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The YES assay as a tool to analyse endocrine disruptors in different matrices in Vietnam

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Table of Contents

List of Tables

List of figures

List of Abbreviations

APnEOs	Alkylphenol ethoxylates
BOD_5	Biological Oxygen Demand
BPA	Bisphenol A
COD	Chemical Oxygen Demand
CPRG	Chlorophenol red-β-D-galactopyranoside
E1	Estrone
E2 eq	17β estradiol equivalent
E2	17β-estradiol
E3	Estriol
ED(s)	Endocrine Disrupter(s)
EDC(s)	Endocrine Disrupting Compound(s)
EE2	Ethinyl estradiol
EHS	The General Environmental, Health, and Safety Guidelines
GSO	General Statistics Office
HCMC	HoChiMinh City
LOD	Limit Of Detection
LOEC	Lowest Observed Effect Concentration
NP	Nonyl phenol
OD	Optical density
OMD	Organic Dry Matter
OP	Octylphenol
PNEC	Predicted No Effect Concentration
PNEC	Predicted No Effect Concentration
SPE	Solid Phase Extraction

STPs	Sewage Treatment Plants
TCVN	Vietnamese Industrial wastewater discharge standard
TOC	Total of Organic Carbon
TSS	Total Suspended Solid
USEPA	The US Environmental Protection Agency
WWTP	Wastewater Treatment Plant
YES	Yeast Estrogen Screen

Abstract

Endocrine disrupting compounds (EDCs) or endocrine disruptors have been a global concern for many years due to their negative impact on wildlife and human health. The main sources of EDCs in the environment are humans and animals. EDCs can appear in a number of environmental matrices such as wastewater, surface water, manure, sludge, sediments, etc. Several techniques are available for analysing EDCs, each with its advantages and disadvantages. When selecting a method, the purpose of the study should be considered, especially an EDC monitoring programme with many samples.

In Vietnam, environmental pollution, especially of surface water, is currently increasing. This is an important issue examined in many studies but information on EDCs is still lacking.

The studies reported in this thesis used the YES assay as a tool to analyse EDCs in different matrices in Vietnam. These included surface water, river sediments, the liquid and solid phases of septage slurry and biogas slurry and wastewater.

The Saigon River showed upstream of HCMC EDC values below the limit of detection (LOD). But in the City and downstream the range of EDCs was 0.02-6.2 ng E2eq/L in the dry season and 0.33-1.17 ng E2eq/L in the rainy season. EDC concentrations in sediments ranged from < LOD (upstream) to 0.28 ng E2eq/g (dw).

Total EDCs in septage slurry ranged from 57.92 to 1506.81 ng E2eq/L, with EDC concentrations in the liquid phase ranging from 11.87 to 184.29 ng E2eq/L and those in the solid phase from 2.21 to 99.16 ng E2eq/g (dw).

EDC concentrations in influent from biogas plants fed with animal manures were in the range 56.98-903.91 ng E2eq/L. Most EDCs were found in the solid phase (77.9-98.7%). The EDC level in fresh cow manure was 3.63 ± 2.23 ng E2eq/g (dw) and decreased by 95.3±3.52 % of the initial value within 2 months of drying or vermicomposting.

The largest EDC discharge into surface waters in the Mekong Delta, Vietnam, was from humans (156 kg E2eq/year), followed by animals (23kg E2eq/year). Wastewater from fish processing companies contributed the least EDCs, 0.025 kg E2eq/year.

The results presented in this thesis can be used to devise strategies to reduce EDC discharges to surface waters in Vietnam.

Zusammenfassung

Endokrine Disruptoren (Eds) sind global vorzufindende Umweltchemikalien, die aufgrund ihrer hormonellen Wirkung das Hormonsystem bei Mensch und Tier negativ beeinflussen können. Die Hauptquellen für EDs sind Ausscheidungen von Mensch und Tier, aber auch industrielle Prozesse. EDs sind beispielsweise in Abwässern, Oberflächenwasser, Gülle, Klärschlamm und Flusssedimenten nachgewiesen worden. Für den analytischen Nachweis von EDs stehen verschiedene Methoden zur Auswahl, so dass je nach Anwendungsfall eine geeignete Methodik ausgewählt werden kann.

In Vietnam nimmt die Wasserverschmutzung zu, es fehlen dort u.a. Informationen von EDs in Oberflächenwässern.

Die Untersuchungen in dieser Arbeit -ED Untersuchungen in verschiedenen Matrizes- wurden alle mit dem YES Assay durchgeführt. EDs wurden in Oberflächenwasser, Flusssediment, in der flüssigen und festen Phase von Abwässern aus sog. „septic tanks“, in Abwasser und Biogassubstraten untersucht.

So wurden flussaufwärts von Ho Chi Minh City im Saigon Fluss keine EDs nachgewiesen. Im städtischen Bereich mit großer Wasserverschmutzung betrugen die ED Konzentrationen 0,02-6,2 ng E2eq/L in der Trockenzeit, in der Regenzeit waren sie geringer: 0,33-1,17 ng E2eq/L. Die ED Konzentrationen im Sediment lagen zwischen nicht nachweisbar (flussaufwärts) und 0,28 ng E2eq/g.

Die ED Konzentrationen in Abwasser aus „septic tanks“ betrugen 57,92-1506,81 ng E2eq/L. In der flüssigen Phase lagen die Konzentrationen zwischen 11,87-184,29 ng E2eq/L und in der festen Phasen zwischen 2,21 und 99,16 ng E2eq/g.

ED Konzentrationen in Gülle, welche Biogasanlagen zugeführt wurden, betrugen 56,98-903,91 ng E2eq/L, wobei 77,9-98,7% der EDs in der Festphase vorzufinden waren. EDs in frischem Kuhmist betrugen 3,63 ±2,23 ng E2eq/L. Durch Trocknen oder Vermikompostierung verringerten sich die EDs innerhalb von zwei Monaten um 95,3 ± 3,52%.

Die meisten EDs werden im Mekong Delta von Menschen in die Oberflächengewässer ausgeschieden (156 kg E2eq/a) gefolgt von Tieren (23 kg E2eq/a). Abwasser aus der Fisch verarbeitenden Industrie spielt beim ED Eintrag eine untergeordnete Rolle (0,025 kg E2eq/a).

Die in dieser Arbeit dokumentierten ED Konzentrationen können dazu beitragen Reduktionsstrategien zu erarbeiten, um den ED Eintrag in Oberflächenwässer in Vietnam zu verringern.

1. General introduction

Endocrine disruptors, including estrogens and other chemical substances with estrogenic activity, have raised concerns as environmental contaminants and EDCs may have potentially wide ecological effects, especially for aquatic organisms. A brief review of the background of endocrine disruptors, their effects and methods of analysis is given below.

1.1 The endocrine system and endocrine disruption

1.1.1 The endocrine system

The endocrine system is one of the two important systems (the other is the nervous system) that control and communicate in the bodies of humans and animals. The endocrine system consists of a group of organs called endocrine glands. Endocrine glands produce chemical messengers called hormones that are transported through the bloodstream to the target cells. These target cells include mammary glands, bones, muscles, the nervous system and the reproductive organs of males and females. A target cell has specific protein molecules that act as receptors to which the hormone can attach. Hormones bind to specialised receptors and these complexes affect gene expression, cell differentiation, hormone secretion and other processes. As a result, biological functions such as growth, development, sexual differentiation and reproduction are regulated (Crisp et al., 1998; Fang et al., 2000). The endocrine system must also regulate the concentration of hormones in a certain range in order to maintain the state of the body's balance (homeostasis).

1.1.2 Endocrine disruption

The great variety of chemicals released into the environment over the past century can disturb normal endocrine system functions and affect wildlife populations and human health. This problem has been evident since the early 1900s (Dobbs et al., 1938), but the concept of endocrine disruption (endocrine disruptors, or endocrine disrupting chemicals - EDCs) was first introduced in 1993 by Colbron and colleagues (Sumpter and Johnson, 2005). Since then, it has become a major issue in environmental science research and policy (Sumpter, 2005).

Many definitions have been proposed for endocrine disruptors, but there is still no globally accepted definition (Fisher, 2004). The U.S. Environmental Protection Agency (1997) has described an endocrine disruptor as: “an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior”. However, the European Commission (2002) has defined it as: “An exogeneous substance mixture, that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub)population”.

Although there are different definitions of endocrine disruptors, the main effect of their presence is considered to be adverse health effects due to alterations to any part of the endocrine system.

The mechanisms of endocrine disruption are complex. Endocrine disruption occurs when EDCs are present, bind to intracellular receptor hormones and alter the natural response patterns of the endocrine system. In general, endocrine disruptors can interfere with the endocrine system in four different ways: (i) They may bind to a receptor and activate a response, acting as a hormone mimic (agonistic effect – agonist). (ii) They may bind to a receptor but there is no response activation. This prevents the binding of a natural hormone (antagonistic effect). (iii) They may interact directly or indirectly with the hormone structure and thus alter the endocrine system. (iv) They may interfere with hormone synthesis or metabolism and may modify hormone receptor levels (Sonnenschein and Soto, 1998; Baker et al., 2001; Vos et al., 2000;).

Most EDCs are small molecules (with molecules weights of a few hundreds) and therefore mimic or antagonise small hormones such as steroid or thyroid hormones (Sumpter 1998). Most EDCs also exhibit estrogenic potency, while only a few have androgenic or anti-androgenic potency (McLachlan et al., 2006). Some EDCs have an influence on thyroid, growth or stress hormones (Burkhardt-Holm, 2010). Sumpter (2005) also states that the most potent estrogenic substances are estrogens such as natural estrogen E2 and synthetic estrogen EE2. All xenoestrogens are much less potent (Table 1.1).

Table1.1 Representative median effective concentrations of estrogenic chemicals for induction of Vitellogenin in fish

Chemical	EC50	Relative potency
Estradiol (E2)	25 ng/L	1
Estrone (E1)	60 ng/L	0.3
Ethinyl estradiol (EE2)	1.2 ng/L	20
4-tert-nonylphenol (NP)	8 µg/L	0.0025
4-octylphenol (OP)	10 µg/L	0.002
Bisphenol A (BPA)	50 µg/L	0.0004
Methoxychlor	8 µg/L	0.0025

Source: Sumpter (2005)

1.2 Endocrine disruption compounds

An endocrine disrupting substance is either a natural or synthetic compound. As mentioned, most EDCs have estrogenic activity. In the European Union, 150 synthetic compounds and seven natural compounds are listed as estrogenic disruptors (Burkhaedt-Holm, 2010). Based on their origins, EDCs can be classified into different groups (Markey et al., 2003; Juvancza et al., 2008; Bryne et al., 2009):

- Natural hormones and their metabolites (e.g. 17β-estradiol (E2) and their metabolites estriol (E3) and estrone (E1));
- Artificial hormones (e.g. diethylstilbestrol, the contraceptive pill and thyroid medicines);
- Phyto- and mycoestrogens (e.g. isoflavones, lignans, genistein, coumestrol);
- Drugs with hormone side-effects (e.g. naproxen, clofibrate);
- Industrial and household chemicals (e.g. alkylphenol ethoxylate detergents, fire retardants, plasticisers such as bisphenol A and phthalates, polychlorinated bis-phenols (PCBs));
- Pesticides and metabolites (e.g. DDT, methoxychlor, kepone, dieldrin, linden, endosulfan, toxaphene);

- By-products of industrial and household processes (e.g. polycyclic aromatic hydrocarbons (PAH), dioxin).

Some heavy metals are also known to affect the endocrine system, for example cadmium and lead.

Selected groups of EDCs are introduced below in more detail, as they may be of concern in Vietnam.

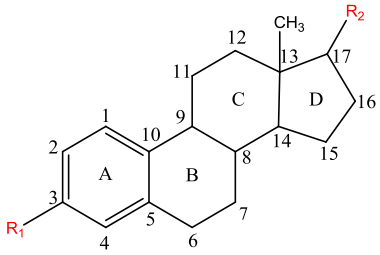
1.2.1 Estrogens

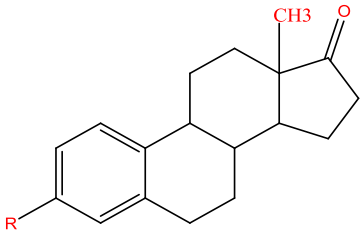
The estrogens (17β-estradiol, estrone, and estriol) are predominantly female hormones, which are important for maintaining the health of the reproductive tissues, breast, skin and brain. In men, small amounts of estrogens are produced in the testes.

Estrogens have an aromatic A ring, a phenolic hydroxyl group at C3 (Fig. 1.1), which is essential for their biological activity. In synthetic estrogens, ethinyl and methyl groups are added to estradiol to form ethinyl estradiol (EE2) and mestranol (MeEE2), which are the compounds used in contraceptives.

The log octanol/water coefficient (log K_{ow}) values for free estrogens range from 2.8 to 4.6 (Tabak et al., 1981; Lai et al., 2000), indicating that these compounds are hydrophobic/lipophilic. Therefore, estrogens dissolve poorly in water. The solubility is about 13 mg/L for natural estrogens, while it is 4.8 and 0.3 mg/L for EE2 and MeEE2, respectively (Lai et al., 2000).

The catabolism of estrogens occurs in the liver, where they are conjugated with sulphate, glucoronide or sulphoglucuronide. These forms are made up by the substitution of hydroxyl groups in the positions C3 and/or C17. This increases the water solubility and they can be discharged via the urine. Free estrogens or unconjugated forms are eliminated via the faeces (Sandor and Mehdi, 1979). Among the free estrogens, 17β-estradiol is the most potent, followed by estrone and estriol. Conjugated estrogens have a very low estrogenic activity compared with unconjugated forms (Bovee et al., 2004). However, once excreted, the conjugated estrogens can be converted into their estrogenically active forms by faecal bacteria activities (Combalbert et al., 2010).





R1 = OH, R2 = OH : estradiol R = OH : estrone

R1 = SO_4, R2= OH : estradiol-3-sulphate R = SO_4: estrone-3-sulphate

R1 = OH, R2 = SO_4 : estradiol-17-sulphate

R1 = SO_4, R2 = SO_4 : estradiol-3, 17-sulphate

Fig. 1.1 Molecular structure of estrogens (E2 and E1) and their sulphate conjugates.

The major source of estrogens in the environment is excretion by humans and animals. The excretion rates and types of estrogens differ from species to species, but also within species. For instance, natural estrogen (E1, E2 and E3) excretion by humans represents an estimated 1 to 6859 µg/day (Table 1.2) (Johnson et al., 2000). A nonpregnant sow excretes approximately 0.6-1.4 mg/day of E1 and a pregnant sow to 10.8 mg/day (Shore and Shemes, 2003).

Table 1.2 Daily estrogen excretion by humans (per person) in µg/day, from Johnson et al., (2000)

	E1	E2	E3	Total estrogens
Males	1.6	3.9	1.5	7
Menstruating females	3.5	8	4.8	16.3
Menopausal females	2.3	4	1	7.3
Pregnant women	259	600	6000	6859

1.2.2 Phytoestrogens

Phytoestrogens are plant chemicals that can act as fungicides, regulate plant hormones, and protect plants from UV radiation. Phytoestrogens are defined as plant

substances that are structurally or functionally similar to estrogens and have been found to bind to estrogen receptors (Breithofer et al., 1998). They may function as agonists and antagonists on the endocrine system (Tham et al., 1998). Many different plants produce phytoestrogen compounds and they have been found in herbs, grains, vegetables, fruits and many alcoholic beverages (Rosenblum et al., 1993; Gavaler et al., 1995; Gavaler et al., 1998).

Phytoestrogens are divided into two main groups: the isoflavones (e.g. genistein, formononetin, daidzein) and the lignans (e.g. matairesinol, enterodiol, enterolactone). The isoflavones are found in soybeans and other legumes and can be taken up by humans. Lignans are products of the breakdown of grains, fibres, vegetables and several fruits by microbial activity. Phytoestrogens exhibit a weak estrogenic activity of the order 10^{-2} to 10^{-3} compared with 17β-estradiol. Bovee et al. (2004) listed them according their estrogenic potency thus: 17β-estradiol >> coumestrol > genistein > zearalenone > 8-prenylnaringen >> daidzein > naringenin > genistin >> daidzin. Although the estrogenic potency of phytoestrogens is very weak, they may be present in the body in concentrations 100 times higher than the endogenous estrogens (Adlercreutz et al., 1986). For instance, the concentrations of the phytoestrogens genistein and daidzein in urine are reported to be about 500-fold higher in infants fed soy milk than in those fed cow milk (Cao et al., 2009). Therefore, the potential for endocrine disruption of phytoestrogens needs to be considered.

1.2.3 Bisphenol

Bisphenol A (BPA) is a substance of the bisphenol group which has received considerable attention in recent years due to the widespread human exposure to BPA. BPA affects the reproduction and development of animals in laboratory studies. It is used in large quantities to produce polycarbonate plastics and epoxy resins. It has many applications in the food and drink packaging industry, bottling, medical devices and household items. It is also used as a coating in food and beverage cans. BPA is described as being weakly estrogenic with a potency of four to six orders of magnitude less than 17β-estradiol (Bergeron et al., 1999; Schafer et al., 1999).

Reflecting the wide use of BPA in households and industries, several studies have found it in raw sewage, wastewater effluents, sewage sludge, surface water and even groundwater (Table 1.3)

Table 1.3 Concentration of bisphenol A (BPA) in various sources from different countries

Location	Matrix	Concentration	Reference
Austria	Surface water	nd - 600 ng/L	Hohenblum et al., 2004
	Ground water	nd - 930 ng/L	
Germany	Surface water	0.5 - 410 ng/L	Fromme et al., 2002
	Sewage effluents	18 - 702 ng/L	
	Sewage sludge	0.004 - 1.363 mg/kg	
	Sediment	0.01 - 0.19 mg/kg	
South Korean	Surface water	7.54 - 335.56 ng/L	Ra et al., 2011
Tokyo Bay	Surface water	20.2 - 30.1 ng/L	Hashimoto et al., 2005
USA	Surface water	nd - 12000 ng/g	Kolpin et al., 2002
Liao River (China)	Surface water	12.3 - 755.6 ng/L	
	Sediment	nd - 33.8 ng/L	
Laos	Surface water	1.8 - 4.9 ng/L	Duong et al., 2010
Cambodia	Surface water	3.3 - 3.4 ng/L	
Vietnam	Surface water	24.2 - 132.5 ng/L	
China	Surface water	2.2 - 2.4 ng/L	
Indonesia	Surface water	7.6 - 7.8 ng/L	
Thailand	Surface water	nd - 2.4 ng/L	
Malaysia	Surface water	7.4 - 10.8 ng/L	
Korea	Surface	3.2 - 15.5 ng/L	

1.2.4 Phthalates

Phthalates are chemical compounds that have been used for a few decades. They are essentially and mainly used as plasticisers in the production of polymeric materials such as polyvinylchloride (PVC). Phthalates are also used in paints, lacquers, pesticides, cosmetics, ammunition and lubricants (Fromme et al., 2002). Plasticisers are used in building materials, home furnishings, transportation, clothing, food packaging and medicinal products (Staples et al., 1997). Phthalates are produced in large volumes. The release of phthalates into the environment occurs during industrial production, use of the products, and on final disposal of the products (Warm, 1987). They have been found in water, sewage water, sediments and air. For instance, di (2-ethylhexyl) phthalate (DEHP), the phthalate occurring in the highest concentrations in the environment, has been found in concentrations of 0.33 to 97.8 µg/L in surface water, 1.74 to 182 µg/L in sewage effluents, 27.9 to 154 mg/kg in sewage sludge, and 0.21 to 8.44 mg/kg in sediments (samples taken in Germany by Fromme et al., 2002). Phthalates have a low water solubility, with a log K_{ow} range from 1.46 to 13.1, indicating great lipophilicity, so they tend to absorb to sediments and suspended solids. Food samples can be contaminated by phthalates at high levels. Because of their lipophilic nature, phthalates mainly infiltrate into foodstuffs containing fat, such as cream, cheese and butter (Sharman et al., 1994). In addition, phthalates have been found in animal feed (pig, cattle and poultry) and can be accumulated in body tissues and organs of pigs and broiler chicks (Jarošová, 2006).

Several studies have revealed that some phthalates have estrogenic activities which mimic endogenous estrogen, but the estrogenic potencies are very weak compared with 17β-estradiol (Jobling et al., 1995, Soto et al., 1995; Harrison et al., 1997; Moore et al., 2000). The relative estrogenic potencies of various phthalates decline in the following order (Harris et al., 1997):

Butylbenzyl phthalate (BBP) > dibutyl phthalate (DBP) > diisobutyl phthalate (DIBP) > diethyl phthalate (DEP) > diisononyl phthalate (DINP).

The most widely used phthalate, DEHP, did not show any estrogenic activity in *in vitro* assays (Harris et al., 1997), but *in vivo* assays it induced an increase in proliferation of MCF-7 cells at a concentration of 10^{-5} M (Choi. et al., 2003). In the body, phthalates are metabolised to toxic metabolites that react with biologically active substances and may impair vital functions of the body (Jarošová, 2006).

Depending on the occurrence and utilisation of phthalates, humans may be exposed to their influence during daily life activities. In 1991, the European Food Commission set a Tolerable Dairy Intake (TDI) level for humans to 25 µg and 50 µg per kg body weight per day for DEHP and DBP, respectively.

1.2.5 Alkylphenols

Alkylphenol ethoxylates (APEOs) are the second largest group of nonionic surfactants in commercial production. They were introduced in the 1940s. They are usually made from a branched-chain nonylphenol or octylphenol, reacted with ethylene oxide. These compounds are widely used in many industrial, commercial, and household functions such as detergents, lubrication, defoamers, cleaners for machinery and materials, paints, pesticides, textiles, and in personal products. APEOs with 8 to 12 ethoxylate groups are commonly used. For instance, more than 300,000 tons of nonylphenol ethoxylates (NPEs) are produced annually worldwide, about 80% of which are APEOs and 20% are octylphenol ethoxylates (OPEs) (White et al., 1994). There are many possible entry routes into the environment for these compounds during their production, use and disposal. Several studies have detected these compounds in wastewater treatment effluents, surface water, soil, sediments, sludge, biota, drinking water and food (Rudel et al., 1998; Lye et al., 1999; Wenzel et al., 2004; Sánchez-Avila et al., 2009).

During wastewater treatment, there is degradation of APEOs first into APEOs with shorter ethoxylates chains and finally into alkylphenol itself, as briefly described by Jobling and Sumpter (1993) and as shown in Fig. 1.2. These compounds tend to participate in the solid phase due to their physicochemical properties, in particular their octanol-water coefficients (log K_{ow}), which range between 4.17 and 4.48 (Ahel et al., 1993).

Several studies have indicated that alkylphenols (AP) such as nonylphenol (NP), octylphenol (OP), butylphenol (BP), nonylphenol diethoxylates (NP2EO), tergitol-NP9, and nonylphenol phenoxycarboxylic (NP1EC) acid are weakly estrogenic (Jobling and Sumpter, 1993; White et al., 1994; Soto et al., 1995; Van den Belt et al., 2004, Cosnefroy et al., 2009;). White et al. (1994) found the estrogenic potencies of four compounds to be in the order: OP > NP1EC > NP > NP2EO.

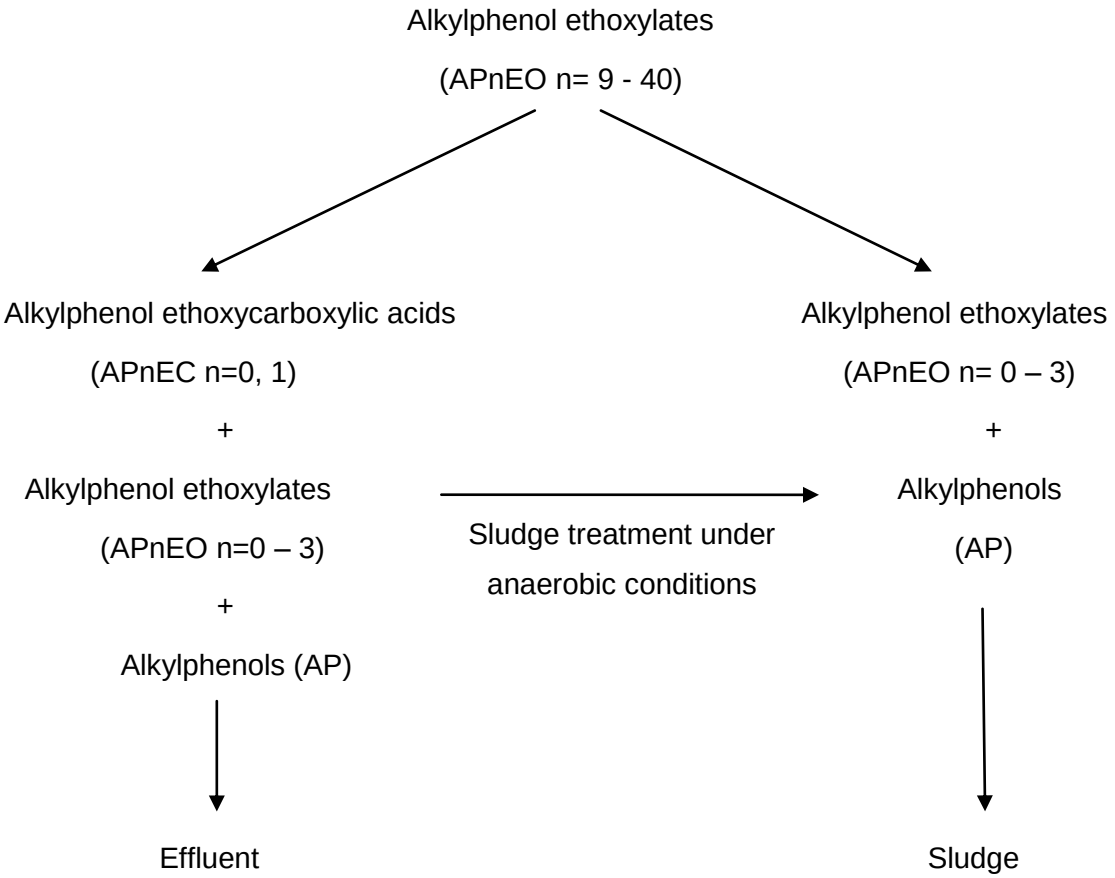


Fig. 1.2 The biodegradation of alkylphenol ethoxylates during sewage treatment. AP is an alkyl phenol. The alkyl chains have the form C_nH_{n+1}. APnEO can refer to any alkylphenol ethoxylate (nonyl, octyl or butyl) (adapted from Jobling and Sumpter, 1995).

The estrogenicity of ethoxylates depends on their chain length – the longer the ethoxylate chain, the lower the estrogenicity (Jobling and Sumpter, 1993). Thus, the metabolites of alkylphenols present in wastewater treatment system effluents may be more estrogenic than their original parent compounds. Regarding this point, Navas and Segner (2006) also ranged the estrogenicity potencies in the order: E2 >> BPA > OP > NP.

The distribution of alkylphenols and APEOs in the environment has been documented in many countries. Among these compounds, NP and OP are the most studied and their detected concentrations can range from the detection limit to several ppm. For instance, Table 1.4 shows the NP levels in surface water from different countries.

Table 1.4 Concentrations of NP in surface water and sediment from different countries

Country	Surface water	Sediment	Reference
USA	<LOD – 95 µg/L	< 2.9 – 2960 µg/kg	Ying et al., 2002
UK	<0.03 – 5.4 µg/L	< 0.1 – 15 µg/kg	Ying et al., 2002
Canada	<0.02 – 4.25 µg/L		Envi. Canada, 2001
Austria	<LOD – 890ng/L		Hohenblum et al., 2004
Italy	8.8 – 158 µg/L		Davi` and Gnudi, 1999
Germany	<LOD – 1.36 µg/L	< LOD – 1364 µg/kg	Grund et al., 2011
Japan	30.4 – 104 ng/L	2.2 – 4560 µg/kg	Hashimoto et al., 2005
Korea	114.63 – 336.14 ng/L		Ra et al., 2011
Liao River (China)	0.122 – 2.07 µg/L	10 – 558.4 µg/kg	Wang et al., 2011
Vietnam	1.76 – 2.09 µg/L		Duong et al., 2010

Nonylphenol can be present in effluents of wastewater treatment plants in the range 0.08-345 µg/L depending on the wastewater sources (Ce'spedes et al., 2005; Sánchez-Avila et al., 2009). In sewage sludge from treatment works, NP concentrations can reach up to 2530 mg/kg. The US EPA recommends that the nonylphenol concentration in fresh water should be below 6.6 µg/L and that in saltwater below 1.7 µg/L (Soares et al., 2007).

1.2.6 Pesticides

The term pesticides include insecticides, herbicides and fungicides. The effects of pesticides on the endocrine system have been known for many years. Gellert (1978) found that mirex, dieldrin and aldrin are estrogenic and can induce persistent vaginal oestrus and anovulation in rats. DDT, heptachlor, endosulfan, chlordance, dicofol and lindane have also been identified as environmental estrogens that can alter the endocrine system and thus affect the reproduction of e.g. alligators. They may also induce abnormalities in alligators (Fry, 1995; Vonier et al., 1996). Many studies have demonstrated the estrogenic potential of *o,p'*-DDT and *p,p'*-DDT because they induce

vitellogenin (Vtg) production in male fish (Sumpter and Jobling, 1995; Mills et al., 2001) By using an E-screen test, Soto et al. (1995) showed that DDT, methoxychlor, chlordecone, lindane, dieldrin, toxaphene and endocsulfan are estrogenic at concentrations of 10 µM. Assessing the estrogenic activity of the organochlorine pesticides in rainbow trout hepatocyte cultures using vitellogenin, Okoumassoun et al. (2002) observed that the estrogenic potency decreased in the order: 17β-estradiol (E2) >> *o,p'*-DDT > dieldrin > aldrin > DDT. However, Smeets et al. (1999) conducted a similar assay and showed that dieldrin did not induce Vtg in carp. Tully et al. (2000) reported dieldrin and aldrin to be non-estrogenic when they studied the transcriptional activation of an estrogen-responsive reporter gene in transfect HeLa cells. Estrogenic responses induced by organochlorine pesticides seem to be model-dependent and the use of a single assay can provide only limited information (Okoumassoun et al., 2002).

Pesticides used in agriculture may reach the water body through agricultural runoff. Once in water, they have the potential to accumulate in aquatic organisms due to their persistence and lipophilicity. They can also accumulate in meat and dairy products at high levels. Pesticides have been determined in both human and animal tissues: Okoumassoun et al. (2002) found dieldrin, aldrin, heptachlor and lindance in a range of 30 to 105 µg/g in tilapia fish. Results from a global survey for the period 1979-1983 showed a mean concentration of DDT ranging from 0.003 to 59.28 ppm in the adipose tissue of humans (Kutz et al., 1991).

1.3 Effects of endocrine disruptors

There have been many papers published on organisms exposed to EDCs:

- Adverse effects observed in wildlife, especially fish;
- Increased incidence of certain endocrine-related human diseases and disorders;
- Laboratory studies showing adverse functional changes due to exposure to endocrine disruptors.

1.3.1 Effects of EDCs on aquatic wildlife

Wastewater effluents are one of the main sources of EDCs, which can affect aquatic wildlife, including invertebrates and vertebrates.

1.3.1.1 Invertebrates

The number of examples of endocrine disruption in invertebrates is limited due to the fact that their hormonal systems are poorly understood compared with those of vertebrates (Oehlmann and Schulte-Oehlmann, 2003). One of the few examples of the effects of EDCs on invertebrates is provided by tributyltin (TBT). TBT compounds are mainly used as biocides and in antifouling paints, but also in various other formulations. The first observation of adverse effects of TBT on molluscs was on *Crassotrea gigas* in the Bay of Arcachon, France, one of the European centres for oyster aquaculture. These organisms showed ball-shaped shell deformations in adults and a decline in annual spatfall (Alzieu et al., 1980). Laboratory and field analyses have revealed that TBT in ambient water is effective at trace concentrations as low as 10 ng/L (Bryan and Gibbs, 1991).

1.3.1.2 Fish

A variety of effects of EDCs on freshwater fish populations have been observed in laboratory and field studies. The exposure of fish to effluent from sewage treatment works containing EDCs has been reported in many rivers in the United Kingdom, Germany, France, Norway and the United States. Jobling et al. (1998) reported that exposure to estrogenic effluents was linked to the stimulation of vitellogenin synthesis in male fish and intersexuality (probably feminisation of males). The protein vitellogenin is normally an egg yolk precursor, produced by oviparous female fish. Experiments have shown that estrogens can cause adverse biological effects in aquatic environments at very low concentrations (ng/L). For instance, EE2 is a very potent inducer of vitellogenesis in fish and is effective e.g. in male rainbow trout (*Oncorhynchus mykiss*) at concentrations of 0.1 ng/L (Purdom et al., 1994). Exposure to a single dose of EE2 (2 ng/L) in water retarded testicular growth by 50% and delayed the maturation of male trout (Tyler and Routledge, 1998). Routledge et al. (1998) reported E2-induced vitellogenesis in rainbow trout at concentrations between 1 and 10 ng/L. The responses of male fish to estrogens vary according to the dose administered and the stage of testicular development at the time of exposure (Tyler and Routledge, 1998).

Alkylphenols such as nonylphenol and octylphenol have been linked to increased mortality rates, reduced reproductive capacity and vitellogenin synthesis in male fish, with yolk degradation in piscine oocytes (White et al., 1994). Soares et al. (2008)

reported that an NP concentration as low as 8.2 µg/L can cause a decrease in female fertility and the survival of juveniles. Earlier, Sumpter (1995) showed that estrogenic alkylphenol chemicals caused a reduction in the rate of testicular development in trout undergoing sexual maturation. Christiansen et al. (1998) also reported that NP and E2 affected the testicular structure and cytology of germ cells and Sertoli cells of male *Z. viviparus*.

Current knowledge about the sensitivity of marine fish to estrogenic chemicals is rather poor. A study by Correia et al. (2007) highlighted the potential danger of joint exposure to E2, EE2 and BPA in marine fish. That study also showed that the combined effects of the chemicals were greater than the sum of its individual components.

The effects of EDCs on reptiles have been well documented in Florida alligators. Pesticides provoke abnormalities of the gonads and induce abnormal sex hormone (estradiol/ testosterone) concentrations in juvenile alligators (Guillette et al., 1994).

1.3.2 Effects on humans

It has been suggested that EDCs in the environment are a risk factor for human health, indicated by decreased sperm counts, testicular dysgenesis syndrome, testicular cancer and breast cancer (Wolff et al., 1993; Rittler and Castilla, 1996; Tas et al., 1996; Harris et al., 1997; Skakkebak et al., 2001). However, concrete evidence on whether EDCs are a real threat to human health is still not available. Different studies have concluded that there is no proof of human health effects caused by EDCs at the current exposure level (Solomon and Schettle, 2000; Sharpe and Skakkeback, 2003; Warning et al., 2005; Waring and Harris, 2011). One of the main complications associated with studying the effects of EDC exposure in humans is the long latency of disease (Cowin et al., 2007).

1.4 Methods for the determination of endocrine disruptors

The analysis of ECDs is an analytical challenge because of the variety of compounds in the environmental samples and because of the very low detection limits required for determination (down to 1 ng/L).

As EDCs are present in water and wastewater at levels of a few ng/L or µg/L, a concentration step is required. In most cases, solid phase extraction (SPE) is used

(de Mes et al., 2005). Because samples usually contain rather high concentrations of organic compounds and suspended solid particles, a filtration step has to be performed before extraction to avoid clogging when loading samples through SPE. The analysis of EDCs is based on either chemical or biological techniques. All techniques have advantages and disadvantages.

Chemical techniques are able to quantify the target estrogenic compounds. The predominant methods used for this application are gas chromatography (GC) or liquid chromatography (LC). However, GC is limited to compounds that are volatile and thermally stable. Recently, mass spectrometry (MS) or MS-MS has been combined with either GC or LC techniques to give higher sensitivity and specificity (Voulvoulis and Scrimshaw, 2003).

Biological techniques are used to identify whether the compound or a mix of compounds is estrogenic rather than directly analysing single EDCs in environmental samples.

Competitive ligand binding assays, cell proliferation assays and *in vitro* gene expression assays are the most common *in vitro* approaches for determination of estrogenic compounds (Snyder et al., 2000). Competitive ligand binding assays are based on the action of estrogens (and xenoestrogens), which bind to a specific, high affinity receptor (ER) within the cell nucleus. This binding to the receptor results in a certain degree of biological activity. These assays measure the binding of agonists or antagonist to the receptor. 17β-estradiol (radiolabelled) is predominantly used as a control to confirm the presence of the receptor and to evaluate the effects of competitive binding to that receptor through addition of the compounds being tested. The greatest limitation of these assays is that the tests do not distinguish between agonistic and antagonistic effects, although compounds may bind to the receptor (reviewed by de Mes et al., 2005).

Cell proliferation techniques, or E-screen assays, are based on human-derived cell lines and utilise a number of end points to measure the cell proliferation induced through exposure to estrogenic compounds. A range of chemical compounds suspected to be estrogenic have been determined by using cell proliferation assays, such as substances belonging the alkylphenols, phthalates and hydroxylated PCBs, and the insecticides dieldrin, endosulfan and toxaphene (Soto et al., 1995). E-screen assays have been evaluated as a suitable technique for testing estrogenic

compounds. They have been applied for the evaluation of estrogenic activity in surface waters, wastewater influents (Körner et al., 2000; Oh et al., 2000; Park et al., 2007; Bicchi et al., 2009; Oh et al. 2009; Ra et al., 2011) and pesticide residues in vegetables and fruits (Schilro et al., 2011). A major disadvantage of cell proliferation techniques relates to the reproducibility of the cell lines (Voulvoulis and Scrimshaw, 2003; de Mes et al., 2005).

For recombinant receptor-reporter assays (also called *in vitro* gene expression assays), genetically engineered mammalian cells or strains of yeast are used. The cells are transformed or transfected with recombinant DNA. Examples of these assays are the E-CALUX (ER-mediated Chemically Activated LUciferase gene eXpression) assay and the YES (Yeast Estrogen Screen) assay. The E-CALUX assay uses luciferase reporter genes in human breast cancer cell lines that express the endogenous estrogen receptor (Legler et al., 2002). The YES assay was developed by Routledge et al. (1996) and is described in more detail in Chapter 2.

In vivo assays: Beside *in vitro* assays, *in vivo* assays have also been developed and applied for the evaluation of EDCs in environmental samples. Fish are often used in these assays. The evaluation of estrogenic activity is based on induction of the synthesis of vitellogenin (Vtg) in the culture medium. Vtg is crucial for the reproductive cycle in female fish. It is normally absent (or present at very low concentrations only) in male fish. However, due to estrogen exposure Vtg can be induced in male fish (Sumpter and Jobling, 1995; Folmar et al., 1996; Tyler et al., 1996). Previous studies have indicated that the presence of Vtg in male fish is a good biomarker of estrogenic activity in wastewater samples (Jones et al., 2000; Sole' et al., 2001; Huggett et al., 2003; Hennies et al., 2003).

Another technique for the quantification of estrogenicity is the immunoassay. Two immunoassay techniques available today are the enzyme-linked immunosorbent assay (ELISA) and the radio immunoassay (RIA). These techniques are based on the reaction of an antigen with a selective antibody. The antigen is the analyte to be determined that will bind to the antibody (reagent) to give a product that can be measured.

The ELISA assay is the most commonly used assay for pesticides and other environmental contaminants (Meulenberg et al., 1995). It is also reported to be a suitable assay for screening estrogenic contamination in a large number of

environmental samples due to its simplicity and ease of execution. However, it mainly provides semi-quantitative results (Viganò et al., 2008), usually designed for single compound analysis (Huang et al., 2001).

Among the biological techniques, *in vivo* assays are unlikely to be used for monitoring or screening of estrogenic compounds in the environment because they are expensive and time-consuming (Salste et al., 2007). Other assays have been applied for the determination of estrogenic compounds in environmental samples in many laboratories, but the sensitivity of these assays differs. Murk et al. (2002) reported ER-CALUX to be the most sensitive, with a detection limit of 0.5 pM for E2, followed by the YES assay, with a limit of 10 pM, and the ER-binding assay, with a limit of 1000 pM. Matsuoka et al. (2005) showed that the sensitivity of the YES assay was 100 to 1000 fold lower than that of the E-screen assay.

Overall, YES has been proposed as an applicable assay for the screening of estrogenic activity because it is easiest to perform, robust and inexpensive (Coldham et al., 1997; Rutishauser et al., 2004, Viganò et al., 2008). For these reasons, the YES assay has been used successfully to assess estrogenic activity in environmental samples (Ingerslev et al., 2005 – Survey of estrogenic; reviewed in Campbell et al., 2006; Nelson et al., 2007;). However, the yeast cell response can be affected by the sample matrix, especially in wastewaters of industrial origin (Roda et al., 2006).

1.5 Thesis hypothesis/objectives

This study forms part of the eco-system monitoring and modelling work package within the project WISDOM (Water related Information system for the Sustainable Development Of the Mekong delta) in Vietnam. Endocrine disruptors monitoring in the Mekong Delta was one of the tasks of the project. An applicable method for EDCs analysis in the monitoring programme needed to take into account the huge number of samples. Based on realistic conditions and in light of the information presented in the preceding sections, we expected that the YES assay could be established and used for analysis of EDCs in different matrices in Vietnam. This expectation led to the specific study objectives:

- To set up, optimise and apply the YES assay method to analyse EDCs in surface water, wastewater, vermicompost, biogas slurry, septage slurry and sediment.

- To survey, determine and estimate the EDCs loads from animal, human and industrial wastewater effluent in the Mekong Delta, Vietnam.

1.6 Thesis outline

The thesis comprises seven chapters. Chapter 1 has presented the basic theory and the hypothesis/objectives of the thesis. Chapters 2-6 are structured as single scientific papers: Chapter 2 introduces the YES assay method and its applicability to determine estrogenic activity in Vietnam. Chapter 3 reports the estrogenic activity levels in surface water and sediment in a part of the Saigon River. The potential estrogen source from humans to surface water is described in Chapter 4. Chapter 5 discusses estrogen excretion by pigs and cows and different techniques for reducing the estrogen content in livestock manure in the Mekong Delta, Vietnam (published online by the Journal of Environmental Monitoring Assessment). Chapter 6 addresses the influence of wastewater treatment of fish processing wastewater on estrogenicity. Finally, the general conclusions and recommendations of the thesis are presented in Chapter 7. Note that the word ‘estrogens’ is used for EDCs in Chapters 4 and 5, because estrogens dominate the estrogenic activity compounds in excreta from humans and animals, while EDCs in surface water and wastewater include a number of other estrogenic chemical compounds.

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2. Optimisation and validation of the YES assay and its applicability in Vietnam

2.1 Introduction

There is increasing evidence that estrogens, including natural or/and synthetic compounds, entering recipient water bodies from various sources can have adverse effects on humans and aquatic organisms. Therefore, there is a need to monitor these compounds and their consequences, which in turn requires the development of analytical techniques.

There are several methods available for the determination of EDCs, falling into two main groups: chemical techniques and biological techniques. The use of chemical techniques allows for analysis of the occurrence of compounds identified as estrogenic in matrices. Quantification of EDCs in aqueous matrices by chemical analytical methods is well documented. The main techniques used are GS-MS (Xiao et al., 2001; Raman et al., 2004; Labadie et al., 2005; Hanselman et al., 2006; Flores et al., 2008; Arditsoglou and Vousta, 2008; Zheng et al., 2008; Sun et al., 2008; Lee et al., 2008; Gong et al., 2009; Velicu and Suri, 2009), LC-MS (Chen et al., 2007; Vulliet et al., 2008; Kuster et al., 2008; Xu et al., 2009; Kumar et al., 2009) and LC-MS/MS (Reddy et al., 2005; Furuichi et al., 2006; Cui et al., 2006; Matthiessen et al., 2006; Shappell et al., 2006; Hutchins et al., 2007; Kim et al., 2007;). There are a few studies on analysis of EDCs in solid samples only using GS-MS (Zheng et al., 2008; González et al., 2010) or GC-MS/MS (Hutchins et al., 2007; Hibberd et al., 2009).

Bioassays are used to identify whether a compound or mixture of compounds is estrogenic. A range of assays are widely used for testing estrogenic activity, such as competitive ligand binding, cell proliferation and recombinant receptor-reporter assays. These bioassays have been described in detail and reviewed by several authors (Arnold et al., 1996; Routledge and Sumpter 1996; Graumann et al., 1999; Oh et al., 2000; Garcia-Reyero et al., 2001; Körner et al., 2001; Witter et al., 2001; Vanderperren et al., 2001; Song et al., 2002; Tashiro et al., 2004; Soto et al., 2006). The simplest assays for measuring estrogenic activities are the recombinant receptor-reporter assays. In particular, in vitro yeast estrogen screening (YES) has been used in many studies due to its advantages of being rapid, sensitive, easy to handle and

not requiring expensive facilities (Gaido et al, 1997; Breithofer et al., 1998; Garcia-Reyero et al., 2001; Lorenzen et al., 2004). Furthermore, yeast cells are more resistant to environmental contaminants such as heavy metals and bacterial endotoxins than mammalian cells. This is why the YES assay has been suggested as a convenient screening tool for the assessment of estrogenic activity in environmental samples (Rutishauser et al., 2004). Several studies have used the YES assay in combination with chemical analysis to assess estrogenic activity and to identify the main contributors to the total estrogenicity in samples (Desbrow et al., 1998; Garcia-Reyero et al., 2001; Céspedes et al., 2004; Rutshauser et al., 2004; Beck et al., 2006; Sarmah et al., 2006; Viganò et al., 2008). Based on combination analysis, Roda et al. (2006) reported that the YES assay showed systematic 20-30% overestimation of estrogenic activity compared with the HPLC-ESI-MS/MS method (HPLC Electro-Spray-Ionisation tandem MS). They suggested that this overestimation was due to the presence of other compounds in the samples that were not detected by the chemical analysis. This is in line with Beck et al. (2006), who combined the YES assay and chemical analysis to quantify estrogenic activity in surface water.

The selection of analytical methods should consider the purpose of the study. For a large number of environmental samples in a survey or monitoring programme, chemical analysis seems not to be a practical choice due to it being expensive, time-consuming and able to report only the known chemicals, while the combined estrogenicity mixture is not taken into account. The YES assay has been suggested and applied in particular as a tool for surveying and initial screening of estrogenicity in the environment because it is the most easily handled and cost-effective assay (Svenson et al., 2003; Rutishauser et al., 2004;).

Sumpter and Johnson (2008) and Strek (2009) stated in a review that estrogenic steroids contribute the bulk of total estrogenic activity in many wastewaters and surface waters. Rutishauser et al. (2004) estimated that alkylphenolic compounds contribute only about 1 to 3% of the total estrogenic activity in sewage effluent samples. According to this information, the existence of estrogenicity in the environment in Vietnam should be considered because most surface water bodies receive untreated wastewater from humans and animals, the main sources of estrogenic steroids. In view of the facilities at research institutions in Vietnam and their budgets, the YES assay is a suitable analytical method for a monitoring programme on estrogenic activity in surface waters in the Mekong Delta, Vietnam.

The aim of this study was to establish the YES assay for different matrices in Vietnam.

2.2 Materials and Methods

2.2.1 Chemicals

All chemicals were purchased from Sigma except chlorophenol red-β-D-galactopyranodise (CPRG), which was obtained from Roche, Germany. The solvents used for the extraction process were purchased from J.T.Backer, USA.

2.2.2 Recombinant yeast estrogen screen (YES)

The yeast was provided by Prof. Sumpter, Institute for the Environment, Brunel University, UK. The recombinant yeast estrogen screen assay and the preparation of the medium compounds are described in detail by Routledge and Sumpter (1996). Yeast cells (*Saccharomyces cerevisiae*) were transferred with the human estrogen receptor gene (hRE) together with expression plasmids carrying the reporter gene *Lac-Z* (encoding the enzyme β-galactosidase). In this system, the hER is expressed in a form capable of binding to estrogen response elements. Active ligands that bind to the receptor cause the expression of the reporter gene *Lac-Z* and produce β-galactosidase, which is secreted into the medium. This causes CPRG to change its original yellow colour to red. The extinction is measured at 540 nm. In this study, the method of Routledge and Sumpter (1996) was modified. To avoid contamination from bacteria on the assay plates, 0.5 mL of an antibiotic mixture (8 mg penicillin and 8 mg streptomycin in 10 mL sterilised distilled water) was added to 50 mL of assay medium. The medium was seeded with 2 mL yeast from a 24-h culture which had reached an absorbance at 630 nm of 0.9-1.0. After running the Routledge and Sumpter yeast assay several times, we observed that the absorbance at 540 nm for the blank wells was almost the same as that for the wells containing standards at low concentrations (2 ng/L, 20 ng/L). This could have occurred because of the influence of CPRG on the yeast assay, as reported previously by De Boever et al. (2001). It indicated that CPRG or its β-galactosidase degradation product chlorophenol red can act in the assay as an estrogenic compound. Therefore, CPRG was not added into the growth medium at the beginning, but after incubation of the assay plates for 1 day.

Preparation of medium components

Minimal medium (pH 7.1) (1 L double distilled water)

- KH_2PO_4: 13.61 g
- $(NH_4)_2SO_4$: 1.98 g
- KOH: 4.2 g
- $MgSO_4$ –0.41g $MgSO_4.7H_2O$ needed to get 0.2 $MgSO_4$
- 1mL $Fe_2(SO_4)_3$ solution 40 mg/50 mL H_2O)
- L-leucine: 50 mg
- L-histidine: 50 mg
- Adenine: 50 mg
- L-arginine-HCl: 20 mg
- L-methionine: 20 mg
- L-tyrosine: 30 mg
- L-isoleucine: 30 mg
- L-lysine-HCl: 30 mg
- L-phenylalanine: 25 mg
- L-glutemic acid: 100 mg
- L-valine: 150 mg
- L-serine: 375 mg
- The solution was stirred on a heated magnetic stirrer to dissolve solids
- 45 mL aliquots were dispensed into 250-mL flasks

The flasks were sterilised at 121^{o}C for 10 minutes and stored at room temperature.

Vitamin solution (180 mL double distilled water)

- Thiamine: 8 mg
- Pyridoxine: 8 mg
- Pantothenic acid: 8 mg
- Inositol: 40 mg
- Biotine solution (2 mg/100 mL H_2O): 20 mL
- The solution was filter-sterilised through 0.2 µm spore size Whatman filters in a laminar flow cabinet and stored at 4^{o}C in sterilised glass bottles.

D (+) glucose: 200mL

- A 20% w/v solution was prepared
- Sterilised at 121^{o}C for 10 minutes
- Stored at room temperature

L-aspartic acid: 200mL

- A stock solution of 4 mg/mL was prepared
- Sterilised at 121^{o}C for 10 minutes
- Stored at room temperature

L-threonine: 100mL

- A stock solution of 24 mg/mL (2.4 g/100 mL) was prepared
- Sterilised at 121^{o}C for 10 minutes
- Stored at 4^{o}C

Copper (II) sulphate: 50 mL

- Prepared a 20 mM solution (159.6 mg/50mL)
- Sterilised by filtering though 0.2 µm pore size Whatman filters in a laminar flow cabinet
- Filtered into sterilised glass bottles in 5-mL aliquots
- Stored at room temperature

Growth medium was prepared by adding:

- 5 mL glucose solution
- 1.25 mL L-aspatic acid solution
- 0.5 mL vitamin solution
- 0.4 mL L-threonine solution
- 125 µL copper (II) sulphate solution to 45 mL minimal medium in a sterile conical flask.

The growth medium was inoculated with 0.25 mL of the concentrated stock yeast and incubated at 28^{o}C on a shaker (250 rpm) for about 24 hours until the absorbance at 620 nm reached approximately 1.0.

2.2.3 Solid phase extraction

Liquid samples such as surface water, wastewater and the aqueous phase of biogas and septage slurry were extracted by solid phase extraction (SPE) using SPE column

Strata™ X Phenomenex polymeric reversed phase 200 mg/6 mL, part no 8B-S100-FCH. All glassware used for extraction was washed, rinsed twice with methanol, then with distilled water, and baked at 180°C for 4 hours. Before extraction, methanol was added to the sample in a suitable volume. For instance, 5 mL/10 mL of methanol were added to 500 mL/1000 mL of surface water sample. The suspended solids were then removed by filtration though cotton wool and two paper filters with pore sizes of 8 µm and 0.2 µm (Millipore glass fibre pre-filters, Cat No, AP 1504 700, Lot No, R 8 S N 49734, and AP 250 4700, Lot No, R 9 AN 64965) to avoid SPE cartridge clogging. The SPE cartridge was activated with 8 mL methanol, and then washed with 8 mL of water:methanol solution (95:5). After that, it was loaded with 500 or 1000 mL of the filtered sample with a flow rate at 5.5-6 mL/min. The cartridge was washed with 10 mL of methanol in water (1:1), followed by 10 mL of acetone in water (1:2), and then dried under nitrogen gas (99.99%) until dryness. Finally, the estrogenic activities were eluted with 10 mL of methanol and the solvent was evaporated in a vacuum oven (Thermosi 3000) at 40°C, 1 atm. The extract was re-dissolved with 500 µL of methanol and stored at 4°C in a 1.5-mL glass bottle with screw cap until final analysis.

The volume for extracting depended on sample characteristics and volume, and was 500-1000 mL for surface water, 30-100 mL for wastewater, and 20-100 mL and 10-25 mL for the aqueous phase of biogas and septage slurry, respectively.

2.2.4 Ultrasonic extraction

Solid samples were extracted by ultrasonic extraction. 2-5 g of the sample were placed in an Erlenmeyer flask (50 mL), covered with aluminium foil and ultrasonicated twice with 5 mL of ethyl acetate at 30°C for 15 minutes each. After ultrasonication, the suspension was transferred into a polypropylene centrifuge tube (Isolab, Germany) and centrifuged for 10 minutes at 12,000 rpm at 4°C. Then 5 mL of the supernatant were evaporated to dryness at 40°C, 1 atm. The residue was dissolved in 500 µL or 1000 µL of methanol and stored at 4°C in a 1.5-mL screw cap vial until final analysis. In parallel, 25-30 g of each sample were weighed, dried overnight at 105°C (14-16 h) and reweighed to determine the dry weight of the sample.

2.2.5 YES assay procedure

Standards (17β-estradiol) ranging from 2 ng/L to 2700 ng/L in methanol and 10 µL aliquots of each concentration, extracted sample and methanol (as well as blanks) were transferred to four wells of a sterilised 96-well optical flat bottom microtitre plate (Nunc, Germany) and the methanol allowed to evaporate until dryness. The wells were then given 175 µL of the assay medium using an 8-channel pipette. Each plate contained at least one row of blanks, as well as standards in addition to 17β-estradiol for calibration. The plates were covered with sterilised lids, shaken for 3 min on a titre plate shaker and incubated at 32^{o}C in an incubator (SANYO) for 1 day. Then 25 µL of CPRG (0.5 mL of CPRG substrate solution in 50 mL of growth medium) were added to each well and the plates were incubated for 2 more days. The plates were shaken for 3 min every day. The chromogenic CPRG stock substrate solution was prepared by dissolving 40 mg CPRG in 1 mL distilled water and sterilised by filtering through a 0.2 µm pore size filter into sterilised glass bottles. There was a change of colour from the original yellow into orange or red after 3 days of incubation. The results were read using a BioRad Benchmark Plus Microplate reader. The colour development was measured at 540 nm and the turbidity of yeast cell biomass was read at 630 nm. The turbidity values were adjusted using the equation:

Corrected value = Test absorbance at 540 nm – Test absorbance at 630nm

The calibration of the 17β-estradiol standard curve was performed with the 4 parameter logistic (4PL) model (Findlay and Dillard, 2007). Concentrations of estrogens in samples are presented as 17β-estradiol equivalents (E2eq).

2.2.6 Extraction recovery

The procedure used for testing recovery is described in more detail in Chapter 4.

2.3 Results and Discussion

2.3.1 Sample extraction

Recovery in distilled water was 89.7% ± 15.9% for E2, 111.2% ± 9.9% for E1, 98.2% ± 27% for EE2 and 83.5% ± 6.6%, 117.9% ± 43.6%, 32.4% ± 10.5% for E2, E1, EE2 in the liquid phase of septage slurry, respectively. Thus, the recovery was not very different between distilled water and the liquid phase of septage slurry except for EE2. EE2 has a higher log K_{ow} (4.15) than E1 (3.43) and E2 (3.94) (Lai et al, 2000).

The higher hydrophobicity of EE2 may have resulted in absorption on solid particles, so that the EE2 spiked into the sample might have been absorbed by the solid phase during filtration. A recovery test with the same extraction method was carried out in another laboratory in CanTho University, Vietnam. The recovery was 87% ± 7.3%, 90% ± 4.3%, and 129.6% ± 16.7% when spiked with 2 ng E2/L, 1 ng E2/L, and 0.1ng E2/L in distilled water, respectively. Not all of our study matrices (surface water, wastewater, liquid phase of biogas slurry) were tested but, based on the above results, it was assumed that the recovery rates for distilled water were representative for surface water. The recovery in the liquid phase of septage slurry represented matrices with a high content of suspended solids, such as the liquid phase of biogas slurry or wastewater.

The recovery for solid extraction obtained with solids of sewage slurry was 86.8% ± 6.1% for E2, 118.4% ± 8.9% for E1, and 72.4% ± 10.8% for EE2. Recovery tests from vermicompost with E2 gave a recovery of 79.2% ± 10.7%. The results are presented in detail in Chapter 4.

2.3.2 Method validation

The YES assay procedure was carried out in a laminar air flow cabinet. However, the assay plates were occasionally contaminated by bacteria, which caused the assay to fail. This problem was solved by adding an antibiotic mixture to the assay medium. This modification step was able to absolutely prevent contamination, since streptomycin and penicillin have been shown to be active against most gram-negative and gram-positive bacteria by interfering with normal protein synthesis (Hanelt et al., 1994).

The modified assay was able to detect estrogenicity at the lowest level of 1 pg E2eq/well in 96-well plates, which was four times more sensitive than the results reported by Li et al., (2004). The detection limit (DL) depended on the volume or weight of sample used for extraction. For instance, the DL in surface water was 0.04 ng E2eq/L with a sample volume of 500 mL and a final extracted volume of 1 mL.

The accuracy is how close a result comes to the true value. Precision is the reproducibility of multiple measurements and is described by the standard deviation. To determine the accuracy and precision of the assay, 5 assay plates were individually tested with E2 standard at concentrations ranging from 20 ng/L to 2000 ng/L. The precision was above 83% and matched the requirements of the Guidance

for Industry, Bioanalytical Method Validation (CDER and CVM, 2001). However the precision at the lowest concentration (20 ng E2/L) was only 58% and was below the acceptable threshold of 80% (Table 2.1). The accuracy was 92-99.2% (confidence levels 95%, Table 2.1) and met the CDER demands for an accuracy of ± 15% of the expected value (Fig. 2.1).

Table 2.1 Precision and accuracy of the YES assay

E2 concentration (ng/L)	**20**	**100**	**250**	**1000**	**1500**	**2000**
SD	7.76	12.62	40.4	169.12	152.45	156.57
X	18.49	107.36	251.95	1086.56	1410.76	2055.45
P (%)	58	88.24	83.96	84.44	89.19	92.38
A (%)	92.43	93.14	99.22	92.03	94.05	97.5

Precision (P%) = (1 – SD/X) x100

Accuracy (A%) = (X/X‘) x 100

SD: Standard deviation

X: Mean of observed test

X‘: Mean of expected test

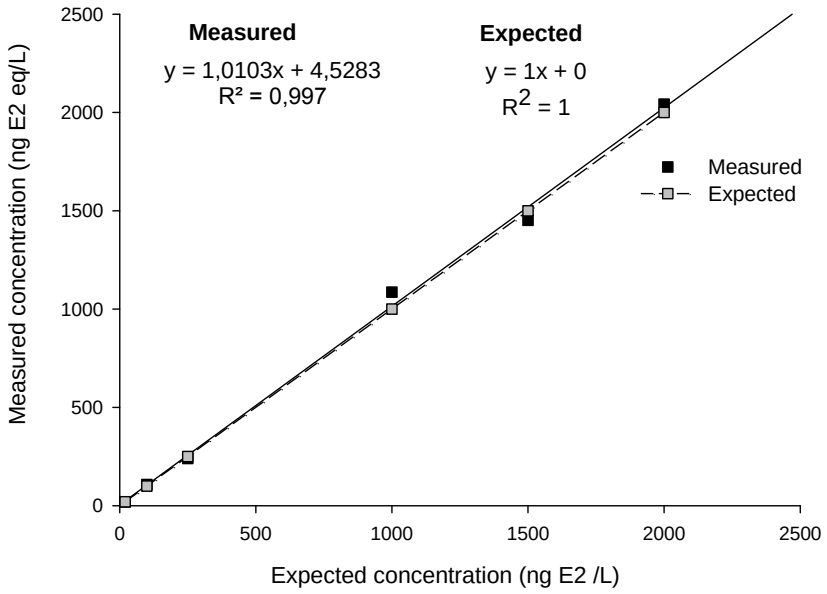

Fig. 2.1 Regression between expected and measured values.

The YES assay was used for different matrices. It turned out that some samples (especially those from small canals in polluted areas) seemed to inhibit the yeast growth and with snake eyes this effect was clearly visible. As a result, there was no reaction of the sample with the receptor and there was no change in colour. This led to underestimation of the estrogenicity.

Fig. 2.2 Photo of YES assay plate with dead yeast cells in yellow wells. Other wells showed normal growth of yeast that resulted in a colour change from orange to red.

For this reason, the turbidity of the samples was analysed as absorbance at 630 nm. The turbidity is an indicator of yeast growth: the higher the density, the more active yeast available.

At the beginning (mixture of growth medium and sample) the absorbance was 0.109. After 3 days of incubation, blanks and 'normal' samples had absorbances >0.5. Samples with inhibited yeast growth or samples where yeast was killed (as confirmed by the control with snake eyes) had absorbances <0.5 (Fig. 2.3).

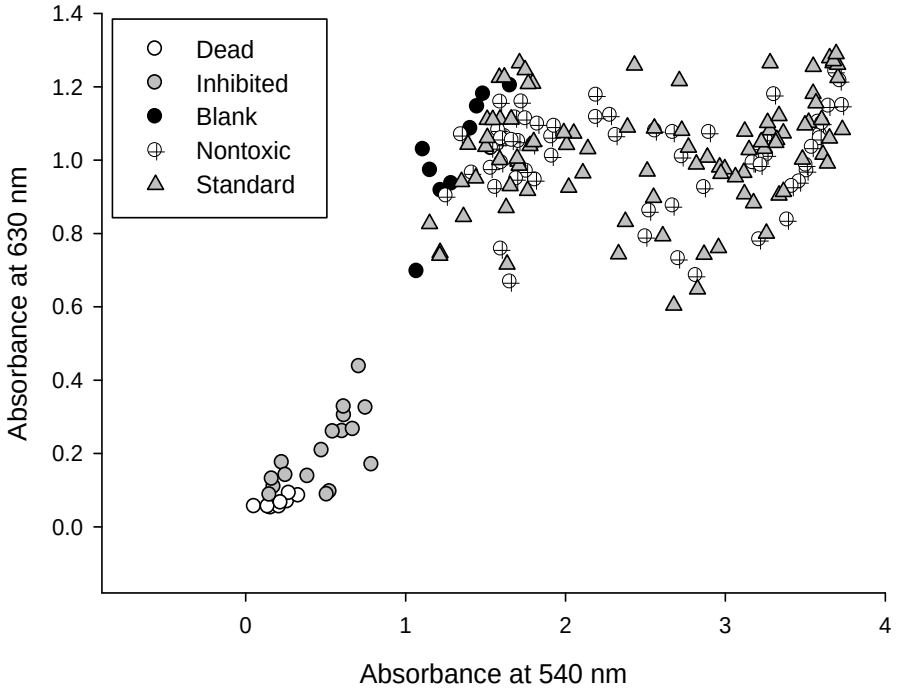

Fig. 2.3 Mean of absorbance at 630 nm and 540 nm of cytotoxic sample compared with normal samples. White circles = wells with dead yeast; grey circles = wells with inhibited yeast; black circles = blank wells; white circles with crosses = wells with nontoxic sample; grey triangles = standard wells.

Previous studies have reported that yeast death and inhibited yeast growth correlates with the concentration of toxic compounds or high estrogenicity concentrations in the samples. This study found that at concentrations up to 20 mg/L of E1, E2 or EE2, yeast growth was not influenced, as the absorbance at 630 nm was 0.822-1.176. Besides, a 5-6 fold dilution of the toxic sample could avoid the effects on cell growth, but its concentration in E2 equivalents was just some ng/L. Therefore, the inhibition of yeast growth was not because of high concentrations of natural estrogens. It is more likely that toxic chemicals influence yeast growth. These chemicals can either have an estrogenicity response or none, as reported by Hurst and Sheahan (2003). They reported insecticides, herbicides and fungicides inducing an estrogenicity response in yeast, including lindane, 2,4-D, diuron, isoproturon, paraquat dichloride, chlorotharonil, dithianon, myclobutanin, penconazole and propiconazole. However, only myclobutanin, penconazole and propiconazole have an estrogenicity response at very low potency. Rutishauser et al. (2004) also found that NP and OP were cytotoxic

to yeast cells at concentrations above 30 µM and 60 µM, respectively. Thus, on the one hand the YES assay cannot express exactly the estrogenic level in the sample causing cell death or inhibition of growth. On the other hand, yeast death or growth inhibition can be a hint of environmental pollution with toxic substances.

2.4 Conclusions

The YES assay was established for the first time in Vietnam. This method proved to be stable and can be used as a tool for screening estrogenic activity in different environmental matrices in the Mekong Delta and Ho Chi Minh City. The sample should be checked at an absorbance of 630 nm. If OD_{630nm} is lower than 0.5, the sample should be categorised as a toxic sample.

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3. A survey of estrogenicity in surface water and sediment in a part of Saigon River, Southern Vietnam

3.1 Introduction

It is reported that xenoestrogenic or estrogenic disrupting compounds (EDCs) can mimic the action of physiological estrogens at estrogen receptors (ERs) to cause reproductive impairment in animals and humans (Soto et al., 1995; Sumpter, 1995; Fu et al., 2007). Therefore, the presence of EDCs in aquatic environments is a global concern due to their impact on the aquatic biota. The first discovery of reproductive disturbances in fish related to EDCs was reported by Purdom et al. (1994) and Harries et al. (1996). Thereafter, several studies have documented similar symptoms in fish populations in many different regions (Sumpter and Johnson, 2005). For instance, Hashimoto et al., (2000) observed vitellogenin (Vtg) induction and intersex gonads, two bioindicators of the effects of environmental estrogens, in male flounder (*Pleuronectes yokohamae*), collected from Tokyo, Japan. The induction and ovotestis of Vtg in Crucian carp (*Carassius auratus*) in the Nackdong River in South Korea was reported by Chung et al. (2007 and 2008; cited by Oh et al., 2009). Widespread sexual disruption in wild fish in the UK has been attributed to hormone steroids and estrogenic chemicals such as alkylphenols originating from sewage treatment plant discharges (Jobling et al., 1998; Sumpter, 1998; Tyler and Sumpter 1998). In addition, laboratory studies showed the lowest observable effect concentration (LOEC) of estrogens affecting fish production to be at the level of ng/L (Metcalfe et al., 2001; Balch et al., 2004; Zha et al., 2008), which might be lower than the concentration detected in some surface waters. Thus estrogenicity in water might be a potential risk for aquatic organisms, especially at areas experiencing pollution.

The Saigon River rises to the north of Ho Chi Minh City (HCMC) and flows through some districts in the city before continuing to the East Sea. This river plays an important role in the life of the city, which has more than 7 million households, because it acts as both a potential water supply and a wastewater drain. Formerly, it supplied domestic water for the city at a rate of up to 10,000 m^3 per day and was planned to supply up to 600,000 m^3 per day by 2010 (Sajor and Minh Thu, 2009). Moreover, the river is a main water source for agriculture and aquaculture activities.

Downstream of HCMC, the river is used by aquatic farms to produce giant freshwater prawns, common pipefish and sculpin (Sajor and Minh Thu, 2009).

With the rapid growth of urbanisation and population together with increased industrial activities in HCMC, the Saigon River has become increasingly polluted. In 2000, domestic and industrial wastewater discharge daily into the canals in HCMC was 710,000 m^3 and 35,000 m^3, respectively. Currently only a small amount of 30,000 m^3/day of municipal wastewater and approximately 40% of industrial wastewater is conventionally treated (Water Resource Management in HCMC, 2007). Due to the importance role of the Saigon River and its pollution problems, many studies and monitoring programmes have been established to examine river water quality. However, they have focused on physical, chemical and bacteria factors only.

This chapter reports the estrogenicity levels in surface water of canals and the Saigon River, as well as in sediment in the river. Three samplings were carried out. The first of these sampling was performed together with a research group from the Department of Environmental Engineering and Management, Institute of Tropical Biology, Vietnam Academy of Science and Technology. Therefore, data on BOD, COD, total nitrogen, total phosphorus in water and total organic carbon (TOC) in sediment are taken from their report (Ecological Health Monitoring to evaluate the Saigon River water quality an environmental hygienic project: The valley of Nhieu Loc – Thi Nghe (in Vietnamese)).

3.2 Materials and Methods

3.2.1 Sampling

First sampling campaign was carried out in March, 2009. It included five sampling sections, namely SG1, SG2, SG3, SG4 and SG5 (Fig. 3.1). Water samples were taken at three sampling points in each section at low tide: left side, right side, and in the middle of the river. At high tide, only one sample was taken, in the middle of the river. All samples from SG1, SG2, SG3 and SG4 were collected on the same day, but SG5 was collected on the following day.

For the second and third sampling campaign, surface water samples were taken in April and May/2009 in order to compare the EDCs during the dry season (April) and the rainy season (May). The sites for the second and third samplings were mostly in

the canals inside the city, coded SG6, SG7, SG8, SG9, SG10, SG11 and SG12. Site SG1 was sampled during every sampling campaign (Fig. 3.2).

Site SG5 was located further upstream of the Saigon River at a site where the population was low and the land on the banks was covered with orchards and wild plants. Sites SG1, SG2 and SG3 were along the Saigon River in the city centre part. Other sampling sites were in the canals. Here, the water quality was strongly affected by human activities. SG4, SG7, SG8, SG9 and SG12 were the areas most polluted by household wastewater in HCMC. Only SG6 was directly affected by industrial activity because it was at the discharge effluent of Tan Binh industrial zones. (All sampling site coordinates are given in the Appendix.)

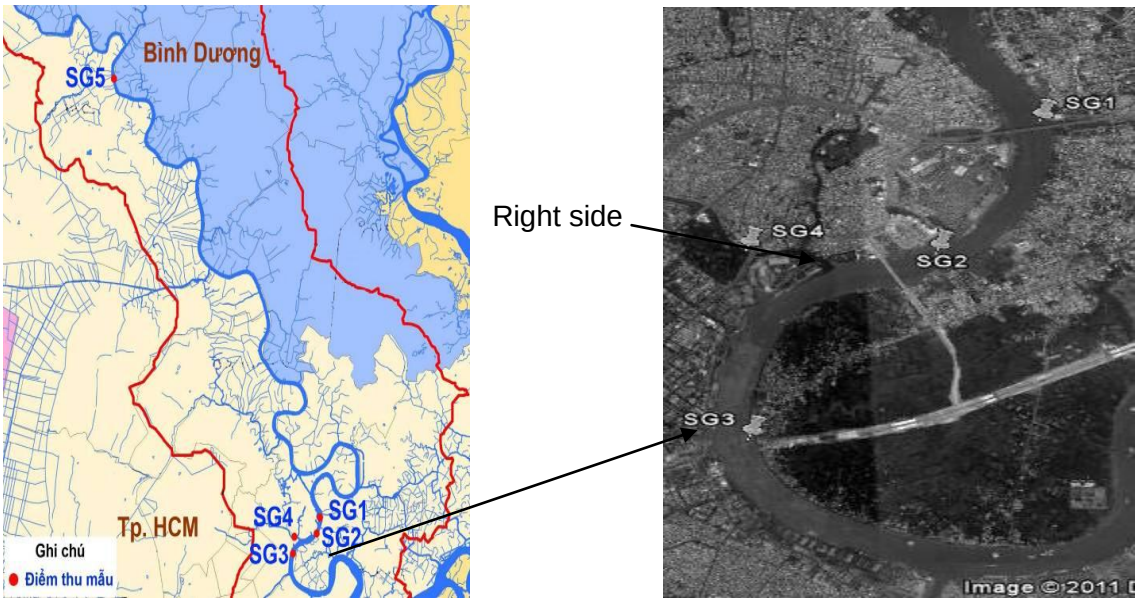

Fig. 3.1 Map of sampling area and sampling sites for the first campaign.

Fig. 3.2 Map of areas and sampling sites for the second and third campaigns (Google map).

Water samples were taken at a depth of 30-50 cm. The water was stored in 1-L glass bottles with Teflon screw caps. Sediment samples were taken at low tide at the left and right side of the river. The SG4 sample was taken only on the left side. A Petersen grab was used for sediment sampling (Fig. 3.3). Around 500-800 g was taken for each sample and stored in a 1-L glass jar with screw cap.

Fig. 3.3 Sediment sampling by Petersen grab.

All samples were kept cool and transported to the laboratory, where they were stored at 4^{o}C in the fridge and analysed within one week.

3.2.2 Analytical methods

Estrogenic activity in the water and sediment samples was analysed by YES assay. Electrical conductivity (EC) and pH value of water were recorded at each sampling point. Only samples from the first sampling were analysed for other physicochemical parameters such as BOD_5, COD, total phosphorus and total nitrogen. Sediment samples were analysed for dry organic matter (DOM), EC and pH at the laboratory.

Extraction

EDCs in water samples were extracted by solid-phase extraction (SPE) using the SPE column Strata^{TM} X phenomenex polymeric reversed phase, and EDCs in sediment samples were extracted by ultrasonic extraction as described in Chapter 2. Sample volumes for extraction were 500 mL for water and 5 g wet weight (44-63.8% of DM) for sediment.

3.3 Results and Discussion

3.3.1 Estrogenicity in surface water

The concentration of EDCs seemed to be influenced by human activities along the canals and the river: Site SG5, with the lowest human influence, had the lowest EDCs concentration (<LOD, Table 3.1).

Table 3.1 Estrogenicity concentrations in surface water (ng E2eq/L)

	Low tide			**High tide**
Sampling point	Left	Middle	Right	Middle
SG1	3.28	0.46	2.56	1.57
SG2	2.9	1.82	2.73	2.88
SG3	1.63	1.22	0.02	LOD
SG4	Died	Died	Died	0.27
SG5	LOD	LOD	LOD	LOD

Died: Yeast cells died due to the toxic compounds in extracted samples

LOD: Lower limit of detection (0.04 ng E2eq/L)

Site SG4 was situated at the confluence of Nhieu Loc-Thi Nghe canal and the Saigon River and EDC analysis was not possible, as the yeast cells died when they were confronted with the water samples. From the canal, polluted water was discharged into the river. This is reported to be the most polluted canal in HCMC, with the dissolved oxygen (DO) always at level of 0 mg/L in both the dry and rainy season (Canh et al., 2010). SG1L (left side of SG1) had the highest EDC concentration (3.28 ng E2eq/L) since it was near a densely populated area and also was close to the effluents of two ditches from a highly populated area. On the right side of SG1, a large restaurant was located and the wastewater from this restaurant could be one reason for the detected EDC concentration of 2.56 ng E2eq/L. At site SG2, there were businesses and tennis courts on the left side and a populated area on the right. The EDC concentration at SG2 was 2.69 ng E2eq/L (left) and 2.73 ng E2eq/L (right). The EDC concentration at SG3L was lower than at SG1 and SG2 since there was depot on this side. At SG3R, there was a park and, about 100 m away from there, a wharf. The activities around this point seemed not to contribute EDCs into the surface water as the EDC concentration was low (0.02 ng E2eq/L).

EDC concentrations in the mid-points of the river were lower than at the left and right side. This might reflect the fact that the sources of EDCs do not originate from the water body, but from activities along the river.

At high tide, samples were taken only in the middle of the river. At SG1 and SG3 the EDC concentration decreased. At SG4 the yeast was alive. This indicated that the high tide diluted the polluted water. Especially at SG4, the decreases in BOD, COD, and total nitrogen were very high (Table 3.2).

EDCs did not correlate with any of the parameters analysed. For the correlation samples < LOD was considered 0 ng E2eq/L. Samples from SG4 during low tide were excluded.

There were significant differences in EDC concentrations between the dry and the rainy season ($p=0.026$; Fig. 3.4). This is in line with Wang et al., (2011) who also reported higher EDCs concentrations in the dry season in the Liao River in China.

Table 3.2 BOD_5, COD, total nitrogen and total phosphorus in surface water in study sections at low tide and high tide (mg/L)

		SG1	SG2	SG3	SG4	SG5
BOD_5	LT	4	5	5	35	3
	HT	5	4	2	22	3
COD	LT	5.66	6.38	4.44	107	4.73
	HT	4.81	6.3	4.97	68	5.33
Total nitrogen	LT	2.16	2.11	2.21	10.32	0.87
	HT	1.7	1.47	1.17	2.05	1.11
Total phosphorus	LT	0.13	0.15	0.15	0.18	0.11
	HT	0.14	0.14	0.14	0.15	0.17

LT Results of samples were taken at low tide

HT Results of samples were taken at high tide

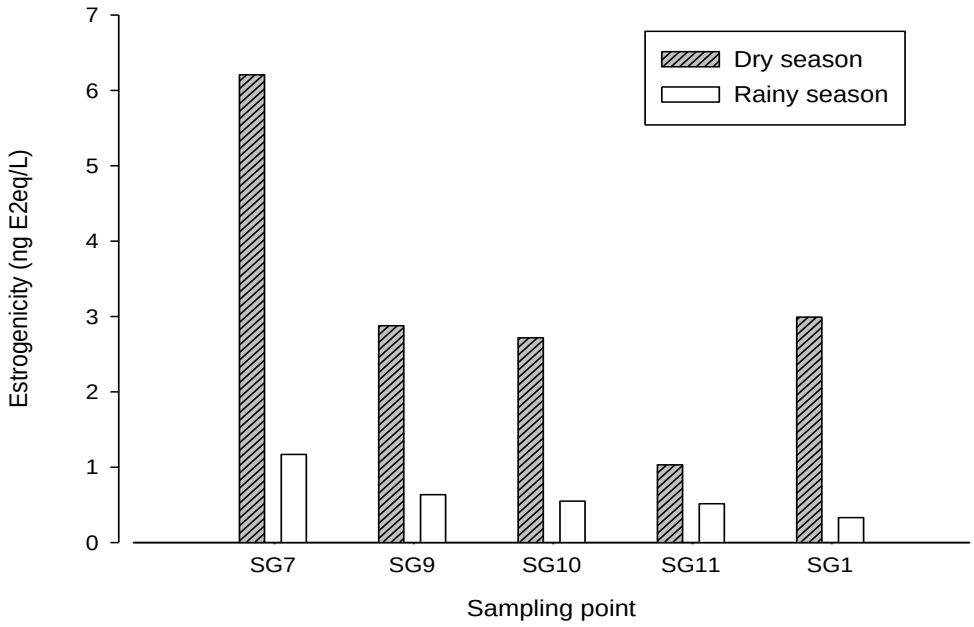

Fig. 3.4 Concentration of estrogenicity in surface water in the dry and wet season.

For SG6, SG8, and SG12, yeast cells in the assay test died both in the dry and rainy season. SG6 was close to the effluent from the Tan Binh industrial zone, so its wastewater may have contained toxic substances that killed the yeast. SG7, SG8, SG12 and SG4 are situated at the canals Tham Luong-Ben Cat, Tan Hoa-Lo Gom, and Nhieu Loc-Thi Nghe, respectively. Those canals have been reported as the most polluted areas in HCMC due to wastewater from domestic, food and textile industries. Because of their length, water cannot be replaced by tidal convection, at least for the upper part of the canals. As a consequence, pollutants may accumulate here. As discussed in Chapter 2, this may be the reason why the yeast died. Similarly, Beck et al. (2006) reported that samples from the Baltic Sea killed yeast in the YES assay.

The concentration of estrogenicity in river water has been reported in studies in many countries, such as Germany (Powlowski et al., 2003), Italy (Vigano` et al., 2008), Australia (Hohenblum et al., 2004), France (Fenet et al., 2003; Cargouët et al., 2004), the Netherlands (Belfroid et al., 1999; Murk et al. 2002; Vethaak et al., 2005), Switzerland (Vermeirssen et al., 2005), Belgium (Witters et al., 2001), Sweden (Svenson et al., 2002), America (Snyder et al., 2001; Soto et al., 2004; Annavarapu et al., 2004), England (Xiao et al., 2001), Japan (Hashimoto et al., 2005; Matsuoka et al., 2005), South Korea (Kim et al., 2007; Oh et al., 2009, Ra et al., 2011), China (Ma et al., 2007; Gong et al., 2009, Yang et al., 2011, Wang et al., 2011) and Taiwan (Cheng et al., 2007; Shue et al., 2009). Recently, Duong et al. (2010) conducted a survey of estrogenicity in river waters in South Korea and in seven other Asian countries (Laos, Cambodia, Vietnam, China, Indonesia, Thailand and Malaysia) and found that estrogenicity levels (calculated estradiol equivalent data) in river water samples from all these countries were higher than those in Europe and America. The EDC levels found in the Saigon River and in the canals are in the same range as reported by Duong et al. (2010) (Table 3.3).

Table 3.3 Comparison of estrogenicity levels in river water samples from different countries

Country (location)	Estrogenicity (ng E2eq/L)	Reference
Belgium	< 2.75 – 81.4	Witters et al., 2001
America (Michigan)	0.86 – 10.9	Sneyder et al., 2001
France	0.3 – 4.52	Cargouët et al., 2004
Japan	0.7 – 4.01	Hashimoto et al., 2005
China (Beijing)	0.12 – 4.66	Ma et al., 2007
China (Shanghai)	2.49 – 40.3	Yang et al., 2011
Vietnam (CanTho)	0.03 – 33.99	Hoa et al., 2011
Vietnam (Saigon)*	0.02 – 6.2[a]	This study
	0.33 – 1.17[b]	

[a] Data for the dry season. Upstream EDC concentration < LOD

[b] Data for the rainy season

* Sampling at high tide

In the rainy season, the concentrations of EDCs in the Saigon River and canals were lower than those in all other rivers. In the dry season, the concentrations of EDCs were in the same range as in France, Japan and Beijing, but lower than those in Belgium, America, Shanghai and Cantho (Vietnam). However, in the dry season all samples from the canals either caused the yeast to die or the EDC concentrations were above 1 ng E2eq/L. This EDC concentration is discussed as a level that might influence aquatic organisms such as fish in the rivers by disrupting the normal functions of these organisms (Jobling et al., 1998; Tyler and Routledge, 1998). Young et al. (2004) suggested a tentative long-term predicted no-effect-concentration (PNEC) for freshwater life of 1 ng E2/L and 0.1 ng EE2/L. Recently, Caldwell et al. (2008) recommended a PNEC for surface water of 0.35 ng EE2/L, equivalent to 0.4 ng E2/L. This means that an estrogenicity concentration > 1 ng E2eq/L in surface water might cause reproductive problems in some fish species. According to the

PNEC (1 ng E2/L), sites SG1, SG2 and the canals in the dry season had the potential to cause estrogenic effects on some aquatic organisms.

3.3.2 Concentration of estrogenicity and other parameters in sediment

Except site SG5, the EDC concentrations in sediments tended to be higher on the left side of the Saigon River. The highest concentration of 0.28 ng E2eq/g (dw) was detected at SG1L and the lowest was <LOD for both sides of SG5 (Table 3.4).

Table 3.4 Estrogenicity concentrations in sediments in study sections (ng E2eq/g)

Sampling point	L	R
SG1	0.28	0.04
SG2	0.2	0.1
SG3	0.09	0.04
SG4	0.13	NA
SG5	<LOD	<LOD

R and L are sampling points of sediment at the right and left of the river.
NA: Data not available.

Several authors have studied the estrogenicity in sediments using different analytical methods (Table 3.5). When the EDC concentration in the Saigon River sediment was compared with that in other studies, the results were lower than those for the river Lambro, Tokyo Bay, Liao River, United Kingdom estuaries and the Netherlands, but similar to that in other rivers.

Table 3.5 Comparison of estrogenicity in sediment from this study and others

Location	Estrogenicity (ng E2eq/g)	Analytical method	Reference
The river Lambro	15.6	YES assay	Viganò et al. (2008)
Tokyo Bay	2.07 – 12.1	Bioassay using MVLN cells	Hashimoto et al. (2005)
The Liao River	< LOQ – 6.04	YES assay	Wang et al. (2011)
The United Kingdom estuaries	0.2 – 13	YES assay	Thomas et al. (2004)
The United Kingdom rivers	0.021 – 0.03	GC-MS	Peck et al. (2004)
Zierikzee in Zeeland	0.005 – 0.34	ER-Calux	Houtman et al. (2006)
Netherlands	0.1 – 1.2	ER-Calux	Legler et al. (2003)
Mobile Bay, United States	Not present	YES assay	Annavarapu et al. (2004)
The upper Danube River, Germany	0.03 – 1.3	YES assay	Grund et al., (2011)
Saigon river	< LOD – 0.28	YES assay	This study

The pH value in the Saigon River was 6.77 (median) with a range of 5.92 (SG5R) to 7.04 (SG3R). Total organic carbon (TOC) in sediment ranged from 0.53% to 1.27%. There was no correlation between TOC and estrogenicity in the sediments. Such a correlation was reported by Hashimoto et al. (2005) and Wang et al. (2011) for sediment samples from Tokyo Bay and the Liao River system, respectively. However, there was a strong correlation between EDC concentration in Saigon River sediment and in water ($p<0.01$, Fig. 3.5), as also reported in study by Petrovic et al. (2002). This hints at a steady state situation between water and sediment EDCs when the samples were grabbed.

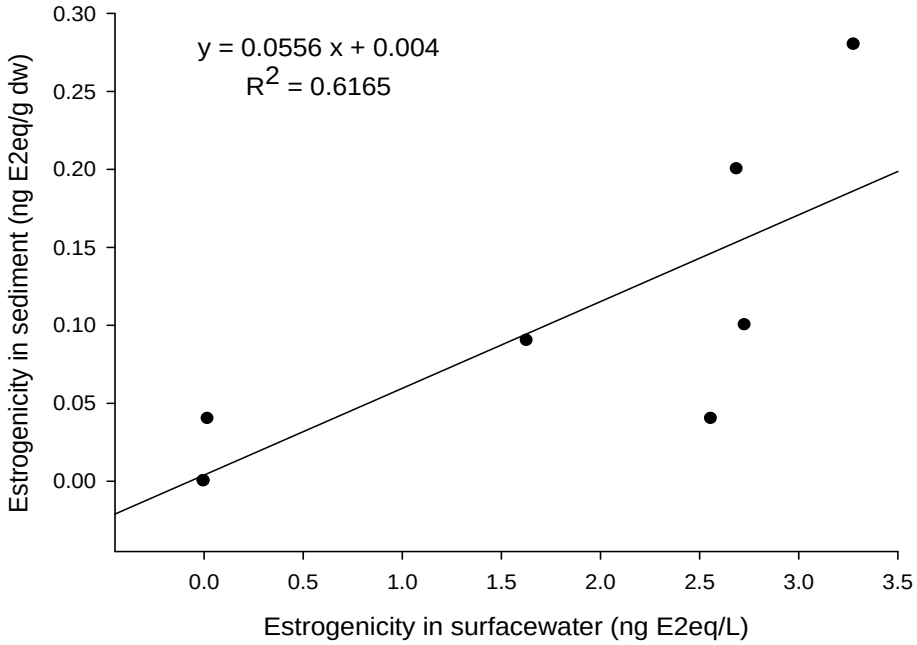

Fig. 3.5 Correlation of estrogenicity concentrations in sediment and water.

Accumulation of EDCs in sediment has been observed in other studies too (Petrovic et al., 2002; Peck et al., 2004;). In addition, studies have shown that not only sediments, but also periphytons and invertebrates can accumulate natural estrogenic compounds (Takahashi et al., 2003; Mäenpää and Kukkonen, 2006; Vigano` et al., 2006; Peck et al., 2007). As these are part of the diet of fish, they can further contribute to the EDC exposure of fish species (Stewart et al., 2001; Pedersen et al., 2003; Pickford et al., 2003).

3.4 Conclusions

The YES assay proved to be a screening tool capable of analysing estrogenicity not only in surface water, but also in river sediment.

Based on these first results of EDC concentrations in surface water and sediments in canals in HCMC and the Saigon River, there is a potential risk to benthic and developing fish and to aquatic biota.

Although there was no correlation between conventional wastewater parameters, the data indicate clearly that the EDCs in the Saigon River are of human origin.

3.5 Acknowledgements

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4. Potential estrogens in septage contributions to surface water in Ho Chi Minh City, Vietnam

4.1 Introduction

Endocrine disrupting chemicals (EDCs) have been increasingly studied in recent decades due to their effects on wildlife organisms and human health. EDCs interfere with the endocrine system and may alter diverse physiological functions such as reproduction and development in different species, including humans (Danzo, 1998). Natural estrogens such as 17β-estradiol (E2), estrone (E1), and synthetic estrogen (17α-ethinylestradiol (EE2)) used as a contraceptive are EDCs and they present the highest estrogenic potency (Johnson et al., 2001; Routledge et at., 1998). Primary sources of human-derived estrogens in the environment are mainly excretion in urine and a small amount in faeces (Adlercreutz et al., 1982; Matsuda et al., 2002; D'Ascenzo et al., 2003). Thus, most of these estrogens appear in blackwater, with the result that E1, E2 and EE2 contribute more than 90% to the estrogenicity in domestic wastewater (Körner et al., 2001; de Mes et al., 2005). Estrogen levels excreted by humans depend on gender, physiological and developmental state. Based on published studies, Combalbert et al. (2010) estimated total estrogen excretion by humans into the environment to be about 4.4 kg/year/million inhabitants. Steroid estrogens can be decomposed during wastewater treatment. However, the presence of estrogens in sewage effluents has been reported in the literature: 48 ng/L E2 and up to 76 ng/L E1 in the UK, (Desbrow et al., 1998), 12 ng/L E2 and up to 47 ng/L E1 in the Netherlands (Belfroid et al., 1999), ranging from <1 to 7.4 ng/L E2 and 8.7-75 ng/L E1 in Canada (Lee et al., 2004), and 1.15-10.70 ng/L E2 and 3.63-69.05 ng/L E1 in Korea (Ra et al., 2011). These estrogens from effluents continuously contribute to surface water bodies at levels sufficient to affect aquatic organisms (Routledge et al., 1998; Jobling et al., 1998, Desbrow et al., 1998). Fish are exposed and negatively affected by these effluents (Larsson et al., 1999; Thorpe et al., 2003; Schlenk et al., 2008; Rani et al., 2010). Wastewater treatment in Vietnam differs from that in other countries where EDCs in wastewater effluents have been studied.

The population in Vietnam is over 86 million (2009 data) but less than 10% of domestic wastewater is treated by municipal wastewater treatment (a talk by Anh,

N.H - Pollution Control Department – VEA – MONRE, http://www.wepa-db.net/pdf/1003forum/6_vietnam_nguyenhoanganh.pdf). Most of the domestic wastewater from houses is pre-treated in septic tanks, then discharged via the sewage network into surface water bodies. In Vietnam, septic tanks receive only blackwater and most septic tanks are not well maintained and may even be damaged (Anh et al., 2002). Normally, septic tanks should be emptied regularly by pumping, once every three to five years (Klingel, 2001), but a survey showed most septic tanks are not maintained as frequently as needed. Some of them were not emptied for more than 30 years. Therefore, in some cases suspended solids in the effluent are eventually higher than in the influent due to floating substances (Anh et al., 2007). The objectives of this study were to collect preliminary data on estrogen concentrations in septage slurry using the YES assay and to estimate estrogen excretion by humans into water bodies in Ho Chi Minh City.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

As described in Chapter 2. Estrone (E1) and 17α-ethynylestradiol were obtained from Sigma.

4.2.2 Sampling

Samples were collected randomly from 30 septic tanks in Ho Chi Minh City, Vietnam. These septic tanks received household blackwater and their effluents were connected to the city's sewer system. The samples were collected when the septic tanks were be emptied by a service company.

For sampling, glass bottles with Teflon caps were used. They were pretreated by rinsing with methanol and distilled water and then baking at 180^{o}C for 4 hours. Samples were taken at 20-30 cm depth from the slurry surface just before the slurry was pumped into a tanker truck. All samples were kept cold during transfer to the laboratory. They were stored at 4^{o}C for no longer than 2 days after collection.

Information on aspects such as volume of septic tank and number of persons in household was recorded while sampling.

4.2.3 Analytical methods

Extraction: All glassware used for extraction was washed, rinsed twice with methanol, then with distilled water, and baked at 180^{o}C for 4 hours. Septage slurry samples were pre-filtered to separate the liquid and solid phase. Total estrogens in the liquid phase were extracted by solid phase extraction (SPE) using the SPE column Strata^{TM} X Phenomenex polymeric reversed phase 200 mg/6 mL, part no 8B-S100-FCH. Solid samples were extracted by ultrasonic extraction. The procedures used for extraction are described in Chapter 2. The volume of samples was 20 mL for the liquid phase and 2 g wet weight (about 12% dry matter) for the solid phase.

Determination of the recovery rate:

Liquid phase: 40 mL of separated liquid of slurry was spiked with 400 µL of the individual estrogen (E1, E2 and EE2) with a concentration of 20 µg/L. The samples were filtered and 20 mL of filtered sample were extracted using the process described in Chapter 2.

Solid phase: Spiked with 100 µL E1/ E2/ EE2 at 20 µg/L. Recovery rate was calculated in relation to the non-spiked samples.

The relative response of E1 and EE2 to E2 was determined. For this purpose, 17β-estradiol (E2) was applied in a range of 2-2700 ng/L and compared with the concentration of E1 and EE2 between 100-2000 ng/L (100, 500, 1000, 2000 ng/L, respectively).

Other parameters such as dry matter (DM), total suspended solids (TSS) and organic dry matter (ODM) were also determined to calculate total estrogens in the septage solid phase and evaluate whether the estrogen concentration correlated with TSS and ODM. pH was determined in all samples before analysing other parameters.

4.2.4 Estimated yearly excretion of estrogens

Assuming that the selected septic tanks represented the population in HCMC, the overall estrogen load to surface waters was calculated as:

$$\sum EEQ = EEQ \times P \times V \times 365 \times 10^{-12} \quad \text{(eq. 1)}$$

∑EEQ: Total discharge of estrogens as 17β-estradiol equivalents (kg E2eq/year)

EEQ: Total estrogens in 1 L septage slurry effluent (median value, E2eq/L)

P: Population: 7165200 persons

V: Daily water consumption (L/person/day)

Based on Vietnamese construction standard (2/2008), the water using for flushing toilets is 60 L/person/d.

The estimated discharge of estrogens according to equation (1) was compared with a model by Johnson (2004):

$$S_T = (1 - k_T) \sum f_i (U^f_i + U^g_i + U^s_i + F_i) + S_s \qquad \text{(eq. 2)}$$

S_T: Total estrogens discharged into the sewage treatment work

k_T: Overall fraction of the steroid lost in transit through the sewage network, assumption k_T = zero for E1 and EE2, 0.5 for E2

U_i: Amount of estrogens excreted via urine

i^{th}: Fraction (f_i) of the population (µg/d).

f: Free estrogens

g: Steroid glucuronides

s: Steroid sulphate

F_i: Amount of estrogens excreted via faeces from the i^{th} fraction of the population (µg/d).

S_s: Generation of estrogens from other estrogens. For E1, (S_{sE1} = 0.5 S_{E2}) due to E1 formed by transformation of E2

i: Fraction of the population: pregnant females, menstrual females, menopausal females, females taking hormone replacement therapy (HRT) and males.

4.3 Results and Discussion

4.3.1 Method validation

The relative potencies were calculated based on the correlation of known concentrations of E1 and EE2 with measured concentrations of E2 equivalent (n=4) (Fig. 4.1).

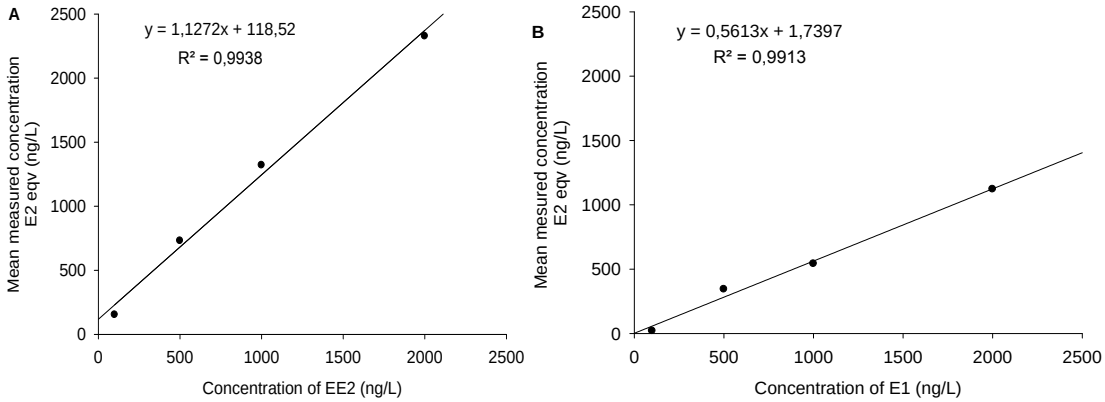

Fig. 4.1 Regression of activity of (A) EE2 and (B) E1 corrected to 17β-estradiol equivalent in YES assay.

Of the three estrogens, EE2 was the most potent, with an estrogenic potency 1.13 times greater than that of E2 (R^2 = 0.99), while the estrogenic potency of E1 was only 0.56 times that of E2 (R^2 = 0.99). The estrogenic potencies relative to E2 obtained using the YES assay are in line with those in other studies (Table 4.1).

Table 4.1 Relative potencies (estrogenic potency relative to 17β-estradiol) in different studies

Reference	E2	E1	EE2
Sumpter, 1997	1	0.5	
Murk et al., 2002	1	0.1	1.11
Segner et al., 2003	1.04	nd	1
Rutishauser et al., 2004	1	0.38	1.19
Svenson et al., 2003	1	0.19	2.2
This study	1	0.56	1.13

nd: not detected

The recovery rate in distilled water was 89.7%, 117% and 108% for E2, E1 and EE2, respectively. The recovery rates in the liquid and solid phases of the septage samples were 83.5% and 87% for E2 and 117% and 118.4% for E1, respectively, i.e. not very different from those in distilled water. However, the recovery of EE2 was lower: 73.9% from the solid phase and only 32.4% from the liquid phase. This was because of the higher octanol-water partition of EE2 (4.15) compared with that of E1 (3.43)

and E2 (3.94) (Lai et al., 2000). Therefore the hydrophobicity of EE2 may have resulted in absorption on solid particles so that the EE2 spiked might have been absorbed by the solid phase during filtration.

4.3.2 Estrogens in septage

The estrogen concentration in the liquid phase was in the range 11.87 to 184.29 ng E2eq/L, with a median of 46.59 ng E2eq/L (n=30). The concentration in solids was in the range 2.21 to 99.16 ng E2eq/g (dw), with a median of 17.16 ng E2eq/g (dw).

Total estrogens in 1 L septage slurry were calculated as sum of estrogens in the liquid and solid phases and ranged from 57.92 to 1506.81 ng E2eq (Fig. 4.1), with a median of 413.05 ng E2eq/L.

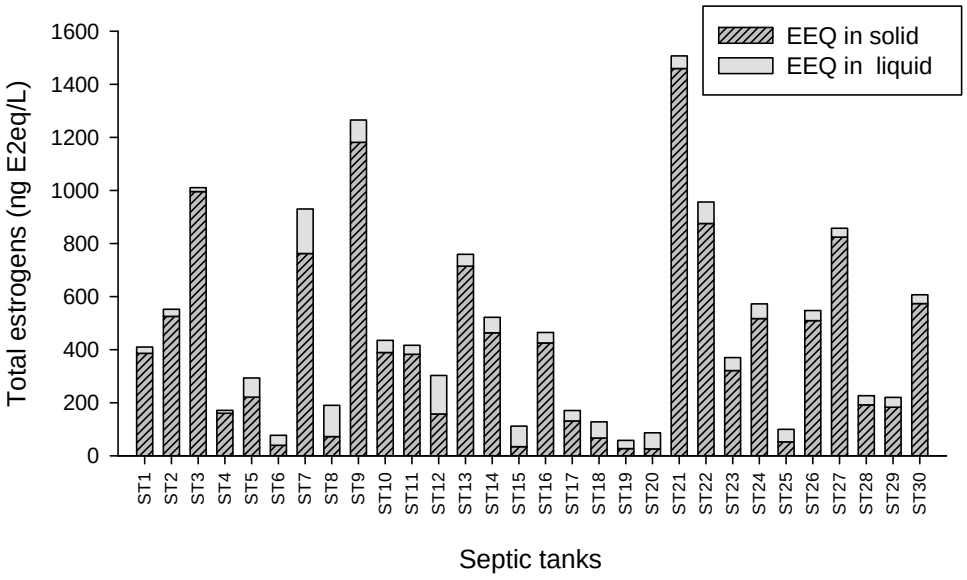

Fig. 4.2 Total estrogens in 1 L septage slurry from 30 samples. Grey bars = estrogens in TSS; grey striped bars = total estrogens in the liquid phase

The estrogen load from the solid phase contributed 75.4-98.5% to the overall estrogen load per L septage slurry except samples from ST6, ST8, ST12, ST15, ST18, ST19, ST20 and ST25. The TSS was low and its contribution to the overall was 29.7-53.1% (Fig. 4.2).

Interestingly, septic tanks ST12, ST15, ST18, ST19, ST20 and ST25 had a volume of only 2 m^3 (no data for ST6 and ST8) and perhaps these septic tanks were not filled with solids as a result of low TSS in septage slurry.

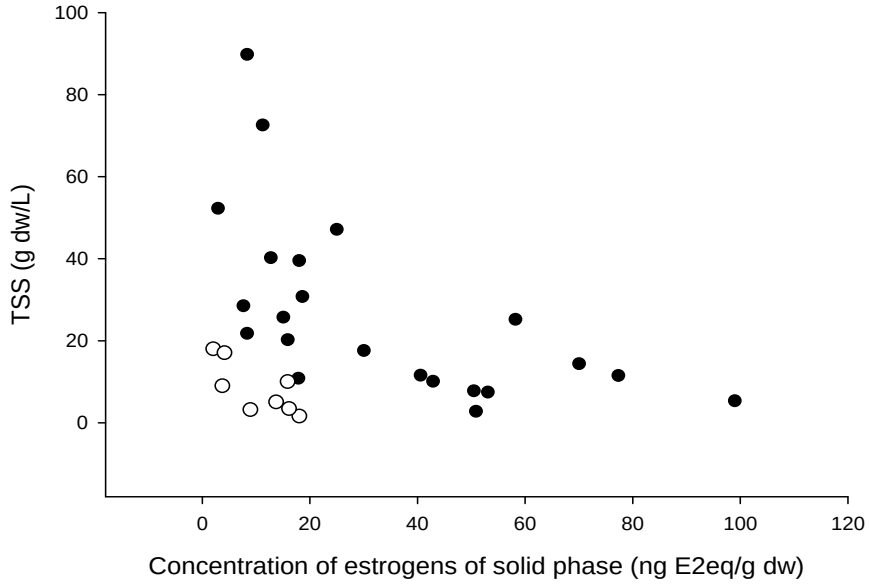

Fig. 4.3 TSS dry matter content and concentration of estrogens in the solid phase. White circles = samples from ST6, ST8, ST12, ST15, ST18, ST19 and ST20; black circles = 22 other samples

The concentration of estrogens in the liquid phase (11.87-184.29 ng E2eq/L) is quite in line with the results from a project report from the Danish Ministry of The Environment (2004), which described different levels of free estrogenic activity in septic tank effluents in Denmark depending on the number of houses connected to the septic tank system. For samples from one household the concentration was in the range not detected to 125 ng E2eq/L, with a median of 31.5 ng E2eq/L, while for samples from seven households the concentration was in range 352-497 ng E2eq/L, with a median of 425 ng E2eq/L. Swartz et al. (2006) reported concentrations of estrogens in the range 40-56 ng E2eq/L from a single septic tank with a capacity of about 7.6 m^3 that served a house and four cottages in Cape Cod, MA, USA.

Studies indicate that approximately 90% of estrogens in urine occur in inactivated conjugated form as glucoronide and sulphate, while 85-90% of estrogens in faeces are in their free form (Adlercreutz et al., 1982; Matsuda et al., 2002; D'Ascenzo et al., 2003). The inactivated estrogens can be activated by the enzyme β-glucuronidase, which is produced by bacteria such as *Escherichia coli* (Desbrow et al., 1998). As *E. coli* is present in septage, it may deconjugate glucuronides. It can be assumed that most of the inactivated estrogens in septage slurry are converted into their

biologically active and free forms. Most of the estrogens (90-95%) are excreted via urine. Free estrogens have a high log K_{ow} and tend to absorb to organic matter. This absorption at dissolved solids with TSS concentrations of about 4 g/L is reported to be approximately 61%, 66% and 70% of the total concentration of E1, E2 and EE2 (Andersen et al., 2005).The absorption is also influenced by pH and is highest at a neutral pH (Chen et al., 2010). In the present study, the pH of septage slurry was in the range 6.32-9.21 (median 7.05) and concentration of suspended solids was in the range 4.8-89.6 g (dw)/L except for the four samples ST17, ST19, ST25 and ST25, where it was < 4 g(dw)/L. Estrogens in septage could be absorbed on faeces and faecal matter may become a preliminary sink for free estrogens in urine too.

The effective volume of the septic tanks was between 1 and 4 m³. It did not correlate with the number of persons in the household or with the total estrogens analysed in septage slurry (Fig. 4.4).

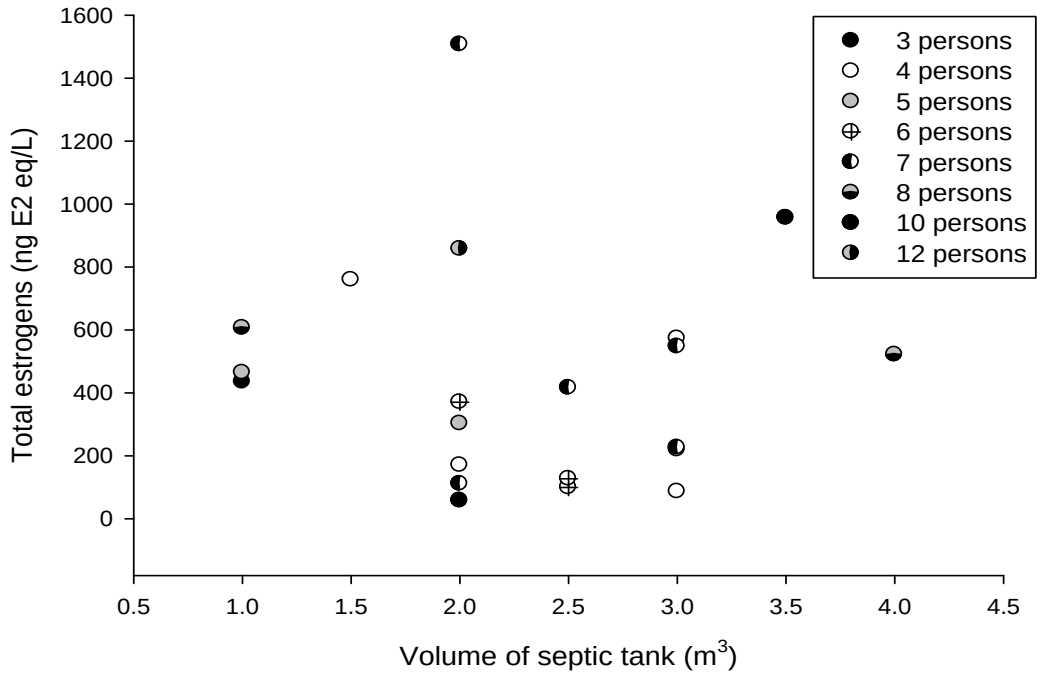

Fig. 4.4 Total estrogens in septage samples in different septic tank volumes with number of persons in households.

It is hard to correlate the estrogen excretion based on number of persons in the household. The amount of estrogens excreted by humans depends on gender,

pregnant/postmenopausal women and age (Adlercreutz et al., 1982). For instance, the E2 equivalent of natural estrogens excreted via urine is 9.937µg/d for premenopausal women, 4.38 µg/d for postmenopausal women, 4336.8 µg/d for pregnant women and 3.103 µg/d for men (Liu et al., 2009_a). The present study lacked information on the gender and health situation of the persons in the households. Likewise, there was no correlation between total estrogens in solid phase and TSS or ODM (Fig. 4.5).

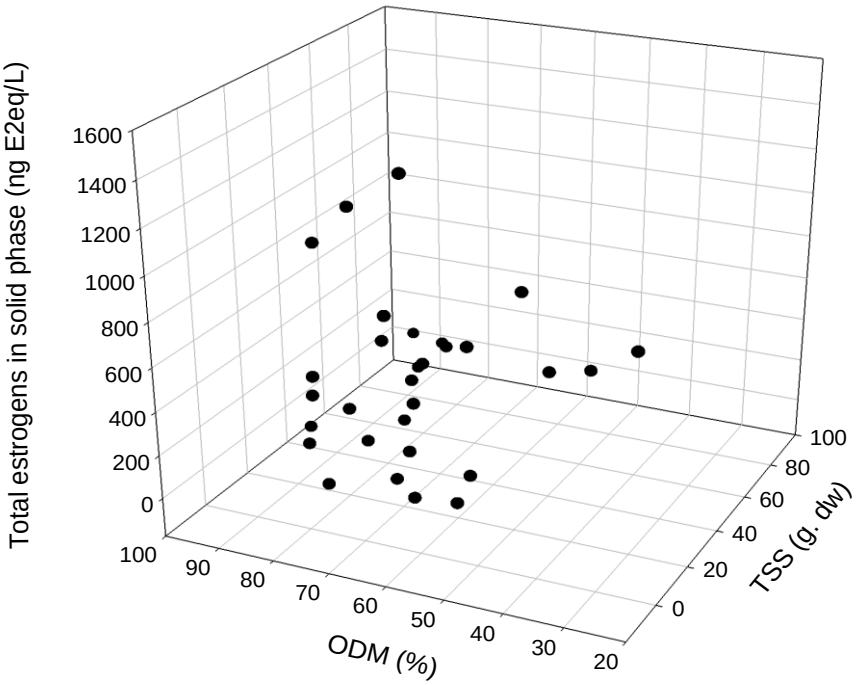

Fig. 4.5 Total estrogens in solid phase, uncorrelated with ODM and TSS.

Estrogen discharge into surface waters can be reduced by different wastewater treatment options such as physical removal, biodegradation and chemical advanced oxidation (Liu et al., 2009_b). However, with the technical status of wastewater treatment in Vietnam, these technologies are not applicable.

Up to now, domestic wastewater treatment plants have not been installed in Vietnam. Only septic tanks have been introduced in cities. In rural areas such as the Mekong Delta 64% of excreta are discharged directly into surface water (Tuan et al., 2005; Herbst et al., 2009). Theoretically, the sludge from septic tanks should be treated before being dumped or applied as fertiliser. However, in order to cut down the

treatment fee, the tanker drivers may directly dump the sludge into the rivers. In this case septic tanks would not have a major reduction effect on estrogens. Nevertheless sedimentation may be an effective way to limit the discharge of estrogens into surface waters: A large septic tank that is emptied on a regular basis and treating the sludge aerobically would significantly reduce the estrogens entering the environment.

4.3.3 Estimated yearly excretion of estrogens

The total estrogen discharge was estimated to be 64.81 kg E2eq/year (following equation 1). Solids contributed 58.27 kg/year. If 50% of the solids could be held back in the septic tanks, then the estrogen discharge would be reduced to 35.67 kg E2eq/year.

The calculated estrogen discharge was compared with the value for estrogen excretion using the model by Johnson et al. (2004). The population data used for calculations were taken from a report by the Vietnam Office of the United Nations Population Fund (UNFPA) for the year 2008. The urban pregnancy/crude birth rate is estimated at 15.8 births per 1000 population (UNFPA Vietnam, 2009). Menstruating females are assumed to be in the age range 15-50, which accounts for 56.6% of the total female population. Among the menstruating group, the group taking the contraceptive pill is assumed to be 68.8%. The mean age of the menopausal woman is set at 51, accounting for 19.7% of the total female population (UNFPA Vietnam, 2009; the 2009 population and housing census). The group of hormone replacement therapy users is 15% (Cuong, 1997) of total menopausal females in the age group 55^{+} (Johnson et al., 2004). Total estrogen discharge is presented as E2 equivalents. Estrogen potency was 0.56 for E1 and 1.13 for EE2 (results from this study), and 0.0033 for E3 (Routledge and Sumpter et al., 1997).

Table 4.2 Estimation details of estrogen excretion by humans in Ho Chi Minh City, Vietnam (g/d) (adapted from Johnson et al., 2004)

	Female population (%)	**Persons**	**E1**	**E2**	**E3**	**EE2**
Pregnant	3	113210	62.27	44.49	3328.72	
Menstrual females	53.6	1992027	23.3	6.37	62.15	
Menopausal females	19.7	732742	1.32	0.73	6.23	
Menopausal females on HRT	2.1	79225	2.25	4.44		
Menstrual females taking contraceptive pills	38.9	1448403	8.96			15.21
Males		3445700		6.2	5.17	
E2 loss				31.12		
Transformation from E2 to E1			31.12			
Total			129.22	31.12	3402.27	15.21
E2 equivalent			72.36	31.12	11.34	17.19
Total sum of E2eq (g/day)			**132.01**			

According to the model, 132.01 g E2eq/day or 48.18 kg E2eq/year would be discharged. This is rather close to the calculated 64.81 kg E2eq/year that was calculated using the septage slurry data. Furthermore, Johnson et al. (2004) found that the model gave an underestimation in comparison with observed values of 22% for E1, 15% for E2, and 20% for EE2. If this were true for the HCMC case too, the estimations would be even closer: 57.82 kg E2eq/year (Johnson et al., 2004) compared with our 64.81 kg E2eq/year.

4.4 Conclusions

Septic tanks are a considerable source of estrogens for the Saigon River.

As most of the estrogens are absorbed on total organic solids, effective solids removal would help to reduce the estrogen discharge. In particular, new designs of septic tank with a layer of activated coal could reduce estrogen discharge.

Septic tanks should be desludged on a regular basis according to the number of persons in the household and volume of the septic tank.

Septic tank sludge should be aerobically treated before it is discharged into surface water. So far, an inspection system is not in place for desludging companies to prevent them discharging untreated sludge into rivers. Such a system would help to improve the treatment efficiency of the current water treatment technology immediately without implementing new treatment technologies.

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5. Estrogens excretion by pigs and cows in the Mekong Delta, Vietnam

5.1 Introduction

Endocrine-disrupting chemicals (EDCs) have been studied for years and are becoming an increasing concern world-wide. Natural estrogens such as 17ß-estradiol (E2), estrone (E1), and estriol (E3) are produced by humans and animals and can potentially contaminate surface water and groundwater sources due to disposal or application of animal manures, wastewater or sewage sludge to agriculture land as fertilisers (Ying et al., 2002; Zheng et al., 2008; Zhao et al., 2009). Previous studies indicate that estrogens can act as endocrine disruptors and affect wildlife such as fish, turtles, frogs, etc. (Jobling et al., 1998; Bjerselius, 2001; Irwin et al., 2001; Thorpe et al., 2003; Gabet, 2007). Roger-Gray et al. (2000) reported that E2 concentrations as low as 1 ng/L are able to induct synthesis of vitellogenin (a protein normally only found in female fish livers in response to estradiol) in male fish. Estrogens have been known to cause human cancers since the 1980s (Henderson et al., 1988) and it has also been suggested that they are linked to a decrease in sperm production in adult males (Xaio-Yai et al., 2001).

Livestock manure is considered a major source of estrogen hormones in the environment (review by Hanselman et al., 2003). Total estrogen excretion by farm animals was estimated to be 33 tons/year in the European Union and 49 tons/year in the USA in 2002 (Lange et al., 2002), while it was 789 kg/year in the UK in 2004 (Johnson et al., 2006).

Data on the excretion of estrogens into surface water depending on different manure treatment systems are non-existent for tropical countries such as Vietnam. As in most Asian countries, milk, meat and livestock production in Vietnam significantly increased at the turn of the 20th century and this increase is expected to continue in the future (Trach, 1998; Gerber et al., 2005). This increasing livestock production will result in a large increase in manure, and an associated higher risk of pollution. Therefore, the management of manure in Vietnam needs to be improved, and this can be done in several ways. Methods such as biogas systems, composting or vermicomposting can be considered.

In Vietnam, pig and cow manure can be treated by biogas plants with a reactor volume of 4-6 m^3 or larger depending on the number of pigs or cows involved. Biogas reactors (or digesters) are constructed of brick walls with covers of concrete, or are made of PVC (polyvinyl chloride). Sediment is removed manually from the sedimentation tank every few months depending on the working capacity of the system and the biogas effluent is discharged into neighbouring surface waters. Cow, pig and chicken manure can also treated by composting, with the process lasting for a period of approximately 2 months. Manure is heaped on a cement pad or on the ground and is mixed with farm residues such as rice husks, rice straw and effective microorganisms (EM) to reduce the odour. The composted manure is then used as an organic fertiliser for crops. Vermicomposting is a process that lasts 5 to 6 months by which earthworms are used to convert organic materials (normally cow manure) into a humus material known as vermicompost. Compost is used as substance for vegetables or ornamental plant production.

The Mekong Delta is located in the lower part of the Mekong river with numerous interlacing rivers and streams. Agriculture is the main economic activity in the Mekong Delta, with rice, fruit and aquaculture and livestock production. The livestock sector comprises 10% of the agriculture industry and is growing annually. Pig production accounts for over 50% of total livestock production, followed by cattle production (Lensink et al., 2008). Livestock production is mostly small-scale and therefore animal waste management is not a major consideration. The farmers manage their animal waste by various methods. They have recently been encouraged to install composting or biogas systems for manure treatment in order to reduce environmental pollution and enable recycling of nutrients to crops. However, large amounts of manure are still stored for several months at a time before being applied to crops or fish ponds, or are directly disposed of in fields, fish ponds and water bodies without appropriate treatment. This study compared various manure treatment practices in the Mekong Delta in terms of their impact on estrogen discharge levels into the water.

5.2 Materials and Methods

5.2.1 Vermicomposting sampling

Sampling was carried out at a vermicomposting farm in O Mon district, Can Tho province, Vietnam, from March to June 2009. The samples included eight samples of fresh cow faeces, eight cow manure samples after 1 month, 2 months, 3 months and 4 months of vermicomposting, and eight vermicompost samples stored for 0.5 month, 1 month, 2 months and 6 months. In addition, eight samples of natural cow faeces dried for 1 month, 2 months, 4 months and 6 months were collected. Thus a total of 32 different samples were taken on four occasions, with duplicates for each. About 600-800 g of each sample were collected in a 1-L glass jar that had been pre-washed, solvent-rinsed and dried at 180^{o}C for 4 hours. All samples were kept cool during transportation to the laboratory and stored at 4^{o}C until extraction.

5.2.2 Biogas slurry sampling

Samples were collected from five household biogas plants in Can Tho City. The biogas plants treated different types of manure from pigs, pigs combined with cows, and cows combined with humans. The survey was carried out from April 2009 to September 2009, with six sampling events. During the study, the number of animals changed in some households as animals were bought or sold. As a consequence, the amount of manure treated in the biogas plant changed. All effluents and where possible the influent into the biogas plant (2 plants) were analysed. Samples were collected in 500-mL glass bottles with Teflon screw caps, kept cool during transportation to the laboratory and stored at 4^{o}C until extraction.

5.2.3 Extraction

All glassware used for extraction was washed, rinsed twice with methanol, then with distilled water, and baked at 180^{o}C for 4 hours. Biogas slurry samples were pre-filtered to separate the liquid and solid phase. Total estrogens in the liquid phase were extracted by solid phase extraction (SPE) using the SPE column Strata^{TM} X Phenomenex polymeric reversed phase 200 mg/6 mL, part no 8B-S100-FCH. Solid samples were extracted by ultrasonic extraction. The procedures are described in Chapter 2. The sample volume was 50-100 mL for the liquid phase of biogas slurry,

3-5 g wet weight (10.4-18.3% dry matter for the solid phase and 16.2-74% for fresh faeces, semicompost and vermicompost).

5.2.4 Determination of other parameters

Dry matter (DM): Sample (20 to 50g) was placed in a weighed aluminium dish and dried at 105^{o}C overnight (14-16 h), then cooled to ambient temperature in a desiccator. After cooling the dish was reweighed and percentage dry matter content (W_{dm}) was calculated as:

$$W_{dm} = [(mc – ma)/(mb – ma)] \times 100 \qquad \text{(eq. 1)}$$

ma: Mass of the empty dish (g)

mb: Mass of the dish containing the sample (g)

mc: Mass of the dish containing dried sample (g)

Total suspended solids (TSS): Samples were shaken well and then 50-80 mL of each sample were filtered through a weighed filter paper. After filtration, the filter paper containing residue sample was dried at 105^{o}C overnight, cooled to ambient temperature in a desiccator and then weighed. Total suspended solids (TSS, g/L) was calculated as:

$$TSS = [(A – B) \times 1000]/ mL\ sample \qquad \text{(eq. 2)}$$

A: Mass of filter paper + dried residue (g)

B: Mass of filter paper (g)

5.2.5 Estimating total annual estrogen discharge by cows to the environment after manure treatment

Total estrogen excretion (TEeq., kg E2eq./year) by cows was estimated using the equation:

$$TEeq.\ excretion = [N \times (F + U) \times 365] \times 10^{-12} \qquad \text{(eq. 3)}$$

$$F = (D \times EEC)$$

$$U = (F \times 67)/33$$

F: Total estrogens excreted via faeces (ng E2eq./day)

U: Total estrogens excreted via urine (ng E2eq./day) (based on an estimation by Lange et al., 2002: 33% of total estrogen excretion)

N: Number of cows in Mekong Delta, Vietnam

D: Amount of faeces produced by a cow (kg/day) (based on faeces wet weight production per day (25 kg) and dry matter content of faeces (23.31%)

EEC: Concentration of estrogens in cow faeces (ng E2eq./g dw)

The excretion of estrogens by pigs was calculated assuming that all pig excreta were treated by anaerobic digestion. In addition, it was assumed that all the water used for pig production flowed through the biogas plant. TEeq. excretion by pigs (kg E2eq/year) was estimated as:

$$TE\ eq. = (N \times EEQ \times Vp \times 365) \times 10^{-12} \quad \text{(eq. 4)}$$

N: Number of pigs in the Mekong Delta, Vietnam,

EEQ: Total estrogen content in 1 L of biogas slurry from biogas systems treating pig excrement (ng E2eq/L)

Vp: Volume of water consumption per day for 1 pig = 40 L of water, including drinking, cleaning, bathing water (Vu et al. (2008) and Sommer et al. (2005) estimated between 30 and 50 L/head/d).

5.3 Results and Discussion

5.3.1 Estrogens from cattle

Fresh cow faeces (F) samples showed the highest level of estrogens, 3.63 ± 2.23 ng E2eq/g dw (Fig. 5.1). The estrogen concentration decreased during drying and during vermicomposting. The estrogen content in naturally dried faeces after 1 month (D1), two months (D2) and 4 months (D4), was 1.28 ± 0.64, 0.31 ± 0.01 and 0.08 ± 0.05 ng E2eq/g dw, respectively. The estrogen content in faeces naturally dried for 6 months was below the detection limit (LOD = 0.004 ng/g wet weight). The estrogen concentration in manure samples after 1, 2, 3 and 4 months of vermicomposting (referred to hereafter as semi-vermicompost SM1, SM2, SM3 and SM4) was 1.57 ± 0.54, 0.22 ± 0.03, 0.11 ± 0.32 and 0.15 ± 0.21 ng E2eq/g dw, respectively.

Vermicompost samples stored for 0.5 month ($V_{1/2}$), 1 month (V1), and 2 months (V2) had an estrogen concentration of 0.28 ± 0.04, 0.32 ± 0.1 and 0.02 ± 0.01 ng E2eq/g dw, respectively. The estrogen level in vermicompost stored for 6 months (V6) was the same as that in naturally dried faeces after 6 months (<LOD). The estrogen level in $V_{1/2}$ and V1 was slightly higher than that in manure samples after 2 months (SM2) of vermicomposting. This could be due to contamination during harvesting and storage at the farm.

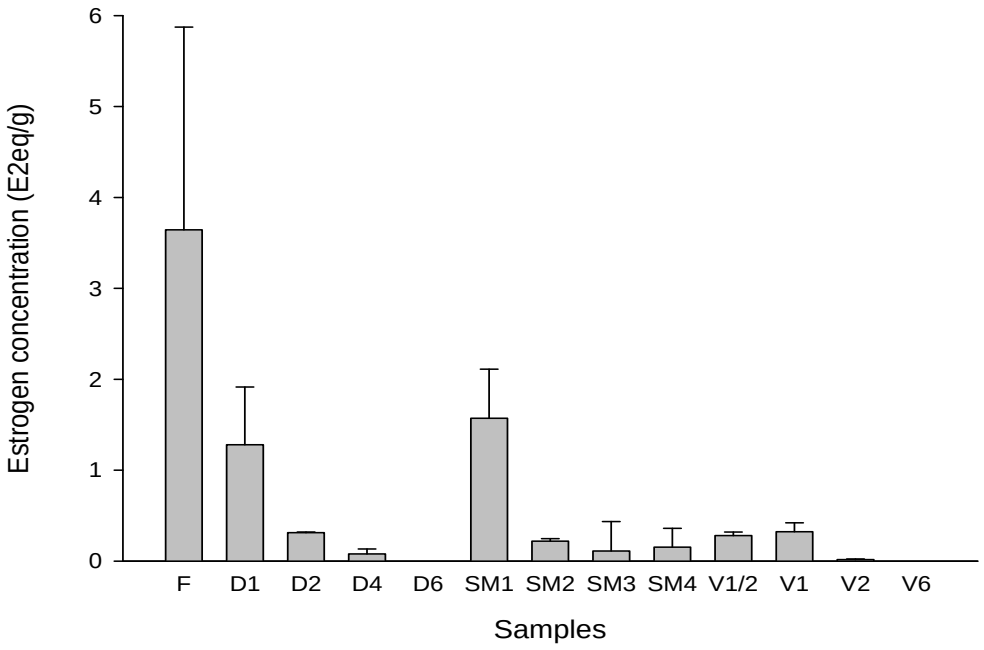

Fig. 5.1 Concentration of estrogens in fresh cow faeces, naturally dried cow faeces, semi-vermicompost, and vermicompost. Results are presented as mean ± SD; F: cow fresh faeces (n=8), D1,2,4,6: dried cow faeces after 1, 2, 4, and 6 months (n=2), SM1,2,3,4: semi-vermicompost (manure after 1, 2, 3, and 4 months of vermicomposting) (n=2), and $V_{1/2}$,1,2, 6: mature stored vermicompost after 0.5, 1, 2 and 6 months (n=2).

The concentration of estrogens in fresh cow faeces in this study was very low, approx 10-fold lower than in previous studies. For example Möstle (1997), using enzyme immunoassay to quantify estrogens, reported estrogen concentrations in a cow manure pile of 27-1000 ng/g. Raman et al. (2004) used GC-MS for quantification and found 31 ng E2eq/g in dairy cow manure. These differences could be caused by animal breed, animal diet or the practices used for manure collection and disposal (Combalbertet al., 2010).

During storage or treatment, the mean decrease in estrogen content was 95.3% ± 3.52 (SD) of the initial value within two months. This is in line with similar studies, e.g. Zheng et al. (2008) reported a 50% removal rate of hormones in heaped cow manure within 2 weeks and more than 80% within 3 months. Schlenker et al. (1999) found that 80% of estrogens were degraded in cattle faeces within 12 weeks.

There was no difference between treatment types ($p=0.771$). However, when cow manure is naturally dried it gets hard and loses its ammoniacal nitrogen. As a consequence, the organic fertiliser value of the manure decreases.

5.3.2 Estrogens in biogas slurry

The 41 samples of the liquid phase of biogas slurries included 11 influent samples and 30 effluent samples. The concentration of estrogens in the liquid phase of biogas slurry influents ranged from 2.04 to 38.84 ng E2eq/L, with a median of 12.54 ng E2eq/L, while that in the biogas slurry effluents ranged between 0.96 and 55.87 ng E2eq/L, with a median of 13.37 ng E2eq/L (Fig. 5.2).

It was possible to sample the inflow and outflow of the digester at two biogas plants. Here, in three of 11 cases, the estrogen concentration in the influent was higher than that in the effluent (P1-30, P1-43, and P2-4). In all other cases, the effluent concentration was significantly higher than the influent concentration ($p<0.05$). This agrees with Zhao et al. (2009) and Holbrook et al. (2002), who reported an increase in estrogenic activity during anaerobic digestion of manure and wastewater treatment systems. The behaviour of estrogens and their fate in the environment is complicated. Estrogens are excreted by animals via urine in conjugated forms that are biologically inactive and via faeces in the unconjugated ‘free’ and biologically active forms (Larsson et al., 1999). Although there is degradation of free estrogens, conjugated forms can be converted into free forms rapidly in the presence of glucuronidase and sulphatase enzymes in manure microorganisms such as *E. coli (*Baronti et al., 2000; Fujii et al., 2002). This could be one reason for the increase in estrogenic activity in effluents.

There was no correlation between the hydraulic residence time of slurry in the biogas plant and the estrogen concentration in the effluent.

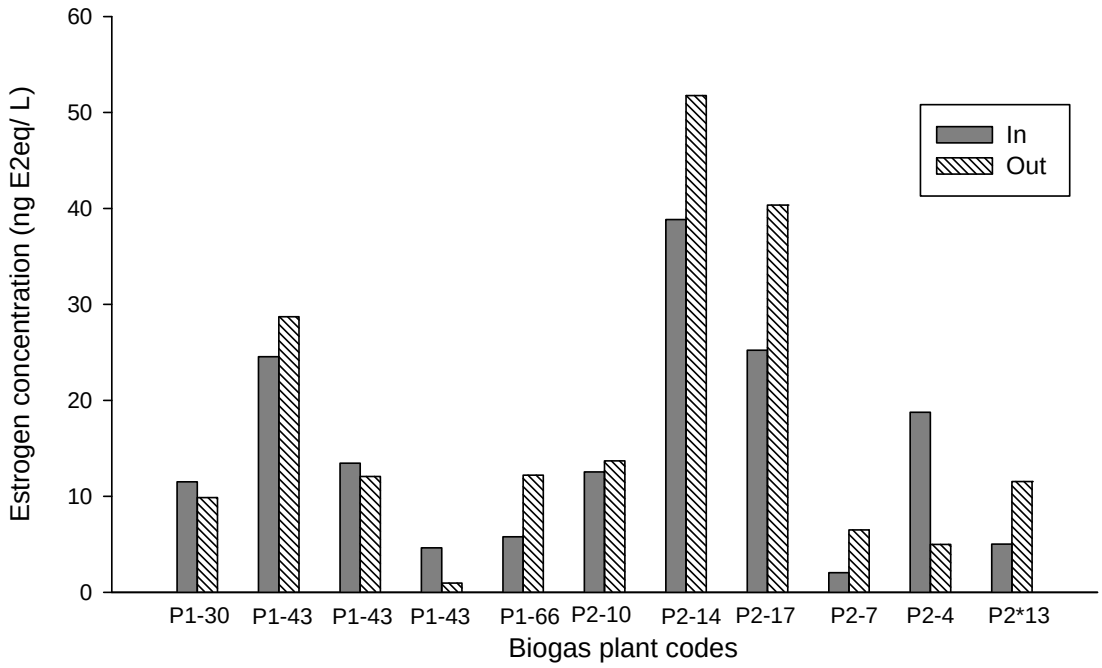

Fig. 5.2 Concentration of estrogens in the liquid phase of biogas slurry influents and effluents from two biogas plants treating pig manure (P1 and P2). The numbers 30, 10, 14, 43, 17, 7, 4, and 13 indicate the number of pigs at the time of sampling. P2* indicates that biogas plant P2 was also treating cow manure on the six sampling occasions (4 pigs and 9 cows).

There was no correlation between estrogens in the effluents from biogas plants P1, P3 and P5 and number of pigs in the system (Fig.5.3). Although the results from biogas plant P2 gave a fair correlation between number of pigs and concentration of estrogens (R^2 = 0.609), the only significant correlation was for biogas plant P4 (R^2 = 0.826, $p < 0.05$, one-tailed). Biogas slurry is a mixture of faeces, urine, water (split between drinking, cleaning, bathing and rain water), feed residues and sometimes bedding material. Therefore estrogen levels in biogas slurry depend on management factors on one hand, but also on the amounts of estrogens excreted by animals depending on species, age, sex and pregnant/non-pregnant period (Lorenzen et al., 2004). All these factors may cause a high variability in estrogen concentration in manure.

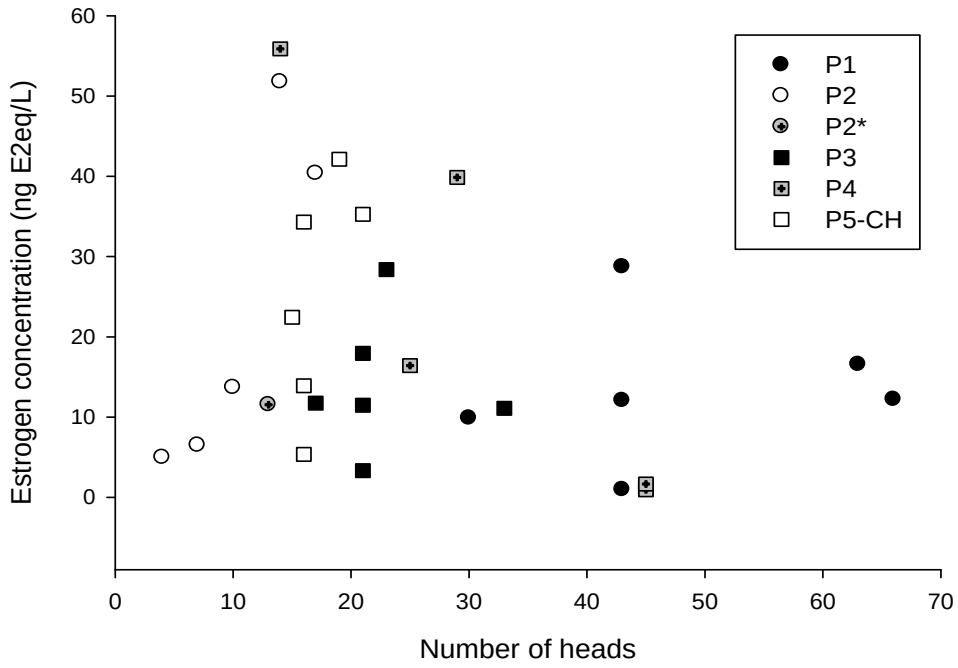

Fig. 5.3 Estrogen concentration and number of pigs at the time of sampling. P1, P2, P3 and P4 are biogas plants treating pig manure only; P2* is biogas plant P2 but with manure from cows (9) at the six sampling events; P5-CH is a biogas plant treating excrement from cows (x) and humans (6).

In the solid phase of biogas slurry (sampled at P1, P3 and P5), the estrogen concentration range at P1, P2 and P3 was 0.18-11.04 (median 6.78), 2.43-39.71 (median 5.76), and 0.09-19.91 ng E2eq/g dw (median 9.23), respectively.

Interestingly, plant P5 showed a very low concentration, 0.09 ng E2eq/g dw, at the fourth sampling event. According to the farmer, the biogas plant was desludged some days before sampling.

Based on the concentrations of estrogens in the aqueous phase and solid phase, together with the results from TSS determination, total estrogens in 1 L of biogas slurry were calculated (Fig. 5.4).

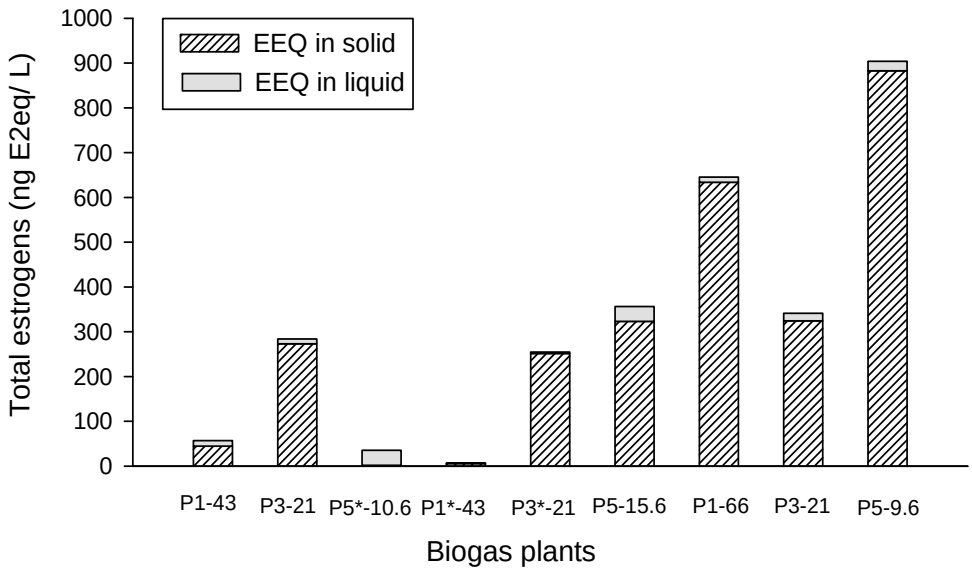

Fig. 5.4 Total estrogens in the liquid and solid phase (TSS) per litre biogas slurry from three biogas plants (P1, P3 and P5) at three sampling events. Suffixes indicate number of pigs at the time of sampling. Samples P1*-43 and P3*-21 were taken after heavy rain and P5*-10.6 after the biogas tank had been desludged a few days previously.

The estrogens in the solid phase far exceeded the estrogens in the liquid phase. The estrogen concentration in the solid phase was low at only one sampling event, at P5 when the plant had been desludged a few days before sampling. In the liquid phase, the estrogen concentration was very low (0.96 and 3.32 ng E2eq/L) at two samplings at biogas plants P1 and P3 (P1*-43 and P3*-21 respectively). Shortly before sampling, there was heavy rainfall that could have diluted the liquid phase in the biogas plant, resulting in a dilution of the estrogen concentration.

Except for the two incidental cases of P1*-43 and P5*-10.6, the total estrogen content in 1 L of slurry ranged from 56.98 to 903.91 ng E2 equivalents, with a median of 341.19 ng E2eq/L. Between 77.9% and 98.7% of the total estrogens were found in the suspended solids phase due to their physicochemical properties, i.e. low aqueous solubility and hydrophobicity (log K_{ow} 2.6-4.0) (Lai et al., 2000). Likewise, Holbrook et al. (2002) found that in a wastewater treatment process, estrogenic activity was significantly higher in the biosolids than in the liquid phase. If suitable technology for sedimentation and sedimentation removal could be introduced to remove TSS, the discharge of estrogens into surface waters could be reduced significantly.

5.3.3 Estimation of estrogen discharge from cows and pigs into surface water

Based on equation (3) and an estimated faeces production of 25 kg wet weight/cow/day (Dan et al., 2003; Johnson et al., 2006), the total discharge of estrogens was estimated at 7.52 kg E2eq/year for the 696,700 cows in the Mekong Delta (General Statistics Office of Vietnam). If slurry were to be used as organic fertiliser, the discharge into surface waters would cease. Adequate treatment of cow slurry could reduce the estrogen concentration to about 94.9% of the original concentration. Estrogen discharge with cow urine was an estimated 2.48 kg E2eq/year.

The pig population in the Mekong Delta provinces was 3,730,000 in 2009 (General Statistics Office of Vietnam). Using the median value of total estrogens in biogas effluent obtained from the biogas slurry study and equation (4), the total estrogen discharge from pigs was calculated to be 15.46 kg E2eq/year.

5.4 Conclusions

Estrogen discharge into surface waters from cows and pigs in the Mekong Delta was estimated to be 22.98 kg E2eq/year. If cow manure were to be used as organic fertiliser in agriculture after appropriate treatment, the estrogen input into aquatic ecosystems could be reduced from 5.04 to 0.26 kg E2eq/year.

The biogas plants treating manure currently do not decrease the amount of estrogens discharged into surface water. However, with optimised settling of suspended solids in the treatment process and their subsequent removal, the estrogen discharge from biogas effluent into surface water could be reduced significantly.

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6. The influence of wastewater treatment of fish processing wastewater on estrogenicity in CanTho, Vietnam

6.1 Introduction

Beside natural and synthetic estrogens, many chemicals also have the potential to disrupt the endocrine system of fish, wildlife and humans, thereby adversely affecting their growth, development, reproduction and health. The presence of EDCs in water is related to human wastes, agricultural practices and industrial activities. The effluents from wastewater treatment plants are reported to be one of the main discharges of EDCs to aquatic environments. Most previous studies have evaluated EDC discharges from wastewater treatment plants that treat domestic wastewater (Kirk et al., 2002). The wastewater treatment technique plays an important role in removal of EDCs. De Mes et al. (2005) and Koh et al. (2008) reported that biological processes, especially biotransformation, biodegradation and absorption, are the mechanisms of EDC removal. The efficiency of removal depends on the hydraulic retention time (HRT), sludge retention time (SRT) and loading rate. Sewage treatment plants (STPs) with primary treatment or enhanced primary treatment remove hormones at rates lower than 14%. The removal efficiency increases if the SPT uses an additional biological treatment with biological or trickling filters (secondary treatment), with a removal mean of 70% and 30% for E2 and E1, respectively. The activated sludge process has been confirmed to be the most efficient technology for the removal of hormones, removing 85% of E2 and E3, as reviewed by Combalbert et al. (2010).

The Mekong Delta of Vietnam is located at the most downstream part of the Mekong river basin, with a population of 17 million habitants. Farmers account for 85% of the total population. As an effect of the recent fast growth in the economy, this area is now facing environmental problems such as surface water pollution by pesticides, fertilisers, animal manure, domestic wastewater and industrial wastewater effluents. Fish processing is reported to be one of the industrial activities that has a strong impact on water quality in the Mekong Delta. About 47 million m^3 of wastewater per year are discharged into the rivers by 119 companies (report by Hien, Southern

Institute for Water Resources Planning – The resources of the Mekong Delta provinces). These companies usually treat their wastewater in their own treatment plants and the discharge of EDCs into the environment is not known. In this chapter the EDC discharge of a fish processing company with a WWTP is analysed and the treatment efficiency in terms of EDC removal is discussed.

6.2 Materials and Methods

6.2.1 Sampling

The samples were taken from a wastewater treatment system in a fish processing plant in Can Tho City in the Mekong Delta, Vietnam. This company produces frozen Tra (*Pangasius hypophthalmus*) products for export.

The wastewater treatment plant has a primary and secondary treatment step. The primary treatment consists of screening to remove large solids, a flow equation tank, and a floatation chamber to remove oils and greases. The oils and greases are removed and recycled. The secondary treatment step consists of an anaerobic tank (without oxygen supply) followed by an aeration tank. Finally a sedimentation tank is used to settle and remove coagulated and flocculated impurities. Part of the biological sludge is recycled to the aeration tank to maintain a high level of suspended solids with active microorganisms. After sedimentation the wastewater is discharged into the river. Excess sludge is removed to a sludge treatment tank. Samples were collected in 6 sampling events at three locations: the inlet to the anaerobic tank (NT1), the outlet of the anaerobic tank (NT2), and the outlet of the sedimentation tank (NT3) (Fig. 6.1).

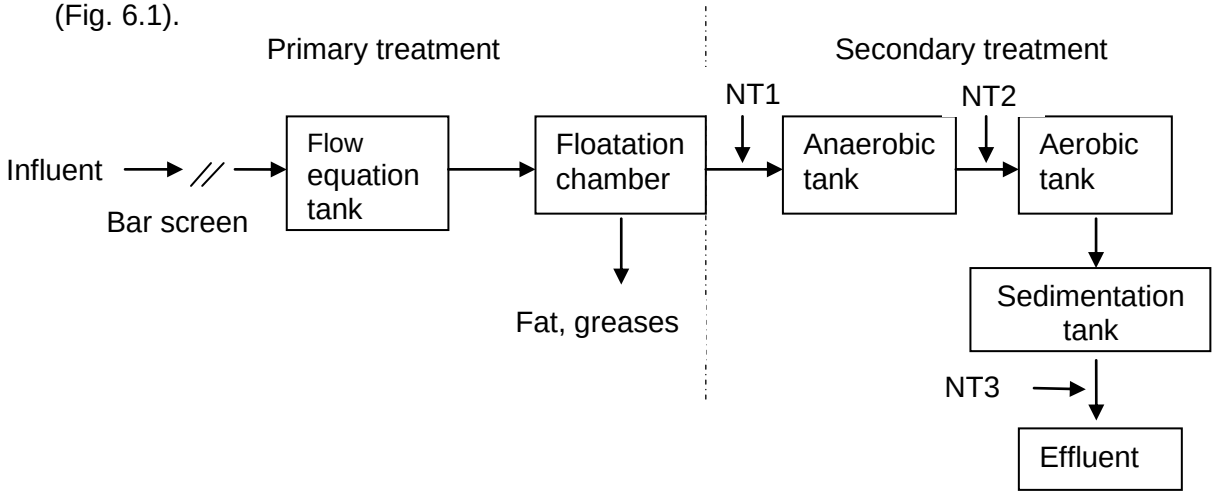

Fig. 6.1 Diagram of the conventional wastewater treatment system of a fish processing plant.

Sampling was carried out from 11 May 2009 to 16 Sept 2009 (11 May, 29 May, 17 July, 23 July, 18 Aug, and 16 Sept). At the last two samplings (before 18 August and 16 September) the anaerobic tank was not under operation and the wastewater bypassed the anaerobic part of the treatment plant.

For sampling, 1-L glass bottles with Teflon caps were used. Prior to sampling they were washed thoroughly with solvents, distilled water and baked at 180^{o}C for 4 hours. Samples were kept cool during transfer to the laboratory and stored at 4^{o}C in the fridge until analysis.

6.2.2 Parameters analysed

In all samples, pH, electrical conductivity, COD, BOD, total nitrogen, total phosphorus, phosphate and ammonia were analysed at the CanTho Centre for Natural Resources and Environment Monitoring laboratory. Estrogenicity was determined on samples extracted (as described in Chapter 2) at the Advance Laboratory, CanTho University, and analysed by YES assay at the Institute of Tropical Biology.

6.3 Results and Discussion

6.3.1 Estrogenicity concentrations

The concentration of estrogenicity in NT1, NT2 and NT3 ranged from 0.98 to 13.04 ng E2eq/L (median 4.73 ng E2eq/L), 0.63 to 14.61 ng E2eq/L (median 4.47 ng E2eq/L), and 0.91 to 16.18 E2eq/L (median 5.26 ng E2eq/L), respectively.

Wastewater influent was only from fish processing operations and did not include human excretion. Therefore, the wastewater mostly contained fish blood, fat, alcohol, salts and surfactant compounds. The estrogenicity detected in fishery wastewater may originate from natural steroidal estrogens, synthetic estrogens in fish residues and perhaps from synthetic compounds such as surfactants using for cleaning and washing.

Except for at the two last samplings, estrogenicity increased from influent to effluent ($p=0.025$, two-tailed) (Fig. 6.2). It is in line with a survey by Servos et al. (2005), which used the YES assay for analysing of EDCs from Canadian municipal wastewater treatment plants. The increase in EDCs may be because of the degradation of alkylphenol ethoxylates (APEOs, from surfactants) into shorter

ethoxylate chains during wastewater treatment. The shorter the ethoxylate chain, the higher the estrogenicity as described in more detail in Chapter 1.

Longer hydraulic retention time (HRT) seems to be a factor in reducing EDCs in treated wastewater. It may have caused the decrease in EDCs at the last two sampling occasions. Likewise, Servos et al. (2005) reported that there was no statistical correlation between HRT or sludge retention time (SRT) and EDC removal. However, treatment plants with high HRT or SRT usually show a higher removal of EDCs.

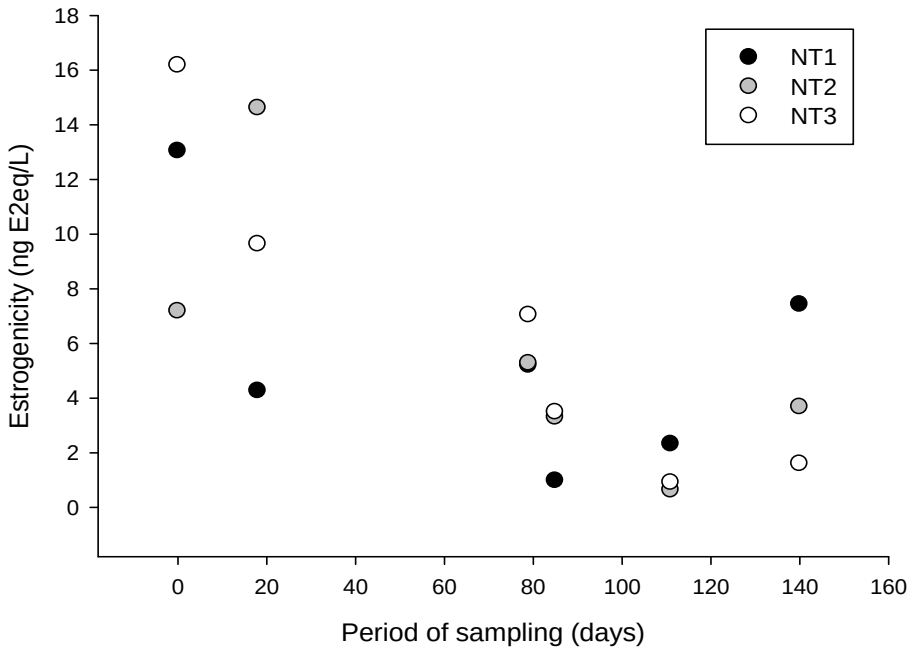

Fig. 6.2 Concentration of estrogenicity at six sampling events at a fish processing wastewater treatment plant. There were 140 days between the first and last sampling. NT1 = inlet into the anaerobic tank; NT2 = outlet of the anaerobic tank; NT3 = effluent after aeration.

6.3.2 Other parameters

The pH, COD and BOD of the treated wastewater met the Vietnamese industrial wastewater discharge standards (TCVN 5945:2005) and the guidelines on EHS (General Environment, Health, and Safety developed by the World Bank, 2007). The removal rate was 92% and 93% for COD and BOD_5, respectively (Table 6.1). For total nitrogen, the removal was 60%. Total phosphorus was removed by 32% and its

concentration in the treated wastewater was twice as high as the threshold in the TCVN. This treatment plan did not play a role in decreasing phosphate.

The concentration of EDCs in the influent wastewater (NT1) and pretreated wastewater after the anaerobic treatment (NT2) did not correlate with other parameters. However, EDCs correlated negatively with COD, BOD_5 and total phosphorus in the treated wastewater (NT3) (Fig. 6.3).

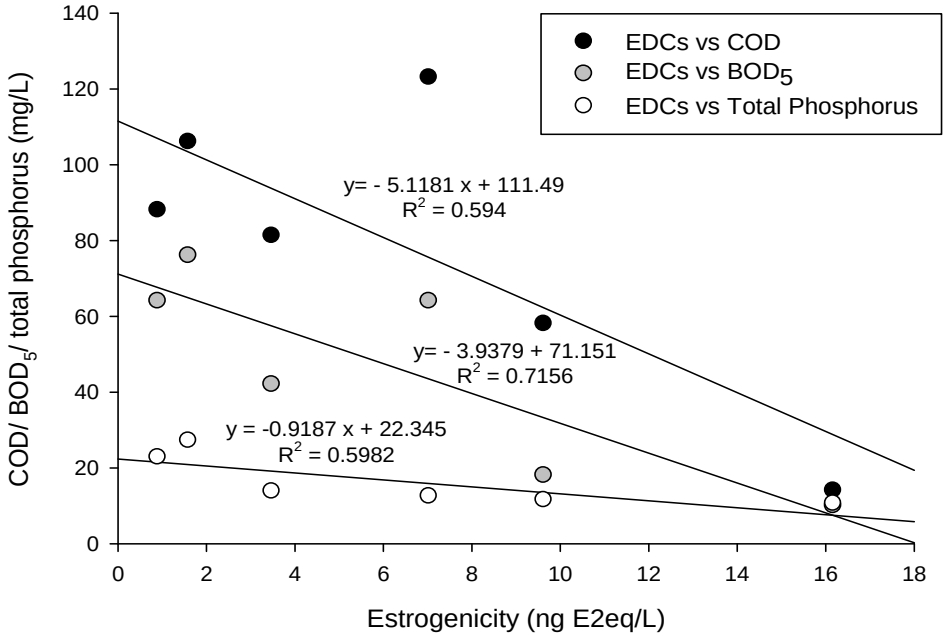

Fig. 6.3 Correlation of EDC concentrations with COD, BOD_5 and total phosphorus in treated wastewater from the fish processing plant.

In addition, it was found that the removal rate of total nitrogen and EDCs were significantly correlated ($p<0.05$, one-tailed). The more nitrogen removed, the higher the estrogenicity (Fig. 6.4). In contrast, the COD, BOD_5 and total phosphorus concentrations were correlated with EDC concentrations in the treated wastewater but their removal rates did not have any relationship.

The correlations showed that EDCs tended to increase with increasingly high quality of the treated wastewater. As EDCs tend to absorb to organic matter, mineralisation of organic matter could release absorbed EDCs into the wastewater.

Table 6.1 Physicochemical parameters in wastewater treatment processes

Parameter	NT1	NT2	NT3	Removal	TCVN	EHS
pH value	6.58 – 7.12	6.74 – 7.53	6.36 – 7.77			
	(6.95)	(7.01)	(7.37)		55.5 – 9	6 – 9
EC (mS/cm)	0.32 – 1.81	0.23 – 1.95	0.20 – 1.82			
	(1.53)	(1.08)	(0.87)			
Ammonium (mg/L)	3.00 – 34.01	22.14 – 99.8	0.29 – 59.90	60%		
	(8.97)	(54.65)	(19.55)		10	--
Total nitrogen (mg/L)	75 – 224	55.5 – 219	26.8 – 76	32%		
	(84.25)	(92.95)	(33.70)		30	10
Total phosphorus (mg/L)	15 – 25.50	15 – 23.40	10.60 – 27.20			
	(18.75)	(18.95)	(13.15)		6	2
Phosphate (mg/L)	35.65 – 122.5	26 – 132.65	45.85 – 164.8	92%		
	(52.83)	(55.85)	(54.80)		--	--
COD (mg/L)	865 – 1592	840 – 1592	14 – 123	93%		
	(1201)	(1003)	(84.6)		80	250
BOD_5 (mg/L)	460 – 1050	235 – 760	10 – 76			
	(685)	(685)	(53)		50	50

In brackets: median values

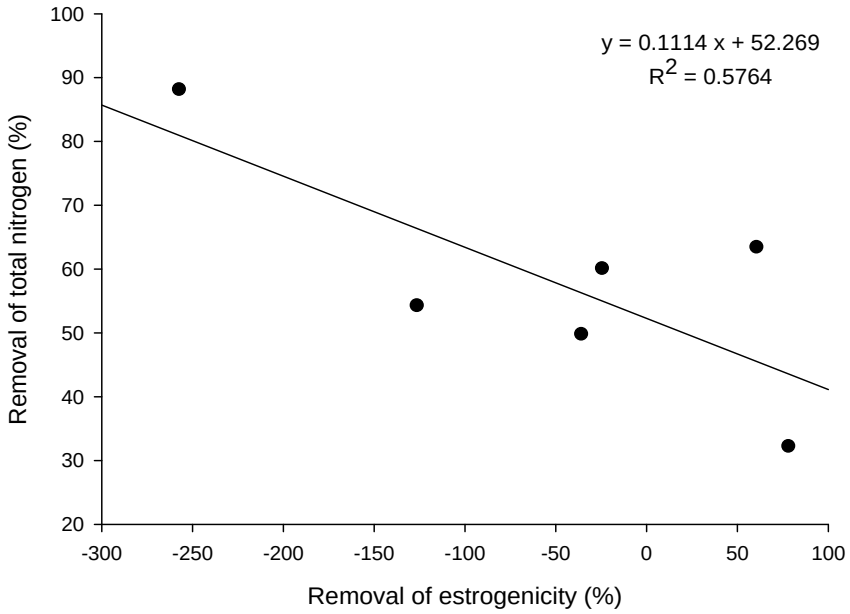

Fig. 6.4 Correlation between percentage removal of total nitrogen and estrogenicity concentration in treated wastewater from the fish processing plant.

6.3.3 Estimated estrogenicity contribution by fish processing plant discharges into surface water in the Mekong Delta, Vietnam

The Ministry of Agriculture and Rural Development for Vietnam reported that Tra fish production in the Mekong Delta in 2010 was around 1.2 million tons and can reach 2 million tons by 2020. Based on data from the fish processing company where the present study was carried out, the plant processed 500 tons of Tra fish per day and discharged 2000 m^3 of treated wastewater. Thus total estrogenicity contribution by Tra fish processing plant discharges to surface water can be estimated as:

Total Eeq (kg E2eq/ year) = $[(V_w \times TF)/ FD] \times EEC \times 1000 \times 10^{-12}$

V_w: Volume of treated wastewater discharge into surface waters (2000 m^3/day)

TF: Total Tra fish production yield (1.2 million tons/year)

FD: Amount of Tra fish processed per day (500 tons)

EEC: Concentration of effluent estrogenicity (5.26 ng E2eq/L)

Most Tra fish processing companies in the Mekong Delta treat their wastewater with the same technique as the wastewater treatment plant surveyed here. Therefore, the median concentration of estrogenicity in effluent, 5.26 ng E2eq/L, was assumed as effluent estrogenicity concentration from Tra fish processing companies. The amount of estrogenicity discharge into surface water was then estimated to be 0.025 kg

E2eq/year (2010) and can reach 0.042 kg E2eq/year (in 2020) if the wastewater treatment technique is not improved.

Compared with the EDC discharge from animals (cattle) and humans in Can Tho, this amount is rather low.

6.4 Conclusions

Wastewater treatment at fish plants eliminates BOD and COD efficiently and meets the thresholds set in Vietnamese industrial wastewater discharge standards (TCVN). However for other parameters, for instance total phosphorus, the treatment efficiency needs to be improved.

The better the COD and BOD reduction, the higher the EDC concentrations in the effluent.

Overall EDC discharge into surface waters in the Mekong Delta, Vietnam, due to fish processing companies is an estimated 0.025 kg E2eq/year. This is lower than the discharge from cattle production, for instance. However, the EDC removal rate may be improved if the hydraulic retention time is increased.

6.5 References

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7. General conclusions and recommendations

The presence of EDCs in the environment is an increasing concern due to their negative effects on human health and wildlife. Surface waters in Vietnam have become increasingly polluted in recent years, posing challenges for Vietnamese society and ecosystems. Systems are in place in Vietnam to monitor water quality and to protect natural resource but in contrast to pesticides, information about EDCs is lacking. This is perhaps because determining EDC concentrations in a large amount of samples using chemical methods is challenging. This thesis sought to resolve this challenge by developing the YES assay method and applying it for the analysis of EDCs. More than 700 samples were analysed to monitor EDCs in surface waters in the Mekong Delta and the Saigon River. In addition, the YES assay was used to analyse EDCs in other matrices such as sediment, manure, vermicompost, biogas slurry and fish processing wastewater.

The YES assay proved to be an appropriate tool for analysis of EDCs within monitoring programmes. However, the assay plates can be contaminated by bacteria during sample preparation or incubating, so use of an antimicrobial in the growth medium is recommended.

The overall EDC discharge from humans into surface waters in the Mekong Delta was estimated to be 156 kg E2eq/year. The second largest discharge was from animal husbandry, with about 23 kg E2eq/year. Fish processing industries contributed only a small share, 0.025 kg E2eq/year.

Wastewater treatment tended to increase EDC concentrations when insufficient hydraulic retention time, or sludge retention time and inappropriate technologies were used. Anaerobic treatment in particular tended to release absorbed EDCs from the mineralised organic matter.

The loads of EDCs reaching surface waters could be reduced if adequate wastewater management were to be introduced. In general, aerobic activated sludge

treatment can reduce EDCs in wastewater. If anaerobic processes are applied, for instance septic tanks or biogas systems, regular desludging would reduce the release of EDCs from organic sediments. The excess sludge from both types of systems could be composted for additional mineralisation of EDCs. In general, human and animal excreta should not be discharged into surface waters directly.

Appendix

Coordinates of sampling points in the Saigon River

Location	Code	Coordinates
Saigon bridge	SG1	10°47’57.72”N
		106°43’39.66”E
Thu Thiem bridge	SG2	10°47’14.56”N
		106°43’15.99”E
Bach Dang park	SG3	10°46’13.53”N
		106°42’32.85”E
Nhieu Loc-Thi Nghe channel gate	SG4	10°47’15.55”N
		106°42’32.25”E
Upstream	SG5	11° 2’45.90”N
		106°33’00.40”E
Tan Binh Industrial zones	SG6	10°49'7.34"N
		106°37'22.68"E
Tham Luong bridge	SG7	10°49'29.77"N
		106°37'41.56"E
Ong Buong bridge	SG8	10°45'17.04"N
		106°38'12.09"E
Ben Nge canal	SG9	10°44'57.07"N
		106°40'35.08"E
Chu Y bridge	SG10	10°45'2.02"N
		106°41'0.03"E
Kenh Te bridge	SG11	10°45'6.06"N
		106°42'6.07"E
Cong Ly bridge	SG12	10°47'29.08"N
		106°40'54.00"E