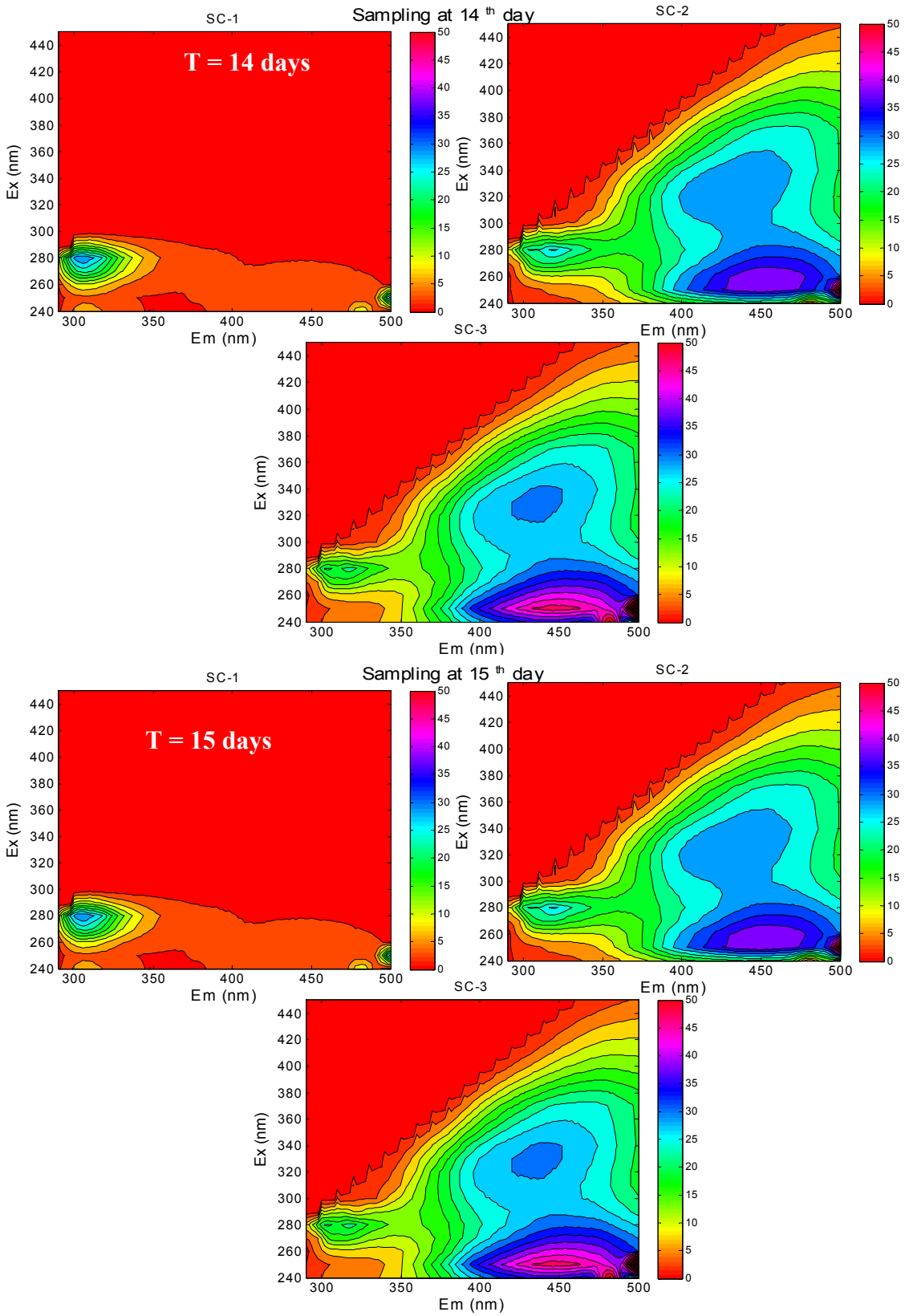

Sampling at 8 th day
SC-1
T = 8 days
SC-2
SC-3
Ex (nm)
Em (nm)


Sampling at 9 th day
SC-1
T = 9 days
SC-2
SC-3
Ex (nm)
Em (nm)


Sampling at 10 th day
SC-1
T = 10 days
SC-2
SC-3
Ex (nm)
Em (nm)


Sampling at 11 th day
SC-1
T = 11 days
SC-2
SC-3
Ex (nm)
Em (nm)


Sampling at 13 th day
SC-1
T = 13 days
SC-2
SC-3
Ex (nm)
Em (nm)


Sampling at 14 th day
SC-1
T = 14 days
SC-2
SC-3
Sampling at 15 th day
SC-1
T = 15 days
SC-2
SC-3
Ex (nm)
Em (nm)