An Assessment of Oestrogenicity in Waste Water Treatment Plant Influent and Effluent using the Recombinant Yeast Screen Assay

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This thesis is
submitted to the Institute of Technology, Sligo
for a Masters of Science Degree.

Research conducted under the supervision of
Mr. John Gault

Submitted to the Higher Education and Training Awards Council,
July 2005.

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Dedication

I would like to dedicate this thesis to my parents, Liam and Frances.

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Declaration

I confirm that the enclosed thesis is all my own work with acknowledged exception.

Signed...Erika....Murray...

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An Assessment of the Oestrogenicity in Waste Water Treatment Plant Influent and Effluent using the Yeast Screen Assay

Ericka Murray

Abstract

The oestrogenic activity of solid phase-extracted (Empore SDB-XC disks) samples of wastewater influent and effluent in four wastewater treatment plants (WWTPs) in Ireland was estimated using the Yeast Oestrogen Screen (YES). Oestrogen activities were expressed as 17β-oestradiol equivalents (EEQ). The oestrogen activity of the effluent was reduced during treatment in the two larger WWTPs by 100% and 45%-56% respectively, the former being equipped with an extended aeration oxidation ditch with diffused air aeration followed by chemical precipitation and the latter equipped with sequential batch reactors with UV disinfection. However the oestrogenic activity increased by 21% and 29% during treatment on two sampling occasions for a smaller older activated sludge (oxidation ditch) WWTP operating at its population equivalent and the oestrogenic activity also increased by 23% on one sampling occasion for another small WWTP with a trickling filter system operating at its population equivalent. It did, however, reduce the oestrogenicity of the influent on the second sampling occasion by 43%. This study demonstrates the varying efficiencies of the different types of treatment systems used in WWTPs. It also demonstrates the importance of the upgrading and proper maintenance of WWTPs which are operating above their capacity and are incapable of sufficiently treating influent with regard to oestrogens and oestrogen mimicking compounds.

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／# Acknowledgements

I gratefully acknowledge my project supervisor, Mr. John Gault, for his guidance and assistance throughout the project.

To Dr. Enda Gibney for his invaluable help especially in the early stages of the project.

To the managers, in particular Jane Biggerstaff, technicians and caretakers at all the wastewater treatment plants who gave me access to the plants and assisted in sampling

To all the science technical staff at Sligo I.T.

To all the library staff at Sligo I.T.

To Thomas Sexton and Noel Sweeney of Sligo County Council.

To Ann Lyons, fellow postgrad in the field at Cork Institute of Technology.

To my fellow postgrads Noel, Pamela, Lydia, and Percy for their help sampling and with calculations and statistics.

Finally, I would also like to thank my parents, brothers and sister and my boyfriend, David, for their support.

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List of Abbreviations

APEO: Alkylphenol polyethoxylate
APEC: Alkylphenol carboxylate
ATAD: Autothermal Thermophilic Aerobic Digestion
ATSDR: Agency of Toxic Substances and Disease Registry
BPA: Bisphenol A
BBP: Butylbenzylphthalate
CPRG: Chlorophenol red-β-D-galactopyranoside
DBP: Dibutylphthalate
DDE: Dichlorodiphenyldichloroethylene
DDT: Dichlorodiphenyltrichloroethane
DEHP: Di(2-ethylhexyl)phthalate
DEP: Diethylphthalate
DES: Diethylstilbesterol
E1: Oestrone
E2: 17β-oestradiol
EE2: Ethinyl-oestradiol
E3: Oestriol
EAWAG: Swiss Federal Institute for Environmental Science and Technology
EC_{50}: Median effective concentration
EEF: Oestradiol equivalency factor
EEQ: Oestradiol equivalent
ELRA: Enyzme-linked receptor assay
EPA: Environmental Portection Agency
ER: Oestrogen recepter
ER-CALUX: estrogen receptor (mediated) chemical activated luciferase gene expression
ERE: Oestrogen response elements
GC-MS: Gas chromatography-mass spectrometry
GLEMEDS: Great Lakes Embryo Mortality, Edema and Deformities Syndrome
GSI: Gonadsomatic index
H_c: Henry's law constant
hER: Human oestrogen receptor
HSI: Hepatic-somatic index
IARC: International Agency for Research on Cancer
K_{oc}: Organic carbon-water partition co-efficient
K_{ow}: Octanol-water partition co-efficient
LAS: Linear alkylbenzene sulphonates
LC-MS: Liquid chromatography-mass spectrometry
MCF7: Human breast cancer line
MELN: Luciferase assay
MVLN: MCF-7-derived cell line
NP: Nonylphenol
NP1EO: Nonylphenol monoethoxylate
NP2EO: Nonylphenol diethoxylate
NP1EC: Nonylphenol monocarboxylate
NP2EC: Nonylphenol dicarboxylate
OH-PCB: Hydroxylated polychlorinated biphenyls
OP: Octylphenol
OPEC: Octylphenol monocarboxylate
PAH: Polyaromatic hydrocarbon
PCB: Polychlorinated biphenyls

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PCDDs: Polychlorinated dibenzodioxins
PCDFs: Polychlorinated dibenzofurans
TBT: Tributyltin
TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin
WHO: World Health Organisation
WWTP: Wastewater treatment plant
YES: Recombinant yeast assay

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Chapter 1.0 Literature Review

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1.1 Hypotheses and Objectives

It is only in recent years in Ireland that awareness about endocrine disruption in the environment has increased. Dempsey and Costello (1998) reviewed oestrogen mimicking chemicals in relation to water quality in Ireland and found no information on the levels of natural or synthetic oestrogens, alkylphenol or alkylphenol ethoxylates, dioxins, phthalates or phytoestrogens in Irish fresh water systems. However, these compounds are continuously entering our freshwater environments through sewage discharge, land run-off, atmospheric deposition and leachate from landfills.

The first hypothesis this study will address is the reduction in oestrogenicity from wastewater influent to effluent and that use of different treatment systems provides varying degrees of removal. Oestrogenic activity is to be assessed using the Recombinant Yeast Oestrogen Screen (YES).

The second hypothesis this study will address is that the YES assay is compatible for the analysis of treated sludge samples extracted by soxhlet extraction. This would allow assessment of the oestrogenic potency of untreated and treated sludge which is important as the latter may be used as biosolids on agricultural land. Many endocrine disrupting compounds are lipophilic and persistent in the environment and may bioconcentrate in the soil and enter the food chain. The presence of these compounds in biosolids poses a threat to human health.

Objectives of the study are to:

- Establish the YES assay by testing known steroidal oestrogens and xenoestrogens.
- Establish an extraction method of oestrogenic compounds from wastewater influents and effluents of four wastewater treatment plants in order to determine the oestrogenic potency of the wastewaters by means of the YES assay.
- Determine the oestrogenic potency of sewage sludge by means of soxhlet extraction and biological analysis with the YES assay.

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1.2 History of Endocrine Disruption

Worldwide contamination by endocrine disruptors is the result of various factors such as deliberate release (incineration, landfill, pulp-mill effluent, industrial and municipal wastewater effluent) or accidental release (agricultural run-off into the environment, accidental pollution incidences) of endocrine disrupting compounds and the incorporation of these compounds into consumer products (for example; contraceptives, pesticides, biocides, dioxins, plasticisers, and surfactants) (Myers *et al*, 2003).

Large volumes of man-made chemicals have been released into the environment since the 1940s, the first of which found to be oestrogenic was the insecticide, DDT. It was shown to affect the reproductive system in birds (Fry and Toone, 1981) and mammals such as seals (Bergman *et al.* 1994) and reptiles (Guillette *et al.* 1994). Incidences of occupational exposure were also identified. In 1949 reduced sperm counts were reported in aviation crop dusters handling DDT (Singer *et al.* 1949). In the 1970s in Virginia, US, factory workers at a plant synthesizing another insecticide, kepone were reported to have headaches, tremors, liver toxicity and low sperm counts (Guzelian, 1982).

From 1948 to 1971, the synthetic oestrogen, diethylstilbestrol (DES) was prescribed to pregnant women to prevent miscarriages. It failed its purpose and in 1971, incidences of unusual vaginal cancers were reported in teenage girls whose mothers had taken the drug (Herbst *et al.* 1971) along with cases of uterine and cervical malformations (Kaufman *et al.*, 1977; Bibbo *et al.*, 1977) and immune system suppression (Vingerhoets *et al.*, 1998). Structural, functional and cellular abnormalities (Gill *et al.* 1976; Klip *et al.* 2002) were reported in exposed male offspring demonstrating that DES affected human reproductive development.

In 1980, a major spill of the organochlorine pesticide Dicofol contaminated with 15% DDT and its metabolites DDD and DDE occurred at Lake Apopka, in central Florida. The population of American alligator in the lake dramatically decreased in the 1980s and still has not recovered. Guillette *et al.* (1994) suggested that the sex organs of juveniles from Lake Apopka have been permanently altered *in ovo* implying that it is improbable that normal sexual maturation will occur. Juvenile male alligators were

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found to have 70% lower plasma testosterone levels and significantly smaller penis size than juvenile males from a control lake (Guillette *et al.*, 1996).

Contaminants of laboratory experiments on oestrogens have led to the discovery of xenoestrogens such as 4-nonylphenol which was being released from plastic centrifuge tubes (Soto *et al.* 1991), and bisphenol-A which was being released from polycarbonate flasks during autoclaving (Krishnan *et al.* 1993) and also from polycarbonate animal cages commonly used to house aquatic laboratory animals (Howdeshell *et al.* 2003).

In 1982 the French restriction of the use of tributyl tin (TBT) formulations in antifouling paint on boats less than 25m in length came into legislation due to the androgenic effect TBT had on female molluscs. This compound induced imposex whereby female molluscs develop male gonads (Alzieu *et al.*, 1991). Regulation was subsequently brought into effect in the USA, UK, Ireland and other European countries in 1987 and application of TBT on vessels has been banned since 2003 under the Marketing and Use Directive (76/769/EEC).

The concept of endocrine disruption in freshwater fish was first realised in the early 1990s when anglers discovered hermaphrodite roach in the lagoons of sewage treatment plants in the UK (Purdom *et al.* 1994). The authors carried out an initial study in which rainbow trout were placed in cages in the effluent of a sewage treatment plant. Plasma vitellogenin concentrations in male fish (usually low to non-existent in male fish as it is produced by sexually maturing female fish) increased more than 1000 times after 3 weeks. An extensive survey of 15 sewage treatment plants in England was carried out and the increase in vitellogenin concentration was found to range from 500-100,000 times compared to the control. The authors suggested that the compound responsible for the oestrogenic effect of the effluent may be ethinyl-oestradiol or alkylphenol ethoxylates which are products of degradation of surfactants and detergents during the wastewater treatment process for example, nonylphenol. Laboratory studies on ethinyl-oestradiol carried out by Purdom *et al.* (1994) demonstrated that concentrations of ethinyl-oestradiol as low as 1-10ng/l could result in the same oestrogenic effect exhibited by fish in the study. Subsequent studies in Britain such as those carried out by Harries *et al.* (1996; 1997), and Jobling *et al.* (1998; 2003) and in other countries; Sweden; Larsson *et al.* (1999), Spain; Lavado *et al.* (2004) reiterated findings of

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Purdom *et al.* (1994) demonstrating the extensiveness of the problem which is the oestrogenic effect of effluent on freshwater fish.

1.3 The Endocrine System

The endocrine system, along with the nervous system, regulates and integrates the functionality of different cells within multicellular organisms and is responsible for growth, sexual maturation, and reproduction. The major vertebrate endocrine glands include the gonads, pancreas, kidney, the adrenal, parathyroid, thyroid and pituitary glands and the hypothalamus. Each gland secretes controlled amounts of specific hormones into the bloodstream that travel to target organs to act as chemical messengers invoking specific target cells to change their growth pattern or activity. These target cells consist of a binding site, or receptor and an effector site. When hormones bind to the receptor (forming ligand-hormone receptor complexes), the effector site is altered triggering gene expression, which consequentially produces the desired response (Cornwell *et al.*, 2004; Fairbrother, 2000).

Some "free" hormone molecules are inactivated before reaching the receptors and are then excreted primarily by the liver and kidneys in a process called metabolic clearance. If the metabolic clearance rate of a specific hormone is low, the hormone remains in the body longer increasing its interaction with receptors resulting in more responses (Birkett, 2003a).

Control of ovulation is the main function of oestrogens and secondary functions include sex determination, development and maintenance of secondary sexual characteristics, regulation of reproductive behaviour and regulation of calcium and water homeostasis (Fairbrother, 2000). The most important oestrogen is oestradiol. The equivalent male hormones to oestrogens are androgens, the most important being testosterone.

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1.4 Natural and Steroidal oestrogens

1.4.1 Steroidal oestrogens

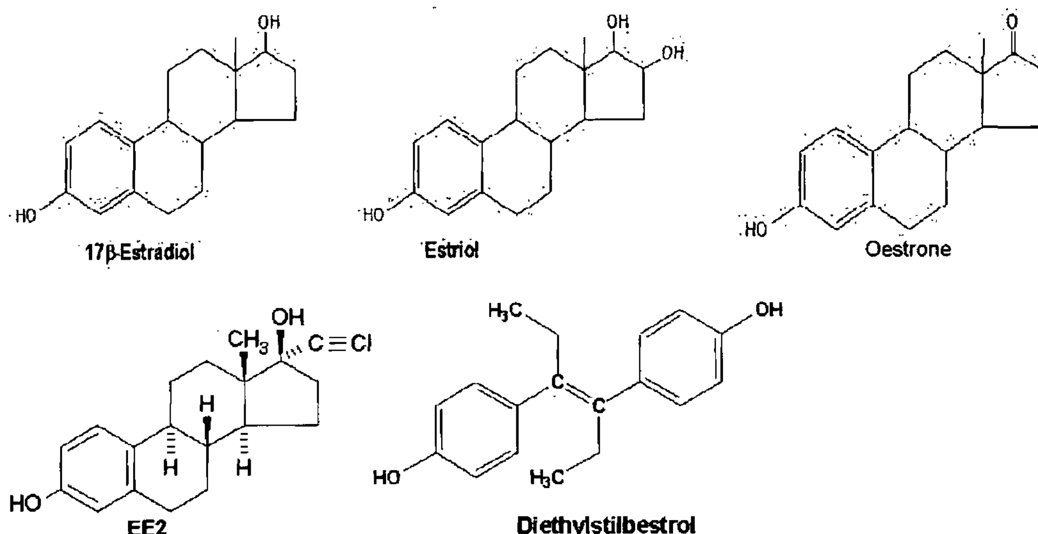

Figure 1.4.1: Chemical structures of 17β-oestradiol (E2), oestriol (E3), oestrone (E1) (http://lpi.oregonstate.edu/infocenter/phytochemicals/lignans/estrogens.html), ethinyl-oestradiol (EE2)(http://www.internal.eawag.ch/~maurer/Nova/bedu/ster_data_body.htm) and diethylstilbestrol (DES) (http://www.aw-bc.com/mathews/ch23/des.htm)

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17β-oestradiol (E2) is the most important naturally occurring oestrogenic hormone (Figure 1.4.1). All steroids share the same lipophilic hydrocarbon ring nucleus as their parent compound cholesterol. This structure consists of three hexagonal rings (ABC) and one pentagonal ring (D) (Figure 1.4.1). All oestrogens contain eighteen carbons. An addition to this structure is the hydroxyl group at position 3 of the phenolic A-ring common in all steroidal oestrogens. Different steroidal oestrogens have different hydrophilic groups bound to the pentagonal D-ring i.e.E2 has an hydroxyl group and E1 has a carbonyl group (Figure 1.1) (Fairbrother, 2000).

Steroidal oestrogens are isolated from the urine of pregnant mares or are synthesised (International Agency for Research on Cancer (IARC), 1999) and have several applications. Conjugated oestrogens, oestradiol and synthetic oestrogens in particular ethinyl-oestradiol (EE2) (Figure 1.4.1) and E2 valerate are commonly used for oestrogen replacement therapy or in combination with a progestogen for hormone replacement therapy. Oestrogens are used in oral contraceptives with combined oral contraceptive formulations typically consisting of EE2 and various progestogens. The synthetic steroidal oestrogen, EE2 was synthesized from E2 to produce a more stable compound which could be used as an oral contraceptive. The current dose of EE2 in oral contraceptives is 20μg (Sütterlin *et al.*, 2003). Oestrogens are also used in the

treatment of prostrate and breast cancer (IARC, 1999). Additionally there is a veterinary application of oestrogens whereby they are used to develop single-sex populations of fish (Piferrer, 2001).

The oestrogenicity of E2 has been assessed by various assays and expressed as EC_{50} values as can be seen in table 1.4.1 whereby the EC_{50} is the concentration of a compound that produces a half-maximal response (median effective concentration) (Rutishauser *et al.*, 2004).

Table 1.4.1: EC_{50} values (M) of 17β-oestradiol determined by various *in-vitro* assays

EC_{50} value (M)	Assay	Reference
2.5×10^{-8}	YES	Matsui *et al.*, 2000
1×10^{-10}	YES	Legler *et al.*, 2002
2.1×10^{-10}	YES	Folmar *et al.*, 2002
2.25×10^{-10}	YES	Gaido *et al.*,1997, Sohoni and Sumpter, 1998
$2.72\text{-}4.39 \times 10^{-10}$	YES	Witters *et al.*, 2001
5.14×10^{-11}	YES	Svenson *et al.*, 2003
4×10^{-11}	YES	Hamblen *et al.*, 2003
2×10^{-11}	luciferase assay	Cargouët *et al.*, 2004
5×10^{-12}	luciferase assay	Gutendorf and Westendorf, 2001
3.2×10^{-11}	E-Screen	Folmar *et al.*, 2002
5×10^{-12}	E-Screen	Gutendorf and Westendorf, 2001
6.2×10^{-12}	E-Screen	Körner *et al.*, 2001
1×10^{-7}	Carp hepatocyte	Smeets *et al.* 1999a

EC_{50} values determined by the luciferase assay with luciferase assay (MELN cells) and the E-Screen bioassay are one to two magnitudes lower than those determined by YES (Table 1.4.1). An EC_{50} value several magnitudes higher (1×10^{-7}M) was determined by a carp hepatocyte assay by Smeets *et al.* (1999a). The EC_{50} values of oestrone (E1) are higher resulting in lower potencies ranging from ten times and 100 times less potent in the E-Screen (Körner *et al.*, 2001, Gutendorf and Westendorf, 2001 respectively) and half the potency of E2 in the YES assay (Pawlowski *et al.* (2004). Matsui *et al.* (2000) determined that E1 exhibited 0.21 times the activity of E2 while

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sulphate and glucuronide conjugates of E2 exhibited activity greater than four orders of magnitude less than E2.

EC_{50} values for oestriol (E3) are typically 2-3 magnitudes higher than that of E2 resulting in potencies approximately 275 times lower determined by yeast assay (Gaido *et al.*, 1997, Matsui *et al.*, 2000, and Svenson *et al.*, 2003).

Potencies determined for EE2 range from 0.5 times the potency (Folmar *et al.*, 2002; Saito *et al.*, 2002) to 0.8 times the potency (Gutendorf and Westendorf, 2001) to approximately equal potency (Körner *et al.*, 2001) of E2 as determined by the E-Screen. The potency of EE2 is reiterated in *in vivo* studies on fish in which an EE2 concentration of only 10ng/l was sufficient to inhibit growth and condition of fathead minnow (*Pimephales promelas*) after three weeks and resulted in vitellogenin levels 500 times higher than the control fish. Also the number of eggs spawned progressively decreased at EE2 concentrations above 1ng/l (Jobling *et al.*, 2003).

Diethylstilbesterol (DES) was the first synthetic oestrogen (Figure 1.4.1). It was widely prescribed as a treatment to prevent miscarriages or premature labour from the early 1940s until 1971 when it was linked to a rare form of vaginal cancer in daughters of women who had taken the drug. DES was prescribed in the 1970s for other uses including control of menstrual disorders, hormone replacement therapy, relief or prevention of postpartum breast engorgement, palliative treatment for breast cancer in postmenopausal women and prostate cancer in men and as a post coital contraceptive. Gradually, the approval of DES for these purposes was withdrawn, however, its use in clinical trials for treatment of prostate and breast cancer continued (Smith *et al.*, 1998; Peethambaram *et al.*, 1999).

Folmar *et al.* (2002) derived EC_{50} values for DES of 1.3×10^{-11}M and 1.9×10^{-10}M from E-screen and YES assays respectively which represented potencies 0.4 and 0.9 times lower than that of the standard. Gaido *et al.* (1997) calculated DES to be over 1.5 times as potent asE2 using a yeast assay. There is a degree of variance among results of studies reviewed since Sohoni and Sumpter (1998) employed the same yeast assay but found DES to be five times less potent than E2. In a carp hepatocyte assay DES was found to be half as potent as E2 (Smeets *et al.*, 1999a).

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1.4.2 Phytoestrogens

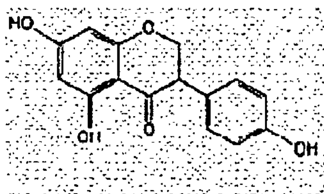

Figure 1.4.2: Chemical structure of genestein (http://www.labmaster.fi/products/tr-fia-kits/genistein-tr-fia.htm)

Phytoestrogens are produced naturally in several classes of plants such as legumes, cereals, grasses and herbs. These substances regulate plant hormones, deter herbivores and they shield plants from damage by UV radiation. There are several groups of phytoestrogens; isoflavones, lignans, coumestans, chalcones and resorcyclic acid lactones (Laganà *et al.*, 2004), the first two groups being given the most attention in terms of endocrine disruption. Isoflavones are synthesized in soybeans and other legumes and include genistein (Figure 1.4.2), daidzein and equol. Lignans are found in flaxseed and include enterolactone and enterodiol. Coumestans are produced in sprouting plants such as alfalfa (Lephart *et al.*, 2005).

The EC_{50} value of the phytoestrogen coumestrol was derived to be 77 times less potent than E2 by the yeast assay (Gaido *et al.*, 1997), and 900-1000 times less potent than E2 by the E-screen assay and luciferase reporter gene assay (Gutendorf and Westendorf, 2001). The authors also analysed genestein and found it to be 7,600 times less potent as E2 in the luciferase reporter gene assay with MVLN cells, 8,000 times less potent in the E-screen and 10,000 times less potent in competitive binding with the ERα receptor. Genestein was found to be approximately 11,000 and 12,500 times less potent than E2 in the YES assay by Matsui *et al.* (2000) and Tanaka *et al.* (2001).

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1.5 Human hormone metabolism

Vertebrates synthesise steroids via a pathway that involves the progressive degradation of cholesterol to progestins, then androgens, for example testosterone, and finally to oestrogens such as E2. This pathway is common to both males and females, and circulating plasma concentrations of androgens and oestrogens relate to the rate of conversion between the two. It is the ratio of androgens to oestrogens that creates a male versus female hormonal environment (Fairbrother, 2000).

The endogenous steroidal oestrogens E2 and E1 are mainly produced in the ovary and testis and in both humans and mammals oestrogens undergo various transformations, mainly in the liver. Frequently they are oxidized, hydroxylated, deoxidised and methylated before conjugation with glucuronic acid or sulphonic acid and excretion as glucuronides and sulphonides (Mao *et al.*, 2004). For instance, E2 is rapidly oxidized to E1. (Ternes *et al.*, 1999b). Oestrogens are principally excreted as inactive polar conjugates. E2 is predominantly found as E2-3-glucuronide, E1 as E1-3-sulphate and E3 as E3-16-glucuronide. Studies on the pharmacokinetics of EE2 have shown that a percentage (1-26%) of the drug is excreted in the free form (Johnson *et al.*, 2000). Metabolites are primarily glucuronides or sulphates and a small proportion of sulpho-glucuronides (Mao *et al.*, 2004).

Free oestrogens were not detected in female urine in a study of oestrogens excreted daily by 72 women (D'Ascenzo *et al.*, 2003). Oestrogen glucuronides accounted for approximately 80% of the total oestrogen derivatives while the remaining 20% was made up of oestrogen sulphonides.

Discrepancies exist among results of studies on oestrogen and oestrogen metabolites excreted in urine. A study carried out on 150 women by Key *et al.* (1996) found that women naturally excrete 3.5μg/day of E2, 7μg/day of E1, and 4.8μg/day E3. Based on earlier studies, Johnson *et al.* (2000) assumed that females excreted approximately 3.5μg/day of E2, 8μg/day of E1 and 4.8μg/day of E3. Pregnant women were assumed to excrete 259μg/day of E2, 600μg/day of E1 and 6000μg/day of E3. Mao *et al.* (2004) examined oestrogen levels in 10 men and 10 women. They detected averages of 2240μg/l E2, 770μg/l E3 and 60μg/l 17α-EE2 among females. Interestingly, a higher concentration of 1,300μg/l 17α-EE2 was detected in males.

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1.6 Classification of Endocrine Disruptors

The European Union defines an endocrine disruptor as an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function (EU, 1996). However, how can an effect be categorized as being adverse? An endocrine disruptor may cause an effect which may not be adverse, but the exogenous substance in question is still an endocrine disruptor. Kavlock *et al.* (1996) provided the following definition "An environmental endocrine or hormone disruptor may be defined 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 behaviour". Endocrine disruptors can be differentiated into two categories; natural and synthetic (Figure 1.6).

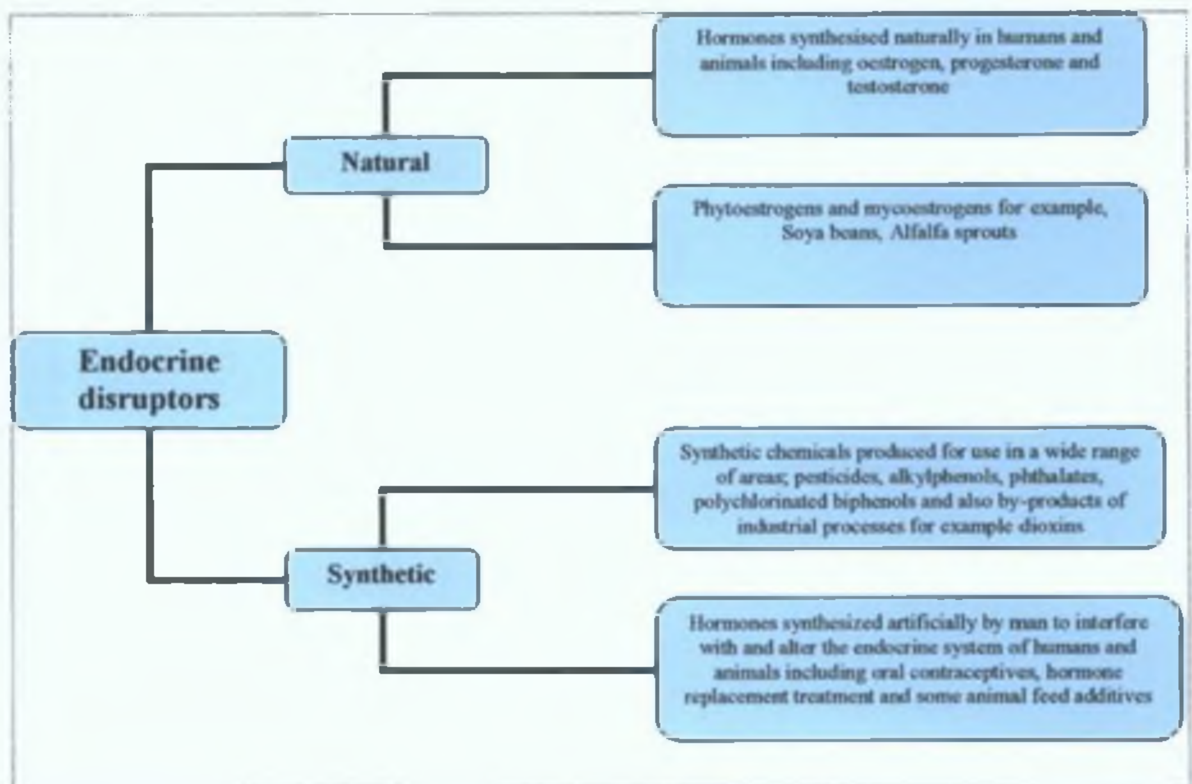

Figure 1.6: Classification of endocrine disruptors.

Endocrine disrupting chemicals can also be differentiated according to those that were intentionally designed to disrupt the endocrine system for example, oral contraceptives, hormone replacement therapy and pesticides, and those that were designed for other purposes or became altered through use or disposal and disrupted endocrine function as a secondary effect.

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1.6.1 Xenoestrogens

1.6.1.1 Bisphenol A (BPA)

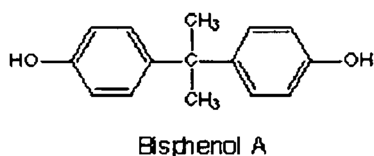

Figure 1.6.1.1: Chemical structure of Bisphenol A (http://website.lineone.net/~mwarhurst/bisphenol.html)

Bisphenols are hydroxylated diphenylalkanes consisting of two phenolic rings joined together by a bridging carbon atom (Figure 1.6.1.1). BPA is used to make epoxy resins and polycarbonate plastics. The resins are used to line the inner surface of food cans and bottle tops. The plastics are used to make food and beverage containers (Goodson *et al.* 2002). BPA is used in some polymers used in dental treatment. BPA can be leached from the coating of tin cans when they are autoclaved after canning. Goodson *et al.* (2002) reported concentrations of bisphenol A in 62 different canned foods ranging from 7μg in creamed rice to 422μg in ham. Leaching of BPA can also occur from reusable containers including polycarbonate baby bottles (Sun *et al.*, 2000) and polycarbonate animal cages used in laboratories (Howdeshell *et al.* 2003).

Using the E-screen bioassay and the luciferase reporter gene assay Gutendorf and Westendorf (2001) determined BPA to be 40,000 times less potent than E2. Körner *et al.* (2001) found it to be 19,5000 times less potent than E2 in the E-screen assay. Its potency was shown to be significantly higher in the YES assay with a potency 10,000-17,000 times lower than that of E2 (Gaido *et al.*, 1997; Sohoni and Sumpter, 1998; Matsui *et al.*, 2000 and Saito *et al.*, 2002).

1.6.1.2 Phthalates

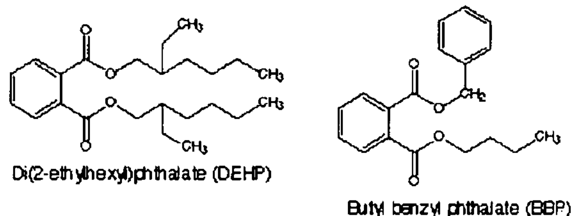

Figure 1.6.1.2: Chemical structure of Di(2-ethylhexyl)phthalate and benzylbutylphthalate (http://website.lineone.net/~mwarhurst/phthalates.html)

Some of the more important phthalate esters regarding oestrogenic potency include benzylbutylphthalate (BBP) (Figure 1.6.1.2), dibutylphthalate (DBP), diisobutylphthalate and diethylphthalate (DEP) and di(2-ethylhexyl)phthalate (DEHP).

Phthalate esters are used in intravenous tubing and as softeners in the manufacturing of polyvinyl chloride to give flexibility and workability. They are also found in vinyl flooring, emulsion paint and frequently in the ink used to print on board, plastic and foil-packed products and some adhesives used in packaging (http://www.checnet.org/healthehouse/chemicals/chemicals). Lower molecular weight phthalates have the least stability as plasticisers and can migrate from a polymer matrix particularly if the plastic is heated or it is in a lipophilic medium (Harris *et al.*, 1997).

Harris *et al.* (1997) found BBP, the most potent phthalate was found to be approximately one million times less potent than E2 in the YES assay. This discrepancy can be attributed to the submaximal dose-response curve produced by BBP which does not lend itself to potency derivation. In the oestrogen receptor (mediated)-chemical activated luciferase gene expression (ER-CALUX) assay BBP was found to have a potency approximately 714,000 times lower than E2 (Legler *et al.*, 2002) which is in closer agreement with results obtained by Harris *et al.* (1997). DEHP, the most extensively used phthalate tested negative in both the ER-CALUX assay and YES assay.

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1.6.1.3 Polycyclic Aromatic hydrocarbons (PAHs)

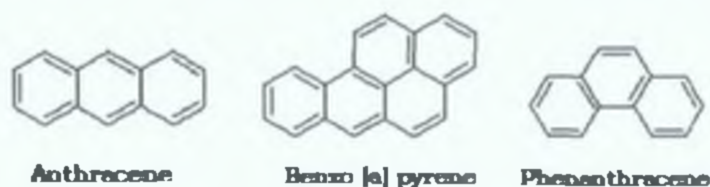

Figure 1.6.1.3: Chemical structure of anthracene, benzopyrene and phenanthracene (www.csmt.ewu.edu/csmt/chem/jcorkill/PAH.html)

The basic structure of polycyclic aromatic hydrocarbons (PAHs) consists of fused benzene rings (Figure 1.6.1.3). Fertuck *et al.* (2001) found that heterocyclic PAHs which contain an oxygen, sulphur, or nitrogen atom in one of the rings possess oestrogenicity.

PAHs are ubiquitous environmental pollutants. All but one PAH is produced intentionally. Naphthalene is produced as a mothproofing agent, in the manufacturing of dye-stuffs and in the synthesis of phthalates (Rogers, 1996). PAHs form upon incomplete combustion of organic compounds, the main source of PAHs in the air being incomplete combustion of wood and fuel for residential heating. PAHs are found in vehicular exhausts, coal tar pitch (used in aluminium smelting, roofing and surface coatings), coke oven emissions, bitumens, industrial smoke and soot, creosote (used in marine pilings, telephone poles and to preserve railroad ties), byproducts of open fires including natural forest fires, refuse burning and cigarette and cigar tobacco and smoke (Environment Australia, 1999).

1.6.1.4 Polychlorinated Biphenyls (PCBs)

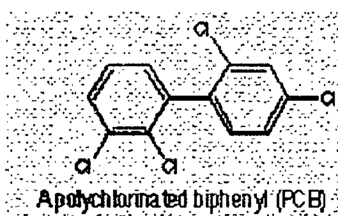

Figure 1.6.1.4: Chemical structure of a polychlorinated biphenyl (http://website.lineone.net/~mwarhurst/pcb.html)

There are 209 congeners. PCB metabolites are hydroxylated PCBs (OH-PCBs) or non-hydroxylated PCBs (Figure 1.6.1.4). PCBs were widely used in electrical capacitors, transformers and electrical insulating materials, as wax polishes, high temperature lubricants and asphalts (Rogers, 1996). Their extensive use was due to their chemical inertness, thermal stability and excellent dielectric properties. PCBs are very resistant to biodegradation, they are highly lipophilic and have the potential for bioaccumulation (Birkett, 2003b).

Soto *et al.* (1995) carried out a study using the E-screen and discovered several novel xenoestrogens among antioxidants, plasticizers, polychlorinated biphenyl congeners and pesticides. The authors found that hydroxylated PCBs were more potent than non-hydroxylated PCBs. Oestrogenic potency of metabolites is dependent on the ortho-Cl and para-OH substitutions on the rings (Birkett, 2003b). Layton *et al.* (2002) determined the potency of several PCBs using a modified YES (in which the plastic 96-well flat-bottom plates were replaced with glass vials) and found that the most potent PCB was five magnitudes of power lower than E2.

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1.6.1.5 Pesticides

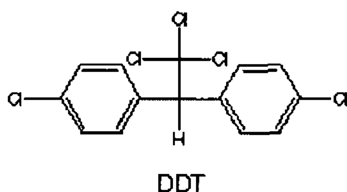

Figure 1.6.1.5: Chemical structure of a pesticide (http://website.lineone.net/~mwarhurst/ddt.html)

Pesticides represent the most extensive group of endocrine disruptors and potential endocrine disruptors. Dichlorodiphenyltrichloroethane (DDT) (Figure 1.6.1.5) was one of the most commonly used insecticides worldwide from 1946 to 1972 until it was banned. It was used to control insect pests such as the Colorado potatoe beetle and the European cornborer. It was also used to control typhus, malaria, body lice and other vector diseases in humans. It was banned due to persistence in the environment and accumulation in the food chain of it and its metabolite, DDE (Agency of Toxic Substances and Disease Registry, 2002). However DDT is still used in African countries such as Morrocco, some parts of Asia and Latin American countries such as Equador and Brazil (Wandiga, 2001; Zumbado *et al.*, 2005). Other insecticides include heptachlor, lindane, kepone and atrazine.

Sohoni and Sumpter (1998) and Legler *et al.* (2002) determined that o'p'-DDT had a potency approximately 100,000 times lower than E2 in the YES Assay and the ER-CALUX assay. In a carp hepatocyte assay o'p'-DDT was found to be 5,000 times less potent than E2 (Smeets *et al.*, 1999a).

1.6.1.6 Dioxins

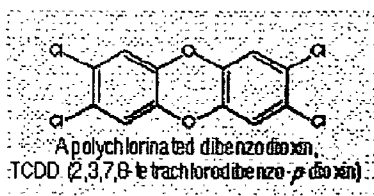

Figure 1.6.1.6: Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (http://website.lineone.net/~mwarhurst/pcb.html)

There are seventeen dioxins; seven are polychlorinated dibenzodioxins (PCDDs) and ten are polychlorinated dibenzofurans (PCDFs). Polychlorinated dibenzodioxins and polychlorinated dibenzofurans are not manufactured purposely but are produced unintentionally through waste incineration, metal production, fossil fuel and wood

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combustion, paper and pulp bleaching and production of certain herbicides (Agency of Toxic Substances and Disease Registry, 1999). The most important group of dioxins are the 2,3,7,8-congeners including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Figure 1.6.1.6) which has the greatest biological activity and toxicity of the group. Dioxins are persistent pollutants and have the potential for bioaccumulation (Birkett, 2003b).

1.6.1.7 Alkylphenolic compounds

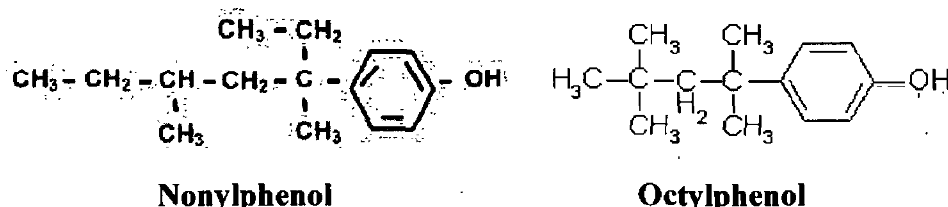

Figure 1.6.1.7: Chemical structure of 4-nonylphenol (www.aperc.org/productinfo.htm) and 4-octylphenol (www.cheric.org/research/kdb/hcprop/showprop.php?cmpid=888)

The basic structure of an alkylphenol is an alkyl group which can vary in size, branching and position joined to a phenolic ring (Figure 1.6.1.7). Alkylphenol polyethoxylates (APEOs) are non-ionic surfactants comprised of a branched-chain alkylphenol which has reacted with ethylene oxide to form a polyoxyethylene derivative (Routledge and Sumpter, 1996). Nonylphenol (NP) and octylphenol (OP) are primarily used to make alkylphenol ethoxylate (APEO) surfactants i.e. detergents and are also used as plasticisers. In Europe APEOs are used in industrial detergents, and outside of Europe they are also used in many domestic detergents. They are also used in the spermicide nonoxynol-9, pesticide formulations and some laboratory detergents (www.aperc.org).

NP and OP (Figure 1.6.1.7), their ethoxylates NPnEO and OPnEO and carboxylates NPEC and OPEC have been analysed in various oestrogen assays producing varied results. Matsui *et al.* (2000) and Saito *et al.* (2002) found that NP had a potency 1,000 times lower than that of E2. Sohoni and Sumpter (1998) determined NP to have a potency approximately 10,000 times lower than E2 in the YES Assay while Folmar *et al.* (2002) derived a potency almost 140,000 times lower than E2 using the same assay. Regarding the E-screen assay, Körner *et al.* (2001) reported that NP was approximately 13,000 times less potent as E2 however Gutendorf and Westendorf (2001) reported a potency 80,000 times lower than that of the standard. Results for

relative potencies of OP did agree ranging from 10,000-13,000 times less potent than 17β-E2 between the two studies using the E-screen.

1.7 Structure and potency

The human oestrogen receptor has relatively low specificity as its binding pocket is nearly twice as large as the molecular volume of oestradiol (E2) (Brzozowski *et al.*, 1997). Thus, space is provided for a variety of other molecules to interact with the oestrogen receptor (ER). It is very likely that ERs of all vertebrate species are rather similar in their non-specificity of the ligand-binding domain (Brzozowski *et al.*, 1997).

There is a wide structural variety between natural steroids, for example, E2, E1, and E3, and their natural (phytoestrogens, mycoestrogens) and anthropogenic impostors such as pesticides, herbicides, polychlorinated biphenyls, phthalates, plasticizers, phenols, anilines, combustion products (for example, dioxins) and breakdown products of surfactants. Environmental endocrine disrupting compounds are not necessarily structurally related to the naturally occurring steroids which make identification of these compounds almost impossible based on chemical structure alone (Routledge and Sumpter, 1996).

Schultz *et al.* (2002) developed structure activity relationships for oestrogenicity based on 120 aromatic chemicals in the *Saccharomyces cerevisiae*- based Lac-Z reporter assay and identified three specific 2-D structural features related to xenoestrogen activity and potency:

(i) The hydrogen-bonding ability of the phenolic ring mimicking the A-ring

(ii) A hydrophobic core of size and shape like that of the B and C rings

(iii) A hydrogen-bond donor imitating the 17β-hydroxyl moiety of the D-ring with an oxygen-to-oxygen distance comparable to that between the 3- and 17β-hydroxyl groups of E2. An aromatic A-ring with an hydroxyl group at position 3 was the most important structural requirement for high affinity binding to the oestrogen receptor (Schultz, 2002).

Fang *et al.* (2001) and Miller *et al.* (2001) reported similar findings in structure-activity relationship analysis on 230 chemicals including natural and xenoestrogens using an ER competitive binding assay.

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1.8 Endocrine disrupting modes of action

Endocrine disruption can occur in many ways when an endocrine disrupting compound alters the natural hormonal processes of the endocrine system by interacting with hormone receptors. A hormonal response is a multi-step process which can be disrupted at any point by stimulation or inhibition of any particular stage in the sequence. There are various mechanisms; some direct and some indirect as are present in Figure 1.8;

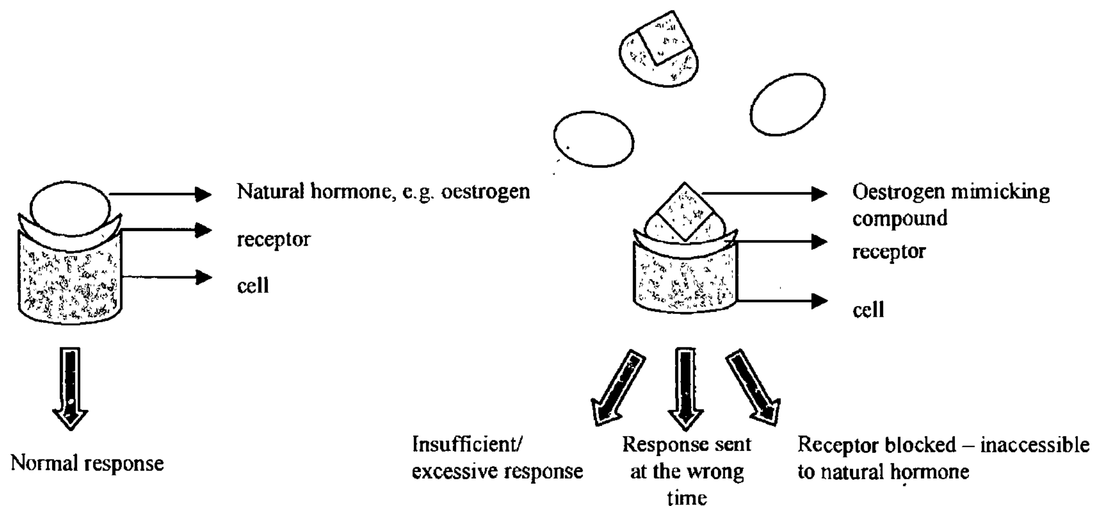

Figure 1.8: Schematic drawing of normal hormonal response and abnormal responses produced by oestrogen mimicking compounds

Direct

- Mimicking or partly mimicking oestrogens and androgens by fitting into the hormone receptor and sending messages to receiving genes. Messages sent at the wrong time or overproduction of messages can badly affect biological functions. These endocrine disruptors are classified as environmental oestrogens, or oestrogen mimicking compounds. This is defined as an agonistic effect.
- Binding to the receptor site without activating it, effectively blocking the natural hormone from binding. The extent of the effect on the genes depends on whether the blocking endocrine disruptor is more or less potent than the hormone being blocked. The reason why they are not agonists is because they cannot provide the molecular interaction required for activation. These endocrine disruptors are anti-oestrogens or anti-androgens. This is defined as an antagonistic effect.

Indirect

- Modifying the number of hormone receptors in a cell by stimulating the formation of extra hormone receptors on or within cells. The number of hormone signals increases, thus amplifying the response of natural and foreign hormones.
- Accelerating the breakdown and elimination of hormones from the body or conversely, destroying the enzymes needed to naturally breakdown hormones allowing surplus hormones to remain in the body. Too many messages are sent to receiving genes and/or sent at the wrong time. (e.g. oestrogens in testis).
- Destroying the hormone in such a way that its structure is altered preventing the hormone from fitting into its receptor site.
- Influencing and altering natural hormone production by interfering with other hormone systems such as the thyroid system or the immune or nervous systems (Birkett, 2003a). Vingerhoets *et al.* (1998) reported associated diethylstilbestrol exposure in female offspring of women who had taken DES with immune system suppression.

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Some endocrine disrupting compounds are capable of acting via more than one mechanism. Some endocrine disrupting compounds are pure agonists, some are pure antagonists while others are partial agonists and partial antagonists and are known as Selective Estrogen Receptor Modulators (SERMs). SERMs can be tissue selective agonsim/antagonism for example tamoxifen is oestrogenic (agonistic) in uterine, liver and osteoblastic cells but it anti-oestrogenic (antagonistic) in breast cells (Inal *et al.*, 2005).

There are a number of factors which affect the potency of a compound including:

- the ability of the compound to permeate membranes and reach the receptor (Hamblen *et al.*, 2003)
- the affinity of the compound for the oestrogen receptor which depends on its structural features and chemical properties
- the accumulation of the compound in the body or environment which can relate to chemical properties such as solubility, and hydrophobicity (Arnold *et al.*, 1996).

1.9 Introduction of oestrogenic compounds to wastewater

The wastewater treatment process acts as a means of access for endocrine disruptors to the aquatic environment due to the partial or complete resistance of endocrine disruptors during the treatment process. Natural oestrogens and progesterones are excreted primarily in the urine of humans and animals but also in the faeces in smaller quantities. The use of oral contraceptives, hormone replacement therapy, surfactants, plasticizers, pesticides and agricultural growth enhancers contribute to the amount of natural oestrogens and progesterones already present. Synthetic oestrogens and progesterones such as ethinyl-estradiol and progestogen respectively are excreted in the urine of females using oral contraceptives. Urban areas are important sources of synthetic chemicals due to industrial emissions, car exhausts, combustion processes and natural background atmospheric deposition. Rainwater run-off collects the organics deposited on the ground and combines with wastewater draining from these areas in the sewerage system. All the aforementioned endocrine disruptors can then enter the aquatic system via the effluent of sewage treatment plants, or industries permitted to discharge effluent directly to waterways. The disposal of dry sludge onto land and the release of volatile organic compounds into the atmosphere also contribute to this source of pollution. Non-point sources include urban and agricultural run-off (Ternes *et al.*, 1999a).

The compounds deemed responsible for the oestrogenic activity of wastewaters are the natural hormones; oestrone(E1), oestradiol (E2), and oestriol (E3), the synthetic hormone, ethinyl-oestradiol (EE2) and to a lesser extent, the alkylphenols nonylphenol (NP) and octylphenol (OP)(Desbrow *et al.*, 1998; Synder *et al.*, 2001; Aerni *et al.*, 2004; Rutishauser *et al.*, 2004). Körner *et al.* (2000) determined that phenolic xenoestrogens accounted for only 0.7-4.3% of the total oestrogenic activity of effluent from a German wastewater treatment plant. NP and OP have exhibited oestrogenic potencies several magnitudes lower than that of E2 in various in-vitro assays for example comparison using the Recombinant Yeast Oestrogen Screen (YES) demonstrated 4-NP to have an oestrogenic potency three magnitudes less than that of E2 (Sohoni and Sumpter, 1998) and using the E-screen assay, Körner *et al.* (2001) assessed the oestrogenic potency of 4-NP and 4-7-OP to be $7.5x10^5$ times less than that of E2. However, when alkylphenols are present at concentrations higher than 1µg/l *in-vivo* studies have indicated that they could have an input similar to that of E1 or E2 (Johnson and Sumpter 2001). This is of concern particularly in areas where sewage treatment

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plants receive large inputs of trade waste containing alkylphenolic compounds. Sheahan *et al.* (2002a) detected 63 µg/l of NP and 230µg/l of nonylphenol metabolite in effluent of a sewage treatment plant being discharged into the Aire River, UK. These concentrations are extremely high compared to levels detected in effluent elsewhere; 0.05-1.31µg NP/l in a study of eight European countries (Johnson *et al.*, 2005) and 0.25-2.3µgNP/l in a German effluent (Spengler *et al.*, 2001).

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1.10 The wastewater treatment process

The function of sewage treatment systems is the rapid conversion of aqueous organic compounds into biomass which is then separated from the aqueous phase by settlement (Johnson and Sumpter, 2001). Sewage treatment is typically comprised of four stages (Figure 1.10) sometimes with an additional stage (tertiary treatment) to produce a higher quality effluent for example, if effluent is to be discharges to a river downstream of which there is an abstraction point for drinking water or if effluent is to be discharged into a lake or into groundwater (Gray, 1998). The population equivalent (p.e.) is based on the wastewater flow and the mass of biochemical oxygen demand that one person excretes per day (Smith and Scott, 2002).

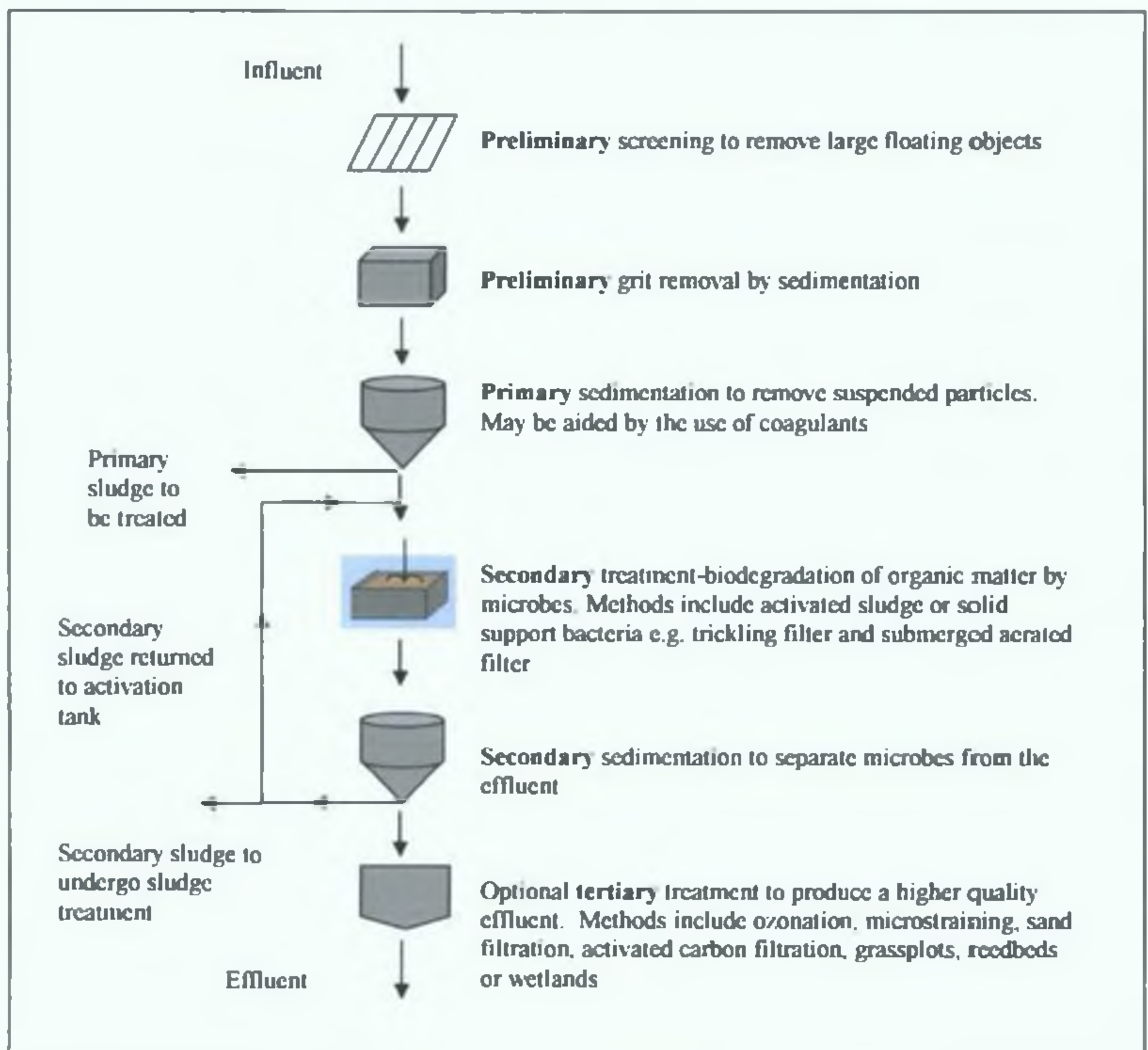

Figure 1.10: Schematic drawing of the wastewater treatment process

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1.10.1 Preliminary treatment

Preliminary treatment involves the removal of:

- Large floating objects (paper, plastic, wood etc) by inclined parallel steel bars in a process called screening
- Grit (dense material such as sand, gravel, and glass, metal, and plastic fragments) by sedimentation usually in cone-shaped separators by centrifugal force or in specialised shallow sedimentation tanks.

Most sewage treatment plants are equipped with storm-water tanks to prevent overloading the plant by retaining storm-water and allowing it to join the influent at a later period. Removal of fats, oils and greases is necessary in sewage treatment plants whose influents contain a large quantity of these substances. Removal occurs by dissolved air flotation tanks which employ the movement of minute air bubbles up through the flotation tank which become attached to the suspended particles of fats, oils and greases and carry them to the surface where they are removed by skimming (Gray, 1998).

1.10.2 Primary treatment

The purpose of primary treatment is to allow sedimentation of the wastewater (Langford and Lester, 2003). The sewage is clarified by flotation and settlement. Suspended matter of lesser density than water for example, fats, oils and greases will rise to the surface and form a scum which is removed by skimmer blades. Primary sedimentation is sufficient for the removal of small amounts of fats, oils and greases. Suspended matter of greater density than water will settle to form primary sludge which is usually scraped into a collection point form where it can be pumped. This can be aided by addition of coagulants. All wastewater treatment plants except very small rural plants have more than one primary sedimentation tank for maintenance purposes with the flow equally divided between the tanks whereas primary sedimentation does not occur in oxidation ditches.

1.10.3 Secondary treatment

The aim of secondary treatment is to allow the primary effluent (the overflow from primary treatment) to mix with a dense microbial population under aerobic conditions to allow the microorganisms, primarily bacteria, to convert organic matter into new cells (Gray, 1998). Three types of secondary treatment processes employed by wastewater treatment plants in this study are activated sludge (of which oxidation

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ditches are one variation), trickling (biological or percolating) filters and sequence batch reactors.

All activated systems consist of two vessels; a reactor tank containing large populations of microorganisms to convert the organic matter and a secondary sedimentation tank to clarify or separate the microorganisms from the final effluent by allowing solids to collect at the base of the tank where they are removed and pumped back into the reactor tank as return sludge to return microorganisms to the reactor tank (Langford and Lester, 2003). The reactor vessel in the trickling filter system consists of a circular tank containing small pieces of medium such as plastic in various shapes to a depth of approximately two metres and a distribution arm equipped with jets which rotates spraying the wastewater evenly over the surface of the bed. The microbial population is present in a biological film over the large surface area of the media and they remove the organic matter as it trickles down through the media. It is collected at the bottom and flows away to the secondary settlement tank. Oxygen is provided by natural ventilation whereas in activated sludge it is pumped into the wastewater by aerators. The secondary sludge in trickling filter systems is not returned to the reactor vessel. The sequence batch reactor activated sludge process consists of a series of reactor tanks in which biological treatment progresses in stages (United States Environmental Protection Agency, 1999c):

1. The filling of the reactor with primary effluent
2. The reaction stage during which biological degradation of the wastewater occurs
3. The settling stage during which solids are allowed to settle to the bottom of the reactor
4. The drawing off stage in which effluent is drawn out of the reactor
5. The idle stage during which there is no activity in the reactor until the cycle restarts once more.

A continuous flow is achieved when different reactor tanks are at different stages of treatment.

The hydraulic retention time is the flow of wastewater in relation to the capacity of the aeration tank. It is typically 5 hours at dry weather flow. The hydraulic retention time in European activated sludge sewage treatment plants is often in the range 4-14

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hours, while trickling filters which normally serve smaller towns and villages may have a hydraulic retention time of only 0.5hours ((Johnson and Sumpter, 2001). The sludge residence time or sludge age is the number of days that the micro-organisms remain both within the aeration tank and while being separated or returned (Gray, 1998). If nitrification (conversion of ammonia to nitrate) is to occur a longer sludge residence and a greater input of oxygen is required to maintain the specific autotrophic bacteria involved; *Nitrosomonas* spp and *Nitrobacter* spp (Lester and Edge, 2000).

1.10.4 Tertiary treatment

The main methods of tertiary treatment are:

- Sand or gravel filtration
- Microstraining
- Irrigation onto grasslands
- Reedbeds or wetlands
- Prolonged settlement in lagoons
- Activated carbon

Ozonation, ultra-violet treatment or membrane filtration are used if disinfection of the effluent is necessary. Nutrient removal by addition of chemical precipitants such as ferric chloride, lime or aluminium sulphate for the removal of phosphorous can be incorporated as a tertiary treatment step. The use of activated carbon employs the process of adsorption whereby soluble molecules become attached to the surface of the activated carbon by Van der Waals forces and electrical attraction facilitating their removal (Gray, 1998). The carbon source (bituminous coal, peat or wood) has a large surface area and a rough external surface. It can be used in a powder or granular form and must be activated before use. Powdered activated carbon can be added to the wastewater at various stages of treatment to improve floc structure of activated sludge to increase settleability (Gray, 1998). Granular activated carbon is used in the form of a filter bed and its regeneration after use is possible.

1.10.5 Sludge treatment and biosolids

As primary and waste activated sludge may be 95-96% and 98% water respectively, the first step in sludge treatment involves thickening to reduce the volume of sludge. Coagulants and polyelectrolytes (such as lime, ferric chloride, ferric chloride and aluminium chloride) are sometimes added prior to dewatering. Thickening is

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carried out using flotation, centrifugation, lagooning and the most common method is gravity settlement, whereby sludge is slowly stirred in a circular tank which enhances particle settlement, while water is forced out at the bottom of the tank under the weight of the sludge. Dewatering of the sludge (the most widely used methods being filter and belt presses, vacuum filtration, drying beds, lagoons, centrifugation and reed beds) can occur after thickening but usually occurs after the following stage of sludge treatment which is stabilization (Gray,1998; Scrimshaw and Lester, 2003). Stabilization results in a stable, inoffensive product with significantly reduced numbers of viable pathogens thereby reducing health risks associated with re-use. There are various stabilization processes including anaerobic digestion, aerobic digestion, composting, lime treatment and heat treatment.

Biosolids- the biological processing of wastewater solids.

Biosolids are the nutrient-rich organic by-product of the stabilization of sewage sludge that have met specific quality criteria and regulations and are therefore, considered safe for use as fertilizer and as a soil amendment to improve and maintain productive soils and stimulate plant growth.

Past disposal practices commonly included land filling, dumping at sea or incineration. Biosolids are rich in organic matter and nutrients and thus recycling through application on land is a means of avoiding the environmental and economic costs of disposal (Bright and Healey, 2003). In agriculture, biosolids applied at approved rates help improve crop growth and yield. Biosolids condition the soil and can supplement or replace commercial fertilizers (Jensen and Jepsen, 2005). Use of biosolids can help to overcome irrigation problems during dry weather and also to improve soil water holding capacity and porosity (USEPA, 1999a). Biosolids can be used to reclaim mining sites as they can help re-establish vegetation at abandoned mine sites where there is little or no topsoil Biosolids which have met the most stringent standards and are of exceptional quality are considered landscape grade, and are used on gardens, flowerbeds, golf courses and city parks (USEPA, 1999a).

However, the use of treated sewage sludge for soil amendment poses potential hazards such as:

- odour problems,
- contamination of groundwater,

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- run-off of contaminants into surface waters,
- possible impact on human health by uptake through crops or grazing and soil contamination by metals, organic compounds and also pathogens (Wilson *et al.*, 1996; USEPA, 1999a; Birkett and Lester, 2003a).

The concentration of pollutants in soils from land application of contaminated biosolids from water and wastewater treatment and agricultural practices is termed terraccumulation (Rooklidge, 2004).

Sewage sludge is the largest by-product of wastewater treatment. The presence and fate of endocrine disrupting compounds in this material is of important concern in the disposal and utilization of sewage sludge. The method of sludge treatment and stabilization will determine the fate of endocrine disrupting compounds in the treated sludge which is to be recycled. For instance, if sludge is incinerated, then it is assumed that any endocrine disrupting compounds present will be oxidized to carbon dioxide and water. Whereas, if the treated sludge is to be recycled, for example applied to agricultural land, it is likely that the parent endocrine disrupting compounds present or their metabolites will be of concern.

Maximum contact of biosolids with soil on application will minimize the risk of subsequent surface or bypass flow into surface or groundwater Colucci *et al.* (2001a). Sludge is incorporated into the plough layer as aggregates of 2-4cm sizes. These sludge aggregates are water saturated and may be anaerobic in their centers for certain periods of time. Hesselsøe *et al.* (2001) demonstrated that sludge aggregate size and oxygen availability will majorly influence degradation of the more lipophilic compounds such as nonylphenol. There is increased degradation if sludge aggregate size is decreased as oxygen can penetrate the aggregates at a faster rate. Hesselsøe *et al.* (2001) carried out experiments which demonstrated the importance of homogeneity in sludge-soil mixtures as radio-labeled nonylphenol was found to degrade within a homogenous sludge-soil mixture within 38 days while a period of over three months was required for similar degradation to occur in a non-homogenous sludge-soil mixture.

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1.11 Alteration/biodegradation of oestrogenic compounds in wastewater and sludge treatment

Since natural and synthetic steroids have been detected in effluents and in rivers in their free form, microbial deconjugation of the compounds must occur somewhere in the sewers prior to entering the sewage treatment plant or else further deconjugation of conjugated compounds occurs within the plant (Baronti *et al.*, 2000). D'Ascenzo *et al.* (2003) determined concentration ratios of free oestrogens to conjugated oestrogen in a condominium septic tank and at the entrance of the sewage treatment plant. The ratio increased from 0.91 to 2.0 suggesting that oestrogen deconjugation continued in the sewers i.e. the concentration of free oestrogens increased from the septic tank to the inlet of the treatment plant while the concentration of conjugated oestrogens decreased.

Panter *et al.* (1999) detected elevated vitellogenin levels in male fathead minnow (*Pimephales promelas*) maintained in simulated sewage treatment effluent to which the oestrogen metabolite E2-3-glucuronide had been added compared to controls containing a solution of E2-3-glucuronide in water. This suggested that E2-3-glucuronide is transformed to a free oestrogen by intense microbial activity in sewage treatment plants. Nasu *et al.* (2001) found a further increase in concentrations of free estrogens from influent to primary effluent in a Japanese study suggesting further deconjugation occurred in the sewage treatment plant. Similar findings were recorded (Ternes *et al.*, 1999a; Kirk *et al.*, 2002) which could be attributed to cleavage of oestrogen conjugates during primary sedimentation (Ternes *et al.*, 1999a). Belfroid *et al.* (1999) did not detect any glucuronide conjugated oestrogens in effluents of activated sludge sewage treatment plants when effluent and effluent treated with the enzyme β-glucuronidase (to deconjugate any glucuronide conjugates present) prior to analysis were compared. This indicates that deconjugation reactions occur during the treatment process.

Escherichia coli is an abundant bacterial strain in domestic wastewaters and Dray *et al.* (1972 cited Baronti *et al.*, 2000 p5064) found that *E. coli* can readily deconjugate steroids while synthesizing β-glucuronidase enzyme, an enzyme commonly found in bacteria in sewage (Ternes *et al.*, 1999b). A laboratory degradation test carried out by D'Ascenzo *et al.* (2003) using septic tank effluent found that oestrogen glucuronides undergo biodegradation in sewers much more readily than oestrogen sulphates. Glucuronated oestrogens were absent from the test liquor within one day while an incubation period of eight days was required for bacteria to completely

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biodegrade E3-3-sulphate, it being the most resistant sulphate species. The authors analysed influent at an Italian wastewater treatment plant and found that oestrogen sulphates represented 60% of the total conjugated oestrogen forms. However in a sub-study on female urine of 72 women oestrogen sulphates only accounted for approximately 20% of total oestrogen forms. This indicates that oestrogen glucuronides undergo deconjugation in the sewers forming free oestrogens leaving the more recalcitrant oestrogen sulphates as the dominant species of oestrogen conjugates on entry to the sewage treatment plant.

It has been reported that *E.coli* has a weak arylsulphatase activity (Baronti *et al.*, 2000) and hence should not biodegrade oestrogen sulphate as well as oestrogen glucuronides. Thus biodegradation of oestrogen sulphates is dependent on other bacterial strains in the activated sludge since the hydraulic retention times commonly employed in wastewater treatment plants (4-14hours) do not allow sufficient time for biodegradation of oestrogen sulphates by *E. coli*, the most prevalent bacteria in sewage.

Since E2 and E3 are primarily excreted as glucuronides and E1 as a sulphide (Ternes *et al.* 1999b; D'Ascenzo *et al.* 2003), the hypothesis exists that large quantities of E1-3-sulphate enter wastewater treatment plants and persist and/or undergo conversion to form free E1. Additional E1 is formed by oxidation of E2 during the treatment process. This could explain the relatively low removal rates of E1 occurring at sewage treatment plants. In a Japanese study, Isobe *et al.* (2003) analysed effluent and surface waters for oestrogen conjugates. Oestrogen glucuronides were not detected but oestrogen sulphates were present in all matrices.

1.11.1 Natural and synthetic steroidal oestrogens

1.11.1.2 Degradation of steroidal oestrogens

Lee and Liu (2002) proposed that biodegradation of E2 begins at the hydroxyl group at C17 on ring D of the molecule, eventually forming E1. Further oxidation of E1 causes cleavage of the D ring producing an hydroxyl acid which then forms an unknown metabolite thought to be a lactone which undergoes cleavage to form tricarboxylic acid.

E1 is often present in effluent at concentrations twice that of E2 (Johnson *et al.*, 2005). Körner *et al.* (2001) found the potency of E1 to be approximately 0.1 times that of E2 using the E-screen assay. Pawlowski *et al.* (2004) and Colucci *et al.* (2001a)

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determined the E2 equivalent (EEQ) to be half that of E2 by means of the YES Assay. E1 is the natural oestrogen which is least effectively removed by the sewage treatment process. Regarding *in-vitro* oestrogen potency and concentration, E1 is the most important endocrine disruptor (Johnson and Sumpter, 2001).

E2 undergoes biodegradation readily in sewage treatment plants forming E1 as a product of degradation. Various analysts have carried out studies demonstrating good removal efficiencies of sewage treatment plants in the removal of E2. Ternes *et al.* (1999b) carried out aerobic batch experiments with activated sludge and demonstrated that after 1-3 hours 95% of unconjugated E2 at concentrations of 1ng/ml and 1μg/ml was oxidised to E1 increasing the concentration of the E1 up to 95% with regard to the initial concentration of E2. After 5 hours neither hormone was measured above the detection limit. Recent studies in Japan on degradation of natural and synthetic oestrogens demonstrated that E2 was most easily degraded via E1 by nitrifying activated sludge (98% elimination of E2 at 1mg/l within 2 hours) compared to E1, E3 and EE2 (Shi *et al.*, 2004). More recently, Li *et al.* (2005) reported a reduction of approximately 99% of spiked E2 at concentrations of 10, 30 and 50μg/l in aerobic batch degradation tests after 1.5 hours.

Colucci *et al.* (2001a) carried out degradation studies of E2 and E1 in agricultural soils in Ontario, Canada and found that both hormones were readily biodegradable (without a lag phase) in soils under a range of temperature and moisture conditions. Similar to what occurs in sewage sludge, E2 was converted to E1 in the soil also. The authors predict that both hormones would undergo rapid dissipation in aerated agricultural soils following application of manures during a temperate growing season. A subsequent study revealed that removal of EE2 in soil was 2-7 times slower than E2 under similar conditions (Colucci *et al.* 2001b) however, both compounds were removed at comparably slow rates when soils were adjusted to their field moisture capacities. Results indicate that hormones will undergo little biodegradation in cold wet soils.

E3 has a lower oestrogenic potency than E1 or E2. It is not analysed for as often as E1 and E2 and there seems to be less concern for its presence in the environment. EE2 is an extremely potent oestrogen based on *in-vivo* studies. Purdom *et al.* (1994) demonstrated that vitellogenesis may arise from levels of EE2 as low as 0.1-0.5ng/l.

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However, *in-vitro*, its potency has been found to be similar to that of E2. Körner *et al.* (2001) found the potency of EE2 to be 0.9 times that of E2 using the E-screen Assay. Using the Recombinant Yeast Screen Assay, Pawlowski *et al.* (2004) found EE2 to have an E2 equivalency of 1.4. EE2 can be difficult to detect due to its minute concentrations which are sometimes very close to the detection limits of chosen analytical methods. Some methods are not sensitive enough to detect it (Johnson *et al.*, 2005). EE2 undergoes very slow biodegradation in the activated sludge system. Ternes *et al.* (1999b) detected elimination of only 20% after 24 hours at a concentration of 1ng/l in an aerobic batch experiment. Layton *et al.* (2000) found that removal of EE2 by mineralization in a Canadian wastewater treatment plant was only 20% compared to 75% removal of E2. This indicates that the ethinyl group inhibits degradation. Cargouët *et al.*, (2003) calculated a mean removal rate of 40% for EE2 in five wastewater treatment plants in Paris.

1.11.1.3 Degradation of phytoestrogens

Splenger *et al.* (2001) detected genestein in effluent at levels up to 38ng/l in a study on 18 German WWTPs. Laganà *et al.* (2004) detected the phytoestrogens dadzein, genistein and biochain A in influents ranging from 8-384ng/l, in effluents; 3-83ng/l and Tiber River water at 1-7ng/l. Genistein was present at the highest concentrations in all matrices. The input of phytoestrogens is possibly due to vegetable material leachate being washed away by rain, irrigation or in tap water (Laganà *et al.*, 2004).

1.11.2 Xenoestrogens

1.11.2.1 Bisphenol A (BPA)

Splenger *et al.* (2001) measured concentrations of bisphenol A up to 1.0μg/l in a study on German effluents. Aguayo *et al.* (2004) detected 0.30 to 5.6μg/l bisphenol A in effluents of seven Spanish wastewater treatment plants. The octanol-water co-efficient of bisphenol A is 3.4 with a relatively high solubility of 120-300mg/l (at 20°C). Rapid biodegradation of bisphenol A in aerobic biological treatment systems have been reported. Vethaak *et al.*, (2005) reported up to 91% removal of bisphenol A in 12 WWTPs in the Netherlands with levels in municipal effluent ranging from <43-4090ng/l and <19-800ng/l in industrial effluent.. Saito *et al.* (2004) reported that an extracellular enzyme, laccase, isolated from a fungus found in soil is capable of

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oxidizing both bisphenol A and nonylphenol and eradicating their oestrogenic potency within 24hours of incubation with the strain.

1.11.2.2 Phthalates

The octanol-water co-efficients of phthalates range from 1.46 to 13.1. Lipophilicity and octanol-water co-efficients of phthalates increases with increasing alkyl chain length (Cousins *et al.*, 2000). The most ubiquitous phthalate is di-(2-ethylhexyl) phthalate (DEHP), it has an octanol-water co-efficient of 7.5 and extremely low water solubility facilitating its sorption to sludge (Langford and Lester, 2003). DEHP was present in 6 out of 7 effluents in a study in Spain (Aguayo *et al.*, 2004). Vethaak *et al.* (2005) reported that phthalates present in the highest concentrations (diethylphthalate (DEP) and DEHP) in influents of 12 Dutch WWTPs were those with the lowest oestrogenic potency and that concentrations were generally reduced to under 1μg/l with the exception of DEHP (1.5 μg/l).

De Jonge *et al.* (2002) reported that DEHP binds strongly to the sludge phase and that the extent of leaching increases with the clay content of the soil. DEHP has poor solubility in water and is degraded relatively easily under aerobic conditions. It has a half life of 5 to 85 days (EC, 2001a). Petersen *et al.* (2003) carried out a study in which sludge bands (two-dimensional sludge particles) containing 55mg/kgDM DEHP among other organic contaminants were placed 6-10cm soil depth in sandy and loamy soil in Southwest Denmark for a period of 3 years. Approximately 40% of the initial concentration of DEHP was still present at six months with only 5-6% remaining after 12 months. Uptake of DEHP by barley grown on the plots was not considered to be significant as there was no relationship between waste and crop concentrations. The EU has set the maximum limit value of di-(2-ethylhexyl)phthalate in sludge for use on land at 100mg/kg dm (EU, 2000).

1.11.2.3 Polyaromatic hydrocarbons (PAHs)

PAHs have log octanol-water co-efficients of approximately 6.0 for example, benzo(k)fluoranthene, benz(a)anthracene and benzo(a)pyrene (Birkett, 2003b) which makes them resistant to degradation.

During primary treatment PAHs tend to partition onto suspended solids while during secondary treatment volatilization and biodegradation are the main methods of

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removal. PAHs of higher molecular mass are more resistant to decay (Manoli and Samara 1999). Pérez *et al.* (2001) analysed sludge from six wastewater treatment plants in Spain and Portugal for 16 different PAH compounds and found phenanthrene to be the most dominant in all wastewater treatment plants. The total load of PAHs in sludge ranged from 1.13-5.52mg/kg.

Most PAHs are very persistent in soils and may have half-lives of up to 10 years, however, uptake by crops is low. They are relatively insoluble in water indicating that the risk of leaching into groundwater is low (EC, 2001a). The maximum concentration of total PAHs in sludge to be used in agriculture is 6mg/kg dm under the Council of the EC Working Directive on Sludge (2000).

1.11.2.4 Polychlorinated biphenyls (PCBs)

PCBs have octanol-water co-efficients ranging from 4.6 to 8.4 indicating that these compounds partition to solids and can accumulate in sludge (Birkett, 2003b). Katsoyiannis and Samara (2004) observed a linear relationship between the removal of PCBs (particularly during primary treatment) and their log K_{ow} values which indicated that compounds with such high log K_{ow} values are principally removed by sorption onto the primary sludge. Removal of PCBs increase as the sludge age increases (Swiss Federal Institute for Environmental Science and Technology (EAWAG), 2003; Langford *et al.*, 2005).

Katsoyiannis and Samara (2004) reported that the total PCB concentration in influent of a Greek activated sludge WWTP decreased from 1000ng/l to 630ng/l following primary sedimentation to 250ng/l following secondary sedimentation with the most abundant PCB congeners being PCB-52, PCB101 and PCB-180 (with total removal rates ranging from 65-81%). Conversely, the total concentration of PCBs increased from the primary sludge (460ng/gdw) to the final sludge (550ng/gdw) which was treated anaerobically. De Souza Pereira *et al.* (2005) reported PCB concentrations of 57.6-145mg/kg in sludge from two Brazilian WWTPs which are at least 70 times greater than the maximum limit value for total PCB concentration (sum of seven indicator congeners) at 0.8mg/kg dm set by the EU (2000). Germany, Switzerland and the Netherlands have set more stringent maximum concentrations of individual PCB congeners in sludge of 200μg/kg (De Souza Pereira *et al.*, 2005).

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PCBs are stable physically, chemically and biologically and due to their lipophilic nature they tend to bind to organic matter in soil and will undergo slow biodegradation. Highly chlorinated PCBs can persist in soil for longer periods than less chlorinated PCBs. They can be introduced into the surface waters via runoff containing soil to which PCBS are bound. Uptake by plants appears to be very low (EC, 2001a).

1.11.2.5 Pesticides

Katsoyiannis and Samara (2004) analysed influent for 19 different pesticides including quntiozene, dieldrin, heptachlor, and *p-p'*-DDE at concentrations ranging from 1.4-330ng/l. Removal rates ranging from 75%-91% were reported from the influent to the secondary sedimentation tank effluent. Chlorinated pesticides such as dieldrin, aldrin, heptachlor and hexachlorobenzene were still detected despite being banned. DDT was detected in approximately 30% of influents and was not detected at any further stage of treatment due to conversion to *p-p'*-DDD and *p-p'*-DDE metabolites. Pesticide concentrations increased from the primary sludge to the final sludge (treated anaerobically) which contained levels ranging from 5-270ng/g dw.

1.11.2.6 Dioxins and furans

De Souza Pereira *et al.* (2005) detected concentrations of 1107pg/g of total PCDDs and 206 pg/g of total PCDFs in a Brazilian WWTP in a semi-rural area with higher concentrations of 3735pg/g of total PCDDs and 414pg/g of total PCDFs in an urban plant.

Like PCBs, dioxins and furans are lipophilic compounds which are physically, chemically and biologically stable. They bind to the organic matter in soil and are found in the topsoil. They are not expected to leach into groundwater due to their highly lipophilic nature. A maximum limit concentration of 100ng toxicological equivalent (TE)/kg dm PCDDs and PCDFs in sludge for use on land has been set by the EU under the Working Draft on Sludge (2000).

1.11.2.7 Alkylphenols and surfactants

Anionic surfactants linear alkylbenzene sulphonates (LASs) and their precursors linear alkylbenzenes (LABs) have been found in sewage sludge at relatively high concentrations (Petersen *et al.* (2003) detected concentrations of LAS of 2870mg/kgdm in sewage sludge in Denmark). There is an average removal of LASs of 15-35% by

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precipitation during primary sedimentation to the primary sludge (Petrovíc and Barceló, 2004).

Levels of 4-nonylphenol, 4-nonylphenol diethoxylate, and 4-nonylphenoxyacetic acid up to 2.3µg/l, 5.5µg/l, and 5.8µg/l were reported in effluents in a study on 18 German WWTPs (Splenger *et al.*, 2001). Solé *et al.* (2000) detected much higher concentrations in effluents of four Spanish WWTPs at 6-289µg/lNP, 24-938µg/LNPEO and 4-80NPECµg/l. Nonionic surfactants alkylphenol polyethoxylates (AP_NEO, n=6-40) are biodegraded via shortening of the ethoxylate chain, the ethoxylate groups being cleaved and/or oxidized one by one. The lipophilicity of the metabolite and its resistance to biodegradation increases as the ethoxylate chain gets shorter due to the presence of the benzene ring and their limited water solubility (Langford *et al.*, 2005). The resulting metabolites include short chain alkylphenol ethoxylates (APEO), their carboxylic acid derivatives (APECs) and alkylphenols (APs) such as octylphenol and nonylphenol. APECs and longer chain APEOs are quite water soluble and can be detected in effluents at high concentrations (Solé *et al.*, 2000; Ahel *et al.*, 2000; Ahel *et al.*, 1994) whereas the shorter chain compounds and the alkylphenols are more likely to adsorb onto particulates. NP has an octanol water co-efficient value of 4.48 indicating it has a high tendency to become adsorbed to the sludge.

In a study by La Guardia *et al.* (2001), 11 biosolids were analysed for OP, NP, NP_1EO and NP_2EO in 10 of which NP was the most abundant APEO metabolite detected representing >84% of the total APs and NPEOs. Average NP concentrations were 49mg/kg while average OP concentrations were 6.2mg/kg. The authors found that biosolids treated by anaerobic digestion obtained the highest levels of NP (754mg/kg) which was almost twice that of biosolids treated by heat (496mg/kg) and lime (470mg/kg) and 12 times higher than composted biosolids (64mg/kg). Approximately 63% of all nonylphenolic compounds that enter wastewater treatment plants are discharged in the form of NP_1EO and NP_2EO and NP, carboxylated deriviatives (NPECs) or untransformed NPEOs. Digested sewage sludge represents 40% of the total output of nonylphenolic compound (Castillo *et al.*, 2000). NP is formed from NPEOs during anaerobic stabilization of sludge resulting in very high concentrations of NP. Concentrations of >1g/kg are often detected in anaerobically digested sludge (Petrovìc and Barceló, 2004). NP is more hydrophobic and recalcitrant than its parent compounds (Ahel *et al.*, 1994) and has a greater oestrogenic potency. Digested sludge is likely to

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contain up to 96% of NP produced during wastewater treatment (Scrimshaw and Lester, 2003).

The high biodegradability of linear alkylbenzene sulphonates (LASs) in the aerobic environment indicates that LASs are not of concern when sludges are applied to land under relevant legislation. Geglsbjerg *et al.* (2003) deduced the half-life of LAS in sludge-amended soil to be 2-3 days at a concentration of 10mg/kg.. Petersen *et al.* (2003) found that concentrations of LAS (2870mg/kgDM) and NP (60mg.kgDM) in sewage sludge applied to soil (6-10cm depth) were degraded by 70% with the first 6 weeks with <5% remaining after 6 months in a study in Southwest Denmark. Uptake of LAS and NP by barley and oats grown on the field sites was not detected. The movement of LAS into groundwater and uptake by plants are not considered significant (Petrovic and Barceló 2004). The limit value for LASs in sludge for use on land is 2,600mg/kg dm according to the Working Document on Sludge (EU, 2000).

NP has been shown to have a mineralization half-life of 16.7 days in loamy soil at a concentration of 1µgNP/g soil. Concentrations as high as 250mg/kg were degraded, however, slower mineralization occurred in sewage sludge amended soil (Topp and Starratt, 2000). Hesselsøe *et al.* (2001) reported that nonylphenol was degraded within 38 days in aerobic homogenized mixture of sludge and soil. Within an aerobic soil environment NP (and LAS) will not accumulate however in anaerobic soils accumulation of their compounds is likely. Hosselsoe *et al.* (2001) suggested that for 2cm sludge aggregate to become aerobic a period of over one year was required indicating significant potential for NP to build up in the anaerobic zones. The working document on sludge set the limit for the concentration of total nonylphenolic compounds (NPs and NPnEOs) at 50mg/kg dm.

1.11.3 Degradation by indigenous bacterial species in sludge

Lee and Liu (2002) conducted tests in both aerobic and anaerobic conditions with an E2 degrading culture (derived from supernatant of activated sludge). Aerobic degradation resulted in 88% degradation of spiked E2 while anaerobic degradation resulted in only 50% degradation after 7 days. A separate biodegradation test was carried out on oestrogen and five of its primary metabolites; E1, E3, 16α-hydroxy-E1, 2-methoxy-E2 and 2-methoxy-E1 was carried out. After a 7hour period 92% of 17β-E2,

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46% of E1, 41% of E3, and 51% of 16α-hydroxy-E1 were degraded and after a period of 49hours all metabolites were completely removed.

Vader *et al.* (2000) investigated the degrading ability of nitrifying activated sludge on EE2 and reported that the nitrifying sludge completely degraded ethinyl-estradiol within 6 days and did not need an acclimatization period. Maximum degradation occurred during the first 2 days. Analysts in Japan isolated an E2-degrading bacterium (named ARI-1) from activated sludge in a sewage treatment plant in Tokyo. ARI-1 is a gram-negative oval-shaped bacterium which is a genus that includes many species capable of assimilating compounds resistant to biodegradation. ARI-1 was found to degrade E2 steadily and subsequent analysis by GCMS did not detect any accumulative metabolites of E2 (Fujii *et al.*, 2002). A separate degradation study using the ammonia-oxidising bacterium *Nitrosomonas europaea* demonstrated similar degradative rates for all four oestrogens and also that E2 degradation by *N. europaea* did not produce E1 (Shi *et al.*, 2004).

1.11.4 Alteration/biodegradation processes

The main processes which determine the fate of a compound during wastewater treatment are advection, sorption, volatilization, air stripping and biotransformation (Byrnes, 2001).

1.11.4.1 Advection

Advection is one of the two processes by which solutes are transported in a fluid. Advection is the bulk movement of solute due to concurrent movement of fluid. Hence solutes which do not react are carried at a rate equal to the linear velocity of the fluid.

1.11.4.2 Sorption

There are two major processes by which sorption of organic compounds takes place; absorption and adsorption. Absorption represents the "hydrophobic interactions of the aliphatic and aromatic groups of a compound with the lipophilic cell membrane of the microorganisms" (Swiss Federal Institute for Environmental Science and Technology (Swiss Federal Institute for Environmental Science and Technology (EAWAG) 2003) and fat components of the sludge. Adsorption represents the "electrostatic interactions of positively charged groups of chemicals with the negatively

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charged surfaces of the microorganisms" (EAWAG, 2003). During primary treatment organic compounds are adsorbed onto primary sludge (composed of suspended solids, few microorganisms and a large fat fraction) while during secondary treatment they are adsorbed onto secondary sludge which consists of suspended and a larger quantity of microorgansims (Langford and Lester, 2003; EAWAG, 2003). Hence, higher absorption of organic compounds is more likely to occur during secondary treatment than primary treatment.

Particle-contaminant interactions greatly influence the fate of contaminants in wastewater treatment plants. Natural particles include clays, sediments, colloids with attached microorganisms while synthetic particles include powdered activated carbon, coagulants and ion exchange resin. Contaminant compounds that are dissolved or associated with dissolved natural organics or unsettleable colloids can pass through treatment systems easily. Contaminants that are adsorbed into activated sludge particles accumulate in the sludge (Filali-Meknassi *et al.*, 2004).

1.11.4.3 Volatilization

In a model to predict the fate of xenobiotic organic compounds in wastewater treatment plants, Byrnes (2001) predicted that compounds with high vapour pressure, low octanol-water co-efficients ($\log k_{ow}$) values and low solubilities are most likely to be removed by volatilization.

1.11.4.4 Degradation

Chemical degradation occurs principally by hydrolysis which is the conversion of organic wastes to more benign compounds through substitution by hydroxide ions. Biological degradation of a compound can occur by partial biodegradation by bacteria by which the compound does not act as a carbon source (co-metabolism) or by total mineralization whereby bacteria derive carbon and energy from the compounds (EAWAG, 2003). Certain structural characteristics influence the degree of biodegradation which can occur. Longer chained molecules are degraded more readily then short chain molecules (Langford *et al.*, 2005) and branched hydrocarbon chains are more resistant to degradation than unbranched molecules.

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1.11.5 Importance of physiochemical properties

The physio-chemical properties of a compound will determine the dominant process(es) involved in its fate in the wastewater treatment system (Byrnes, 2001). Physio-chemical properties of oestrogens and selected xenoestrogens are presented in table 1.11.5.

Table 1.11.5: Physiochemical properties of oestrogens and selected xenoestrogens

Compound	Molecular weight (g)	Water Solubility (mg/l at 20°C)	Octanol-water co-efficient (log K_{ow})
E1	270.37	13 [b]	3.13 [a]
E2	272.39	13 [b]	4.01 [a]
E3	288.39	13 [b]	2.45 [a]
EE2	296.40	4.8 [b]	3.67 [a]
NP	220.00	5.43 [c]	4.48 [c]
NP1EO	264.00	3.02 [c]	4.17 [c]
NP2EO	308.00	3.38 [c]	4.21 [c]
NP3EO	352.00	5.88 [c]	4.2 [c]
OP	206.00	12.6 [b]	4.12 [b]
BPA	228.29	120-300 [d]	3.4 [d]

References
a: Kuster *et al.*, 2004
b: Lai *et al.*, 2000
c: Ahel and Giger, 1993
d: Langford and Lester, 2003

1.11.5.1 Solubility

The solubility of a solute is the maximum quantity of solute that can dissolve in a certain quantity of solvent or quantity of solution at a specified temperature. The degree of solubility that occurs depends on the size of the particles, agitation and temperature. Compounds with high solubilites are most likely to remain dissolved in the wastewater and less likely to adsorb to suspended particles or sludge.

1.11.5.2 Octanol-water partition co-efficient (K_{ow})

The octanol-water partition co-efficient is the ratio of a compounds concentration in octanol to that in water at equilibrium and is expressed logarithmically (EC, 2001b). Compounds with large logK_{ow} values tend to be large hydrophobic molecules with a greater tendency to associate with solid organic matter while compounds with small log K_{ow} values tend to be smaller more hydrophilic molecules (Langford and Lester, 2003). In sorption potential, compounds with a logK_{ow} <2.5 have low sorption potential, compounds with a logK_{ow} between 2.5 and 4.0 have medium sorption potential while those with a logK_{ow} of >4.0 have high sorption potential (Rogers, 1996).

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1.11.5.3 Organic carbon-water partition co-efficient (K_{oc})

The organic carbon-water partition co-efficient is the ratio between the concentration of the organic compound on organic carbon (mg/g) and its concentration in water (mg/l) at equilibrium (EC, 2001b). In general, compounds with a high $logK_{oc}$ (>3) will tend to absorb to organic carbon such as sewage sludge while compounds with low $logK_{oc}$ (<3) tend to remain in the aqueous phase (Langford and Lester, 2003).

1.11.5.4 Henry's law constant (H_c)

Henry's law constant is the ratio of a chemicals concentration in air to its concentration l in water at equilibrium. Compounds with high H_c values tend to volatilize while those with low H_c values tend to remain in the aqueous phase (Wilson *et al.*, 1996). A compound with a H_c value of $>1x10^{-4}mol/m^3$ and a H_c/K_{ow} quotient of $>1x10^{-9}$ has a high tendency to be volatilized while a compound with a H_c value of $<1x10^{-4}mol/m^3$ and a H_c/K_{ow} quotient of $<1x10^{-9}$ will tend to remain in solution.

1.12 Removal of oestrogenic compounds in wastewater treatment

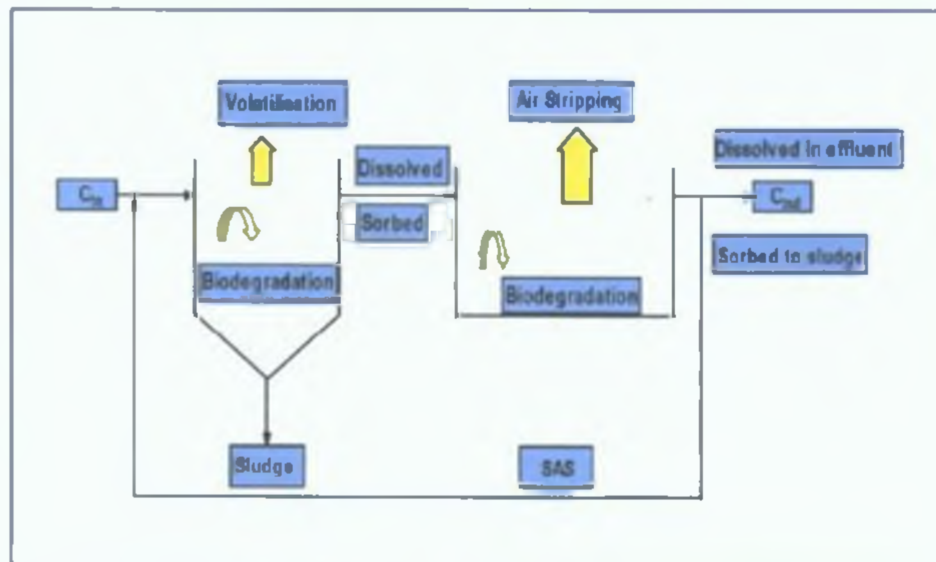

Figure 1.12: Processes of removal of organic compounds in an activated sludge system (Byrnes, 2001).

1.12.1 Treatment stages

1.12.1.1 Primary treatment

The major removal pathways during primary treatment are advection in the dissolved or adsorbed phase, sorption to particulate matter and subsequent sedimentation and to a lesser extent, volatilization and biotransformation (Figure 1.12). Advection of the dissolved phase is the major removal pathway for compounds with octanol-water coefficients below 4. Advection to suspended matter and sorption to

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sludge are the main removal pathways for compounds with octanol-water coefficients above 4. The extent to which removal occurs depends on the removal of suspended solids which is determined by settling characteristics of the particles (their density, size and ability to flocculate), the sludge retention time and the loading into the plant (Langford and Lester, 2003).

Volatilization accounts for a small degree of removal during primary treatment with maximum removal occurring for compounds with an octanol-water coefficient of approximately 2.0 (Byrnes, 2001). As can be observed in Table 1.11.5, E3 has the highest solubility and the lowest octanol-water co-efficient of the four oestrogens; E1, E2, E3 and EE2 indicating that it is most likely to exist in the dissolved phase. EE2 has the lowest solubility of the four oestrogens and the highest octanol-water co-efficient indicating that it is most likely to become sorbed to sludge or suspended matter than to remain in the dissolved phase.

1.12.1.2 Secondary treatment

Compounds with low octanol-water co-efficients continue to be removed by advection into the dissolved phase and volatilization (Byrnes, 2001) (Figure 1.12). Volatilization represents a greater proportion of the total removal of compounds during secondary treatment than during primary treatment due to an increased air:water interface during aerated treatment processes (EC, 2001b). Biodegradation is the major removal pathway with maximum removal occurring for compounds with an octanol-water co-efficient between 3.0 and 3.5. The process of nitrification may occur during secondary treatment which is beneficial for the degradation of organic pollutants such as oestrogenic compounds and xenoestrogens as it requires growth of slow-growing microorganisms. Compounds with higher octanol-water co-efficients are more likely to become sorbed to the sludge.

1.12.1.3 Tertiary Treatment

Numerous studies have been carried out on different forms of advanced treatment to assess their efficiency in the removal of endocrine disrupting compounds, A study on the effects of chlorination on the oestrogenic potencies of E2, nonylphenol and bisphenol A demonstrated that complete elimination of oestrogenic activity of E2 with a chlorine concentration of 1.5mg/l necessitated a time period of 36 hours whereas a higher concentration of chlorine (7.5mg/l) was used to assess bisphenol A and resulted

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in elimination of oestrogenic activity after ten minutes. No oestrogenic activity was observed in the by-products after chlorination after 24hours. This reduction in potency is attributed to oxidation of the phenolic ring by the addition of free chlorine (Lee *et al.*, 2004). However advantageous use of chlorination is with respect to endocrine disrupting compounds the disadvantage of increasing formation of chlorination by-products still persists.

Ternes *et al.* (2003) demonstrated that ozonation using 5-15mg/l of ozone is sufficient to eliminate E1 with >80% removal. The ozonation process increases both the polarity and the number of functional groups of E1 which results in a weaker binding affinity for the oestrogen receptor.

The efficiency of addition of absorbents (ferric chloride coagulant, powdered activated carbon and magnetic ion exchange resin) for the removal of E1 in jar tests was assessed by Schäfer and Waite (2002) with removal rates of <10%, >90% and 40%-70% respectively.

The ability of nanofiltration and reverse osmosis to remove E1 from wastewater and synthetic surface water was examined by Nghiem *et al.* (2004a). E1 was effectively retained by tighter nanofiltration and reverse osmosis membranes while lower retention was observed for more porous membranes. The study also reiterated the fact that hormone retention is enhanced by the presence of organic matter. In a different study Nghiem *et al.* (2004b) reported that lower absorption and retention occurred at higher pH levels.

Splenger *et al.* (2001) and Körner *et al.* (2001) found that the use of activated charcoal filtration as a tertiary treatment can greatly reduce the oestrogenic potency of an effluent (the EEQs of the effluent following activated carbon filtration were 5-10 fold lower than those of effluents of WWTPs without activated carbon filtration). De Rudder *et al.* (2004) carried out studies to assess the treatment efficiency of sand, granulated activated carbon (GAC) and Manganese dioxide (MnO_2) granules. Removal of EE2 was carried out using upstream bioreactors filled with sand, granulated activated carbon (GAC) and Manganese dioxide (MnO_2) granules in which the oestrogenic activity was determined by the Yeast oestrogen screen (YES) bioassay. Granular activated carbon is highly adsorptive for EE2 at µg/l concentrations however its

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adsorptive capacity decreases for EE2 at environmentally relevant concentrations (0.1-20ngEE2/l). It also has to be replaced or regenerated regularly whereas self-regeneration of manganese dioxide is possible making it a more cost-effective option for tertiary treatment. Removal efficiencies of EE2 for the sand, granular activated carbon and manganese dioxide upstream bioreactors were 17.3%, >99.8% and 81.7% respectively.

1.12.2 Factors that affect removal

On entering wastewater treatment plants oestrogenic compounds can persist due to their resistance to microbial degradation and may be present in one or more phases within the plant and leave the plant unaltered in the effluent or they can undergo biodegradation producing metabolites which may be less potent or indeed, more potent than their parent compounds. Various factors exist which influence the removal efficiency of organic compounds including endocrine disrupting compounds in a wastewater treatment plant.

It has been shown that oestrogen-degrading organisms accumulate more readily in wastewater treatment plants receiving domestic waste than in those receiving industrial waste only as Layton *et al.* (2000) reported that the rate of mineralization of ^{14}C-E2 in municipal activated sludge was 84% while that of industrial activated sludge was only 4%. Interestingly, mineralization of testosterone occurred in both municipal and industrial activated sludges. The treatment process itself is also important as some compounds are more recalcitrant under anaerobic conditions (Environment Agency, 1999). Temperature, pH and dissolved oxygen content can affect the growth of aerobic microorganisms which in turn affects biodegradation of oestrogenic compounds. Since wastewater treatment plants are operated at ambient temperatures, effects due to temperature changes are inevitable. Lower temperatures reduce the metabolic rate of microorganisms inhibiting biodegradation. Ternes *et al.* (1999a) assessed the removal rates of E1, E2 and EE2 in a Brazilian sewage treatment plant (operating at ≈20°C) and a German sewage treatment plant (operating at ≈2°C) and found the Brazilian sewage treatment plant to have much greater removal efficiency. Rodgers-Gray *et al.* (2000) reported seasonal variation in the oestrogenicity of effluent in a study on Chelmsford WWTP in the UK in which mean E2 and E1 concentrations for months November-March were approximately three times higher than the mean concentrations for months June-December (1998-1998). An increase in temperature, nitrate levels and bacterial

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count were observed for the latter months. Following aerobic batch degradation experiments of E2 by activated sludge, Li *et al.* (2005) reported that biodegradation was reduced at lower temperatures.

A neutral pH is maintained in most activated sludge treatment plants. PH level in sludge can influence "the bioavailability of compounds due to their changing solubility, sorption potential and aerobic growth" (Langford and Lester, 2003). Changes in pH influence the extent and type of bonding involved in sorption. Clara *et al.* (2004) demonstrated that adsorption of bisphenol A and EE2 to suspended particles and sludge is likely to occur at pH levels over approximately 10.5 while both compounds are likely to remain dissolved at pH levels below 10.5.

Increased rainfall has the effect of increasing the flow rate which in turn reduces the retention time which can lead to an overall reduction in treatment efficiency (Rodgers-Gray *et al.*, 2000). However, increased rainfall also dilutes the effluent hence weakening its oestrogenic potency and its oestrogenic effects on aquatic organisms (Harries, 1996).

Biodegradation increases with increasing sludge retention time as specific degrading microorganisms are slow-growing and require time to become established within the activated sludge (Andersen *et al.*, 2003). With increasing sludge age the bacteria compete for more complex, less easily degrading compounds (EAWAG, 2003; Langford *et al.*, 2005). Ternes *et al.* (1999a) and Andersen *et al.* (2003) demonstrated the increased removal efficiency of a German WWTP once the sludge age was increased (from 4 days to 11-13 days) and all year nitrification and denitrification was incorporated into the treatment process. Ternes *et al.* (1999a) detected average concentrations of 24ng/l E1, 5mg/l E2 and 2ng/l EE2 in the effluent in 1997 while Andersen *et al.* (2003) detected concentrations of less than 1ng/l of E1, E2, and EE2 in 2001.

Holbrook *et al.* (2002) noticed a positive correlation between the oestrogenic activity of mixed liquor suspended solids and aerobic sludge age. Li *et al.* (2005) reported that higher concentrations of mixed liquor suspended solids gave rise to the most marked decreasing trends in E2 biodegradation in aerobic batch degradation experiments. Clara *et al.* (2005) determined the critical sludge retention time at 10°C

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for the natural oestrogens to be between five and ten days. Effluent concentrations in the range of the limit of quantification (1ng/l for E1, E3 and EE2 and 5ng/l for E2) were measured at sludge retention times longer than ten days at 10°C. The degree of removal that occurs in a typical activated sludge WWTP depends on the capacity of the wastewater solids to adsorb the contaminants and how quickly the microorganisms present can degrade these compounds (Schäfer *et al.*, 2002).

In a study on five WWTPs in the United Kingdom, Kirk *et al.* (2002) examined the oestrogen activity of wastewater at different stages of treatment using the Yeast Oestrogen Screen (YES) and found that maximum removal occurred during secondary treatment while some additional removal occurred during tertiary treatment. The overall removal rate of a WWTP with only primary treatment was only 10%. Studies have shown that WWTPs equipped with only primary treatment are not very efficient at removing oestrogenic compounds. Three Swedish WWTPs with primary treatment and direct precipitation by aluminium or iron were not effective in reducing the oestrogenic potency of the effluents but actually increased the oestrogenic potency (Svenson *et al.*, 2003). Lime was found to be a more effective precipitate with a removal rate of 73% in a fourth WWTP with only primary treatment in which the effluent was of a higher pH (pH11.4).

Primary treatment may actually increase the concentration of oestrogenic compounds in the effluent as demonstrated by Servos *et al.* (2005) in which the average E1 concentration increased by 28.6% in the effluent of a Canadian plant equipped with primary treatment only. Similarly, Carballa *et al.* (2004) reported a 20% reduction of E2 with a concurrent increase in E1 (42%) during primary treatment in a Spanish WWTP in Galicia, Spain. Ternes *et al.* (1999a) found that E1, E2, and 16α-hydroxyestrone all increased during primary treatment. E2 and E1 levels are known to increase during primary treatment due to cleavage of glucuronides while oxidation of E2 to E1 may also contribute to the increase in E1. E1 is much more slowly degraded than E2 (Ternes *et al.*, 1999b).

Studies carried out on the removal efficiency of different varieties of secondary treatment have indicated that the activated sludge and lagoon systems exhibit a greater removal efficiency than solid support bacteria systems such as trickling filters and biorotors. Ternes *et al.* (1999a) found that E1, E2, and 17α-ethinylestradiol were

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removed at rates of 67%, 92% and 64% respectively in a trickling filter system in a Brazilian WWTP while higher removal rates of 83%, 99.9% and 78% occurred in an activated sludge system in the same plant. A German study reported that the oestrogenic potency of effluent from a trickling filter WWTP was approximately four times higher than that of the activated sludge plants assessed in the study (Körner *et al.*, 2001).

Svenson *et al.* (2003) reported an average removal rate of 81% for activated sludge and 28% for solid support bacteria systems in a study on Swedish WWTPs. Average removal rates of 93% and 83% respectively were reported from four lagoon treatment systems by Servos *et al.* (2005) while a treatment plant with trickling filter produced increases in 17β-E2 and E1 concentrations of 18.5% and 62.4% respectively. Joss *et al.* (2004) reported greater than 90% removal of all oestrogens in 3 activated sludge processes consisting of conventional activated sludge treatment, a membrane bioreactor and a fixed-bed reactor.

It has become apparent that there may be higher removal rates in secondary biological treatment if denitrification is employed. In a study on twenty Swedish WWTPs two of the highest removal rates occurred in activated sludge treatment plants in which denitrification occurred (Svenson *et al.*, (2003). Servos *et al.* (2005) made an association between the degree of nitrification in the treatment system and removal of E1, E2 and the overall reduction in oestrogenic potency as measured by the YES assay. This may be due to the higher hydraulic retention time (HRT) required to allow nitrification and denitrification to occur. Two Swedish WWTPs with HRTs of 12hours and 20hours and a third plant employing wetland treatment with a HRT of 7 days had removal rates ≥97% while the HRT of the remaining WWTPs (2-8hours) produced removal rates of 0-94% (Svenson *et al.*, 2003). Similarly, the greatest degree of removal of oestrogen activity in an English study on five WWTPs occurred in the plants with the longest retention times (13-13.5hours) (Kirk *et al.*, 2002). The removal rate of EE2 increased to approximately 90% at a German WWTP in Wiesbaden after the plant was upgraded to remove nutrients and its sludge retention time was increased from less than 4 days to 11-13 days (Andersen *et al.*, 2001).

Percentage removal rates of oestrogens in selected studies reviewed in literature are presented in table 1.12.2. Frequently, higher removal rates are reported for E2 than

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E1. E3 and EE2 are seldom analysed for, however, the studies presented which included these compounds indicate efficient removal.

Table 1.12.2: Percentage removal rates of oestrone (E1), oestradiol (E2), oestriol (E3) and ethinyl-oestradiol from different countries

Removal of E1 (%)	Removal of E2 (%)	Removal of E3(%)	Removal of EE2(%)	Country	Reference
	65			Spain	Carbella et al., 2004
	64-69			Japan	Nasu *et al.*, 2001
61	85	97		Italy	D'Ascenzo *et al.*, 2003
61	87	95	85	Italy	Baronti *et al.*, 2000
74	88	77		Italy	Johnson *et al.*, 2000
46-98	39-98			Canada	Servos *et al.*, 2005
>98	>98		>90	Germany	Andersen *et al.*, 2003
83	64-99.9		78	Brazil	Ternes *et al.*, 1999a

With regard to xenoestrogens, Laganà *et al.* (2004) determined that bisphenol A (BPA) was removed by 90% in a Roman activated sludge plant while 75% of nonylphenol (NP) was removed. In a Japanese study the removal rate for BPA ranged from 85% to 96% while that of NP ranged from 93-94% (Nasu *et al.*, 2001).

1.13 Introduction of oestrogenic compounds to surface water

The aqueous concentrations of oestrogens in surface waters are decreased by dilution, degradation and sorption (Environment Agency, 1999). Most endocrine disrupting chemicals will undergo partitioning to the solid phase due to their low solubility and their hydrophobic nature. Sedimentation of particulates occurs in lakes and slow moving waters and to a lesser extent in faster moving rivers.

There are several environmental consequences of the partitioning of endocrine disrupting chemicals to suspended particulates and/or sediment. Firstly, it allows endocrine disrupting compounds to persist for a longer period of time in the aquatic environment. Secondly, particles laden with endocrine disrupting chemicals in river systems may be carried downstream meaning that the oestrogenic potential of point source pollution for example sewage effluent may persist several kilometres downstream, however, dilution does occur (Harries *et al.*, 1996, 1997, Sheahan *et al.*,

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2002a). Thirdly, the availability of an endocrine disrupting compound for biodegradation is decreased if it is bound to particulate matter and the presence of endocrine disrupting compounds in the sediment means they may have endocrine disrupting effects on benthic (bottom-dwelling) organisms for example copepods (Marciel *et al.*, 2003; Andersen *et al.*, 2001; Forget-Leary *et al.*, 2005) and demersal fish and may become biomagnified in the food chain (Gomes *et al.*, 2003).

Lai *et al.* (2000) carried out sorption tests on natural oestrogens; E2, E1, and E3 and synthetic oestrogens mestranol and EE2 and observed that the synthetic oestrogens bound to the sediments to a greater degree (4.5-5.5µg/g) than the natural oestrogens (3.2-4.1µg/g). Sorption occurred within the first 30 minutes of contact. It was evident that the compounds with higher octanol water co-efficients and lower water solubilities exhibited greater binding affinity for the sediment and competed with other oestrogens. Holthaus *et al.* (2002) also found that EE2 showed a greater affinity for bed sediments than E2. These findings imply that in natural systems sorption to particulates occurs rapidly and there is competition for sorbent binding sites between oestrogens and all other hydrophobic compounds implying that compounds of lower hydrophobicities such as the natural oestrogens will remain in the aqueous phase where they are gradually degraded (Lai *et al.*, 2000; Environment Agency, 1999). Holthaus *et al.* (2002) estimated that suspended solids would remove less than 1% of E2 and EE2 from the water column.

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Lai *et al.* (2000) assessed the effect of salinity on sorption and found that after one hour sorption to the sediment had increased with addition of sodium chloride to reverse osmosis water implying that oestrogens bound to suspended or dissolved organic matter are more likely to be deposited with sediments in estuarine areas. Bowman *et al.* (2002) found that sorption onto estuarine sediment particles was relatively slow.

In a study for the Environment Agency (1999), Jürgens *et al.* reported the half-lives of E2 to range from <3-4days in river water samples and 6-27 days in estuary water samples while 46 days was required for EE2 to undergo 50% degradation. Williams *et al.* reported a half-life of 1.2 days for E2 in Thames river water and a half-life of 17 days for EE2 in the same water (Environment Agency, 2001).

Bisphenol A is rapidly degraded by bacteria in river water as reported by Kang and Kondo (2002) in a Japanese study in which BPA degradation was found in all samples taken from 13 different rivers. It was also found that faster biodegradation occurred at higher temperatures.

An Australian study on the sorption of BPA, E2, EE2, 4-tert-octylphenol and 4-n-nonylphenol onto sediments demonstrated that the alkylphenols had the strongest binding affinity for the sediment. Degradation studies were carried out in groundwater under aerobic conditions and it was found that E2 and NP had estimated half-lives of 81 days whereas BPA and OP did not undergo degradation. The lack of degradation of BPA may be due to the fact that riverine bacterial populations may have developed their ability to degrade BPA over gradual exposure over time whereas these bacteria may be absent from groundwater hence degradation may not occur. Little or no degradation was observed for all five endocrine disrupting substances under anaerobic conditions (Ying *et al.*, 2003).

Concentrations of E2 measured in surface waters in worldwide studies reviewed to date range from non detectable in Germany (Ternes *et al.*, 1999, and the U.K (Fawell *et al.*, 2001) to 25ng/l in the U.K. (Fawell *et al.*, 2001). Levels of E1 are typically higher (as can be seen in Table 1.17.1 (Appendix B)) due to oxidation of 17β-E2 and range from non detectable (Ternes *et al.*, 1999a) to 27.8ng/L in a Japanese study (Furuichi *et al.*, 2004). A high concentration of EE2 (37ng/l) was also detected on the same study. The E2 equivalents of surface water determined by in-vitro assays in studies reviewed to date range from non detectable to 81.4ng/L in Belgium (Witters *et al.*, 2001) and are presented in Table 1.17.2 (Appendix C).

Median concentrations of 66.7ng/l 4-nonylphenol (NP), 31.4ng/l 4-tert-octylphenol (OP) and 72.1ng/lBPA were determined in Körsch Stream in Southwest Germany which receives effluents from six wastewater treatment plants (Bolz *et al.*, 2001). Higher concentrations of alkylphenols and bisphenol A were detected in surface waters in the Netherlands with <110-4,100ng/LNP, <50-6,300ng/LOP, and <8.8-1,000ng/lBPA. Concentrations of 4- NP in sediments from various streams and rivers in Southwest Germany ranged from 10-259μg/kg dry matter (Bolz *et al.*, 2001) while levels of BPA in the sediment were low (n.d.-15 μg/kg dry matter) since this compound

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is readily degraded in river systems. Solé *et al.* (2000) detected higher levels of NP up to 644μg/l in the Anoia Tributary in Northeast Spain.

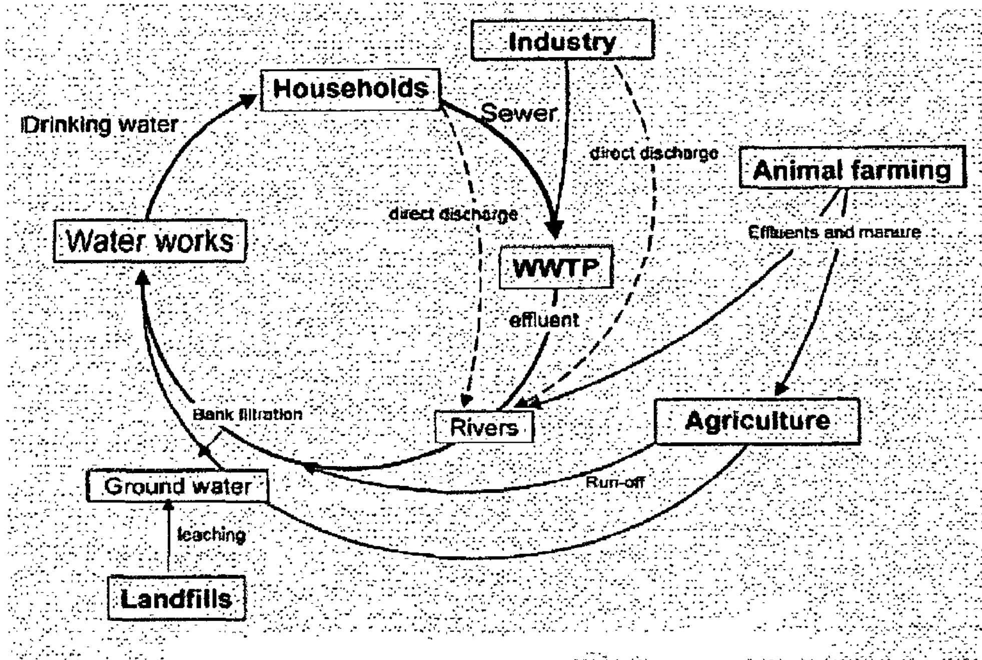

Figure 1.13: Components of a (partially) closed water cycle with indirect potable re-use (Petrovic *et al.*, 2003).

Endocrine disrupting chemicals do not need to be persistent in the environment to cause endocrine disfunction because their transformation and removal rates are negated by their continuous re-introduction into the environment (Petrovic *et al.*, 2003) (Figure 1.13).

Studies carried out to determine if endocrine disrupting compounds were present in the raw intake and in the reservoirs of drinking water treatment plants have shown that endocrine disrupting compounds were present (1.8-4.1ng/L E1 and <0.3ng/LE2 in Severn Trent in the UK (Fawell *et al.*, 2001) and 0.30-0.44ng/L E2 equivalent in two Parisian drinking water treatment plants (Cargouët *et al.*, 2004). NP, NP_nEOs, BPA and bisphenol F were non detectable in all samples, however, low concentrations of phthalates, primarily bisethylhexylphthalate, were measured in all samples of intake, reservoir and final drinking water. Levels of steroid oestrogens were below the detection limit in treated drinking water (Fawell *et al.*, 2001).

Rodriguez-Mozaz *et al.* (2004) did not detect oestrogens in drinking water from a waterworks in the Llobregat area of Barcelona which supplies approximately one third of Barcelona's drinking water in a study carried out in 2002. Herbicides were also

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analysed for in raw river water and at different stages of treatment and in the final drinking water. Concentrations of the herbicides Simazine and Atrazine up to 2.218μg/l and 0.463μg/l respectively were detected in river water with levels up to 0.032μg/l and 0.018μg/l respectively detected in final drinking water (Rodriguez-Mozaz *et al.* 2004).

1.14 Effects of endocrine disruption on wildlife

Numerous cases have been reported worldwide where different species of wildlife have been affected by endocrine disruptors.

Exposure of animals to endocrine disruptors depends on their habitat and occurs via air, water, soil, sediment (bioconcentration), in their food (biomagnification) and also from the mother *in utero* or *in ovo.* Most endocrine disruptors are fat-soluble and accumulate in animal body tissues. This has two implications; the first being that these compounds are passed on from mother to offspring via egg yolk or breast milk, for example, cetacean females off-load >60% of their organochlorine burden to their calf during reproduction and lactation (Borrell *et al.*, 1995). The second being that these compounds are passed on from prey to predator with increasing biomagnification occurring as trophic levels increase (Gray, 2002; Clotfelter *et al.*, 2004).

Various factors such as the degree, timing and duration of exposure relative to the developmental stage of the organism make assessment of exposure to endocrine disruptors difficult (World Health Organisation, 2002). Critical periods of development exist (for example the first 10 days after hatching for salmon) wherein exposure to endocrine disruptors at these times causes permanent organizational change in anatomy (WHO, 2002; Clotfelter *et al.*, 2004). The effects of endocrine disruption on adult individuals are sometimes reversible if the exposure is stopped (Sonnenschein and Soto, 1998). Many studies of endocrine disruption have concentrated on developmental (Iguchi *et al.*, 2001) and reproductive endpoints (Guillette *et al.*, 1994) since the early stages of life are the most sensitive and since hormones such as oestrogen regulate many reproductive processes in vertebrates meaning the reproductive system is prone to attack by endocrine disruptors, particularly oestrogen mimicking compounds.

Effects of endocrine disruption are not exclusively due to the presence of synthetic compounds in the environment. They can also be due to an altered distribution of natural hormonal products in the environment, for example, elevated

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concentrations of phytoestrogens such as β-sitosterol which is a wood-derived compound in the effluent of pulp mills has caused vitellogenesis in fish (Karels *et al.*, 1999) and reptiles (Shelby and Mendonça, 2001).

In vertebrates, oestrogens are involved in reproduction, somatic cell function, sexual differentiation, development of secondary sex characteristics, ovulation, regulation of mating and breeding behaviours and the regulation of calcium and water homeostasis (Fairbrother, 2000).

Exposure to hormonal oestrogen and oestrogen mimics during sexual differentiation has been shown to induce sex reversal and/or intersexuality, while exposure during sexual maturation can inhibit gonadal growth and development (Jobling *et al.*, 1996).

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1.14.1 Mammals

Fish-eating mammals are more vulnerable to the effects of endocrine disruption than other mammals as they depend on an aquatic/marine foodweb (of which they represent the higher trophic levels); also certain aspects of their reproductive physiology may make them more vulnerable to endocrine disruption; and the influence of industry and agriculture on aquatic and marine systems means that the load of endocrine disruptors in these systems is ever increasing (WHO, 2002). The majority of information on endocrine disruption in wildlife by chemicals has been derived from aquatic ecosystems.

Baltic Seal Disease Syndrome is a disease syndrome in Baltic Grey (*Halichoerus grypus*) and Ringed Seals (*Phoca hispida*) linked to high body burdens of PCBs, DDT and their metabolites and has resulted in a decline in seal numbers (Bergman *et al.*, 1994; Van den Berg, 2000). Effects of exposure include interruptions during early pregnancy, uterine stenosis and occlusions and partial or complete sterility of Baltic ringed seals (70%) and grey seals (30%). In 1986, Reijnders carried out a semi-field reproductive study with harbour seals (*Phoca vitulina*) in which one group of twelve female seals were fed flatfish from the polluted Wadden Sea for two years while a second group were fed mackerel from the Atlantic Ocean. The former seals had lower concentrations of 17β-E2 and had lower reproductive success than seals in the latter group (Reijnders, 1986). Exposure to PCBs is thought to have impaired the immune

systems of seals in the Dutch Wadden Sea, Sweden, the Baltic Sea and the British Isles in the 1980s which exacerbated the spread of a deadly virus, phocine distemper virus which killed 20,000 seals by mid 1989 (Olsson *et al.*, 1994).

High concentrations of PCBs have been reported in European otter (*Lutra lutra*) and mink (*Mustela vison*) and are generally held responsible for reproductive impairment in these species (Brunström *et al.*, 2001; Roos *et al.*, 2001). In 1988 a low reproductive rate in a population of Beluga Whales (Delphinus leucas) in the polluted Gulf of St. Lawrence in Canada was associated with high body burdens of organochlorines (Martineau *et al.*, 1994). More recently, levels of total PCBs (dominated by congeners 153 and 138) four to five times higher than that of the Beluga Whales were found in transient killer whales in British Columbia. These whales can now be considered to be among the most contaminated cetaceans in the world (Ross *et al.*, 2000).

1.14.2 Reptiles

Crocodiles, most turtles and many lizards employ temperature dependent sex determination whereby the sex of the offspring is determined by the incubation temperature of the eggs (Lance (1994 cited WHO, 2002 chapter 4 part 4.3.1). Studies have shown that oestrogenic compounds administered to eggs incubated at a temperature which would normally give rise to male offspring result in female sex determination (Wibbels and Crews, 1995). Crews *et al.* (1995) reported that PCBs altered sex determination of turtles.

The most infamous case of endocrine disruption in reptiles occurred in the Great Lakes, North America. In 1980, a major spill of the organochlorine pesticide Dicofol contaminated with 15% DDT and its metabolites DDD and DDE occurred at Lake Apopka, in central Florida. The population of American alligator in the lake dramatically decreased in the 1980s and still had not recovered. Guillette *et al.* (1994) investigated the reproductive development of alligators from Lake Apopka and a control lake, Lake Woodruff by incubating and hatching eggs taken from both sites and raising the alligators in captivity for 6 months. At six months of age, plasma 17β-E2 concentrations two times greater were present in Lake Apopka female alligators than in Lake Woodruff female alligators. Ovaries of Lake Apopka females were abnormal containing polyovular follicles and polynuclear oocytes. Male alligators from Lake

Apopka had poorly organised testes and abnormally small phalli. Juvenile males had plasma testosterone concentrations three times lower than males from the control lake. Guillette *et al.* (1994) suggested that the sex organs of juveniles from Lake Apopka have been permanently altered *in ovo* implying that it is improbable that normal sexual maturation will occur.

1.14.3 Fish

Exposure of fish to oestrogenic compounds in the aquatic system occurs via respiration and osmoregulation. Fish gills consist of thin epithelial membranes of high surface area and there is also a counterflow of blood and water (World Health Organisation, 2002). However, the discovery of hermaphrodite roach in lagoons of sewage treatment plants in the United Kingdom in the 1990s marked the realization that some component of wastewater effluent had the capacity to affect gonadal development of fish.

Oestrogens are involved in many processes in fish, one of which is vitellogenin synthesis. Vitellogenesis is a physiological process for the production of yolk in all female oviparous vertebrates. Sexually maturing female fish produce E2 from their ovaries, which is transported to the liver where it diffuses into hepatocytes and binds to the receptor resulting in expression of vitellogenin gene(s) i.e. vitellogenin production. Vitellogenin then travels to the ovary where it is taken up by developing oocytes to become yolk (Tilton *et al.*, 2002). Male fish do not require vitellogenin; hence vitellogenin concentrations in males are low to non-existent. Levels are low in immature female fish also. Expression of vitellogenin is controlled by the oestrogen receptor cascade meaning that any ER agonist that can activate the ER results in expression of vitellogenin. Aquatic contaminants that can increase E2 levels in the blood or increase gonadotrophin releasing hormone levels or inhibit the negative feedback mechanisms related to these compounds have the ability to increase vitellogenin levels in fish (Tilton *et al.*, 2002).

Induction of plasma vitellogenin, intersex (typically testis-ova which is the presence of ovarian tissue in the testes), reduced testicular development, and reduced fertility in fish downstream of sewage treatment plant outfalls have been linked to the presence of oestrogenic substances in the effluent (Purdom *et al.*, 1994; Harries *et al.*, 1996; 1997; Jobling *et al.*, 1998; Rodgers-Gray *et al.*, 2000). *In-vivo* studies have

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demonstrated that exposure of fish to as little as 1-10ng/l of E2 (Routledge *et al.*, 1998) and 0.1ng/l of EE2 (Purdom *et al.*, 1994) is sufficient to induce intersex in some species of male wild fish. A study on juvenile Japanese medaka (*Oryzias latipes*) demonstrated that E1, E2, E3, and EE2 induced testis-ova and altered sex at nanogram per litre concentrations from the time of hatching to 100 days afterwards (Metcalfe *et al.*, 2001). Madsena *et al.* (2004) demonstrated that short-term exposure to E2 and nonylphenol can delay smolt development and downstream migration in Atlantic salmon (*Salmo salar*) by inhibition of gill Na+, K+-ATPase.

A myriad of studies have presented evidence of endocrine disruption in field studies in many countries for example flounder (Kleinkauf *et al.*, 2004), rainbow trout (Purdom *et al.*, 1994, Harries *et al.*, 1996;1997, Sheahan *et al.* 2002a;2002b), and roach (Jobling *et al.*, 1998) in the UK; carp in Spain (Lavado *et al.*, 2004); channel catfish (Tilton *et al.*, 2002) and mosquito fish (Toft *et al.*, 2003) in the USA, perch and roach in Sweden (Noaksson *et al.*, 2001) and swordfish off the Italian coast (Fossi *et al.*, 2004).

1.14.4 Birds

Many lipophilic chemicals such as chlorinated pesticides, PCBs, PCDFs, and PCDDs accumulate in the egg yolk of laying birds which exposes the avian embryo during its early developmental stages.

In the 1970s in Southern California, it was observed that 8.14% of pairs of western gulls (*Larus occidentalis*) in the population consisted of pairs of females and low numbers of reproductively competent males were reported (Hunt *et al.*, 1980). The probable cause was exposure to the pesticide o,p'-DDT. This pesticide has been shown to induce abnormal development of ovarian tissue and oviducts in male gull embryos and interfere with their breeding (Fry and Toone, 1981). Testicular feminization was reported in fifty-seven percent of male gull (*Larus occidentalis*) embryos collected from Scotch Bonnet Island, Canada in 1975 and 1976 (Fox, 1992).

Studies on Laysan Albatross (*Diomedea immutabilis*) and Black-footed Albatross (*Diomedea nigriceps*) on Midway Island in the Pacific have revealed detectable levels of PCBs, PCDDs, PCDFs, DDT and metabolites in their tissue despite the fact that this island is isolated. A decrease in the population of Black-footed

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Albatross has been observed since the 1980s due to poor hatching success and eggshell thinning (Auman *et al.*, 1997).

Exposures to high concentrations of organochlorines in the 1970s and 1980s have resulted in depleted populations of the Herring gull (*Larus argentatus*), Common tern, Forster's tern (*Sterna forsteri*), Bald eagle (*Haliaeetus leucocephalus*) and Double-crested cormorant (*Phalacrocorax auritus*) due to low hatchabilities, deformities in embryos and hatchlings, eggshell thinning and "chick-edema" disease. Exposure to PCBs has also been linked to behavioural effects such as poorer nest construction observed in PCB contaminated tree swallows along the Hudson River in New York (McCarty and Secord, 1999).

Eggshell thinning primarily due to exposure to DDE which is a degradation product of DDT, has been reported in many avian species such as Peregrine falcon (*Falco peregrinus*) (Johnstone *et al.*, 1996) and Guillemot (*Uria aalge*) (Bignert *et al.* 1995) causing eggs to break and adversely impacting on reproduction.

A syndrome prevalent among fish-eating birds in the Great lakes region involving skeletal and beak malformations, cardiac edema, and subcutaneous edema has been named GLEMEDS (Great Lakes Embryo Mortality, Edema and Deformities Syndrome) (Gilbertson *et al.*, 1991). With some decline of concentrations of DDT, PCBs, and PCDDs/Fs there have been reductions in the symptoms of GLEMEDS and the rate of reproductive failure among some species.

1.14.5 Invertebrates

The most prominent association of endocrine disruption to invertebrates is imposex, which is masculinization of female prosobranch gastropods due to use of tributyltin (TBT), a commonly used antifouling agent on hulls of ships. Many different species of prosobranch gastropods have been affected by organotins worldwide in countries such as Ireland (Minchin *et al.*, 1997), France (Alzieu *et al.*, 1991), Malta (Axiak *et al.*, 2003) and Northern Europe (Vos *et al.*, 2000). Detrimental effects were first noticed in the early 1980s in France (Alzieu *et al.*, 1991). There are varying degrees of imposex from females with a tiny phalli but no penis duct to females with almost complete male reproductive organs with sperm-like structures present in the

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ovaries. In the earlier stages of imposex, normal reproduction is not affected; however, imposex is irreversible (Axiak *et al.*, 2003).

In 1987 an Irish bye-law prohibited use of organotins on vessels under 25 metres and on all other structures (Anon, 1987). A recent study of six Irish bays have concluded that the effects of TBT contamination on wild molluscs and cultivated oysters are negligible from the point of view of commercial production and that the bye-law of 1987 has been effective in reducing contamination by TBT (Minchin, 2003). Application of TBT to all vessels is forbidden in all EU countries since 1/1/2003 under the Marketing and Use Directive (76/769/EEC) and antifouling biocides will be reviewed in 2006 under the EU Biocidal Products Directive (98/8/EC) which may result in removal of products containing antifouling biocides from the EU market.

Various studies have been carried out on invertebrates to evaluate the impact of oestrogenic compounds on their development. Marciel *et al.* (2003), Andersen *et al.* (2001), and Forget-Leary *et al.* (2005) suggested that naupliar development of copepods *Tigriopus japonicus, Acartia tonsa* and *Eurytemora affins* respectively could be affected and/or inhibited by environmentally relevant concentrations of oestrogenic compounds.

1.15 Effects of endocrine disruption on humans

There has been increasing concern that chemicals in the environment that are capable of disrupting the endocrine system are affecting human health and may be associated with altered reproductive capacity for example the supposed decrease in sperm count in adult males, the increasing incidence of cancers of the breast, uterus, prostate and testes (Anon, 2000).

The human routes of exposure to environmental toxicants include inhalation, ingestion and dermal contact. The endocrine disrupting contaminants with greatest potential for exposure via inhalation are low mass endocrine disrupting compounds such as halogenated compounds, for example, lindane and non-halogenated aromatic hydrocarbons, phthalate esters and phenols (WHO, 2002). Drinking water is not considered a major source for the ingestion of endocrine disrupting contaminants unless unusual contamination has occurred. The primary exposure route to endocrine disrupting contaminants for humans is ingestion. Exposure to potential endocrine disrupting contaminants varies considerably due to geographic region and food culture

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for example, Zumbado *et al.* (2005) associated very high mortality rates for breast cancer in the province of Las Palmas, Gran Canaria with the fact that higher DDT:DDE ratios and higher values of parental DDT isomers were reported in the population of Gran Canaria. DDE is the metabolite of DDT and high DDT: DDE ratios indicate that there could be an active source of the banned pesticide, DDT on the island despite it being banned in Spain since 1977.

Dermal exposure occurs when the skin is in immediate contact with toxicant contaminated water, soil, or surfaces (Rice *et al.*, 2003). This is of particular concern with regard to children chewing or sucking teething rings, toys, stuffed animals etc. in their mouths that may have endocrine disrupting contaminants on their surface which are solubilised in the mouth and swallowed (Latini *et al.*, 2004).

The most critical periods of development susceptible to disruption appear to be *in utero* (Newbold, 2004), in the first few months postnatally (Pohl *et al.*, 2004) and puberty (WHO, 2002; Teilmann *et al.*, 2002). Exposure before conception can jeopardise normal foetal differentiation and development. Exposure to toxicants at these stages may result in permanent damage to organ function. The degree of exposure *in utero* depends on the placental barrier and neonatally, on the blood-brain barrier which is not fully developed until one year of age (Damgaard *et al.*, 2002). The effects of exposure during foetal life and childhood may not become apparent until adult life, for example, decreased sperm count and motility.

Studies comparing the effects of endocrine disrupting compounds on males and females are generally not carried out because the cyclical nature of hormones in females complicates studies on hormonal processes (Rice *et al.*, 2003). Developing males are susceptible to demasculinizing and feminizing effects from oestrogens and anti-androgens. Developing females may have an increased cancer risk due to exposure to oestrogens; however, the potential effects on anti-androgens on females are not yet known (Damgaard *et al.*, 2002).

Documented evidence of effects of endocrine disrupting contaminants on humans has come from "specific cases of accidental high-dose exposure to known endocrine disruptors through industrial accidents, food contamination, or

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pharmacological dosing during critical periods of development" (Filali-Meknassi *et al.*, 2004). The following are some examples of these incidences:

1.15.1 Polychlorinated biphenyl exposure in Yucheng, Taiwan

In 1978-1979 in central Taiwan a leak from a heating pipe in a rice oil production factory led to the contamination of cooking oil by high concentrations of PCBs and their combustion products, the polychlorinated dibenzofurans (PCDFs). Over 2,000 people, several of whom were pregnant ingested contaminated cooking oil resulting in *utero* exposure to PCBs and furans and subsequent neonatal exposure through breast milk. Increased foetal loss and low birthweight were reported immediately (Lione, 1988). Abnormal menstrual bleeding and higher stillbirth rates were reported in exposes women in a long-term study (Yu *et al.*, 2000). The children that were exposed prenatally had delayed developmental milestones, intrauterine growth retardation, abnormal skin pigmentation, lower IQs and adverse effects on cognitive development (Lai *et al.*, 2001).

1.15.2 TCDD exposure in Seveso, Italy

In 1976 in Seveso, Italy, an explosion at a plant that manufactured the herbicide 2,4,5-trichlorophenol (TCP) exposed the whole community to high concentrations of 2,37,8-tetrachlorodibenzo-p-dioxin (TCDD) Mocarelli *et al.*, 1996; 2000). An increase in cancer in the area of highest exposure has been linked to the incident along with increases in chronic circulatory and respiratory diseases and a higher incidence of diabetes particularly in exposed women (Bertazzi *et al.*, 2001).

1.15.3 Diethylstilbesterol (DES) exposure

Diethylstilbesterol is a synthetic non-steroidal oestrogen that was used in hormone replacement therapy, inhibition of lactation, control of menstrual disorders, as a postcoital contraceptive, and in treatment for prostrate cancer in men and in breast cancer treatment in postmenopausal women (U.S. Department of Health and Human Services, 2004). From 1948 to 1971 DES was prescribed to pregnant women in order to prevent miscarriages and premature labour. The drug failed and in 1971 Herbst *et al.* (1971) linked a rare type of vaginal cancer "vaginal clear cell adenocarcinoma" in a number of adolescent girls (<0.1%) whose mothers had taken the drug. A study by Swan (2000) found that the gestational age of the foetuses at exposure related strongly to the degree of abnormalities induced. 50% of prenatal females exposed to a total dose

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of up to 1000mg DES went on to develop vaginal adenosis while those exposed to over 3000mgDES all developed adenosis (Swan, 2000). Additionally, malformations in the uterus such as T-shaped uterus, constricting bands in the uterus, hypoplastic uterus and also abnormalities of the cervix were reported in exposed female offspring (Kaufman *et al.*, 1977; Bibbo *et al.*, 1977) and structural, functional and cellular abnormalities such as hypospadias, epididymal cysts, hypotrophic testes, retained testes, microphallus, Gill *et al.*, 1976; Klip *et al.*, 2002). Kaufman *et al.* (2000) carried out a follow-up study in 1994 of pregnancy outcomes in DES-exposed offspring. Preterm births and spontaneous abortion occurred at higher rates of 19.4% and 19.2% respectively in DES-exposed women than in unexposed women (7.5% and 10.3% respectively).

1.15.4 Decreased sperm counts

In 1992 a meta-analysis of sixty-one studies on 14,947 men was published by Carlsen *et al.* (1992) which demonstrated a major decrease in sperm concentrations from 113×10^6/ml to 66 $\times 10^6$/ml from 1938 to 1990. However, the results were considered invalid by some researchers due to highly variable data, validity of statistical methods, bias due to changing study populations and confusion by factors such as age or abstinence. Swan *et al.* (1997) reanalysed the studies carried out by Carlsen *et al.* (1992) including more studies from the same period and taking factors such as abstinence time, age, and specimen collection method into account. They determined that the decline in sperm density per year in the US and Europe/Australia was 1.5 times and 3 times greater than the average decline of 1% per year Carlsen *et al.* (1992) had reported. A subsequent study was carried out with 47 additional studies published between 1934 and 1996 in which the average decline in sperm count was almost exactly the same as that reported by Carlsen *et al.* (1992) (a slope of -0.94 versus a slope of -0.93) (Swan *et al.*, 2000).

Abell *et al.* (2000) reported lower sperm counts and percentages of morphologically normal sperm in greenhouse workers in Denmark with higher/longer exposure to pesticides. In an American study, Swan *et al.* (2003a; 2003b) reported a lower sperm count (58.7×10^6/ml) and a reduced number of motile sperm in Columbia, Missouri than in New York (102.9×10^6/ml), Minneapolis (98.6×10^6/ml) and Los Angeles (80.8×10^6/ml). This decline was associated with exposure to herbicides alachlor and atrazine and the insecticide diazinon.

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1.16 Legislation and regulations

Development of policies and regulations must be concurrent with research in progress and legislation must be able to be readily adjusted to advances in research. In a communication from the Commission to the Council and the European Parliament in 1999 (COM(1999)706), the commission proposed a strategy consisting of short, medium and long term actions to address the problem of endocrine disruption. Short-term measures included among other actions; establishment of a priority list for evaluation, use of existing legislation and establishment of monitoring programmes. Medium-term measures included identification and assessment of endocrine disruptors and further research and development and long-term measures entailed adaptation/amendment of current legislation to take account of endocrine disruption.

A priority list of substances was compiled in 2000 made up of 553 man-made substances, evidence of endocrine disruption was found for 118, a further 9 of which were not addressed under legislation at that time. A more in depth study on these 9 substances and also on 3 natural/synthetic hormones (oestrone, oestradiol and ethinyloestradiol) was carried out in 2001 (EC, 2002a). However, there was insufficient data to assess endocrine disruption of 435 of these substances on the priority list and a subsequent study in 2001 led to the finding that there was evidence/potential evidence of endocrine disruption for 147 of these substances (EC, 2002b). The Organization for Economic Co-operation and Development Endocrine Disruptor Testing and Assessment Task Force led the program of validation of agreed test methods to ensure all countries (European and non-European) adopted the same testing and assessment approaches.

In 1993 the Council adopted the Existing Substances Regulation (93/793/EEC) to control use of "existing" chemical substances. This regulation was intended to complement regulations already in place for "new" chemical substances (Council Directive 67/548/EEC). However, more recently, in 2003, the European Commission approved a proposal for a new regulatory framework for chemicals, which will re-address and greatly improve the EU's existing chemical substances policy. The proposed system is titled REACH (Registration, Evaluation and Authorisation of Chemicals). This proposal requires any company that manufactures or imports more than one metric ton of an existing substance to register it in a central database. Registration includes information about the properties of the substance, its uses and guidelines on handling the chemical.

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The Water Framework Directive (2000/60/EC) sets a framework of common objectives, principles and measures for the management of water resources across the EU. It concerns inland surface waters, groundwater, estuarine and coastal waters and it aims to achieve at least "good status" in all waters by 2015. It includes a list of 33 priority substances that represent a significant risk to or via the aquatic environment at EU level. Among other contaminants listed are nonylphenols and octylphenols, di(2-ethylhexylphthalate (DEHP), C10-C13 chloroalkanes and brominated diphenylethers.

1.16.1 Biosolids

By the end of 1998, under the Urban Wastewater Directive 91/271/EEC (Article 14 Irish Sea Act), disposal of sludge to surface waters for dumping from ships should have been phased out but dumping continued through 1999. Application of sewage sludge to the land is regulated under Council Directive 86/278/EEC, 1986, on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture however organic contaminants were not considered in this directive. In 2000, the European Community drafted regulations specifying maximum concentrations for a range of contaminants in sludges to be used for agriculture which are presented in table 1.16. Some European countries have assigned more stringent regulations for some organic contaminants in sludge for agricultural use. Germany, Switzerland and the Netherlands set values of 200μg/kg for individual polychlorinated biphenyl (PCB) congeners (De Souza Pereira *et al.*, 2005) and the Danish Ministry of the Environment and Energy lowered the Danish limit value for nonylphenols, nonylphenol-1-ethoxylate and nonylphenol-2-ethoxylate from 30mg/kg to 10mg/kg.

Table 1.16 Limit values for organic compounds and dioxins in sludge for use on land designated by the EU Working Document on Sludge in 2000.

Organic compounds	**Limit value (mg/kgdm)**
AOX[a]	500
Linear Alkylbenzyl Sulphonates	2600
Di(2-ethylhexyl)phthalate	100
NPE[b]	50
PAH[c]	6
PCB[d]	0.8

Dioxins	**Limit value (ng TE[e]/kgdm)**
Polychlorinated dibenzodioxins and dibenzofuranes	100

[a] Sum of halogenated organic compounds
[b] Nonylphenol and nonylphenolethoxylates with 1 or 2 ethoxy groups

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[c]Sum of the following polyaromatic hydrocarbons: acenapthene, phenanthrene, flourene, flouranthene, pyrene, benzo (b+j+k)flouranthene, benzo(a)pyrene, benzo(ghi)perylene, indeno(1,2,3-c,d)pyrene.
[d] Sum of polychlorinated biphenyl congeners 28,52,118,138,153,180.
[e] toxicological equivalent

1.17 Oestrogen assays

In the analysis of wastewater when it is not definite which chemicals are present and in what quantities they are present in, an assay that can measure the total oestrogenic activity of influents and effluents can indicate the degree of removal taking place within the plant but also gauges the ecotoxicological consequences of exposure to the effluent (Kirk *et al.*, 2002). Some researchers have carried out both chemical analysis and *in-vitro* studies on environmental samples in order to determine whether or not the selected xenoestrogens for chemical analysis are responsible for all of the oestrogenic potency of the sample (Desbrow *et al.*, 1998; Synder *et al.*, 2001; Cargouët *et al.*, 2004; Furuichi *et al.*, 2004).

The results of the chemical analysis for each compound analysed can be expressed as theoretical E2 equivalent (EEQ) values which can be summed up to obtain a total EEQ value for the sample assuming additive effects within the sample mixture. If this value is lower than the total EEQ value determined by *in-vitro* analysis then there may be other compounds present in the sample which were not chosen for analysis that are responsible for the extra oestrogenic activity. If the value is higher than the total EEQ value determined by *in-vitro* analysis then there may be unknown active antagonists in the in-vitro assay or calculation of EEQ values based on detection limits has overestimated the EEQ of chemical analysis (Murk *et al.*, 2002). Different methods of chemical analysis have been used to determine oestrogen potency in various environmental matrices (Table 1.17.1, Appendix B).

1.17.1 *In vivo* assays

1.17.1.1 Rodent Uterotrophic assay and vaginal cornification assay

Allen and Doisy developed one of the first assays to measure oestrogenicity in the 1920s to identify potent oestrogens and to allow structural elucidation (Hertig, 1983). In subsequent years the assay was modified for use in the discovery of drugs for oestrogen agonists and antagonists.

Growth of the uterus following administration of a chemical to either sexually immature or ovariectomised rodents is determined by increase in wet weight (Ashby,

2001). Immature or ovariectomised female rats and mice are used as they have low endogenous oestrogen levels (Owens and Koëter, 2003). In a comparison of the rodent uterotrophic assay to the Yeast Oestrogen Screen assay (YES), Odum *et al.* (1997) found both assays had similar sensitivities to E2, nonylphenol and methoxychlor.

The vaginal cornification smear is another original method developed for studying oestrogenicity in the mid 1900s. This assay was used to detect histological changes in the vaginal epithelium. A positive result is indicated by the formation of nucleated (cornified) cells (Combes, 2000).

These assays are significant in that they mark the beginning of oestrogen analysis however they are unsuitable for large scale screening due to relatively poor sensitivity and labour-intensive end point measurements (Zacharewski, 1997).

1.17.1.2 Vitellogenin assays and reptile egg assay

Vitellogenesis is a physiological process for the production of yolk in all female oviparous vertebrates. Male fish do not require vitellogenin; hence vitellogenin concentrations in males are low to non-existent. Levels are low in immature female fish also. However, aquatic contaminants that can increase 17β-E2 levels in the blood or increase gonadotrophin releasing hormone levels or inhibit the negative feedback mechanisms related to these compounds have the ability to increase vitellogenin levels in fish (Tilton *et al.*, 2002). The increase of vitellogenin in male fish is used as a biomarker to assess endocrine disruption by oestrogenic compounds (Jones *et al.*, 2000).

Vitellogenin studies have been carried out on various species of fish exposed to xenoestrogens for example carp (*Cyprinus carpio*) (Smeets *et al.*, 1999a; Smeets *et al.*, 1999b), brown trout (*Salmo trutta*) (Sherry *et al.*, 1999), flounder (*Platichthys flesus*) and bream (*Abramis Brama*)(Vethaak *et al.*, 2005) with determination of vitellogenin levels by subsequent *in-vitro* assay. Studies have been carried out in effluents both in the laboratory and on site. Other tissue indices such as the hepatic-somatic index (HSI) and the gonadsomatic index (GSI) used to identify tissue level effects from effluent exposure are usually carried out alongside vitellogenin determination. The HSI is a ratio of the total body weight by the different between total body weight and liver weight (Tilton *et al.*, 2002) while the GSI is the ratio of the size of the gonad to the total body weight (Jobling *et al.*, 1998).

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The reptile egg assay is based on the temperature-dependent sex determination of turtles and lizards. Exposure of turtle eggs to a putative xenoestrogen by spotting or painting them with compounds exhibiting oestrogenic potency interferes with the temperature-dependent sex determination. It can result in a high proportion of feminization or intersex conditions of hatchlings at a temperature that would normally result in 100% males (Bergeron *et al.*, 1994).

1.17.2 *In vitro* assays

A range of *in-vitro* assays have been developed for the detection of potential oestrogens at several stages in the primary mechanism of actions. In general, there are three types of assay: ER competitive binding assays; recombinant receptor-reporter gene assays; and cell proliferation assays.

1.17.2.1 ER competitive binding assays

Competitive ligand binding assays rely solely on the binding ability of an oestrogen or xenoestrogens to bind to the oestrogen receptor (ER). ER binding assays are significantly less sensitive than other *in-vitro* assays. Murk *et al.* (2002) compared an ER binding assay and two receptor-reporter gene assays; YES and ER-CALUX in a study to determine oestrogenic potency in wastewater and surface water. The detection limit for the ER binding assay was 272ng/l E2 while it was 2.7ng/lE2 and 0.1ng/l E2 for the YES assay and the ER-CALUX assay respectively. The ER binding assay is expected to produce higher measurements because it cannot distinguish agonists from antagonists, hence, the EEQ is increased. However, this can be seen as an advantage because adverse effects can be produced from both agonist and antagonist activity (Li *et al.*, 2004). Compounds that can not permeate cell membranes or would otherwise be metabolized can still bind to the ER (Murk *et al.*, 2002).

Binding of a substance to the ER does not imply that it can initiate the molecular cascade of events leading to gene transcription and protein synthesis associated with adverse effects (Zacharewski, 1997; Joyeux *et al.*, 1997; Birkett, 2003a). ER binding assays are fast but they are not amenable to automation and they require specialised laboratory equipment for analysis of radioactive substances. A new ER binding assay named ELRA (Enyzme-linked receptor assay) has been developed which is sensitive, non-radioactive, has a rapid analysis time of less than four hours and is cost effective (Seifert *et al.*, 1999). Li *et al.*, (2004) compared ELRA to the YES assay in the

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determination of oestrogenic potency of E2, tamoxifen, bisphenol-A (BPA), and resveratrol. Results showed that ELRA was approximately one order of magnitude more sensitive to17β-E2 then the YES in samples.

1.17.2.2 Cell proliferation assays

These assays measure the increase in cell number of target cells (for example, MCF-7 or T47-D human breast cancer cells) induced by exposure to oestrogenic compounds. The E-SCREEN assay is a cell proliferation assay which measures the oestrogen-induced increase of cell number of MCF-7 cells (Soto *et al.*, 1995). In 1995, novel xenoestrogens were discovered by Soto *et al.* among antioxidants, plasticizers, pesticides and polychlorinated biphenyl congeners in an extensive study using the E-SCREEN assay. Körner *et al.* (1999) miniaturised the assay from 24-to-96 well plates without compromising sensitivity (detection limit of 0.27ng/l) which reduced time and labour. The authors analysed nine effluent samples from five different municipal sewage treatment plants in South West Germany and detected oestrogenic activity ranging from 2.5ng EEQ/L to 25ng EEQ/L. They also demonstrated the additive behaviour of the oestrogenicity of single compounds in mixtures using the E-SCREEN assay.

Folmar *et al.* (2002) compared the oestrogenic potencies of E2, EE2, diethylstilbestrol, nonylphenol and methoxychlor in the E-SCREEN assay, YES assay and two *in-vivo* vitellogenin assays. The E-SCREEN assay was found to be one order of magnitude more sensitive than the YES assay for all the chemicals tested. Relative oestrogenic potencies for EE2 were equal for the E-SCREEN assay and the vitellogenin assay. Diethylstilbestrol was somewhat higher in the E-SCREEN assay but methoxychlor was three and five orders of magnitude more potent *in-vivo* than in the E-SCREEN assay and YES assay respectively.

1.17.2.3 Recombinant receptor-reporter gene assays

Reporter gene assays are either mammalian based (i.e. the ER-CALUX, MVLN cell assay and chimeric receptor-reporter gene assays) or yeast based (i.e. the YES assay). In these assays the test chemical not only binds to the ER but also activates the receptor causing transcription and expression of reporter genes thereafter. The assay can be carried out with cell lines containing an endogenous receptor (MCF-7cells or

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T47D cells) or a cell line devoid of an endogenous receptor (yeast cells or HeLa cells). Popular reporter genes used code for firefly luciferase and β-galactosidase.

Following interaction of the test compound with the ER, conformation of the ER is changed and it becomes activated. The ligand-ER complex binds to the ERE on the reporter gene plasmid. This initiates expression of the reporter gene and production of the enzyme. This newly synthesised enzyme metabolises a suitable substrate in the incubation medium (some reporter gene assays require prior lysis of the cells for metabolism to occur) producing an easily detectable product (Danish Environmental Protection Agency, 2003; Combes 2000).

In the determination of oestrogenic potency cells are exposed to a test substance(s) and a concentration gradient of a positive control i.e. E2. The response is measured and compared to the standard to determine its E2 equivalent (EEQ),

1.17.2.3.1 ER-mediated chemical activated luciferase gene expression (ER-CALUX)

This assay uses T47D human breast cancer cells expressing endogenous ER stably transfected with an oestrogen responsive luciferase reporter gene (pEREtata-Luc) containing three EREs. On expression of the luciferase gene, the cells are lysed, the substrate luciferin is added and then light output is measured using a luminometer. It has a detection limit of 0.5pM and can be used to assay anti-oestrogenic compounds (Legler *et al.*, 1999).

1.17.2.3.2 MCF-7-derived cell line assay (MVLN)

The cell line is derived from the MCF-7 breast cancer line expressing endogenous ER and is stably infected with an oestrogen- responsive luciferase reporter gene. This cell line was originally developed and characterised by Pons *et al.* (1990). The level of luciferase activity following lysis of MVLN cells is measured in a luminometer

1.17.3 Recombinant yeast oestrogen screen assay

Different yeast based receptor-reporter assays have been developed by various researchers:

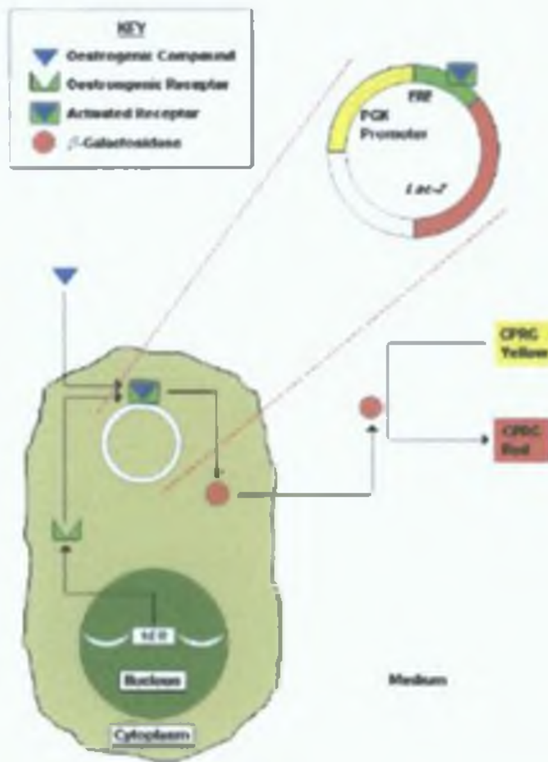

Figure 1.17: Schematic of the oestrogen-inducible expression system in yeast (Routledge and Sumpter, 1996)

The Glaxo Welcome-derived *Saccharomyces cerevisiae* strain developed by Routledge and Sumpter (1996) is a molecular toxicology assay used to identify compounds that can interact with the human oestrogen receptor (hER) (figure 1.17). The DNA sequence of the human oestrogen receptor was stably integrated into the yeast genome. The hER is expressed in a form capable of binding to oestrogen-responsive sequences (ERE). On a separate expression plasmid, the ERE controls the expression of the Lac-Z reporter gene. This reporter gene encodes the enzyme β-galactosidase. Natural or xeneoestrogens interact with the bound receptor on the hybrid promoter to activate transcription of the genes i.e. expression of the reporter gene Lac-Z which results in the production of β-galactosidase. This enzyme is secreted into the medium where it metabolizes the chromogenic substance present in the medium, chlorophenolred-β-d-galactopyranoside (CPRG) causing a colour change from yellow to red, the intensity of which relates to the oestrogenic activity of the compound or mixture being tested and can be measured by absorbance at 540nm.

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The YES system developed by Gaido *et al.*, (1997) and used by researchers recently (Servos *et al.*, 2005) employs yeast containing two plasmids, one plasmid contains the CUP1 metallothionien promoter and human oestrogen receptor cDNA for copper inducible oestrogen receptor production; the second contains two oestrogen-responding elements liked to the lacZ gene. This assay requires lysis of yeast cells before assessment of galactosidase activity.

The yeast construct used by Rehmann *et al.* (1999) did not contain a complete oestrogen receptor or a reporter gene linked to oestrogen responsive elements. The yeast strain used "expresses a fusion protein carrying only the hormonal binding domain of the human oestrogen receptor connected to the yeast GaL4-DNA binding domain. When a suitable compound binds to the hormone binding domain the fusion protein recognises a responsive DNA element upstream of a β-galactosidase reporter gene" Rehmann *et al.* (1999). The test cultures are incubated for two hours only and β-galactosidase activity was determined by Miller units.

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1.17.3.1 Advantages and disadvantages

The use of yeast as a recipient organism is advantageous in that it has a rapid growth rate, it is easy to culture, and is amenable to genetic engineering. Since yeast does not normally contain an oestrogenic receptor or other endogenous receptors, potential problems regarding the effects of other gene targets or the complex interaction between the oestrogen receptor and other receptors are avoided (Routledge and Sumpter, 1996; Joyeaux *et al.*, 1997). The expression and reporter plasmids in the yeast strain are maintained by growth in selective media, the product of the receptor is secreted into the medium and cell lysis is not required for the assay developed by Routledge and Sumpter (Legler *et al.*, 2002). In other yeast based assays (Coldham *et al.*, 1997; Gaido *et al.*, 1997), the β-galactosidase remains within the yeast cell requiring lysis followed by an enzyme assay. Routledge and Sumpter (1996) reported the assay to be highly reproducible with a concentration of 3ng/L E2 producing a desirable increase in β-galactosidase production. The assay allows analysis of multiple compounds over a wide range of concentrations. The colour change can be seen by the naked eye allowing results to be qualitative or quantitative.

However, there are also disadvantages associated with the YES system including variations in ligand selectivity compared to mammalian cells in genetic drift of the yeast

cells over time (Joyeaux *et al.*, 1997). Some researchers have recorded false negative data due to lack of uptake of test chemicals which may be due to toxic effects. (Andersen *et al.*, 1999). The YES assay cannot detect all anti-estrogens (Beresford *et al.*, 2000; Graumann and Jungbauer, 2000).

Various researchers have used various yeast cell clones, ER constructs and reporter genes which makes an assessment of the overall performance of the assay quite difficult. Researchers have made different procedural modifications to the assay also. Beresford *et al.*, (2000) demonstrated the effects of modifying various aspects (incubation time, mode of addition of sample, carrier solvent and initial cell number) of the standard procedure developed by Routledge and Sumpter (1996). The authors reported that the assay became more sensitive to 17β-E2 and also to nonylphenol with increased incubation time, and with the addition of the sample directly to the medium containing yeast instead of allowing the sample to evaporate and then adding the media. Two different carrier solvents were assessed; ethanol and dimethylsulfoxide and were found to give rise to almost the same sensitivity in the assay. The assay could also be made more sensitive by increasing the initial cell number of yeast. Consequently, various analysts have used dimethylsulphoxide as the carrier solvent in place of ethanol (Witters *et al.*, 2001) in addition to adding the samples to the media containing yeast rather than adding the media to the evaporated samples (Murk *et al.*, 2002; Legler *et al.*, 2002). Other analysts have increased the incubation period, for example, Holbrook *et al.*, (2000) incubated samples at 32°C for 3 days and then a further 3 days at room temperature.

Yeast based receptor-reporter assays have been used to determine oestrogen potency in various environmental matrices (Table 1.17.2, Appendix C).

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Chapter 2.0 Methods

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2.1 Materials

2.1.1 Chemicals

- ultra pure water
- Methanol (99+), acetone, ethanol, hexane and nitric acid (purchased from Sigma-Aldrich Ireland Ltd. Dublin, Ireland)
- Dichloromethane (purchased from Lennox Laboratory Supplies Ltd, Dublin 12)
- KH_2PO_4 (purchased from Lennox Laboratory Supplies Ltd, Dublin 12)
- $(NH_4)2SO_4$ (1.98g), KOH pellets (4.2g), $MgSO_4$ (0.2g), $Fe_2(SO4)_3$ solution (1ml of 40mg/50ml H_2O), L-leucine (50mg), L-histidine (50mg), adenine (50mg), 2L-arginine-HCl (20mg), L-methionine (20mg), L-tyrosine (30mg), L-isoleucine (30mg), L-lysine-HCl (30mg), L-phenylalanine (25mg), 1L-glutamic acid (100mg), L-valine (150mg) and L-serine (375mg) all purchased from Sigma Aldrich (Sigma-Aldrich Ireland Ltd. Dublin, Ireland)
- *D-(+)-Glucose* (prepared at 20%w/v solution)
- *L-Aspartic acid* (prepared at 4mg/ml)
- *L-Threonine* (prepared at 24mg/ml)
- Thiamine (8mg), pyridoxine (8mg), pantothenic acid (8mg), inositol (40mg) and biotin solution (20ml of 2mg/100ml H_2O)
- *Copper (II) sulphate* (20Mm solution)
- *Chlorophenol red-β-D-galactopyranoside (CPRG)* (purchased from Fannin Healthcare, Dublin 18) (prepared at 10mg/ml)
- Glycerol (15%-8ml of sterile glycerol was added to 45ml minimal media)
- 17β-oestradiol (97%), norethindrone (98%), diethylstilbestrol (97%), bisphenol-A (99%), benzylbutylphthalate (98%) and genestein (all purchased from Sigma Aldrich (Sigma-Aldrich Ireland Ltd. Dublin, Ireland).

2.1.2 Culture

The recombinant yeast strain, *Saccharomyces cerevisae*, was kindly provided by Professor Sumpter of Brunell University, Middlesex, UK.

2.1.3 Apparati and materials

- 1L borosilicate glass bottles
- Buchner funnel, Whatman GF/C filters , 0.45µm filters and filter unit
- Sonication bath

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- 47mm Empore SDB-XC (styrenedivinylbenzene) extraction disks (purchased from JVA Analytical, Dublin 12) , 47mm glass filtration apparatus, 20ml Quickfit conical flask collection vessels, 20ml volumetric flask, and vaccum pump
- Blender, 1mm mesh sieve, stainless steel dishes
- Soxhlet apparati, cellulose Whatman thimbles,
- 0.2-µm pore size disposable filter (Lennox Laboratory supplies Ltd, Naas Road, Dublin 12), 25ml glass universals
- Laminar air flow cabinet (Astec Microflow Advanced Bio Safety Cabinet class 2)
- Orbital shaker, Incubator, Anthos 2010 microplate reader
- 1.2ml sterile cryovials, 50ml sterile centrifuge tubes, centrifuge,
- Sarstedt 96-well microtitre plate, micropipettes, Petri dishes, sterile 50ml conical flasks

2.1.4 Wastewater treatment plants to be sampled

Details of the four wastewater treatment plants (WWTP) chosen for analysis are presented in table 2.1.4 (overleaf). These wastewater treatment plants are representative of both small (WWTPs A and B) and large-scale (WWTPs C and D) treatment plants. They are all domestic treatment plants with some industrial waste contributing to the input into WWTPD. The choice of treatment plants to analyse was influenced by the different treatment technology employed at the plants which would allow for comparison of treatment efficiency of each type.

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Table 2.1.4: Details of wastewater treatment plants analysed

Parameter	WWTP A	WWTP B	WWTP C	WWTP D
Population equivalent	Original:1400 Present:1900	Original:1700 Present: 2100	20,000	1.5 million
Input	Domestic	Domestic	Domestic	Industrial and domestic
Preliminary & primary treatment	Screening and primary sedimentation imhoff tank	Screening only	Primary sedimentation	Screening and aerated grit removal and primary sedimentation
Secondary treatment	Trickling filter	Oxidation ditch and clarifier	Extended aeration oxidation ditch with diffused air aeration	Sequential batch reactors
Tertiary treatment	Grass plots	n/a	Chemical precipitation with ferric chloride	UV disinfection
HRT (hrs)	≈18	≈7	≈12	≈15
Sludge treatment	None. Sludge is dried on drying beds and transported to Portarlington for treatment	None. Sludge is dried on drying beds and transported to Portarlington for treatment	Autothermal thermophilic aerobic digestion producing Class A biosolids	Anaerobic digestion and thermal drying producing Class A biosolids

Abbreviations

WWTP = Wastewater treatment plant

HRT = Hydraulic retention time

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2.2 Method

2.2.1 Sampling and preparation

2.2.1.1 Influent and effluent

Sampling was carried out during the summer of 2004. Samples were collected in 1L borosilicate glass bottles, previously washed twice with methanol and once with ethanol. Grab samples of influents were taken first with grab samples of effluents taken at a period of time later equivalent to the retention time of the wastewater treatment plant. Samples were chilled on transit to the laboratory. 50ml methanol was added to each sample to prevent bacterial growth (Desbrow *et al.*, 1998; Kirk *et al.*, 2002; Aguayo *et al.*, 2004) and samples were acidified to approximately pH2 using nitric acid to so as to aid subsequent filtration (Ternes *et al.*, 1999a; Servos *et al.*, 2005; Pawlowski *et al.*, 2004). A series of filters of decreasing pore sizes were used. Samples were filtered twice using GF/C filters and once using 0.45μm filters. Sample bottles were rinsed with methanol which was then used to sonicate spent filters for 10 minutes. The methanol was filtered and added to the sample as was carried out in several studies (Baronti *et al.*, 2000; Johnson *et al.*, 2000; Laganà *et al.*, 2004). This process of washing and sonicating was repeated a second time.

2.2.1.1 Sludge

Activated sludge and treated sludge were collected in suitable containers previously washed with methanol and ethanol. Samples were chilled on transit to the laboratory. Samples were then dried in stainless steel dishes (previously washed with methanol and ethanol) at 35°C for approximately 48 hours.

2.2.2 Extraction of oestrogenic compounds

2.2.2.1 Influent and effluent

Samples were extracted within 24hours of arrival to the laboratory. An overview of the extraction and analysis steps is presented in Figure 2.2.2

2.2.2.1.1 Apparatus set-up

Extraction was carried out using 47mm Empore SDB-XC (styrenedivinylbenzene) (purchased from JVA Analytical, Dublin 12) extraction disks with 47mm glass filtration apparatus. These extraction disks have been used in several studies (Synder *et al.*, 2001; Murk *et al.*, 2002; Hugget *et al.*, 2003; Furuichi *et al.*, 2004). The method employed was a modified method of that of the manufacturers, 3M

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(http://www.3m.com/empore/library/envirodisks/instructions.html). The extraction disk was centred on the base of the filtration apparatus. The sample reservoir was placed on top and secured with a clamp. Three different collection vessels were used; one for solvents, another for water, and a third for collecting eluate following extraction. One disk was used for each sample. A method blank was carried out on each disk prior to extraction of the sample. Spiked influent and effluent were also extracted. Percentage recoveries for influent ranged from 78%-147% and 58%-139% for effluent.

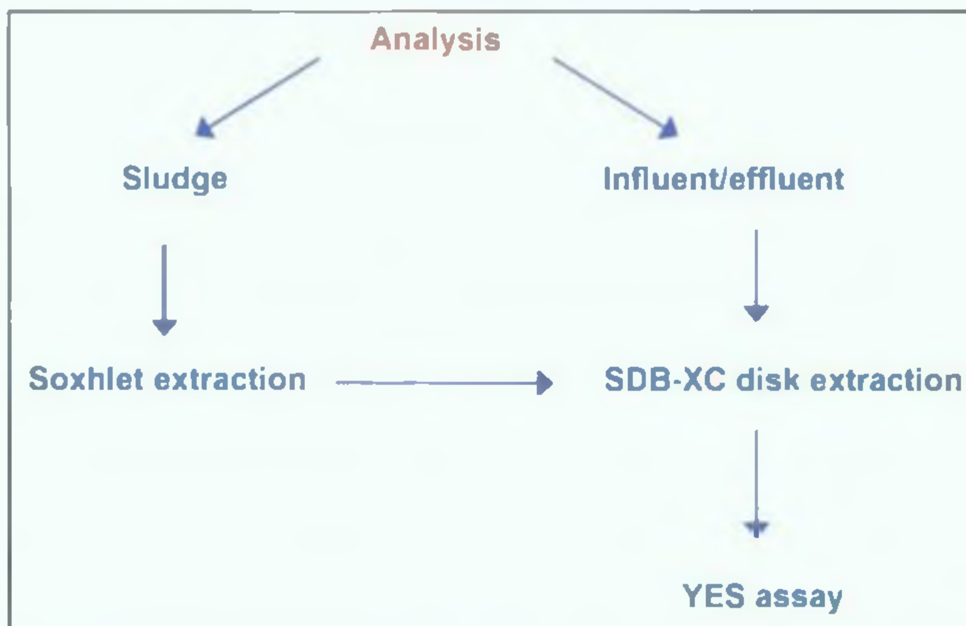

Figure 2.2.2 Schematic of the procedure for analysis of oestrogens in wastewater and sludge samples

2.2.2.1.2 Extraction disk conditioning

5ml acetone was pipetted into the sample reservoir. The vacuum was applied and the disk let to dry. 5ml of methanol was pipetted into the sample reservoir. The vacuum was applied and the disk let to dry. 10ml of methanol was added to the disk. The vacuum was applied and several ml were drawn through the disk. The vacuum was vented and the disk was allowed to soak for 5 minutes. The vacuum was applied and methanol pulled through the disks until the methanol surface was 3-5mm above the disk surface. 10ml of ultra pure water was added to the reservoir. The vacuum was applied and several ml were drawn through the disk. The vacuum was vented and the disk was allowed to soak for 5 minutes. The vacuum was applied and water pulled through the disks until the water surface was 3-5mm above the disk surface.

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2.2.2.1.3 Sample extraction and elution

500ml of sample were poured into the sample reservoir and the drawn through the filter. Recoveries are not affected by flow rate. The vacuum was left to dry the disk to remove as much residual water as possible.

The filter base was placed into the collection vessel, 20ml Quickfit conical flask. The chosen elution solvent was methanol as was used by Kirk *et al.* (2002) and Murk *et al.* (2002). 5ml of methanol was added to the sample container to rinse the container. Solvent was transferred to the sample reservoir while washing the walls of the sample reservoir in the process. The vacuum was applied and a little methanol pulled through the disk. The vacuum was vented and the remaining methanol was let sit on the disk for 5 minutes before applying the vacuum to dry the disk. This process was repeated four times. The eluate was transferred quantitatively to a 20ml volumetric flask and then evaporated and reconstituted with 2.5ml ethanol and refrigerated for further analysis.

2.2.2.2 Sludge

Dried sludge was ground using a blender and sieved using a 1mm mesh sieve. 5g aliquots were weighed into cellulose Whatman thimbles. Spikes of both sludge types were also extracted. Soxhlet apparati were set up. An experiment was carried out twice to determine the most suitable solvent for extraction. Methanol, hexane:acetone, (1:1), dichloromethane, and dichloromethane:acetone (1:1) were used to extract organic compounds from 5g of a common sludge and it was found that dichloromethane resulted in the highest recovery on both occasions. Soxhlet extractions ran for 20hours after which extracts were collected, filtered and refrigerated. Further extraction was carried out the same day using Empore SDB-XC extraction disks in order to purify and concentrate the extracts. The same procedure as for influent and effluent samples was applied.

2.2.3 Recombinant Yeast Oestrogen Screen Assay

2.2.3.1 Preparation and storage of minimal medium and medium components

All ingredients were purchased from Sigma Aldrich (Sigma-Aldrich Ireland Ltd. Dublin, Ireland) unless otherwise stated. All glassware, spatulas and stirring bars were washed twice with methanol and then once with ethanol prior to use. Minimal medium and medium components were prepared according to Routledge and Sumpter (1996). Minimal media was prepared by adding 13.61gKH_2PO_4 (purchased from Lennox

Laboratory Supplies Ltd, Dublin 12), 1.98g $(NH_4)2SO_4$, 4.2g KOH pellets, 0.2g $MgSO_4$, 1ml $Fe_2(SO4)_3$ solution (40mg/50ml H_2O), 50mg L-leucine, 50mg L-histidine, 50mg adenine, 20mg L-arginine-HCl, 20mg L-methionine, 30mg L-tyrosine, 30mg L-isoleucine, 30mg L-lysine-HCl, 25mg L-phenylalanine, 100mg L-glutamic acid, 150 mg L-valine and 375mg L-serine to 1L distilled water. It was dispensed into 45ml aliquots into 100ml Erlenmeyer flasks which were then autoclaved and stored at 4°C.

D-(+)-Glucose: Prepared at 20%w/v solution, autoclaved and stored at 4°C.
L-Aspartic acid: Prepared at 4mg/ml, and autoclaved and stored at 4°C.
L-Threonine: Prepared at 24mg/ml, and autoclaved and stored at 4°C.
Vitamin Solution: 8mg thiamine, 8mg pyridoxine, 8mg pantothenic acid, 40mg inositol and 20ml biotin solution (2mg/100ml H_2O) were added to 180ml distilled water. They were sterilized by filtering through a 0.2-µm pore size disposable filter (Lennox Laboratory supplies Ltd, Naas Road, Dublin 12) in a laminar air flow cabinet. They were filtered into sterile glass bottles and stored at 4°C.
Copper (II) sulphate: A 20Mm solution was prepared and sterilised by filtering through a 0.2-µm pore size disposable filter in a laminar air flow cabinet. It was filtered into sterile glass bottles and stored at 4°C.
Chlorophenol red-β-D-galactopyranoside (CPRG): CPRG was purchased from Fannin Healthcare, Dublin 18. A 10mg/ml solution was prepared and sterilised by filtering through a 0.2-µm pore size disposable filter in a laminar air flow cabinet. It was filtered into sterile glass bottles and stored at 4°C.

2.2.3.2 Preparation and storage of yeast stocks

All yeast work was carried out in a laminar flow cabinet (Astec Microflow Advanced Bio Safety Cabinet class 2). Yeast was stored on a short term basis with a maximum storage period of four months.

2.2.3.2.1 Short term storage (-20°C)-10X concentrated yeast stock culture

Day 1

Growth medium was prepared by adding 5.00ml of glucose solution, 1.25ml aspartic acid solution, 0.4ml threonine solution, 0.5ml vitamin solution and 0.125ml copper sulphate solution to 45ml of minimal media. 0.5ml of 10X concentrated yeast stock stored at -20°C was added. The solution was incubated at 32°C for approximately 24 hours on an orbital shaker.

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Day 2

Growth medium was added to two conical flasks of 45ml minimal media. 1ml of the 24hour-old yeast culture was added and incubation resumed for another 24 hours.

Day 3

Each 24-hour culture was transferred to a sterile 50-ml centrifuge tube and centrifuged at 2000g for 10 minutes at 4°C. The supernatant was then decanted and each culture was resuspended in 5ml minimal media with 15% glycerol (8ml of sterile glycerol was added to 45ml minimal media). 0.5ml aliquots of the 10X concentrated stock solution were transferred to1.2ml sterile cryovials which were stored at -20°C for a maximum of four months (Routledge and Sumpter, 1996).

2.2.3.2.2 Preparation and storage of chemicals

The steroid hormone, 17β-oestradiol (97%) to be used as the standard solution was purchased from Sigma Aldrich (Sigma-Aldrich Ireland Ltd. Dublin, Ireland). Synthetic hormones, norethindrone (98%) and diethylstilbestrol (97%) were also analysed. The ability of the assay to identify xenoestrogens was assessed by examining the activity of known xenoestrogens such as bisphenol-A(99%), benzylbutylphthalate (98%) and phytoestrogen, genestein (all purchased from Sigma-Aldrich Ireland Ltd. Dublin, Ireland).

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All glassware, spatulas and stirring bars were washed twice with methanol and then once with ethanol prior to use. Chemicals were weighed directly into bottles. The standard solution, 17β-oestradiol was made up to a concentration 54.48μg/l in absolute ethanol (Routledge and Sumpter, 1996). Bisphenol-A, benzylbutylphthalate and genestein were made up to a concentration of 2g/l in absolute ethanol. Diethylstilbesterol was made up to a concentration of 1E-06M and norethindrone was made up to 0.08g/l as these concentrations gave rise to optimum dose response curves parallel to that of 17β-oestradiol.

2.2.3.3 Assay procedure

All yeast work was carried out in a type II laminar flow cabinet to minimise aerosol formation. The assay procedure is that of Routledge and Sumpter (1996). Analysis was carried out on three different occasions for Ringsend WWTP and Killarney WWTP and twice for Strandhill WWTP and Tubbercurry WWTP.

Day 1

Growth medium was prepared by adding 5.00ml of glucose solution, 1.25ml aspartic acid solution, 0.4ml threonine solution, 0.5ml vitamin solution and 0.125ml copper sulphate solution to 45ml minimal media in a sterile conical flask. The media was then inoculated with 0.5ml of 10X concentrated yeast stock frozen at -20°C. The yeast suspension was then incubated at 37°C.

Day 2

The standard was serially diluted across one row of a Sarstedt 96-well microtitre plate to give a concentration range of 2724ng/l to 3ng/l as follows: 100µl of ethanol were put into each well except the first well. 100µl of 17β-oestradiol (54.48µg/l) were placed into the first well containing ethanol. The solution was mixed ten times within the pipette, and 100µl of the solution were transferred into the next well and so on. 10 µl of each concentration was transferred to the first row in the test well plates. The second row was an assay blank. 10µl aliquots of ethanol were transferred to the last row in the test well plates as a solvent blank (Figure 2.2.3).

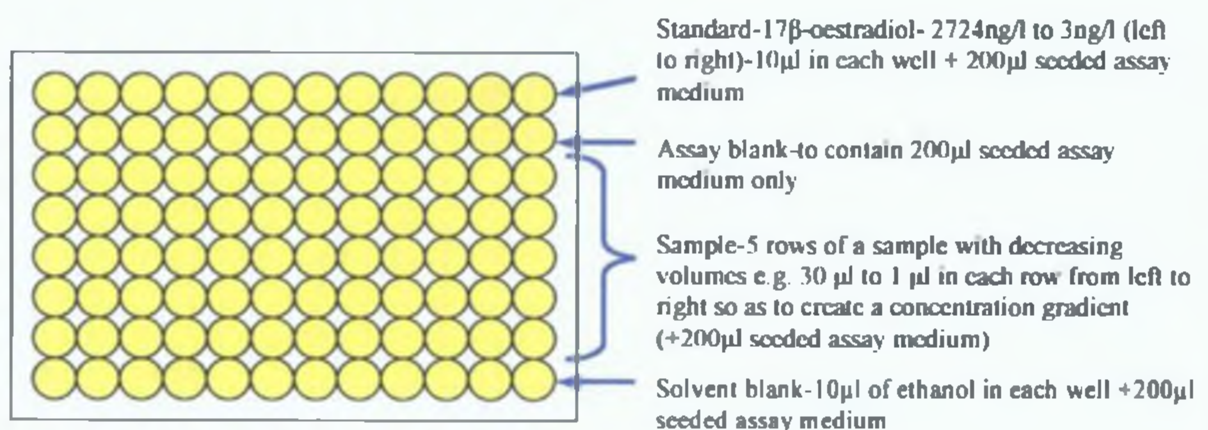

Figure 2.2.3: 96-well plate layout for the Yeast Oestrogen Screen assay

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Varying volumes of sample extract were transferred to the remaining five rows in order to dilute the sample (Kirk *et al.*, 2002). Extracts were shaken between each transferral. Volumes of effluent extract typically ranged from 30µl to 1µl and for influent; 20µl to 1µl.

The different solutions in the assay plates were left to evaporate. The absorbance of the yeast suspension from the previous day was measured using a microplate reader at 640nm. If necessary, the suspension was diluted with minimal media to obtain an absorbance of 1.0 at 640nm. Fresh growth medium was prepared by adding 5.00ml of glucose solution, 1.25ml aspartic acid solution, 0.4ml threonine solution, 0.5ml vitamin solution and 0.125ml copper sulphate solution) to a minimal media solution. 0.5ml of CPRG was also added. Finally, 1ml of yeast culture (absorbance 1.0 at 640nm) was added. The solution was then poured into a Petri dish and continuously mixed to ensure homogenous distribution of the yeast cells while 200µl of the solution was transferred into each well on the test plate using a multi-channel pipette (Figure 2.2.3). The plates were then shaken for two minutes and incubated at 32°C.

Days 3 + 4

The test plates were shaken for two minutes and re-incubated at 32°C.

Day 5

The test plates were shaken for two minutes approximately one hour before readings were taken and allowed to settle (colour development of the standard and the negative control are presented in Appendix A). Assay plates were measured using an Anthos 2010 microplate reader. Absorbance was measured at 540nm and at 620nm. The plates were read at 620nm to measure background turbidity of the yeast.

2.2.3.4 Quantitative analysis

The corrected absorbance values were calculated as follows (Routledge and Sumpter, 1996):

(chemical absorbance (540nm) minus chemical absorbance(620nm)) plus average absorbance of solvent blank@620nm

Oestradiol equivalency factors (EEFs) of the synthetic oestrogens and xenoestrogens, were determined by dividing the EC_{50} of 17β-oestradiol by the EC_{50} of the compound being tested (Tanaka *et al.*, 2001; Beresford *et al.*, 2000; Coldham *et al.*, 1997). Each compound had its own standard oestradiol curve each time it was assayed. EC_{50} values were obtained by plotting graphs of log concentration (M) versus absorbance of the most vertical portion of the standard curve and each compound. In this way, line equations could be attained for the linear portions only of the graphs whereas a line equation of a sigmoidal graph would take into account the whole curve and not just the required linear portion.

Equal minimum and maximum response values of the test compound and the standard and their parallelism are required for the derivation of accurate relative potency (Villeneuve *et al.*, 2000). In some studies, authors designate minimum and maximum absorbance values between which they calculate EEQ values at various points (Kirk *et al.*, 2002) while in others, authors have selected one absorbance point only (Witters *et al.*, 2001). An absorbance mid-range in the dose response curves was chosen (whereby the maximum absorbance of 17β-oestradiol is assumed EC_{100} and the lowest absorbance of 17β-oestradiol is assumed EC_0) and the EC_{50} values were determined for 17β-oestradiol and for each of the five replicate concentration gradients of the compound being tested at the chosen absorbance (Tanaka *et al.*, 2001; Coldham *et al.*, 1997). Five EC_{50} values were derived each time a compound was tested.

Microsoft Excel was used to graph the data and to extrapolate EEQ of wastewater extracts by plotting graphs of log concentrations (M) versus absorbance of the vertical portion of the standard curve. EEQ values of the five replicate sample gradients were determined at dilutions which were within the most vertical portion of the standard curve (Kirk *et al.*, 2002) using the equation of the line which was typically between 1.0 and 1.8. Then, the dilution on the plate relative to the standard and the concentration of the sample that occurred due to extraction from 500ml to 20ml and evaporation to 2.5ml was accounted for. For example, the EEQ value of a sample extract volume of 20μl that was diluted 10 times in the assay (i.e. 20μl in 200μl seeded assay medium) of which the extract was concentrated 200 times (from 500ml to 2.5ml) prior to the assay was multiplied by 10 and divided by 200. All of the results were averaged to produce a mean for that particular sample i.e. influent or effluent at that particular sampling period. Results were statistically analysed using Sigma Stat.

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Chapter 3.0 Results

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3.1 Steroidal oestrogens and xenoestrogens

Validation of the Recombinant yeast Screen assay was carried out regularly using 17β-oestradiol (Table 3.1.1). The average EC_{50} for 17β-oestradiol was $1.16x10^{-10}$M or 26.76ng/l. Five different compounds exhibiting oestrogenic potency was assessed to further validate the assay and these compounds consisted of a phytoestrogen; genestein, two synthetic oestrogens; diethylstilbestrol and norethindrone and two xenoestrogens bisphenol A and benzylbutylphthalate. The EC_{50} concentrations and Oestrogen Equivalency Factors (EEF) for these compounds are presented in Table 3.1.2

Table 3.1.1: EC_{50} values and 95% confidence intervals for 17β-oestradiol (54.48μg/l)

n	Average EC_{50} value (M)	95% confidence interval (M)	Average EC_{50} value (ng/l)	95% confidence interval(ng/l)
50	$1.16x10^{-10}$ ($1.28 x10^{-10}$)	8.07E-11-1.52 $x10^{-10}$	26.76 (6.79)	24.86-28.66

EC_{50}: concentration of a compound that produces a half-maximal response (median effective concentration) (Rutishauser *et al.*, 2004).
Values in parenthesis represent standard deviation.

Table 3.1.2: EC_{50} values and oestrogen equivalency factors for one phytoestrogen, two synthetic oestrogens, and two xenoestrogens tested.

Compound	Effective concentration	n	Average EC_{50} value (M)	Average EC_{50} value (w/v)	EEF
Genestein (2g/l)	EC_{50}	20	$2.70x10^{-6}$ ($2.46x10^{-7}$)	0.65mg/l (0.02mg/l)	$4.14 x10^{-5}$
Diethylstilbestrol ($2.6x10^{-4}$g/l)	EC_{50}	20	$1.68x10^{-10}$ ($3.72 x10^{-11}$)	44.34ng/l (9.80ng/l)	$6.03 x10^{-1}$
Norethindrone (0.08g/l)	EC_{50}	20	$1.68 x10^{-7}$ ($3.96 x10^{-8}$)	50.20μg/l (11.81μg/l)	$5.33 x10^{-4}$
Bisphenol A (2g/l)	EC_{50}	20	$2.20 x10^{-6}$ ($3.69 x10^{-7}$)	0.50mg/l (0.08mg/l)	$5.33 x10^{-5}$
Benzylbutylphthalate (2g/l)	EC_{10} [1]	5	$2.86 x10^{-5}$ ($1.89 x10^{-5}$)	8.95mg/l (5.91mg/l)	$8.22 x10^{-7}$

Values in parenthesis represent standard deviation.
[1] An EC_{10} concentration was extrapolated for benzylbutylphthalate due to its submaximal potency

The EC_{50} values are expressed as both molar concentration and weight/volume as both values are often presented in literature. Genestein, bisphenol and benzylbutylphthalate were assayed at 2g/l. Benzylbutylphthalate produced the highest EC_{50} value meaning it is the least potent compound assayed. Similar EC_{50} values were derived for Genestein and bisphenol. The most potent compound tested was diethylstilbestrol with an EC_{50} value of

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1.68×10^{-10} M and an oestradiol equivalency value of 6.03×10^{-1} meaning it is 1.6 times less potent than oestradiol.

3.2 Wastewater influents and effluents

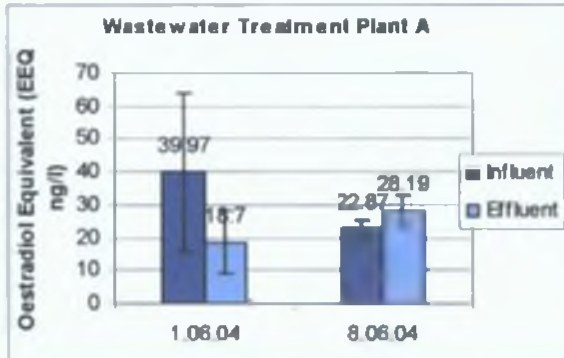

Figure 3.2.1: Oestradiol equivalent (EEQng/l) of influent and effluent of Wastewater Treatment Plant A

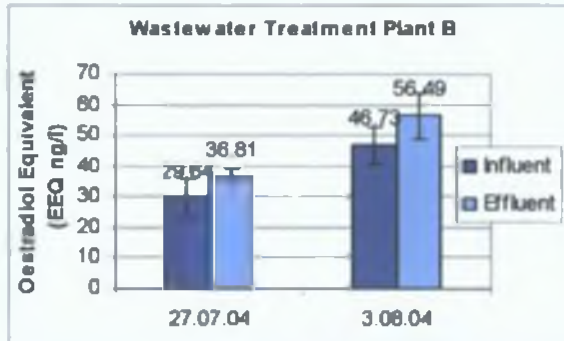

Figure 3.2.2: Oestradiol equivalent (EEQng/l) of influent and effluent of Wastewater Treatment Plant B

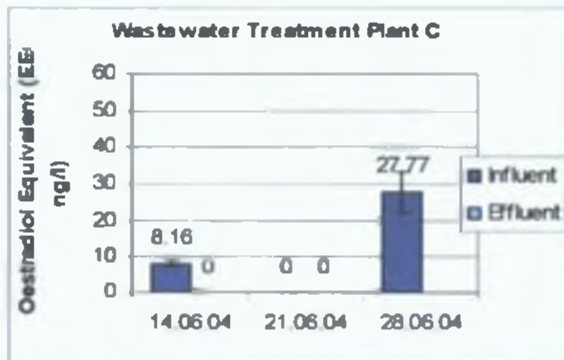

Figure 3.2.3: Oestradiol equivalent (EEQng/l) of influent and effluent of Wastewater Treatment Plant C

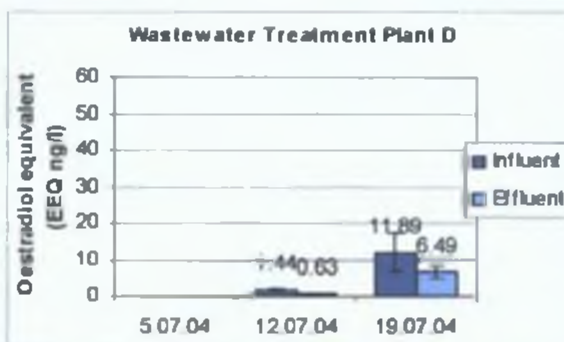

Figure 3.2.4: Oestradiol equivalent (EEQng/l) of influent and effluent of Wastewater Treatment Plant D

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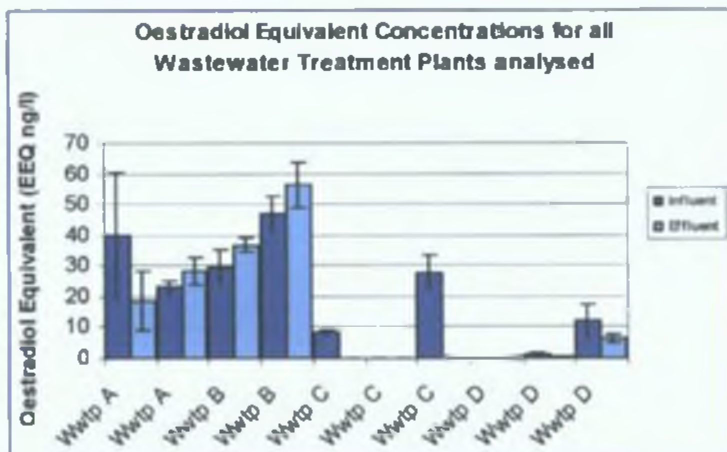

Figure 3.2.5: Oestradiol equivalent (EEQng/l) of influent and effluent of all Wastewater Treatment Plants

Table 3.2: Oestrogen equivalent concentrations and percentage removal rate for each wastewater treatment plant

WWTP	Date	Influent EEQ (ng/l)	Effluent EEQ (ng/l)	Percentage Removal rate
WWTP A	1.06.04	39.97 (26.82-50.12)	18.7 (12.76-24.64)	53.21%
WWTP A	8.06.04	22.87 (21.92-23.82)	28.19 (25.92-30.46)	-23.26%
WWTP B	27.07.04	29.64 (25.67-33.61)	36.81 (35.44-38.18)	-24.19%
WWTP B	3.08.04	46.73 (44.25-47.21)	56.49 (53.25-59.73)	-20.89%
WWTP C	14.06.04	8.16 (7.8-8.52)	0	100.00%
WWTP C	21.06.04	0	0	n/a
WWTP C	28.06.04	27.77 (25.76-29.74)	n/a	n/a
WWTP D	5.07.04	n/a	n/a	n/a
WWTP D	12.07.04	1.44 (1.27-1.63)	0.63 (0.60-0.66)	56.25%
WWTP D	19.07.04	11.89 (9.24-14.54)	6.49 (5.83-7.11)	45.42%

Values in parenthesis represent 95% confidence intervals
n/a: not applicable

These results indicate that wastewater treatment plant (WWTP) B actually increases the oestrogenicity by the influent by 21-24% (Table 3.2) rather than decreasing it (Figure 3.2.2). Reduction (53%) is observed on one sampling occasion for WWTPA while

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oestrogenicity increases by 23% on the second sampling occasion (Figure 3.2.1 and Table 3.2). Lower oestrogenic potency is detected in the influents and effluents of WWTPs C and D (Figure 3.2.5) with a removal rate of 100% observed for WWTPC on one sampling occasion (Table 3.2). The removal rates for WWTPD range from 45-56% (Table 3.2).

3.2.1 Statistical analysis

The influent and effluent data were statistically compared by T-test using Sigmastat 2003 software. The influent and effluent values of each WWTP were statistically different from each other ($P<0.05$) except those of WWTPA for which there was no statistical difference. This was due to the high standard deviation (24.20) of the influent values on 1.06.04.

Comparison between the four WWTPs (one-way anova) showed that all four plants were significantly different to each other regarding their influent mean values at a significance level of $P<0.050$. This was also the case regarding the effluent mean values (at a significance level of $P<0.050$) except that effluents of WWTPC and WWTPD were not significantly different ($P=0.479$) to one another. This was due to the fact that oestrogenic activity was not detected in WWTPC on two occasions i.e. there was no oestrogenic response. On a third occasion the result was invalid due to toxicity in the sample as the response of the sample extract was below that of the solvent blank indicating that something in the sample hindered the growth of yeast. Invalid results were also recorded for both influent and effluent of WWTPD on one occasion and oestrogenic activity detected for the remaining sampling dates were very low. For these reasons, the effluent values of WWTPC and WWTPD were similar to one another and did not give rise to a statistical difference.

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3.2.2 Toxic effects of samples

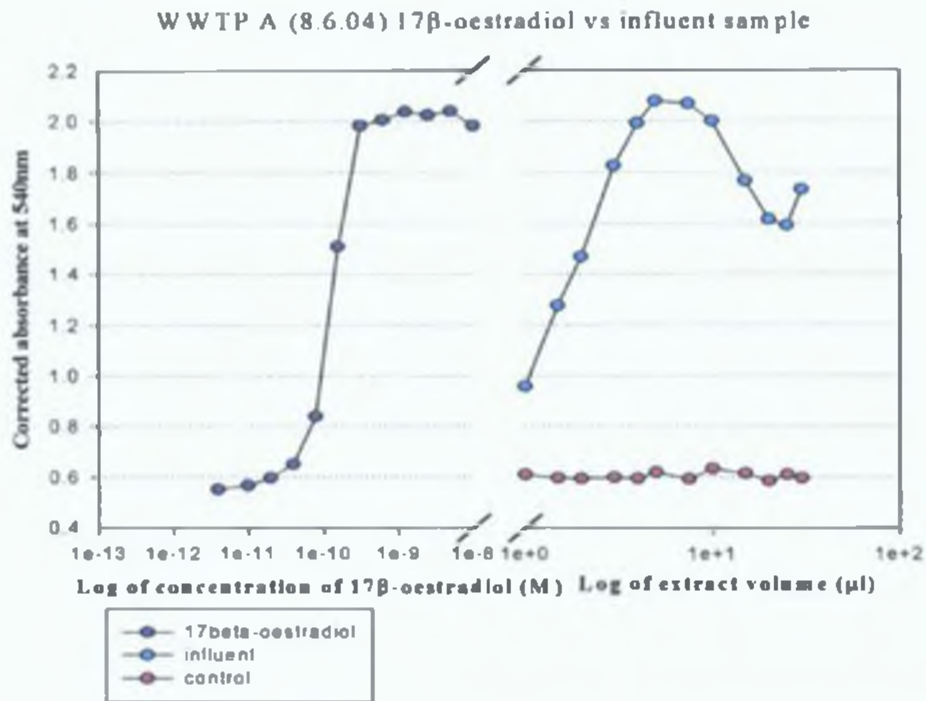

Figure 3.2.6: Graph of WWTP A (8.06.04) influent sample exhibiting toxicity at higher concentrations

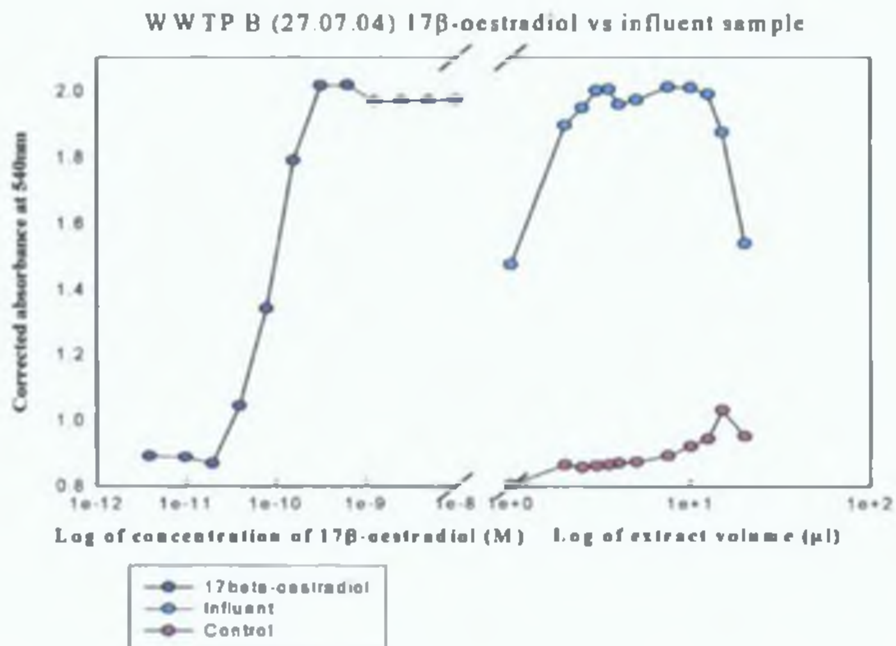

Figure 3.2.7: Graph of WWTP B (27.07.04) influent sample exhibiting nonmonotonic characteristics

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Figure 3.2.6 and Figure 3.2.7 display examples of toxicity observed in two samples from WWTPA and WWTPB. At higher concentrations cytotoxicity occurs due to something unknown in the samples preventing growth of yeast.

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Chapter 4.0 Discussion

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4.1 Steroidal oestrogens and xenoestrogens

The EC_{50} concentration derived for 17β-oestradiol (1.16 x10^{-10}M) (table 3.1.1) agrees with those of Legler *et al.* (2002); 1x10 x10^{-10}M and Folmar *et al.* (2002); 2.1x10 x10^{-10}M. The oestradiol equivalency factor calculated for genestein indicates that it is almost 24,200 times less potent than 17β-oestradiol. This does not agree with EEFs derived by Matsui *et al.* (2000) and Tanaka *et al.* (2001) who reported the potency of genestein to be 10,000 and 12,500 times less potent than 17β-oestradiol in the same assay. Diethylstilbestrol was the compound with the EC_{50} value (1.68 x10^{-10}M) closest to that of the standard meaning that out of the compounds tested it had the greatest potency (table 3.1.2). The EEF value (6.04 x10^{-1}M) indicated DES to be approximately 1.6 times less potent than 17β-oestradiol. This result is similar to that of Folmar *et al.* (2002) who reported an EC_{50} value of 1.9E x10^{-10}M and indicated DES to be 0.9 times less potent than 17β-oestradiol. Tanaka *et al.* (2001) found DES to approximately 3.3times less potent than the standard in the same assay.

Norethindrone was found to be almost 1,900 times less potent than 17β-oestradiol with an EC_{50} value of 1.68 x10^{-7}. Bisphenol A gave rise to an EC_{50} value of 2.20x10^{-6}M and an EEF of 5.33 x10^{-5}M when compared to the standard which indicated that it is almost 19,000 times less potent than 17β-oestradiol (table 3.1.2). This is in agreement with Tanaka *et al.* (2001) and Körner *et al.* (2001) who reported bisphenol A to be almost 16,700 and 19,500 times less potent than 17β-oestradiol in the YES and E-screen assays respectively. However, relative potencies for bisphenol A in the YES assay reported in literature vary between 10,000-15,000 times weaker than 17β-oestradiol (Gaido *et al.*, 1997; Sohoni and Sumpter, 1998; Matsui *et al.*, 2000). The response curve produced by benzylbutylphthalate was submaximal (as found by Sohoni and Sumpter (1998) and Beresford *et al.* (2000)) i.e. it did not produce a response high enough to allow derivation of an EC_{50} value even if the initial concentration of the compound was increased. However, on one occasion the response produced was sufficient to derive an EC_{10} value (2.86 x10^{-10}M) which gave rise to an EEF of 8.22 x10^{-7}M indicating that BBP was almost 1,220,000 times less potent than 17β-oestradiol. Harris *et al.* (1997) and Tanaka *et al.* (2001) also found that BBP produced submaximal dose response curves in the YES at concentrations up to 2g/l. Harris *et al.* (1997) and Murk *et al.* (2002) indicated that BBP was approximately one million times less potent than 17β-oestradiol.

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4.2 Wastewater influents and effluents

Higher oestradiol equivalent concentrations were determined overall for influents and effluents of wastewater treatment plants A and B than for wastewater treatment plants C and D (Table 3.2 and Figure 3.2.5). This may be attributed to the fact that treatment plants C and D have higher treatment efficiencies as they are medium to large scale treatment plants with more advanced treatment technologies. The biological treatment method employed in both wastewater treatment plants B and C is sludge activation in an oxidation ditch however wastewater treatment plant C uses a diffused air aeration system while wastewater treatment plant B has two alternating surface aerators (table 2.1.4). Lower volatile organic compound emissions have been reported with the use of fine bubble diffusers than with coarse bubble diffusers or mechanical surface aeration (United States Environmental Protection Agency, 1999c). Also, wastewater treatment plant C is the only plant of the two to have primary sedimentation and chemical precipitation in the form of ferric chloride. However, use of ferric chloride as a coagulant is not itself efficient for the removal of oestrogenic potency as has been shown by Schäfer and Waite (2002) in a study in which poor removal of oestrone (<10%) was observed after ferric chloride coagulation compared to a removal rate of >90% with powdered activated carbon.

WWTP B did not reduce oestrogenic potency on either sampling dates (Figure 3.2.2) but actually increased the oestrogen potency by approximately 24% and 21% while WWTP C was the most efficient WWTP giving rise to removal rates of 100% on one occasion with a second sampling date on which no oestrogenic potency was determined in either the influent or effluent (Table 3.2). However, on a third sampling date the effluent was found to be toxic to the yeast (the response of the yeast was below that of the solvent control meaning that something in the sample was toxic to the yeast) and the oestrogenic activity of the effluent could not be determined. Removal rates of oestrone of >99% and 89% were estimated for Belgian and German WWTPs employing oxidation ditches based on oestrone levels in the effluent and estimated oestrone levels in the influent (Johnson *et al.*, 2005). These plants had total hydraulic retention times of 17.5hours and 41hours respectively and served population equivalents (6,500 and 15,000 respectively) intermediate of those of WWTP B and C. The poor treatment efficiency of WWTP B can be attributed to the fact that it was designed to serve a population capacity of 1,700 but it operating at a population capacity of 2,100. This means that the load entering the plant was

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too high, the biodegrader microorganisms are overwhelmed and the hydraulic retention time (approximately 7 hours) is not long enough to allow sufficient treatment. This problem may be worse in the summer months (when sampling occurred) due to less rainfall and higher input of people to the area as it is a popular seaside village.

Wastewater treatment plants A and B are located locally and samples were extracted within an hour of sampling whereas wastewater treatment plants C and D are considerably further away meaning that the length of time between sampling and extraction was longer for these samples. Samples from wastewater treatment plant D were extracted within 5 hours of sampling. Samples from wastewater treatment plant C were transported by courier and were delivered the day after sampling meaning a loss of oestrogenicity may have occurred via the degradation of 17β-oestradiol to oestrone.

The biological treatment method employed in WWTP A is a trickling filter system followed by tertiary treatment via grassplots (table 2.1.4). This treatment plant is serving approximately 1,900 people when it is designed for 1,400 but it has a longer hydraulic retention time (18hours) than WWTPB (7hours) which may have contributed to its better treatment efficiency (Andersen *et al.*, 2003; Langford *et al.*, 2005). The oestrogenicity decreased during treatment on one occasion by approximately 53% and increased by approximately 23% on another occasion (Table 3.2). Such increases in oestrogenicity following trickling filter systems have been reported elsewhere. Servos *et al.* (2005) reported a 62% increase in the YES response from the influent to the effluent of a Canadian WWTP (equipped with primary treatment but devoid of tertiary treatment) in which the hydraulic retention time in the filter itself was 1 hour and the total system hydraulic retention time was 6-8hours. Instrumental analysis determined that the concentrations of 17β-oestradiol and oestrone increased by 18.5% and 62.4% respectively. Splenger *et al.* (2001) measured concentrations of natural and synthetic oestrogens, phytoestrogens and xenoestrogens in effluents of 18 German WWTPs employing various treatment methods. Only one WWTP did not employ an activated sludge system but consisted of a trickling filter with nitrification and phosphate removal. The effluent of this plant contained the highest concentrations of oestrone, ethinyl-oestradiol, genestein, bisphenol A, 4-nonylphenol and 4-nonylphenoxyacetic acid among all 18 WWTPs. Using the YES assay Svenson *et al.* (2003) determined the percentage removal of oestradiol equivalent of three

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WWTPs, the sole biological treatment of which were trickling filters. The effluent of one plant was approximately 3.5 times more potent than the influent while removal efficiencies of the second and third WWTP were 33% and 75% respectively. Specific analysis of the second plant at intermediate treatment stages demonstrated that the biological treatment step accounted for only 8% removal of the total 33% while post-precipitation using aluminium was mainly responsible for removal of oestrogenic potency demonstrating the poor removal efficiency of trickling filter systems.

Studies carried out on other systems of solid support bacteria have also reported poor removal efficiencies. A Swedish WWTP employing a rotating biological contactor (a system method in which media i.e. circular disks, plates or tubes attached to a rotor shaft rotates in the wastewater flowing through the tank) as biological treatment was not effective in removing oestrogenic potency as the effluent was 2.6-4.6 times more potent than the influent (Svenson *et al.*, 2003). The authors reported an average removal rate of only 28% for solid support bacteria systems (trickling filter and rotating biological contactor systems) in a study on Swedish WWTPs while activated sludge systems resulted in an average removal rate of 81%.

The biological treatment in WWTP D consists of sequence batch reactors with a hydraulic retention time of approximately 15 hours (table 2.1.4). Ozonation is used as a form of tertiary treatment. Removal rates of approximately 56% and 45% were observed for this WWTP. Results could not be calculated on a third sampling date as both the influent and the effluent were toxic to the yeast i.e. oestrogenic activity could not be deduced (Table 3.2 and Figure 3.2.4). Ozonation using 5-15mg/l of ozone has been shown to be effective in the removal of oestrone with removal rates of >80% as reported by Ternes *et al.* (2003). Ozonation has the ability to weaken binding affinity for the oestrogen receptor.

Oestrogens are excreted as glucuronide and sulphonide conjugates (80% and 20% respectively as determined by D'Ascenzo *et al.* (2003) in a study on 72 women. Oestrogen glucuronides (for example, 17β-oestradiol-3-glucuronide, oestriol-3- glucuronide) have been shown to undergo biodegradation in the sewers allowing oestrogen sulphonides (for example, 17β-oestradiol-3-sulphate, oestrone-3-sulphate) to dominate influent (D'Ascenzo

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et al., 2003). Oestrogen sulphonides are also more recalcitrant in the wastewater process, as *E. coli*, the most prevalent bacterial species in wastewater, can not sufficiently degrade sulphonide conjugates in the given hydraulic retention time of most wastewater treatment plants (4-14hours). Hence efficient biodegradation of oestrogen sulphonides may only take place in WWTPs with a long enough hydraulic retention time to allow diverse bacterial species other than *E. coli* to flourish. Glucuronide conjugated oestrogens were not detected in the effluents of activated sludge WWTPs in a Dutch study carried out by Belfroid *et al.* (1999) indicating that deconjugation reactions of glucuronides occur during treatment. Oestrogen sulphonides were detected in effluent, river water and lake water in a study carried out by Isobe *et al.* (2003) whereas oestrogen glucuronides were not detected.

If complete degradation of oestrogen conjugates does not occur these conjugates may be cleaved producing their free (more potent) form. This may account for the observed increase in oestrogenic activity from the influent to the effluent on both sampling dates for wastewater treatment plant B (Figure 3.2.2) and on one occasion for WWTP A (Figure 3.2.1). Ternes *et al.* (1999a), Nasu *et al.* (2001) and Kirk *et al.* (2002) reported increases in concentrations of oestrogens and/or potency from the influent to the primary effluent (that is the wastewater following primary treatment). The authors attributed this increase to the deconjugation of conjugated oestrogens.

Furthermore, oxidation of 17β-oestradiol to oestrone during the treatment process may also account for the reported increase in oestrogenic potential as although oestrone is not as potent as 17β-oestradiol it is more recalcitrant to biodegradation and it is the oestrogen species found in the highest concentrations in influent and effluent worldwide (Table 1.17.1, Appendix B). Matsui *et al.* (2000) found oestrone to be 0.21 times as potent as 17β-oestradiol by the YES assay while Pawlowski *et al.* (2004) found oestrone to be 0.5 times as potent as 17β-oestradiol using the same assay. Therefore, if oestrone was present in wastewater at a concentration 2-5 times that of 17β-oestradiol present in the influent then it could contribute greatly to the oestrogenic potency of the effluent determined by the YES assay resulting in associated poor removal efficiency of the WWTP. As can be seen in Table 1.17.1 (Appendix B), this is quite probable as oestrone was present in most effluents at concentrations on average 4-8 times that of 17β-oestradiol while two authors reported

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maximum oestrone levels approximately 23 times higher than maximum 17β-oestradiol levels (Baronti *et al.*, 2000; Ternes *et al.*, 1999a).

The findings (of reviewed studies in Table 1.17.1 Appendix B) that oestrone was present at concentrations 3-5 times that of 17β-oestradiol in some influents (Johnson *et al.*, 2000; Baronti *et al.*, 2000; Andersen *et al.*, 2003; Servos *et al.*, 2005) in addition to the deconjugation of oestrone conjugates, oxidation of 17β-oestradiol to oestrone and its recalcitrance to biodegradation in the wastewater treatment process suggest that oestrone may be largely responsible for the oestrogenic potency of effluent with regard to steroidal oestrogens. Of course, xenoestrogens in personal care pharmaceutical products such as cosmetic surfactants and in industrial discharges such as alkylphenols, polyaromatic hydrocarbons and dioxins will also contribute to the oestrogenic potency of an effluent, however, these substances were not quantitatively analysed for in this study and since WWTPs A, B and C are domestic WWTPs it is likely that steroidal oestrogens are primarily responsible for oestrogenicity in the influents and effluents of these WWTPs. Solé *et al.* (2000) suggested that the higher the percentage of domestic influent in wastewater, the greater the amount of endocrine disrupting compounds released into the environment. Körner *et al.* (2000) reported that phenolic xenoestrogens represented only 0.7-4.3% of the total oestrogenic activity of effluent from a German WWTP. Aerni *et al.* (2004) calculated that the highest NP concentration (1.74μg/l) in samples measured in a French WWTP equivalent to 0.04ng/l oestradiol accounted for only 0.8% of the total oestrogenic activity of the sample (4.9ng/l) measured by the YES assay.

4.2.1 Toxic effects of samples

As the yeast assay associates gene expression with a reporter element that has a delayed response (secretion of β-galactosidase into the medium), the intensity of the colorimetric change and then time it takes to occur will be affected by initial cell density and temperature of incubation. Any toxic effects which hinder transcription and translation will affect subsequent β-galactosidase activity. A chemical must exhibit oestrogenic activity at a concentration well below its acute toxicity otherwise the yeast will be functionally impaired or die before transcription, translation and expression can occur i.e. β-galactosidase would not be produced hence there would be no colour change (Hamblen *et al.* 2003). In a study on the oestrogenicity of anilines using the YES system Hamblen *et al.*

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(2003) attributed the lack of oestrogenic activity of large aniline derivatives to their acute toxicities as calculated EC_{50} values were at, or near, the concentration inferred to cause mortality.

Toxic effects result in irregular concentration response curves and there were occasions where the concentration response curves produced by sample extracts were not parallel to the standard s-shaped curve. At times yeast growth was inhibited in certain wells containing higher concentrations of sample as found by Witters *et al.* (2001). Cell lysis occurred which was observed as a clear yellow colour in the medium opposed to an opaque yellow due to turbidity as a result of yeast growth (Routledge and Sumpter, 1996) and also a reduced absorbance at 620nm (background turbidity of yeast).. This is exemplified in Figure 3.2.6 where cell lysis was observed at the higher concentrations of sample. On occasions where ideal concentration response curves were not produced but cell lysis was not observed, it is thought that competition between oestrogenic compounds and/or the presence of anti-oestrogenic compounds and/or partial agonists may be responsible. Toxicity in a fraction of municipal waste landfill leachate (Kawagoshi *et al.*, 2003) was attributed to the presence of anti-oestrogens. Different oestrogenic compounds have varying affinities for the oestrogen receptor such that an oestrogen or an oestrogen mimicking compound with a greater affinity for the receptor but present in a low concentration may become displaced by a compound present in higher concentration but with a weaker affinity for the receptor and vice versa. If anti-oestrogenic compounds were present and had a greater affinity for the oestrogen receptor they may have occupied and blocked the receptors making them unavailable to the oestrogenic compounds (antagonist effect). Also, if the oestrogen mimicking compounds present were partial agonists they may not have produced responses sufficient enough to increase with increasing sample concentration.

The fundamental assumption that dose-response relationships are linear is contradicted by these compounds with the observation of U-shaped and inverted U-shaped curves (Alworth *et al.*, 2002). Difficulties arise in the extrapolation of oestrogenic activity as both response to oestrogens and receptor occupancy become saturated as concentration increases meaning that the relationship between response and concentration cannot be linear in the high concentration range. For many responses the concentration-response

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relationship is nonmonotonic (inverted-U) i.e. responses increase until a saturation plateau is reached and then responses decrease at concentrations higher than those at which saturation occurred due to toxicity (Welshons *et al.*, 2003). The graph in Figure 3.2.7 represents a nonmonotonic concentration response. Welshons *et al.* (2003) demonstrated that growth of MCF-7 cells exhibits monotonic tendencies as the cells were stimulated in the presence of 0.1pM to 100pM of oestradiol; cell growth response was saturated and did not show an increase between 100pM and 1μM oestradiol and cell growth response decreased due to cytotoxicity at concentrations above 1μM oestradiol.

4.3 Sludge

An extraction procedure was developed for dried sludge samples incorporating soxhlet extraction followed by solid phase extraction using Empore SDB-XC in order to purify the extracts prior to analysis by the Recombinant Yeast Screen Assay. Studies in which sludge extraction is coupled with biological analysis could not be found in literature at the time of submission of this thesis. Experiments were carried out to determine the most appropriate solvent for extraction among methanol, dichloromethane (DCM): acetone (1:1), acetone and hexane:acetone (1:1) of which DCM gave rise to the highest oestrogenic potency. Experiments demonstrated the benefit of solid phase extraction to clean and purify the extract and also to concentrate it to a smaller aliquot which could be more easily evaporated prior to YES assay. It also facilitated a necessary solvent change from DCM to methanol for the purpose of analysis by the YES assay as DCM is not compatible for use in 96-well plates as it does not readily evaporate in the wells and it leaves a residue on the bottom of the wells which could affect the subsequent absorbance measurement.

However, once the sampling programme had started, oestrogenic activity was not detected in sludge samples. In all other samples, the response given by the yeast was below that of the control meaning that something in the samples was inhibiting yeast growth. Several measures were taken to solve this problem such as increasing the period of time and the temperature during which/at which soxhlet extraction occurred; increasing the concentration of oestradiol in the spiked samples, increasing the weight of dried sludge to be extracted and use of different soxhlet apparati. It is possible that the extracted samples required further cleaning and purification than SDB-XC extraction provided.

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When experiments were carried out to determine the suitability of the solvents, the solvent volume used was 150ml and this was allowed evaporate in a fume cupboard to approximately 5ml before being drawn through a SDB-XC disk and tested by YES. However, larger soxhlet apparati had to be used for analysis of samples which required 250ml of solvent. Rotary evaporation of five 250ml samples (control, activated sludge, spiked activated sludge, treated sludge and spiked treated sludge) was impractical as the risk of contaminating the samples from previous material which may have been on the inside of the evaporator was too high. All glassware had to be meticulously washed twice with methanol and once with ethanol to remove contamination. It was impractical to do this with a rotary evaporator. Instead, extracts were filtered and then purified and concentrated to 20ml by SDB-XC extraction and then they were allowed to gently evaporate in a water bath before being re-constituted with 2.5ml ethanol and analysed by the YES assay. It is possible that retention on the disks was compromised by the larger sample volume and oestrogenic compounds were lost by breakthrough.

4.4 Outcome of hypotheses

The first hypothesis this study examined was the reduction in oestrogenicity from wastewater influent to effluent and would use of different treatment systems provide varying degrees of removal. Reduction in oestrogenicity was observed (100% in WWTPC and 45-56% in WWTPD) but not in all cases. It has been shown that removal of oestrogens and oestrogen mimicking compounds is greatly reduced if wastewater treatment plants continue to operate at or above their capacities. This is represented in the increases in oestrogen activity which occurred for WWTPB (21% and 23%) and WWTPA (23%). These WWTPs are old and are serving sprawling villages with ever increasing population numbers much higher than what the treatment plants were originally designed to cater for. They were chosen to represent small-scale WWTPs typical all over Ireland. The fact that these treatment plants were operating above their treatment capacity made comparison of different removal rates by different treatment technologies difficult as it is probable that more efficient removal would have occurred if these plants had not been overloaded. However, plans to upgrade/extend wastewater treatment plants throughout the county including Strandhill WWTP and Tubercurry WWTP are underway.

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WWTPs C and D are newer medium and large-scale WWTPs respectively equipped with improved treatment technologies. WWTPC has extended aeration oxidation ditch with diffused air aeration and also chemical precipitation. WWTPD has sequential batch reactors with UV disinfection. Less oestrogenic activity was recorded in the influent and effluent of these WWTPs and these treatment plants also gave rise to higher removal rates (100% and 45-56% respectively). However, invalid results were recorded on two sampling dates which hindered the comparison of these modern WWTPS with the two smaller WWTPS.

The second hypothesis the study addressed is the compatibility of the recombinant yeast screen assay for the analysis of treated sludge samples extracted by soxhlet extraction. Unfortunately, it was unsuccessful. Soxhlet extraction of oestrogenic compounds from sludge coupled with further solid-phase extraction did not purify the samples to the extent that would allow subsequent assessment by the recombinant yeast screen assay despite several attempts to solve the problem.

4.5 Recommendations

- It is recommended that all studies of oestrogenic activity on wastewaters and surface waters in Ireland carried out to date are compiled together to provide a bigger picture on the state of the environment with regard to oestrogens and oestrogen mimicking compounds.

- A comprehensive nationwide study should be carried out using uniform standardized test systems and methods (possibly involving collaboration between several postgraduate students) on wastewater, sludges and surface waters to ascertain the current levels of oestrogenic compounds in our environment at present.

- Removal of oestrogens and oestrogen mimicking compounds should be taken into consideration in the design phase of wastewater treatment plants as different treatment systems and combinations of treatment systems differ in their removal efficiency.

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- It is essential that wastewater treatment plants are upgraded to deal with increasing population equivalents as the degradation and removal of oestrogenic compounds is severely compromised if the treatment system is overloaded.

- It is recommended that assessment of endocrine disruption in the environment be brought into Irish legislation such that the Environmental Protection Agency and perhaps the county councils are required to regularly sample and analyse wastewater influents, effluents and also surface waters for oestrogenic activity. In view of the use of stabilized sludges as biosolids, analysis of halogenated organic compounds, LAS, DEHP, NP and NPEOs, certain PAHs, PCDDs and PCDFs, and certain PCB congeners for accordance with limit values in sludge to be used on land should already be taking place (EU, 2000).

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Appendix A

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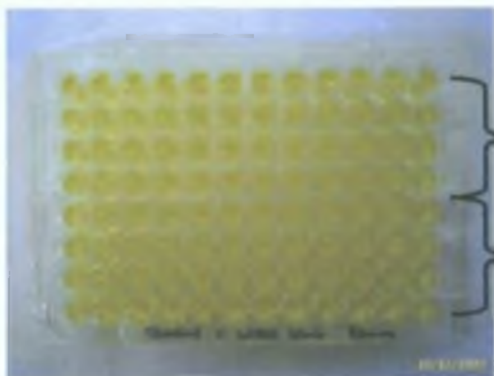
Colour development of standard and negative control over 72 hours



0 hours

Standard: 17β-oestradiol
Four replicates diluted from 54.48μg/l to 3ng/l (left to right).

Negative control: ethanol

24 hours

48 hours

72 hours

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Appendix B

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Table 1.17.1: Oestradiol equivalent concentrations determined by chemical analysis for various matrices in different countries

Influent EEQ (ng/l)				Effluent EEQ (ng/l)				Surface water EEQ (ng/l)				Country	Analytical method	Reference
E1	E2	EE2	E3	E1	E2	EE2	E3	E1	E2	EE2	E3			
15-60	10-31	n.d.	23-48	5-30	3-8	n.d.	n.d.-1	5-12	2-6	n.d.-1	2-5	Italy	Oasis cartridge.Tandem LC-MS-MS	Laganà *et al.*, 2004
<0.5-75	0.5-20	-	-	n.d.-54	nd-7	n.d.-2.2	n.d.-28	-	-	-	-	Italy	Carbograph cartridge. LC-MS-MS	Johnson *et al.*, 2000
25-132	4-25	0.4-13	24-188	2.5-82.1	0.35-3.51	N.d.-1.7	0.57-18	0.33	0.11	0.04	1.5	Italy	Carbograph cartridge.LC-NIS-MS-MS	Baronti *et al.*, 2000
54.9-76.6	12.2-19.5	6.2-10.1	-	<1	<1	<1	-	-	-	-	-	Germany	RP-C18 cartridge GC-ion trap-MS	Andersen *et al.*, 2003
-	-	-	-	1.2-19	1-5.6	<1-1.5	-	<1	3.9	<1	-	Germany	1:RPC18 column + GC-MS 2:GC-QUAD MS + LC-ESI-MS-MS	Pawlowski *et al.*, 2004
-	-	-	-	n.d.-70	n.d.-3	n.d.-15	-	n.d.-1600	n.d.	n.d.	-	Germany	Lichrolut EN + RP-C18 columnsGC-MS-MS	Ternes *et al.*, 1999a
-	-	-	-	n.d.-22	n.d.-6.4	n.d.-4.1	-	-	-	-	-	Germany	C18 SPE cartridge. GC-MS	Splenger *et al.*, 2001
-	-	-	-	51	6	2	-	-	-	-	-	Switzerland	Lichrolut EN + RP-C18 columnsGC-MS-MS	Aerni *et al.*, 2004

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Continuation of Table 1.17.1: Oestradiol equivalent concentrations determined by chemical analysis for various matrices in different countries

Influent EEQ (ng/l)				Effluent EEQ (ng/l)				Surface water EEQ (ng/l)				Country	Analytical method	Reference
E1	E2	EE2	E3	E1	E2	EE2	E3	E1	E2	EE2	E3			
-	-	-	-	5.8	1.1	4.5	-	-	-	-	-	Sweden	SDB Isolute ENV GCMS	Larsson *et al.*, 1999
-	-	-		4-50.5	<LOQ-6.4	<LOQ-2.8	2.3-17.5	-	-	-	-	Switzerland	Lichrolut EN + RP-C18 columnsGC-MS-MS	Rutihauser *et al.*, 2004
-	-	-	-	<0.4-47	1.1-12	<0.2-7.5	-	<0.1-3.4	0.3-5.5	<0.1-4.3	-	Netherlands	SDBXC disks columns HPLC + GC-MS-MS	Belfroid *et al.*, 1999
20-130	17-150	<0.3-5.9	-	<-3-11	<0.8	<0.3-2.6	-	<0.3-7.2	<0.8-1.0	<0.3-0.4	-	Netherlands	SDBXC disks columns HPLC + GC-MS-MS	Vethaak *et al.*, 2005
-	-	-	-	<50	<50-540	<50-240	-	-	-	-	-	Spain	C18 cartridges GC-MS	Aguayo *et al.*, 2004
9.6-17.6	11.1-17.4	4.9-7.1	11.4-15.2	4.3-7.2	4.5-8.6	2.7-4.5	5.0-7.3	2.2-3.0	3.0-3.2	1.8-2.9	2.1-2.5	France	Bakerbond speedisk DVB GC-MS	Cargouët *et al.*, 2004
-	-	-	-	6.4-29	1.6-7.4	n.d.	2.0-4.0	0.2-10	n.d.-7.1	n.d.-3.1	n.d.	UK	SDBXC disks LC-NCI-MS	Xiao *et al.*, 2001
-	-	-	-	1-80	1-50	n.d.-7	-	-	-	-	-	UK	C18 cartridges GC-MS	Desbrow *et al.*, 1998
-	-	-	-	-	-	-	-	1.8-7.1	n.d.-25	n.d.	-	UK	C18 GC-MS	Fawell *et al.*, 2001
-	-	-	-	-	1.9-14.6	n.d.-3.0	-	-	1.0-10.0	1.0-2.0	-	US	NP + RP HPLC	Synder *et al.*, 2001
-	-	-	-	1.6-18	0.77-6.4	-	-	-	-	-	-	US	Immunoaffinity extraction + LC-ESI-MS	Ferguson *et al.*, 2001
-	-	-	-	<1-42	<1-20	<1	-	-	-	-	-	US	SDBXC disks+ GC	Hugget *et al.*, 2003

Abbreviations: - not analysed; n/d: not detected

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Continuation of Table 1.17.1: Oestradiol equivalent concentrations determined by chemical analysis for various matrices in different countries

Influent EEQ (ng/l)				Effluent EEQ (ng/l)				Surface water EEQ (ng/l)				Country	Analytical method	Reference
E1	E2	EE2	E3	E1	E2	EE2	E3	E1	E2	EE2	E3			
19-78	2.4-26	-	-	1-96	0.2-14.7	-	-	-	-	-	-	Canada	RPC18 supelco cartridge GCMS	Servos *et al.*, 2005
12-15	17-20	-	3.6-4.7	<0.2-0.3	<0.4-0.9	-	1.2-2.1	-	-	-	-	Japan	SDBXC disks silica column cleanup LC-MS	Sunardi *et al*, 2001
-	-	-	-	2.5-34.0	0.3-2.5	-	-	3.4-6.6	0.8-1.0	-	-	Japan	SPE cartridge LC-MS-MS	Iosbe *et al*, 2003
-	-	-	-	107.6	14.7	<0.2	<0.2	6.4-26.3	0.5-5.9	<0.2	<0.2	Japan	SPEcartridge LCMS + LC/MS/MS	Furichi *et al*, 2004

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Appendix C

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Table 1.17.2: Oestradiol equivalent concentrations determined by *in-vitro* assay for various matrices in different countries

Influent EEQ (ng/l)	Effluent EEQ (ng/l)	Surface water EEQ (ng/l)	Country	*In-vitro* assay	Reference
1.1–119.9 (av 41.7)	0.03–16.1 (av 2.59)	0.07-0.47	Netherlands	ER-CALUX	Murk *et al.*, 2002
n.d–86.4 (av 31.9)	0.1–15.8 (av 3.1)	n.d–1.1	Netherlands	YES	Murk *et al.*, 2002
-	≈0.55– ≈70	-	Switzerland	YES	Rutishauser *et al.*, 2004
-	0.4-53.0	-	Switzerland	YES	Aerni *et al.*, 2004
av =100	av =12.9	0.01–31 (av =1.6)	Japan	YES	Tamamoto *et al.*, 2001
35–72	4–35	-	Japan	YES	Onda *et al.*, 2002
-	25.9	16.7–17.2	Japan	MVLN	Furuichi *et al.*, 2004
57–63	2–24	0.3–4.52	France	MVLN	Cargouët *et al.*, 2004
-	1.90–14.9	0.86–10.9	USA	MVLN	Synder *et al.*, 2001
17.7–24.0	4.9–10.6	-	USA	YES	Holbrook *et al.*, 2002
-	21–147	-	USA	YES	Tilton *et al.*,2002
-	<1–15.0	-	USA	YES	Huggett *et al.*, 2003
n.d.–145 (av 79)%	n.d.–106 (av 50.1)%	-	Canada	YES	Servos *et al.*, 2005
1.1–29.8	<0.1–16.4	<0.006–0.083	Sweden	YES	Svenson *et al.*, 2003
-	n.d –4.46	n.d.–81.4	Belgium	YES	Witters *et al.*, 2001
-	-	<15	UK	YES	Fawell *et al.*, 2001
≈12– ≈75	n.d.– ≈40	-	UK	YES	Kirk *et al.*, 2002
-	4.0-35.8	0.3-7.1	UK	YES	Thomas *et al.*, 2001
-	34.1–65.9	11.8–19.4	Germany	YES	Pawlowski *et al.*, 2004
-	2–25	-	Germany	E-SCREEN	Körner *et al.*, 1999
58–70	5.6–6.4	-	Germany	E-SCREEN	Körner *et al.*, 2000
-	0.2–7.8	-	Germany	E-SCREEN	Körner *et al.*, 2001
-	-	0.0005-7.4	Korea	E-SCREEN	Oh *et al.*, 2000

Abbreviations
av: average
-: not analysed
n/d: not detected

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