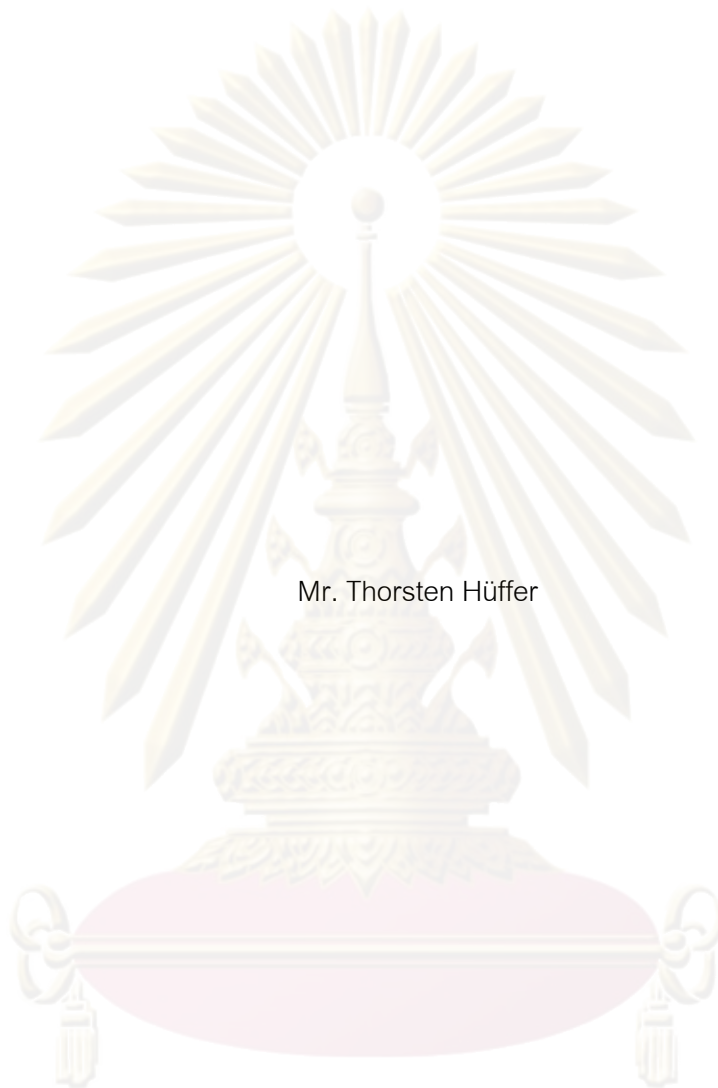DETERMINATION OF 17α-METHYLTESTOSTERONE IN SEDIMENT SAMPLES FORM A

NILE TILAPIA NURSERY POND

Mr. Thorsten Hüffer

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in

Environmental Management

(Interdisciplinary Program)

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การตรวจวัด 17α-METHYLTESTOSTERONE และสารเมตาโบไลท์หลักในน้ำและดินตะกอน

จากบ่ออนุบาลปลานิล

นายฮอร์สเตน ฮัฟเฟอร์

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Thesis Title   DETERMINATION OF 17α-METHYLTESTOSTERONE IN

                    SEDIMENT SAMPLES FROM A NILE TILAPIA NURSERY POND

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It is a common practice in the aquaculture of Nile tilapia to use 17α-methyltestosterone (MT) impregnated in fish feed to obtain an all male population. However, only little is known about the occurrence and fate of MT in the environment. Therefore, one objective of this study was to qualitatively and quantitatively determine MT in sediment samples obtained from a Nile tilapia earthen nursery pond as MT has been shown to preferably sorb onto sediment. In addition as the second objective of this study, a method for the extraction and purification of MT from sediment samples had to be developed, as there is no comprehensive protocol reported in literature. The developed method was based on liquid-phase extraction in combination with sonication. Optimized parameter of the extraction procedure included extraction solvent, extraction time, and sonication time. A recovery rate of 97.01% ± 3.36% RSD (n= 5) could be achieved and additional residues of MT in sediment could be extracted with sonication. Moreover, sediment samples were taken during hormone treatment period to monitor concentration of MT during its application. The obtained results indicated that MT could be found in all sediment samples; although, the concentrations of MT measured were in the low μg/kg-range. This is the first study reporting the occurrence of MT in samples taken from the environment.

Field of Study: Environmental Management   Student's Signature...............

Academic Year:....2009..........................   Advisor's Signature..................

Co-Advisor's Signature....................

V

ฮอร์สเตน ฮัฟเฟอร์:   การตรวจวัด 17α-METHYLTESTOSTERONE   และสารเมตาโบไลท์หลักในน้ำและดินตะกอนจากบ่ออนุบาลปลานิล. (DETERMINATION OF 17α-METHYLTESTOSTERONE IN SEDIMENT SAMPLES FROM A NILE TILAPIA NURSERY POND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.ตะวัน ลิมปิยากร, 74 หน้า.

การเพาะเลี้ยงปลานิลนิยมใช้สาร 17α-methyltestosterone (MT) เติมในอาหารปลาเพื่อให้ได้ปลาเพศผู้ แต่ไม่มีการศึกษาว่ามีการตกค้างของสารนี้ในสิ่งแวดล้อมหรือไม่ ดังนั้นวัตถุประสงค์หนึ่งของงานวิจัยนี้เพื่อบ่งชี้ปริมาณการปนเปื้อนของสารนี้ในตัวอย่างดินที่ได้จากบ่ออนุบาลลูกปลานิล เนื่องจากสาร MT มีแนวโน้มที่จะถูกดูดซับบนผิวดินตะกอนได้ดี นอกจากนี้อีกวัตถุประสงค์หนึ่งของการศึกษาคือการพัฒนาวิธีการสกัดแยกสาร MT จากตัวอย่างดิน ซึ่งยังไม่มีรายงานวิธีการวิเคราะห์สารนี้ในดิน การพัฒนาวิธีการสกัดใช้หลักการสกัดแยกแบบของเหลวร่วมกับการเขย่าด้วยคลื่นอัลตราซาวน์ ศึกษาตัวแปรที่เหมาะสมในการสกัด ได้แก่ ตัวทำละลาย เวลาในการสกัด และเวลาในการใช้คลื่นอัลตราซาวน์ ผลการสกัดตัวอย่างดินที่เติมสารมาตรฐานที่ระดับความเข้มข้น 100 ไมโครกรัมต่อลิตร ได้ร้อยละการคืนกลับ 97.01±3.36 (n=5) นอกจากนี้ได้ทำการเก็บตัวอย่างดินตะกอนจากบ่ออนุบาลปลานิลในช่วงที่มีการให้ฮอร์โมนโดยใช้สารที่สนใจศึกษานี้ ผลการตรวจวิเคราะห์สามารถตรวจพบการปนเปื้อนของสารนี้ในทุกตัวอย่างดินในระดับความเข้มข้นต่ำช่วงไมโครกรัมต่อกิโลกรัม ระดับความเข้มข้นที่ตรวจพบนี้อาจมีผลต่อระบบนิเวศวิทยา รายงานนี้นับเป็นรายงานแรกของการศึกษาตัวอย่างดินที่แสดงการปนเปื้อนสาร MT ในสิ่งแวดล้อม

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ACKNOWLEDGEMENTS

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ABBREVIATIONS

AAS	Anabolic androgenic steroid
CEC	Cation exchange capacity
EDC	Endocrine disrupting compound
EE_2	17α-ethinyloestradiol
ELISA	Enzyme-linked immunosorbent assay
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
HPLC-MS	High performance liquid chromatography-mass spectrometry
HPLC-UV	High performance liquid chromatography-UV/vis detector
K_d	Distribution coefficient
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDR	Linear dynamic range
LL-SPE	Liquid-liquid solid phase extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPE	Liquid phase extraction
MRM	Multi-selected reaction monitoring
MSTFA	*N*-methyl-*N*(trimethysilyl)trifluoroacetamide
MT	17α-methyltestosterone
NADPH	Nicotinamide adenine dinucleotide phasphate
OECD	Organisation for Economic Co-operation and Development
OM	Organic matter content
PAH	Polycyclic aromatic hydrocarbon
RIA	Radioimmunoassay
RSD	Relative standard deviation
SPE	Solid phase extraction
SPME	solid phase microextraction
STP	Sewage treatment plant

TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry
WWTP	Wastewater treatment plant
YAS	Yeast androgen screen
Z	Fugacity capacity

CHAPTER I

INTRODUCTION

1.1 Theoretical Background

Treatment of newly hatched Nile tilapia fry with 17α-methyltestosterone (MT), an anabolic androgenic steroid, for sex reversal has become a common aquaculture practice. Nile tilapia is a popular farmed-raised fish with aquaculture farmers because in addition to its high market value, it is tolerant, easy to spawn, and easy to grow with various types of feed. However, growth of both male and female tilapia in the same pond can cause overpopulation and stunting. The culturing of only male Nile tilapia, which can have double the growth rate of female tilapia and hence a larger body size, is therefore more desirable (MacIntosh and Little, 1995). 17α-alkyl anabolic synthetic steroids are popular because alkylation at the 17-position prevents the rapid oral inactivation that occurs with other anabolic steroids and, therefore, eliminates the need to inject the drug (Stanley et al., 1997; Gonzalo-Lumbreras and Izquierdo-Hornillos, 2003).

There are several methods available for the production of monosex tilapia, such as manual sexing, interspecific hybridization, hormonal sex reversal, and YY male technology (Beardmore et al., 2001). However, the benefits of these technologies have not been consistently documented in literature and none have proven to be cost-effective or sustainable (Kamaruzzaman et al., 2009). A number of studies (Macintosh et al., 1988; Little et al., 2003) have reported that Nile tilapia treated with MT via a feed supplement had higher body weight or a faster growth rate than mixed sex populations.

In hormonal sex reversal, MT is fed as a food additive in a concentration of 60 mg of MT per kilogram of feed in the early stages of sexual differentiation in Nile tilapia. This diet is administered to the fish at an average of 15-20% of their body weight per day for 28 consecutive days (Barry et al., 2007). Uneaten or un-metabolized food may, therefore, leak significant amounts of MT into the masculinization pond environment and are released into receiving waters.

Furthermore, in some countries, pond sediments are dredged and used to "prepare soil" for crop production; thereby, spreading the risk of MT exposure to terrestrial organisms (Contreras-Sánchez et al., 2001).

Some researchers have warned about the unintended effects of steroid administration, such as the fish-to-fish transfer of steroids (Budworth and Senger, 1993), biased sex ratios in untargeted organisms (Abucay et al., 1997), and paradoxical feminization (Piferrer and Donaldson, 1991; Piferrer et al., 1993; Rinchard et al., 1999; Eding et al., 1999). Studies have reported that exposure of untargeted organisms to MT can result in biased sex ratios. Significant masculinization of common carps (*Cyprinus carpio*) exposed to water used in MT-impregnated feeding trials was reported by Gomelsky et al. (1994). Their findings suggest that MT or its metabolites can persist in the water at concentrations capable of causing sex inversion. Abucay and Mair (1997) and Abucay et al. (1997) reported incidental sex inversion in Nile tilapias kept in aquaria and concrete tanks. These authors reported that sex ratios were significantly biased when non-targeted fish were housed in the same tank where groups of fish were fed with MT.

MT is a questionable human carcinogenic, producing nonmalignant tumors in the liver. It is a poison by the intraperitoneal route, and it causes developmental abnormalities in the urogenital system (Lewis, 1999). In laboratory studies, it has been demonstrated that long-term exposure to MT can act as a weak hepatocarcinogen in male and female rats (Taylor et al., 1984) and can eliminate male sexual behavior and suppressed serum testosterone levels in male rats (Clark et al., 1997).

A number of analytical protocols have been described for the detection and quantification of MT residues in a variety of matrices. Among them are methods based on radioimmunoassay (Daeseleire et al., 1991), chemiluminescence (Jansen et al., 1985; Van Peteghem et al., 1989), gas chromatography-mass spectrometry (Bowden et al., 2009), and liquid chromatography-mass spectrometry (Chu et al., 2006). Despite the abundance of methods developed for urine, hair, and other matrices, few have been developed for aquatic matrices (Fritzpatrick et al., 1999; Fitzpatrick and Contreras-Sánchez, 2000). Therefore, there is a need for developing a sensitive and reliable method to analyze MT in environmental matrices such as water

and sediment. In addition, no comprehensive protocol is available for the determination of MT and its metabolites in such matrices.

This study focuses on the determination of MT in sediment samples. Parameters of extraction of the analyte from sediment and its further enrichment in such matrices were investigated. In addition, the occurrence of MT in earthen nursery ponds was monitored during a hormone treatment period. The hypothesis that is tested is that MT can be determined in sediment samples taken from earthen ponds where MT is used for sex reversal in Nile tilapia.
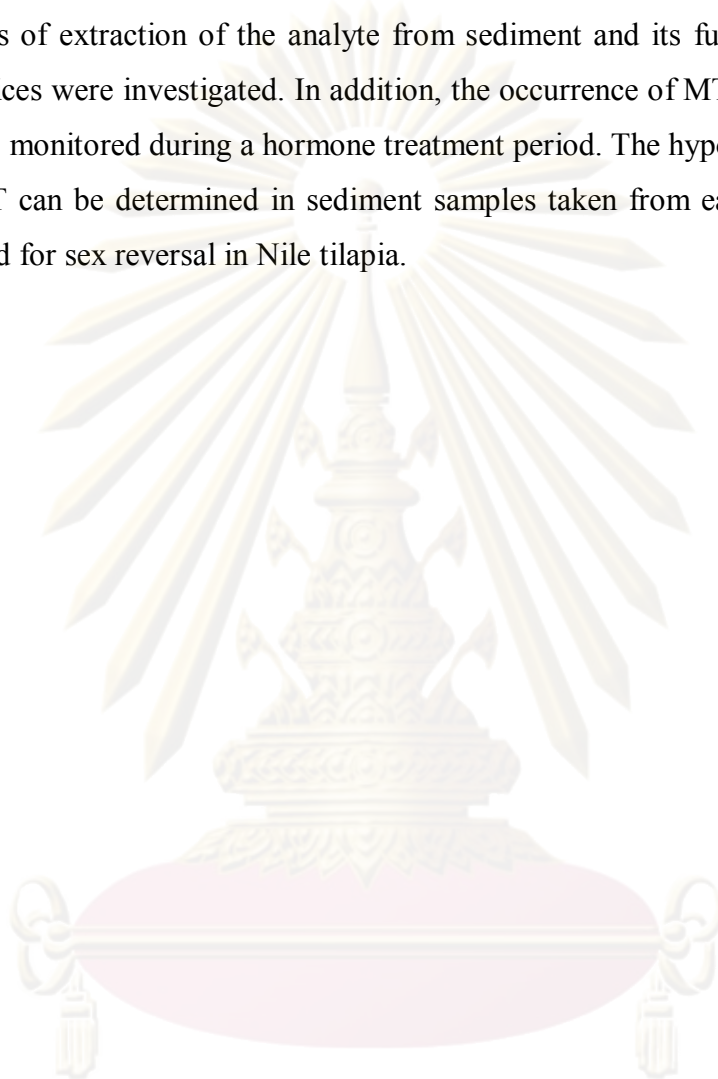
1.2 Objectives

- To develop a method for the extraction and purification of MT from sediment by liquid-phase extraction (Objective 1).
  - o To study the effects of solvent on the performance of LPE.
  - o To study the effects of extraction time on the performance LPE.
  - o To study the effects of sonication on the performance LPE.
- To quantitatively determine the concentrations of MT in sediment from an earthen nursery pond during the hormone treatment period (Objective 2).

1.3 Hypotheses

- MT can be determined in sediment samples in a Nile tilapia nursery pond.
- MT can be extracted from sediment samples by LPE.

1.4 Scope of the Study

- Basic properties (organic matter, CEC, pH, and texture) of sediment taken at the study site were determined.
- Parameters of LPE for the extraction of MT from sediment were optimized.
- Concentrations of MT were monitored in two earthen nursery ponds during and after the treatment period.
- MT was analyzed by ultra performance liquid chromatography-tandem mass spectrometry.

CHAPTER II

LITERATURE REVIEW

2.1 Endocrine Disrupting Compounds (EDCs)
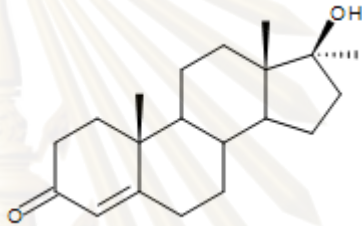
The occurrence of active estrogenic compounds influencing the sexual development of fish in an English river was reported 15 years ago (Purdom et al., 1994). Since then, the presence of chemicals that have the potential to interfere with the endocrine system of humans and wildlife has become a major concern worldwide. Gradually, the attention of researchers and regulators broadened from estrogenicity to other endocrine pathways (e.g., the androgen or thyroid hormone system). The most potent active endocrine compounds present in the environment belong to the chemical class of steroids, which are formed naturally by humans and wildlife or produced synthetically (e.g., for use in contraceptives). Estrogenic steroids have often been found in the aquatic environment, and their ability to impair wildlife has been demonstrated (Sumpter and Johnson, 2008). Although research on progestagenic and androgenic compounds has only begun recently, a few studies have reported the presence of androgenic or progestagenic steroids in the environment (Vulliet et al., 2008).

Steroids comprise a skeleton of three hexagonal and one pentagonal carbon rings, generally arranged in a 6-6-6-5 structure, to which various functional groups and side chains are attached. All steroids can be derived from cholesterol. Androgens are C19 steroids that stimulate or control the development of masculine characteristics. Like other steroid hormones, natural androgens generally enter the environment via wastewater-treatment plants (WWTPs). Like all groups of steroids, natural and synthetic androgens have been used as growth promoters and in human and veterinary therapy. Furthermore, the microbial degradation of phytosterols to progestagens and even further to androgens has been proposed as a natural androgenic steroid source (Jenkins et al., 2004).

2.2 *Physiochemical properties of MT*

From the physiochemical properties of MT, which are displayed in Table 1, it can be seen that it is a hydrophobic organic contaminant. From this, it is expected that its sorption on soil or sediment will be a significant factor in reducing concentrations in the aqueous phase.

Table 1: Physiochemical properties of MT.

Property	17α-methyltestosterone[1]
Structure	
CAS	58-18-4
Molecular weight	302.46
Melting point	163 °C
Water solubility	3.39 mg/L[2]
Vapor pressure	1.85 10^{-8} mmHg[2]
Henry's constant	4.68 10^{-9} atm m^3/mole[2]
Log K$_{ow}$	3.36

[1] Data obtained from SRC PhysProp Database

[2] at 25 °C

2.3 *Occurrence of MT in the environment*

Neither MT nor its metabolites have not been reported in any environmental sample. This fact is mainly due to two reasons. Firstly, there are very few studies available that take MT into account when analyzing hormone concentrations in environmental waters. Laganà et al. (2001) developed a method with characteristics seen in Table 3; however, in this study the method was not applied to a real sample. In another more recent study, Chang et al. (2008) performed a trace analysis of

androgens and progestogens in environmental samples. The developed procedure was applied to samples taken from the influent and final effluent of two sewage treatment plants (STPs) in Japan. Unless spiked, MT could not be detected in any of the samples. However, since both STPs were stated to receive mainly domestic wastewater, an elevated concentration of MT could not have been expected. In addition, samples from surface waters were analyzed without determining the presence of MT. Despite the fact that these sampling sites were known to be located in a major farming area, it was not said whether MT was applied in any of the farming activities.

The second reason for the rare abundance of studies focusing on analyzing MT is the fact that MT has been known more as an AAS compound of concern in doping analysis than in environmental issues. However, MT concentrations have been monitored in earthen ponds for growing Nile tilapia by the Aquaculture Collaborative Research Support Program (ACRSP). Fitzpatrick et al. (1999) determined MT in water and soil samples in a model pond by radioimmunoassay (RIA). Water and samples were taken before the onset of the 28-day treatment with MT and weekly starting from the last day of treatment. In addition, water samples were taken weekly during the treatment period. The study revealed that the MT concentrations in water were highest between 1 and 2 μg/L at 14 and 21 days of treatment, whereas its concentration decreased to background levels one week after the treatment. In contrast, the MT concentration in soil ranged from between 1.4 and 1.7 μg/kg at the offset of the treatment and remained detectable up until three weeks after the end of the treatment at concentrations of between 0.8 and 1.6 μg/kg.

In a similar study with a larger container size, Fitzpatrick and Contreras-Sánchez (2000) determined elevated MT concentration levels during the treatment period, compared to that of background levels. In addition, concentrations were compared between model ponds containing soil, gravel, or no soil. The results showed that MT concentrations in water were highest when there was no soil present, followed by ponds containing soil, and concentrations were lowest for ponds containing gravel. This indicates that the sorption of MT has to be taken into account. Moreover, the results suggested that sediment acts as a trap for MT because while in containers without substrate, MT remained in suspension for a longer period of time. It was shown that between 2.8 and 2.9 μg/kg of MT still remained in soils nearly three months after the cessation of the treatment.

In a more recent study performed by this group, no MT concentrations were determined at any sampling point throughout the experiment. Concentrations in soil were determined to average 146.7 ng/kg. The results were explained as being due to the use of the new antibody RIA, the detection limit of which was established to be 10 pg/tube. It should be noted that their study focused on the use of MT in earthen ponds with no record of hormone usage. In general, the large variability detected in that study was attributed to active bacterial degradation and an uneven distribution of MT in the pond due to dominant winds or uneaten food deposition (Contreras-Sánchez et al., 2001).

2.4 Fate of MT in the environment

2.4.1 Sorption

The distribution and partitioning of androgenic steroids in the environment are determined by their physiochemical properties and site-specific environmental conditions. There is no specific study on the sorption of MT available in the literature. However, as previously described (see "Physiochemical properties of MT"), Fitzpatrick and Contreras-Sánchez (2000) reported that MT has lower concentrations in water when soil is present in the system. However, soil characteristics were not determined in that study. From its physiochemical properties shown in Table 1, it can be anticipated that MT has a tendency to sorb onto soil or sediment.

Sorption experiments performed in a previous study of this group showed that distribution coefficients (K_d) of MT greatly depend on organic matter content of the solid phase, whereas as pH and salinity had no effect on sorption of MT on solid matrices (Chotisukarn, 2008).

2.4.2 Biodegradation

Little is known about the fate of MT in fish farms and receiving streams. Fitzpatrick and Contreras-Sánchez (2000) showed that between 2.8 and 2.9 µg/kg of MT still remained in soils nearly three months after the cessation of MT treatment. In a recently published study, Homkiln et al. (2009) investigated the biodegradation of MT under aerobic conditions in sediment of a Nile tilapia masculinization pond. The results suggest that MT is biodegradable under aerobic conditions. In addition, MT-degrading bacteria were isolated and identified as similar to the *Pimelobacter simplex* strain S151 using DNA sequencing. The first-order degradation rates were found to decrease with increasing initial MT concentrations and ranged from 0.52 to 0.10 d^{-1}. These results are consistent with the results obtained by Druzhinina et al. (2008), who investigated the conversion of MT to methandrostenolone by *Pimelobacter simplex* VKPM Ac-1632.

2.4.3 Metabolism

Since MT is an anabolic androgenic steroid that can be misused by human athletes, its metabolism has broadly been studied in humans. The metabolism of 3-keto-4-ene steroids, such as testosterone and MT, was summarized by Schänzer and Donike (1993). The initial and rate-limiting step in the A-ring metabolism of such steroids is the reduction of the double bond between C_4 and C_5. The reaction is catalyzed by the enzymes 5α- and 5β-reductase and yields two isomers with 5α- and 5β-configuration, as can be seen in Figure 1. Both enzymes require NADPH as a co-factor. The amount of 5α- and 5β-isomers formed depends on the structure of the parent compound, whereas even the D-ring has a strong influence on the enzymatic activity of the C-4,5 double bond reducing enzymes.
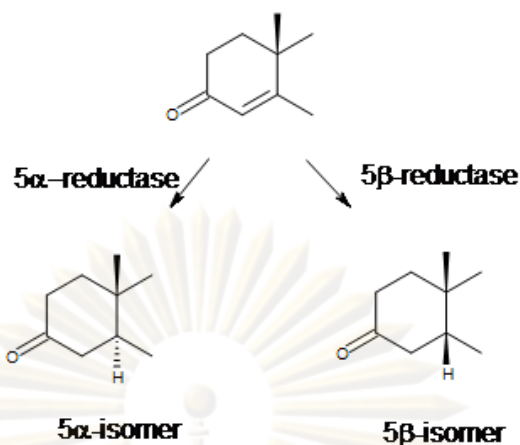
Figure 1: A-ring metabolism: 5α- and 5β-Reduction of 3-keto-4-ene steroids (adapted from Schaenzer 1997).

Once the double bond is reduced, the 3-keto group is immediately transformed, mainly to a 3α-hydroxy structure. This leads to 17α-methyl-5α-androstane-3α,17β-diol (5α3α17β) and 17α-methyl-5β-androstane-3α,17β-diol (5β3α17β), the two major urinary metabolites identified in humans (see Figure 2).
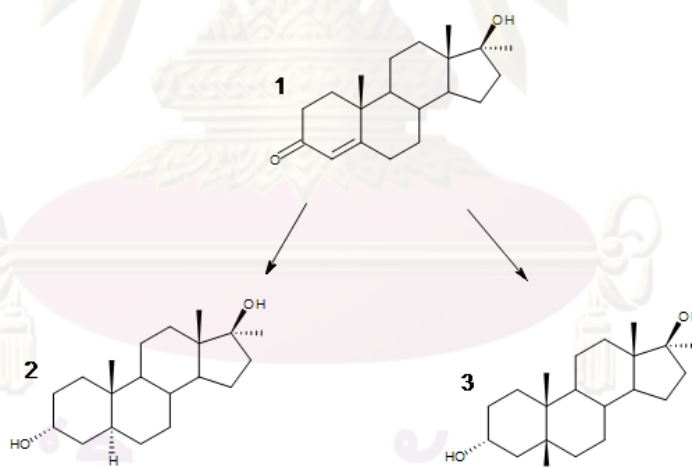
Figure 2: Metabolism of MT (1), main excreted metabolites: 5α3α17β (2) and 5β3α17β (3) (adapted from Schaenzer 1997).

The metabolism of MT has mainly been investigated in humans, horses, and dogs due to its possible usage as a doping agent in sports. An overview of these studies and the detected metabolites of MT are shown in Table 2.

Table 2: Identified metabolites of 17α-methyltestosterone in various organisms.

Compound	Target organism	Metabolites[a]	Analysis[b]	Reference
MT	Greyhound Dog	6β-Hydroxy 16α-Hydroxy 2β-Hydroxy	HPLC-MS	William et al., 2000
MT	Dog	5αA3β17βD 5βA3α17βD	GC	Mosbach et al., 1968
MT	Human	5αA3α17βD 5βA3α17βD	GC-MS	Shinohara et al., 2000
MT	Human	5βA3α17βD 5αA3a17βD	TLC	Rongone and Segaloff, 1962
MT	Equine	5αA3β17βD 5βA3α17βD 5αA3β16α17βT 5αA3β16β17βT 5βA3α16α17βT 5βA3α16β17βT	GC-MS	McKinney et al., 2007
MT	Bovine	5βA3α17βD 5αA3α17βD	ELISA	Lu et al., 2006
MT	Heifer	5βA3α17βD 5αA3β17βD 5βA3β17βD	GC-MS	Blokland et al., 2005

[a] 6β-Hydroxy: 6β-Hydroxymethyltestosterone; 16α-Hydroxy: 16α-Hydroxymethyltestosterone; 2β-Hydroxy: 2β-Hydroxymethyltestosterone; 5αA3β17βD: 17α-methyl-5α-androstane-3β,17β-diol; 5βA3α17βD: 17α-methyl-5β-androstane-3α,17β-diol; 5αA3α17βD: 17α-methyl-5α-androstane-3α,17β-diol; 5αA3β16α17βT: 17α-methyl-5α-androstane-3β,16α,17β-triol; 5αA3β16β17βT: 17α-methyl-5α-androstane-3β,16β,17β-triol; 5βA3α16α17βT: 17α-methyl-5β-androstane-3α,16α,17β-triol; 5βA3α16β17βT: 17α-methyl-5β-androstane-3α,16β,17β-triol; 5βA3β17βD: 17α-methyl-5β-androstane-3β,17β-diol. [b] HPLC-MS: high performance liquid chromatography-mass spectrometry; GC: gas chromatography; GC-MS: gas chromatography-mass spectrometry; TLC: thin layer chromatography; ELISA: enzyme-linked immunosorbent assay.

2.4.4   Abiotic Degradation

The elimination of MT applied as a masculinization agent in Nile tilapia as been subject to investigation. Contreras-Sánchez et al. (2004) studied the effects of activated charcoal on the elimination of MT from water used in intensive sex-reversal systems when charcoal was placed in the filter. In general, MT was not detectable in the water at the end of the trials in any of the systems that had a filtration system. MT levels were determined in the two types of charcoal tested in this study. It was shown that vegetal charcoal had a higher adsorption capacity than mineral charcoal, with the detected MT levels at 100 and 50 μg/kg, respectively. However, low MT levels in water were detected at different times, but the compound was never seen consistently at all sites within the tank. MT levels detected in this study varied between 0.14 and 9.17 μg/L. In addition, MT levels showed the same pattern of variability and low values in the systems with and without charcoal. It was suggested that either the biodegradation of MT in the biological portion of the filter with no charcoal contributed to this effect or sunlight degraded the MT molecules. As already mentioned the biotransformation of MT in sediments has been studied and confirmed by Homkiln et al. (2009). To further investigate the effects of sunlight on the elimination of MT, Schreck et al. (2005) exposed different concentration of MT diluted in water to UV and sunlight. The results indicate that MT can be partly eliminated by sunlight and completely eliminated by UV-light. The authors suggest the further research of treatment methods for masculinization effluents to eliminate the risk of unintended exposure to humans and other non-target organisms, though no further literature was available of such research at the time of this study.

MT is a light sensitive hormone, which is subject to photodegradation (Budavari et al., 1989). The type of light most likely responsible for photodegradation is UV-B (wavelengths of 280-315 nm). MT absorbs UV light strongly at a wavelength of 254 nm, which is in the UV-C part of the spectrum (100-280 nm), and absorbs UV weakly in the UV-B area of the spectrum. Unlike UV-B, UV-C is quickly absorbed in the atmosphere and does not reach the earth's surface. Since MT does not absorb UV-B very effectively, treatment with irradiation at 254 nm should be much more effective than exposure to sunlight or UV-B. Virtually nothing is known about the amount of exposure to UV needed to remove MT or of possible metabolites

produced during photodegradation. Some growers to destroy pathogens currently use commercial ultraviolet water sterilizers. These sterilizers emit UV light at a wavelength of 254 nm.

2.5 Ecological effects of MT

Snails

Schulte-Oehlmann et al. (2004) studied the effects of 17α-ethinyloestradiol (EE$_2$) and MT in the fresh-water ramshorn snail *Marisa cornuarietis*. Exposure experiments were conducted for sexually mature adult and sexually immature juvenile specimens with nominal concentrations of 0.1-1 μg/L EE$_2$ and MT over a period of 6 months. It was shown that in the tested concentration range, MT and EE$_2$ lead to the induction of imposex, the development of additional male sex organs in females; in addition, MT showed higher virilization capabilities than EE$_2$. For MT, this effect was significant almost independent from the tested concentration. It was indicated that EE$_2$ does not induce imposex directly but through androgens. Additionally, both steroid substances affected the formation of germ cells in male and female gonads, and resulted in the impairment of spermatogenesis in the male specimen of this prosobranch gastropod.

Czech et al. (2001) used MT as an androgenic test compound to expose sexually mature specimens of the hermaphroditic snail *Lymneaea stagnalis* over an 8-week period. The shell height and weight and mortality of the adults, egg production, hatching rate of the eggs, and histopathology of the adult snails were examined. MT was neither found to affect the shell height and weight nor the mortality of the adults, number of egg masses or hatching rates. However, it caused weak histological damage in the albumen and prostate glands at the concentration of 100 ng/L.

Mice

The effects of anabolic androgenic steroids (AAS) on the estrous cycle of adult rats (Long-Evans) were examined by Blasberg et al. (1996), whereas sexual receptivity, vaginal cytology, and body weight were monitored throughout a 2-week baseline. Administered doses of MT, methandrostenolone, and nandrolone decanoate were selected to mimic the human abuse levels of each compound. The highest doses of 17a-methyltestosterone (7.5 mg/kg) and nandrolone decanoate (5.6 mg/kg) disrupted behavioral and vaginal cyclicity, while AAS effects on body weight were found to be minimal.

A 28-day oral toxicity study in rats based on the "Enhanced OECD Test Guideline 407" was conducted by Wason et al. (2003) to detect the endocrine effects of MT. Daily dose levels of MT for males ranged from 0-200 mg per kg of body weight, while those for females ranged from 0-600 mg of MT per kg of body weight. In males, alterations to the genital organs consisted of decreased testis and epididymis weights with associated histological changes, a decreased number of Leydig cells and germinal epithelial degeneration/vacuolation in the testis together with degenerated germ cells in the epididymis at 200 and 40 mg/kg. Prostate weights and seminal vesicles were markedly increased at 200 mg/kg per day. In females, genital organ effects consisted of lower ovary weights at 600 and 100 mg/kg. Dose-related histological changes at these two levels mainly composed of epithelial hyperplasia and metaplasia, with an associated increase in uterine weight at the high dose. The results, therefore, indicate the presence of endocrine-mediated effects of MT at high- and mid-level doses.

Rojas-Ortiz et al. (2006) studied the behavior of adult mice in an automated elevated plus maze after 17 days of exposure to 7.5 mg/kg MT. A reduction of stretch attended postures was observed among the MT-exposed animals. However, locomotor activity and light-dark transitions in additionally tested activity chambers were not altered. The outcomes of this study suggest that exposure to a supraphysiological dose of MT has minimal effects on the exploratory-based anxiety of adult mice.

The androgenic activity of MT at 0.5, 2, 10, and 40 mg/kg per day was evaluated in the rodent Hershberger assay. Sexually immature Sprague-Dawley rats were castrated, andstatistically significant changes in sex accessory tissue were detected. However, the results reveal a clear dose-related androgen agonist effect of

MT (Kennel et al., 2004). A more recent and very similar study additionally revealed an MT induced increase of gland weight.

Fish

The heart growth in rainbow trout (*Oncorhynchus mykiss*) was quantified by Davie and Thorarensen (1997). Cocoa butter and silicone rubber pellets were used as vehicles to elevate plasma levels of testosterone and MT in immature male and female rainbow trout. Relative ventricle masses were stimulated over 1.7 times the controls in a 42-day study period. MT showed about a 2-fold higher potential for anabolic effects on the heart.

2.6   Analytics

Analytical methods to determine MT and its metabolites are show in Table 3. Besides commonly used analytical instruments such as GC or LC coupled with different types of detectors such as mass spectrometers (direct measurements), there are alternatives approaches for the determination of endocrine disrupting compounds (indirect measurements).

2.6.1 Direct Measurement

The rapid development of the chemical analysis of steroids in the environment in recent years has lead to a broad abundance of analytical techniques, which have been applied in various studies. Methods based on GC-MS have been complemented by LC-MS analysis. The major advantage of GC-MS is its high separation power in combination with good identification capabilities. However, since GC usually requires a derivatization step, it has been reported that derivatization with some silylation reagents can cause artifacts, especially the failure to derivatize the hydroxy group at position C17 (Zhang and Zuo, 2005; Shareef et al., 2006). The introduction of LC-MS/MS has lead to great advantages due its ability to analyze real samples with complex matrices. In addition, LC-MS/MS is a more sophisticated technique

because of its low LODs (sub-ng/L) as well as its high selectivity, which avoids false positives when analyzing complex matrices (Diaz-Cruz et al., 2003). Since LC-based techniques do not require a derivatization step, a potential source of analytical errors is eliminated. The analysis of steroids in the environment has extensively been reviewed by Streck (2009).

2.6.2 Indirect Measurement

Yeast Androgen Screen (YAS)

The main disadvantage of immunochemical and analytical chemical methods is that they can only determine target compounds. Moreover, they are not capable of detecting the biological activity of unknown compounds and their metabolites. Biological assays such as the receptor-based transcription assay are promising approaches to solve this problem since they can be used to detect all compounds having an affinity for a certain receptor whether agonist or antagonist. On the other hand, this method is unable to determine a specific target analyte (Rijk et al., 2009). In comparison to animal *in vivo* studies such as the previously mentioned one by Herschberger- and also the Allen-Doisy tests, the latter are highly valuable for assessing the overall biological effect of a compound but are not suitable for large-scale screening (Bovee et al., 2008).

Such specific yeast androgen screens (YAS) are available to detect androgenic and antiandrogenic activities in a variety of sample matrices. In addition, assays have been developed using both mammalian and yeast cells, but human cell lines seem to be more sensitive. Compared with yeast cells, human cells may, therefore, be better capable of identifying compounds that require human metabolism for activation. On the other hand, in addition to their robustness and ability to survive in extracts from dirty sample matrices, yeast cells are cheaper, easier to handle, and they do not possess media containing steroids (Witters et al., 2001; Bovee et al., 2006).

Table 3: Analytical methods for the determination of MT in various matrices.

Analyte	Sample	Preparation	Derivatization	Analysis	LOD	LOQ	LDR	Recovery (%)	Reference
MT Metabolites	Bovine Urine	-	-	ELISA	266 ng/L	n.a.	n.a.	n.a.	Lu et al., 2006
MT Metabolites	Human Urine	SPE	yes	GC-MS^2	130 ng/L	n.a.	n.a.	n.a.	Viryus, 2007
MT Metabolites	Horse Urine	SPE	MSTFA	GC-MS^2	n.a.	50 µg/L	0.05-5 mg/L	70-110	Yamada et al., 2007
MT+ Metabolites	Horse Urine	SPE	TMS	GC-MS^2	5-50 µg/L	n.a.	n.a.	71-105	Yamada et al., 2008
Steroids (MT)	Pig Urine	SPME	-	GC-MS	2-8 ng/L	n.a.	16-510 ng/L	71-120	Zhang et al., 2009
MT Metabolites	Equine Urine	SPE	TMS	GC-MS	n.a.	n.a.	n.a.	n.a.	McKinney et al., 2007
Steroids (MT)	Hair	LL-SPE	TMS	GC-MS^2	n.a.	n.a.	n.a.	n.a.	Gambeunghe et al., 2007
Steroids (MT)	Human Urine	LLE	-	HPLC-UV	12-110 µg/L	n.a.	n.a.	n.a.	Lumbreras, 2000
MT	Fish Feed	LL-SPE	-	HPLC-UV	mg/kg	n.a.	n.a.	n.a.	Marwah et al., 2005
Androgens (MT)	Environmental Waters	SPE	-	UPLC-MS^2	1 ng/L	n.a.	n.a.	84	Chang et al., 2008
Steroids (MT)	Animal Muscle	SPE	-	LC-MS^2	0.06 µg/kg	0.16 µg/kg	0.1-20 µg/kg	65-89	Xu et al., 2006
Steroids (MT)	Environmental Waters	SPE	-	LC-MS^2	n.a.	1 ng/L	n.a.	89	Lagana et al., 2001
MT	Fish Muscle Tissue	LL-SPE	-	LC-MS^2	0.04 ng/g	0.09 ng/g	n.a.	78-84	Chu et al., 2006
Steroids (MT)	Horse Urine	SPE	-	HPLC-MS^2	µg/mL	n.a.	n.a.	74	Yu et al., 2005

ELISA: enzyme-linked immunosorbent assay; HPLC-MS: high performance liquid chromatography-mass spectrometry; HPLC-UV: high performance liquid chromatography-UV/vis detector; GC-MS: gas chromatography-mass spectrometry; GC-MS^2: gas chromatography-tandem mass spectrometry; LC-MS^2: liquid chromatography-tandem mass spectrometry; LDR: linear dynamic range; LLE: liquid-liquid extraction; LL-SPE: liquid-liquid solid phase extraction; LOD: limit of detection; LOQ: limit of quantification; MSTFA: *N*-Methyl-*N*(trimethysilyl)trifluoroacetamide; SPE: solid phase extraction; SPME: solid phase microextraction; TMS: trimethylsilyl derivatization reagent; UPLC-MS^2: ultra performance liquid chromatography-tandem mass spectrometry.

CHAPTER III

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

Chemicals used in this study are displayed in Table 4. All solvents were used as delivered without further purification.

Table 4: Chemical list.

Chemical	CAS	Manufacturer	Grade
Acetone	75-05-8	Merck, Darmstadt, Germany	LC-Grade
Diethyl ether	60-29-7	Carlo Erba, Rodano, Italy	Analytical Grade
Ethylacetate	141-78-6	Merck, Darmstadt, Germany	LC-Grade
n-Hexane	110-54-3	Merck, Darmstadt, Germany	LC-Grade
Methanol	67-56-1	Carlo Erba, Rodano, Italy	Analytical Grade
17α-methyltestosterone	58-18-4	Fluka, Basel, Switzerland	Analytical standard

A 10 mg/L stock solution of MT was prepared by dissolving the appropriate amount of MT in methanol. The solution was stored in a refrigerator at -20 °C. To prepare the calibration standards and for spiking sediment samples, two working solutions were prepared daily by diluting the stock solution to the desired concentrations. Working solutions 1 and 2 contained 10 and 100 μg/L MT in methanol, respectively. All calibration standard solutions were prepared prior to sample extract analysis.

3.1.2 Instruments and Glassware

Balance

An analytical balance model TE214S purchased from Satorius (Elk Grove, IL, USA) was used in this study.

Centrifuge

A Heraeus Sorvall Biofuge Stratos Refigerated High Speed Centrifuge (Hanau, Germany) was used to separate the sediment from the extraction solvent.

DI water

Deionized water was obtained from a PURELAB ultra (18.2 MΩ-cm) purchased from ELGA Veolia Water STI (St. Maurice Cedex, France).

Rotary evaporator

A Laborota 4001-efficient purchased from Heidolph Instruments GmbH (Schwabach, Germany) was used to remove excess solvent during the extraction procedure.

Oven

Sediment samples were dried in a model FD 115 drying oven purchased from BINDER GmbH (Tuttlingen, Germany).

pH meter

A pH meter model sensION 1 portable pH meter with gel-filled pH electrode from HACH LANGE (Düsseldorf, Germany) was used for pH measurements.

Shaker

A mechanical shaker model Sseriker II PNP (Bangkok, Thailand) was used for shaking extraction batches.

Sonication

An ultra sound bath from Elma (Singen, Germany) model Transsonic T 700 H was used for sonication of sediment samples.

Ultra performance liquid chromatography-tandem mass spectrometer

Analysis of MT from sediment samples was carried out with an Acquity ultra performance liquid chromatography coupled to a Micromass Quattro Premier XE triple quadrupole mass spectrometer. (Waters, Milford, USA). MassLynx 4.1 software was used for data processing and transition confirmation.

Vortex

A Vortex Genie 2 purchased from Scientific Industries Inc. (Bohemia, NY, USA) was used to homogenize spiked sediment samples and re-constitute the analyte after evaporation of solvent.

The used pipettes and glassware used in this study are listed in Table 5.

Table 5: Pipettes and glassware used in this study.

Pipettes and glassware	Supplier
Eppendorf Research® (500-5000 μL) with epTIPS pipette tips	Eppendorf AG, Hamburg, Germany
Eppendorf Research® (100-1000 μL) with epTIPS pipette tips	Eppendorf AG, Hamburg, Germany
Eppendorf Research® (10-100 μL) with epTIPS pipette tips	Eppendorf AG, Hamburg, Germany
DURAN® Laboratory bottle 2000 mL clear with screw-cap and pouring ring from PP (blue)	SCHOTT AG, Mainz, Germany
DURAN® Laboratory bottle 250 mL amber with screw-cap and pouring ring from PP (blue)	SCHOTT AG, Mainz, Germany
DURAN® Volumetric flask: 25 mL ($\pm$0.04 mL) and 50 mL ($\pm$0.06 mL) with stopper from PE	SCHOTT AG, Mainz, Germany
Oak Ridge Centrifuge Tubes (50 mL)	NALGENE Labware, USA

3.2   Experimental Framework

The experimental framework of this study can be seen in Figure 3.
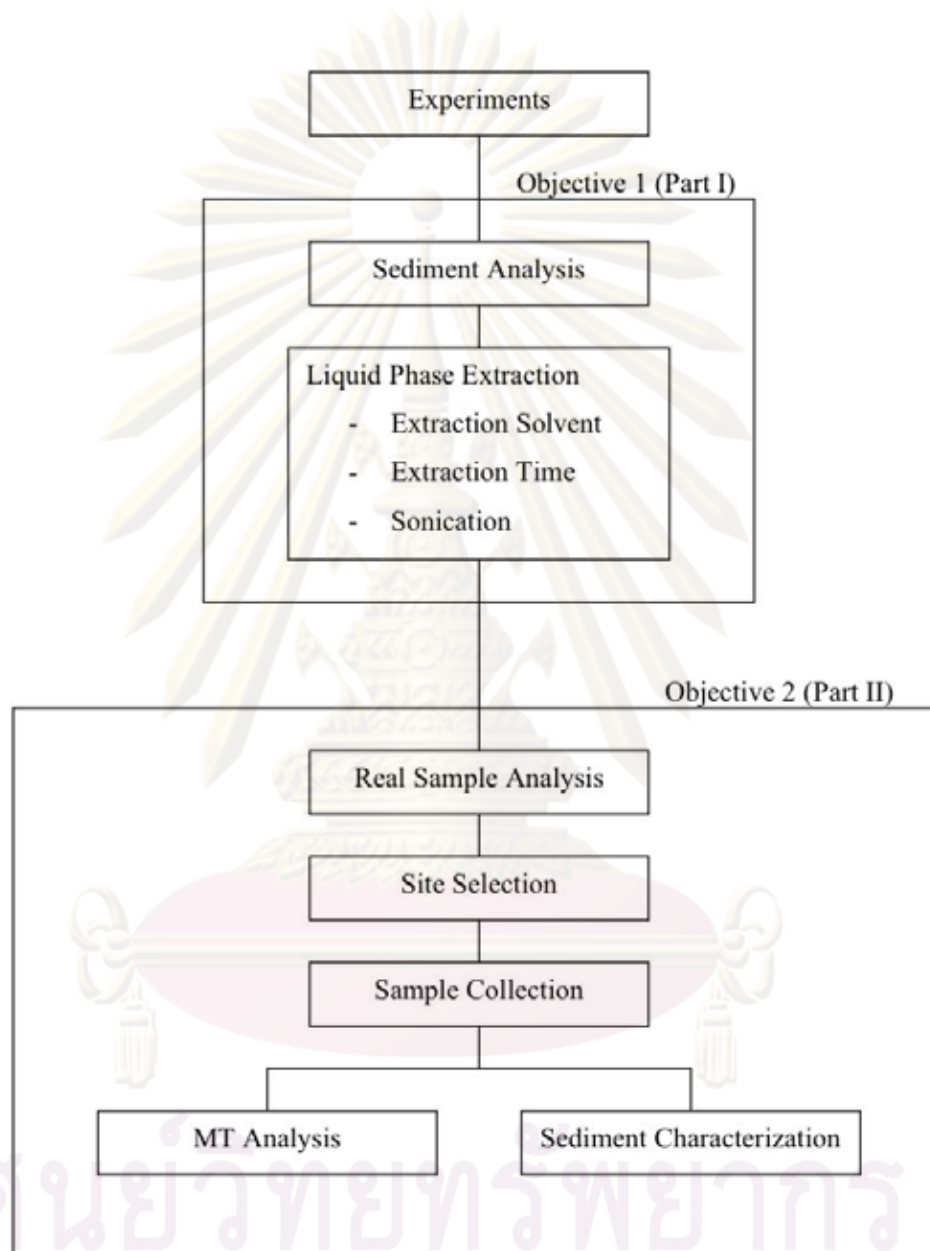
Figure 3: Experimental framework of this study.

3.3   Sediment Analysis (Part I)

Sediment extraction

The analyte was extracted from sediment by liquid-phase extraction (LPE). LPE parameters were optimized as displayed in Figure 4. After drying, sediment samples were grinded and sieved through a US Sieve Size 60 (250 μm). For extraction, 5 g sediment sample were weighted into 50 mL-Oak Ridge PP centrifuge tubes (Nalgene Labware, USA). Samples were extracted three times with 10 mL solvent. For each extraction, samples were horizontally shaken at 320 rpm, centrifuged at 8000 rpm for 10 min, and the supernatants were collected in new tubes. The sediment extracts were then evaporated to near dryness at 55 °C. After their re-constitution in 0.5 mL of methanol, samples were filtered through 0.45 μm Mini-Uniprep^{TM} PVDF filter media with propylene housing (Whatman, USA). These prepared sediment sample were subjected UPLC-MS/MS for analysis.

The following parameters for the extraction of analyte by LPE were optimized: extraction solvent, extraction time (5, 10, 30, 60, 150 min), and sonication (5, 10, 20, 30 min).
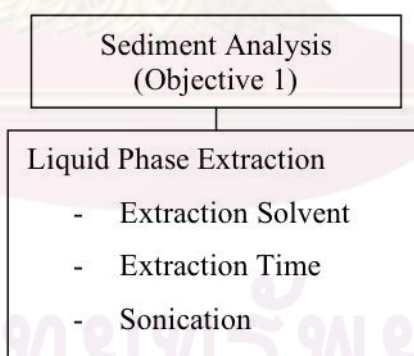
Figure 4: Part I of this study.

Extraction solvent

Methanol, acetone, diethyl ether, and n-hexane were studied as extraction solvents because they do not only reflect a broad variety of dielectric constants (Table 6), which can be used as an indicator of their polarity, but also as they are most

widely used for liquid phase extraction. The extraction time for this experiment was set to 60 min.

Table 6: Dielectric constants of solvents used for LPE.

Solvent	Dielectric constant ε_r[1]
Methanol	32.66
Acetone	20.70
Diethyl ether	4.34
n-Hexane	1.89

[1] Data obtained from SRC PhysProp Database

Extraction time

After identifying the optimal solvent for the extraction of MT from sediment samples, extraction times for such solvent were investigated as 5, 10, 30, 60, and 150 min. Each extraction was performed in triplicate steps and the extracts were pooled as previously described.

Sonication

In addition to solvent extraction, the effects of sonication on the extraction performance were studied. Sonication times of 5, 10, 20, and 30 min were investigated using optimized solvent. All samples were sonicated in water bath with a frequency of 35 kHz and HF peak of 320 W. As the liquid extraction, sonication was performed in triplicate steps and the extracts were pooled.

Analysis

All samples were analyzed using an Acquity Ultra Performance LC (Waters, Milford, USA) equipped with an Acquity UPLC HSS T3 column (2.1 x 100 mm, 18 µm particle size) (Waters, Milford, USA). The UPLC, gradient, and multi-selected reaction monitoring (MRM) conditions are shown in Table 7, Table 8, and Table 9, respectively. Mobile phases contained 0.1% formic acid (solution A) and 0.1% formic acid in methanol (solution B).

Table 7: UPLC conditions for MT analysis.

Parameter	Condition
Ionization Mode	ES+
Capilary (kV)	3
Extractor (V)	3
RF Lens (V)	0.1
Source Temperature (°C)	120
Desolvation Temperature (°C)	350
Cone Gas Flow (L/h)	50
Desolvation Gas Flow (L/h)	1000
LM 1 Resolution	15
HM 1 Resolution	15
Ion Energy 1	0.5
LM 2 Resolution	13.8
HM 2 Resolution	13.8
Ion Energy 2	0.5
Multiplier	650
Pressure (mbar)	3.66E-03
Collision Gas Flow	0.18

Table 8: Gradient table for MT analysis.

Time (min)	Flow rate	%A	%B
Initial	0.25	30	70
1	0.25	5	95
3.2	0.25	5	95
3.7	0.25	30	70

Table 9: Multi-selected reaction monitoring (MRM) conditions for MT analysis.

MRM Transition	Dwell Time (s)	Cone Voltage (V)	Col. Energy (eV)	Compound
303.24 > 96.78	0.25	30	24	MT
303.24 > 108.82	0.25	30	27	MT

Sediment spiking

To demonstrate the efficiency of the extraction procedure, sediment samples were spiked at levels of 10 or 100 µg/kg. For this purpose, the weighted samples were spiked with 2.5 mL working solution 1 or 2, containing 25 or 250 ng MT, respectively. To allow MT to sorb onto sediment, samples were vortex mixed for homogenization and left alone for 6 h. Then, solvent was completely evaporated at 55 °C. Sorptionexperiments previously performed indicated sorption equilibrium time for MT onto sediment of 6-9 h (Pawittra Chotisukarn, 2008).

Quality assurance

One laboratory blank was run with each set of samples to check for contamination from preparative steps and to demonstrate laboratory background levels. Using the proposed procedure, background levels of laboratory blanks were not detected.

Statistical analysis

All data were statistically analyzed using one-way ANOVA at a significance level of $P < 0.05$ with Microsoft Excel. Mean separations were compared using ANOVA ($P < 0.05$) to test the difference between background and sample taken during MT treatment.

3.4 Study site and sample collection (Part II)

As can be seen in Figure 5, part II of this study incorporated site selection, sample collection, soil characterization, and real sample analysis.
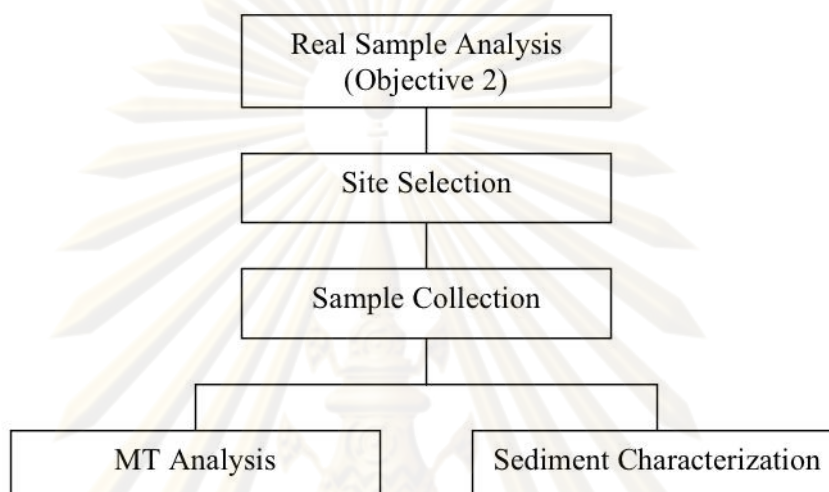
Figure 5: Part II of this study.

A fish farm was chosen for the determination of MT in sediment samples. The farm was located Petchaburi province, southwest of Bangkok, Thailand. Two sites for monitoring MT concentrations in sediment were utilized for this study. The first site, pond A, was a 7200 m^2 earthen unlined masculinization pond with an average depth of 1.5 m, which is common nursery management practice. The pond contained approximately 180 net cages each containing 10000 fish fry each. Water from the pond was regularly drained to a reservoir by a small canal, which surrounded the facility, to allow natural attenuation. Pond A was stopped for using as nursering pond prior to sampling. According to the management history provided for the second site, pond B, no MT had been used for masculinization of Nile tilapia prior to sampling. The size of pond B was estimated to be approximately 25000 m^2. Ponds A and B were chosen as sampling sites because results obtained from pond A could have yielded information of the fate of MT in sediment after the usage of this pond for the masculinization of Nile tilapia, whereas in pond B the accumulation of MT in sediment of such a pond could have been studied in order to assess the risk to the

ecosystem. For neither of the ponds a liner was used to protect the ecosystem from potential damage.

Samples were taken according to the schematic drawings of ponds A and B as seen in Figure 6 and Figure 7, respectively. For pond A, samples were taken in the middle of the pond indicated with an X in Figure 6, whereas samples in pond B were taken next to net cages 1, 15, and 30 as indicated in Figure 7. Note that in both figures the drawing does not reflect the pond's dimension nor its aspect ratios.
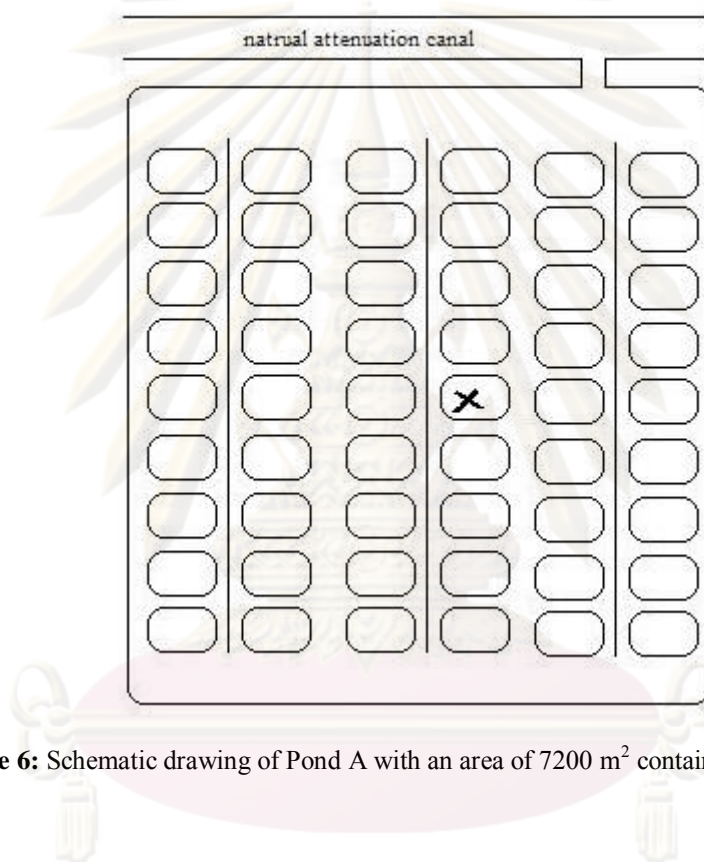
Figure 6: Schematic drawing of Pond A with an area of 7200 m^2 containing 180 net cages.
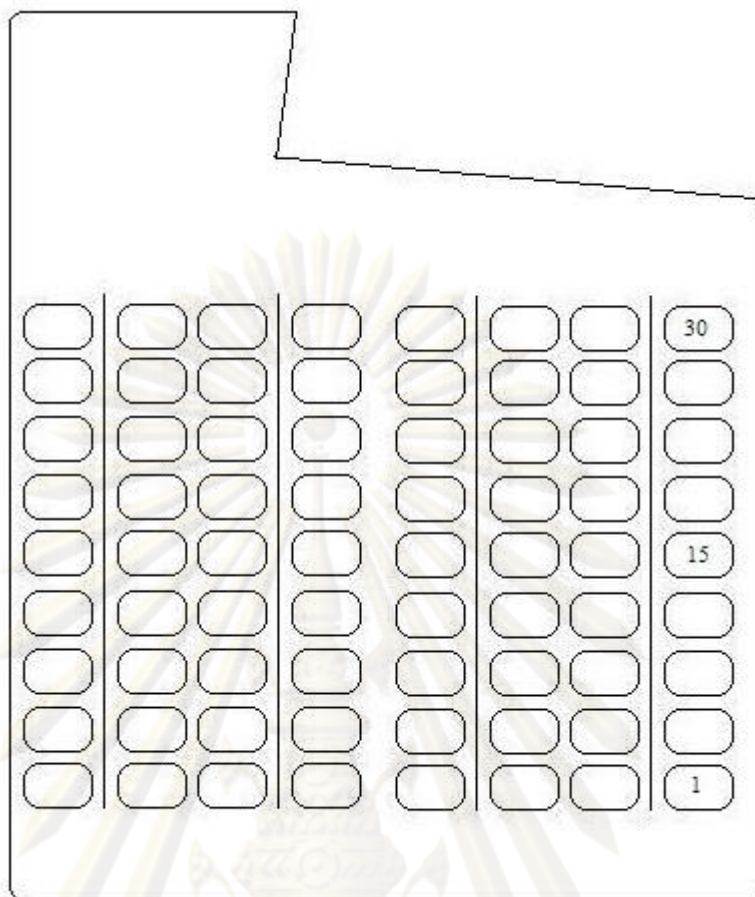
Figure 7: Schematic drawing of pond B with an area of 25000 m² containing two sets of 400 net cages.

Sediment samples were taken of the upper layer at each sampling site. Sediment core samples were collected with a 5-cm-diameter self-constructed PVC pipe, which was cleaned between the samples with water to prevent cross-contamination. The top 5 cm of the sediment was collected in plastic bags. All samples were kept on ice until they reached the laboratory (<2 h). Sediment samples were oven-dried at 50 °C and, then, stored at ambient temperature in the dark until further processing.

All samples were taken according to Table 12. No precipitation was reported during sampling duration and average daytime temperature could be assumed to range between 30 and 35 °C.

Sediment characterization

After drying, sediment samples were grinded, sieved through a US Sieve Size 60 (250 μm), and stored at ambient temperature. Basic soil properties were

determined, such as cation-exchange capacity (CEC), pH, soil texture, and organic matter content. The methods of the sediment characterization are displayed in Table 10. Sediment characterization was performed at the Soil Plant and Agriculture Material Testing and Research Unit at Kasetsart University, Thailand (Kamphaengsaen Campus).

Table 10: Methods of sediment characterization.

Sediment property	Method
CEC	NH_4OAc at pH 7.0
pH	$CaCl_2$ method
Soil texture	Pipette method
Organic matter	Walkley & Black

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Initial method development (Objective 1)

Optimization of the method used in part II of this study was carried out during the initial stages of this research, as there is no comprehensive protocol for determination of MT in soil or sediment reported in literature. The variables considered during this part included extraction solvent, the length of extraction, as well as the effect of sonication on the extraction efficiency.

4.1.1 Extraction solvent

In general, solvents for extracting target analytes are examined based on differences either in pH or polarity (Ramirez et al., 2007). In this study, methanol, acetone, diethyl ether, and hexane were examined to extract the target compound with the procedure described in Chapter 3.3. These solvents were selected due to their variety in dielectric constants as an indicator for their polarity (see Table 6) as well as for their broad application as solvents for the extraction of organic contaminants in soil and sediment matrices (Xu et al., 2008; Hibberd et al., 2009). Diethyl ether was also included as extraction solvent in this part because it was used for this purpose in the only study available in literature reporting the determination of MT in soil and sediment samples (Fritzpatrick et al., 1999; Fritzpatrick and Contreras-Sánchez, 2000). The results obtained from this experiment are shown in Figure 8 indicating recoveries for the investigated extraction solvents with relative standard deviations from five replicates analysis.
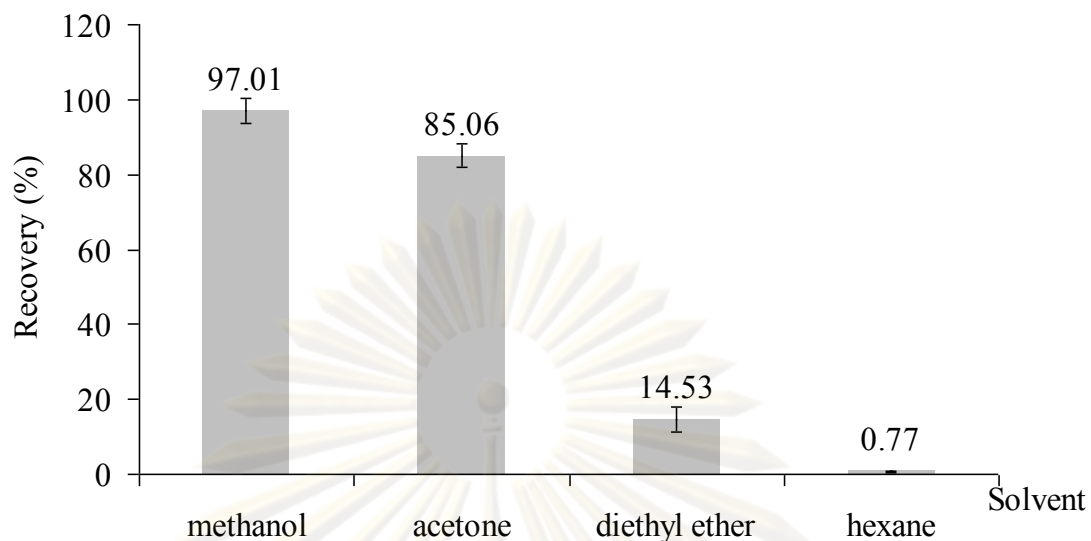
Figure 8: Recovery rates of MT from sediment spiked at 100 μg/kg by different extraction solvents.

The percent recoveries and relative standard deviation (RSD) of the five replicate for methanol, acetone, diethyl ether, and hexane were 97.01 ± 3.36, 85.06 ± 3.89, 14.53 ± 23.54, and 0.77 ± 24.62, respectively. These recovery values reveal that the less polar solvent such as diethyl ether and hexane had less MT extraction potential than the more polar ones. This may have been due to the high polarity of MT, which, therefore, favored methanol as a solvent. Both methanol and acetone yielded sufficient extraction recoveries, which correlates well with the polarities of both solvents (see Table 6). From the results obtained in this experiment, it seems unclear why diethyl ether was used as extraction solvent in previous studies as diethyl ether is a solvent of moderate polarity (Fritzpatrick et al., 1999). Although water is even more polar than methanol, it was not examined as an extraction solvent in this study due to potential difficulties in the subsequent evaporation of solvent.

The tendency in recoveries indicated in Figure 8 is consistent with results from other studies. Methanol was shown to be the most appropriate solvent for the extraction of EDCs in river sediment yielding recoveries higher than 74% for the tested compounds (Liu et al., 2004). Urbatzka et al. (2007) applied methanol as solvent for LPE prior to a recombinant YAS for the analysis of androgens in sediment samples from a river in Italy. However, as the choice of extraction solvent greatly depends on the nature of the solvent, as well as sample matrix and the properties of the target compound, and the fact that there is no comparable study available in

literature, these results can only be compared with the results obtained in this study to some extent.

While methanol and acetone indicated sufficient extraction recoveries from the spiked sediment samples, methanol was chosen as solvent for further studies as it yielded a relatively slight but yet significant increased recovery percentage over acetone.

4.1.2 Extraction time

The extraction duration is another potential important parameter affecting the extraction efficiency of MT from sediment samples. To obtain the highest recovery of MT from sediment, the extraction period was varied from 5 to 150 min and the results are displayed in Figure 9.
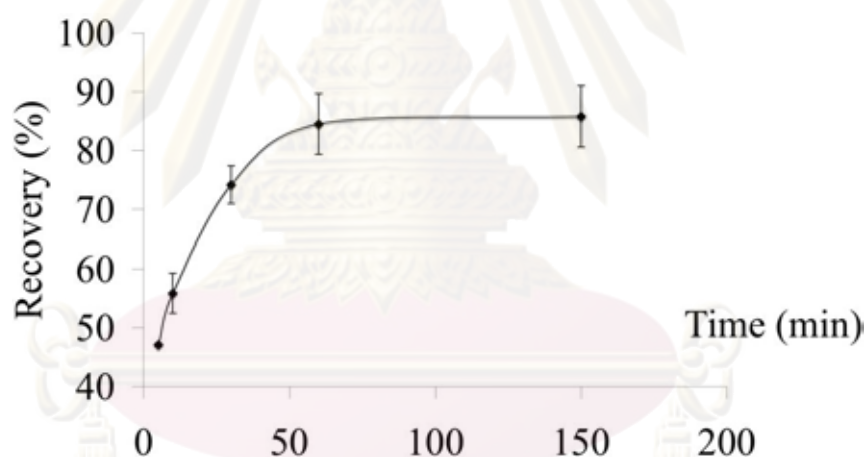
Figure 9: Recovery rates of MT from sediment samples spiked at 100 µg/kg after different extraction times.

It can be seen in Figure 9 that the MT recovery continuously and sharply increased over the extraction times of 5, 10, and 30 min. After that, between the extraction times of 60 and 150 min, there was no significant change in the recovery of MT from the sediment.

The time required for the extraction of the analyte from the solid matrix greatly depends on the strength of the bonds between the solid and target compound. Compared with PAHs, upon which there has been numerous studies on their extraction from solid matrices, it becomes apparent in Figure 9 that the extraction of

MT from sediment is a rather time consuming step. As PAHs are compounds of low polarity their interactions with solid matrices are based on weak hydrophobic van-der-Waals interactions, whereas MT as a more polar compound that contains an -OH functional group, can form strong bonds (e.g., hydrogen bonds) with the functional surface sites of the sediment. As for the choice of extraction solvent, the extraction kinetics greatly depends on the structural properties of the compounds and phases involved, as well as a proper mixing of extractant and solid phase.

For further experiments an extraction time of 60 min was selected as it yielded highest recovery rates for the extraction of MT from sediment samples.

4.1.3 Sonication

In order to study the effects of sonication on the extraction of MT from the sediment samples, un-spiked sediment samples taken from pond A as indicated in Figure 6 were prepared. The sediment used for this experiment was taken from a highly contaminated site with a long history of MT application as masculinization hormone (pond A). Prior to their extraction on a horizontally shaker, samples were placed in a sonication water bath and the duration of sonication was varied between 5 and 30 min. The results of the experiment are shown in Figure 10.
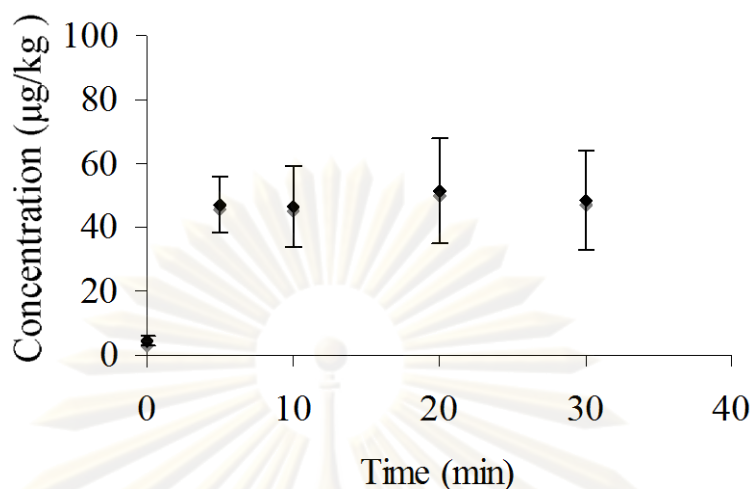
Figure 10: Effect of sonication on the extraction of MT from un-spiked sediment samples.

It must be stressed that for this experiment, un-spiked sediment from pond A was chosen to study the effects of sonication on MT extraction. The results in Figure 10 show that sonication prior to solvent extraction greatly increased the measured concentration of MT as MT concentrations of about 50 μg/kg were extracted throughout the durations of sonication investigated. In order to assess the benefit of sonication, one sample was extracted without sonication; the result is indicated in Figure 10 as t_0. Without sonication, MT could be extracted from the sediment at concentrations of less than 5 μg/kg. Therefore, sonication prior to solvent extraction significantly increased the extraction of MT from sediment.

Robinson et al. (2009) successfully extracted estrogenic compounds (bisphenol A, 17β-estradiol, and 17α-ethinylestradiol) from sediment obtained from Halifax Harbor (Nova Scotia, Canada) by applying sonication extraction. Samples were further analyzed by liquid chromatography-tandem mass spectrometry and concentration of these compounds in the sub μg/kg-range could be determined.

Sonication assisted extraction is unique as it aids in the chemical as well as physical extraction of analytes from solid matrices and it is employed to ensure close contact between the extraction solvent and the solid matrix. The cavitation bubbles produced during the sonication process may lead to an increased surface area being exposed to the extraction solvent and, therefore, yield a higher extraction of the target compound in comparison to other methods (Capelo and Mota, 2005).

4.1.4 Evaluation of the extraction

The optimized method for the determination of MT from sediment was not only characterized by the extraction of MT from solid matrix, but also the target compound was pre-concentrated in re-constitution step of the extraction procedure. A tool to quantitatively evaluate the pre-concentration is by calculating the enrichment factor, the equation used to calculate it is as follows:

$$EF = \frac{C_i}{C_e}$$

where EF is the enrichment factor and C_e and C_i represent the concentration before and after the enrichment, respectively. With regard to an initially 10 μg/kg spiked sediment sample, the enrichment factor of this procedure could be calculated to be 10. According to Sutherland (2000) this enrichment factor represents a significant enrichment.

In general, ultrasonic extraction is a simple extraction method. However, potential problems are re-adsorption of the analyte onto the solid phase, heating of the sample and thus loss of extraction solvent, and decomposition of the analyte due to zones of high energy. Compared to other extraction methods, ultrasonic extraction is characterized by its low solvent volume per sample and rapid extraction time.

4.2   Real sample analysis (Objective 2)

4.2.1   Sediment characterization

Properties of sediment samples taken from pond A and pond B were characterized and the results are shown in Table 11. The determined properties were sediment pH, organic matter content (OM), cation exchange capacity (CEC), and texture (clay, sand, and silt). As displayed in Table 11 sediment in both ponds are characterized by similar properties. However, organic matter content of pond B is higher than organic matter content of pond A.

Table 11: Properties of sediment samples.

	pH	OM (%)	CEC (me/kg)	Clay (%)	Sand (%)	Silt (%)	Texture[1]
Pond A	6.75	1.61	3.04	38.81	7.84	53.35	SCL
Pond B	6.43	2.94	3.48	39.45	4.31	56.24	SCL

[1]SCL: Silty Clay Loam

4.2.2 MT concentration in Nile tilapia nursery ponds

Sediment samples were taken according to Table 12. The original sampling plan for pond A of taking samples during a period of five weeks had to be changed as there was no more water present in the pond from week four as water from pond A was removed for treatment in an aerobic facultative pond. Note that the dates indicated under treatment in Table 12 refer to the hormone treatment schedule of pond B. This is due to indicate the duration of one nursering cycle.

Table 12: Sampling plan.

Date	Treatment	Pond A	Pond B
27.11.2009	Background		X
02.12.2009	Day 2	X	X
08.12.2009	Day 8	X	X
15.12.2009	Day 15	X	X
22.12.2009	Day 22[1]	X	X
29.12.2009	Week 1[2]	n.w.[3]	X
05.01.2010	Week 2[2]	n.w.[3]	X

[1]Last day of hormone treatment

[2]Time after hormone treatment

[3]no water in pond

To monitor MT concentrations in pond A and pond B, sediment samples were taken and prepared as described in Chapter 3.3. Five replicates of each sample were analyzed and the results are discussed in the following section.

Pond A

The results of MT analysis in sediment obtained from pond A are shown in Figure 11. As can bee seen, MT concentration gradually increased from 0.12 on day 2 to almost 0.2 µg/kg over the complete duration of sampling.
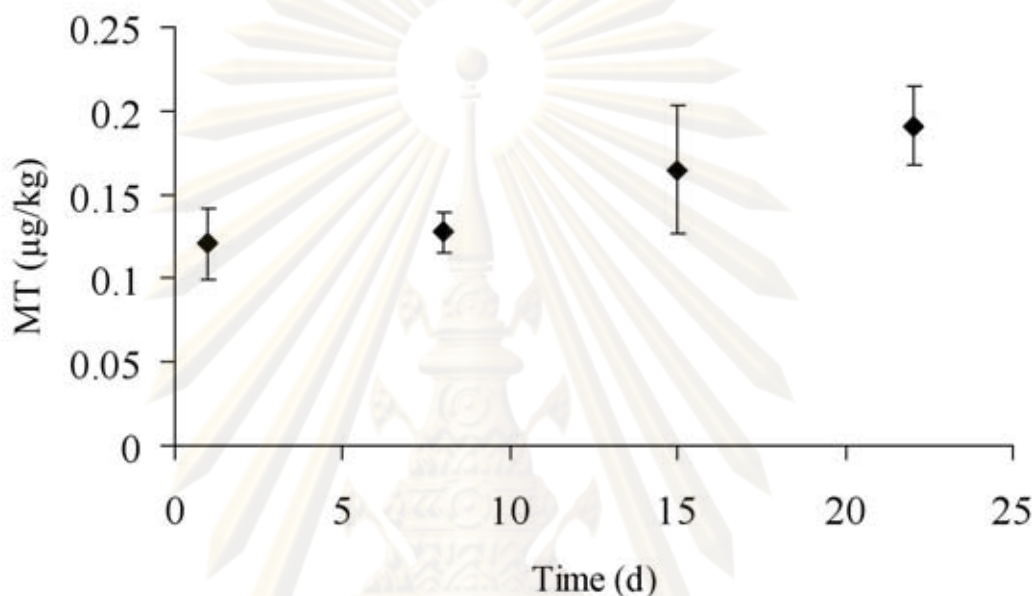
Figure 11: Concentration of MT determined in pond A.

As previously mentioned, usage pond A as a Nile tilapia nursery pond was stopped prior to sampling. In addition, due to this, the amount of water present in pond A decreased due to evaporation and infiltration. Additionally, water from pond A was removed to an aerobic facultative treatment pond The effects of this could clearly be seen especially on days 15 and 22 of sampling. Therefore, this may have had an effect on MT concentration to be increased. As these two effects cannot be quantified, their contribution to an increased concentration of MT in the sediment of pond A remains unknown.

The elevated concentrations of MT on days 15 and 22 of sampling could also be a result from a decreased water volume in the pond. As the water volume decreased, the concentration of MT in water increased, resulting in an increased amount of MT sorbed onto the sediment as MT preferably sorbs onto sediment.

On the contrary, as shown in the study of Homkiln et al. (2009), MT could be aerobically biodegraded in sediment taken from a masculinization pond at a rate of 0.5 d^{-1}. Their results indicate that depending on the initial MT concentration, no MT could be detected (< 0.1 mg/L) after a period of 3 to 28 days. It was also found that the biodegradation rate decreased with increasing initial MT concentration. As pond A has a long history as masculinization pond and, therefore, MT application, it can be assumed that MT concentration in pond A was high; hence, resulting in a low biodegradation rate.

Regarding to one of the hypotheses of this study, MT could clearly be determined by optimized extraction procedure followed by UPLC-MS/MS as displayed in Figure 12, which shows a chromatogram obtained from the analysis of MT in sediment samples from pond A. The analysis of the background sample taken from pond A revealed no MT present in soil from that site as seen in Figure 13.
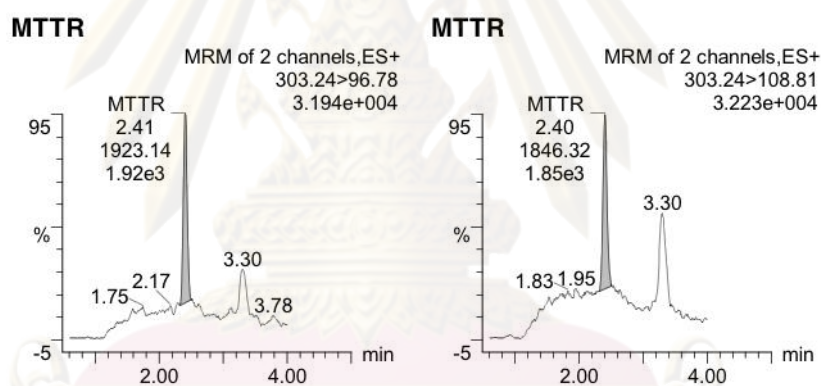

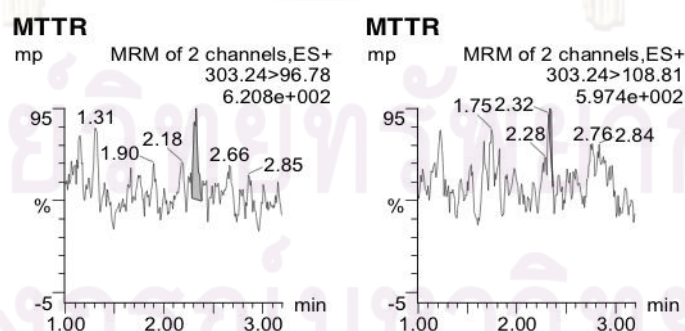**Figure 12**: Chromatogram of MT from sediment of pond A for two MRM.

Figure 13: Chromatogram of background from sediment of pond A for two MRM.

Pond B

Three net cages were chosen for sampling in pond A because they were among the first to be loaded with Nile tilapia fry. Sampling locations of cages 1, 15, and 30 are indicated in Figure 7. All samples were processed in five replicates and the results for MT analysis in sediment from these cages are shown in Figure 14, Figure 15, and Figure 16, respectively, whereas one exemplarily chromatogram of MT in sediment from pond B is shown in Figure 17. The dashed line in Figure 14, Figure 15, and Figure 16 indicates the end of the treatment. The chromatogram of the background sample taken from pond B is shown in Figure 18.

MT could be determined and identified as the chromatogram in Figure 17 indicates; however, the determined concentrations of MT in sediment samples were low and relatively close to the detection limit of the analytical instrument.

For all three observed net cages the measured MT concentration in sediment are relatively constant over the hormone treatment period between day 2 and day 22. On day 22 the masculinized fish fry were harvested and the pond was not further used. After that the determined MT concentrations for all three cages monitored increased on sampling days 29 and 36. Even though there were outliers for cage 1 on day 36 and for cage 15 on day 29 of sampling, there was an obvious tendency of a significantly increasing concentration of MT after the hormone treatment had stopped. This increase in concentration may be due to sedimentation of particulate matter to which MT is likely to sorb onto after its application via fish feed (K_d = 300 L/kg found by Pawittra Chotisukarn, 2008). As the water motion was reduced after the fish fry was harvested, sedimentation of this suspended matter was more favorable. However, to evaluate the contribution of sedimented matter to which MT sorbed onto, further confirmation by experimental data would be required.

Figure 14: Concentration of MT determined in sediment from cage 1 in pond B during hormone treatment period.

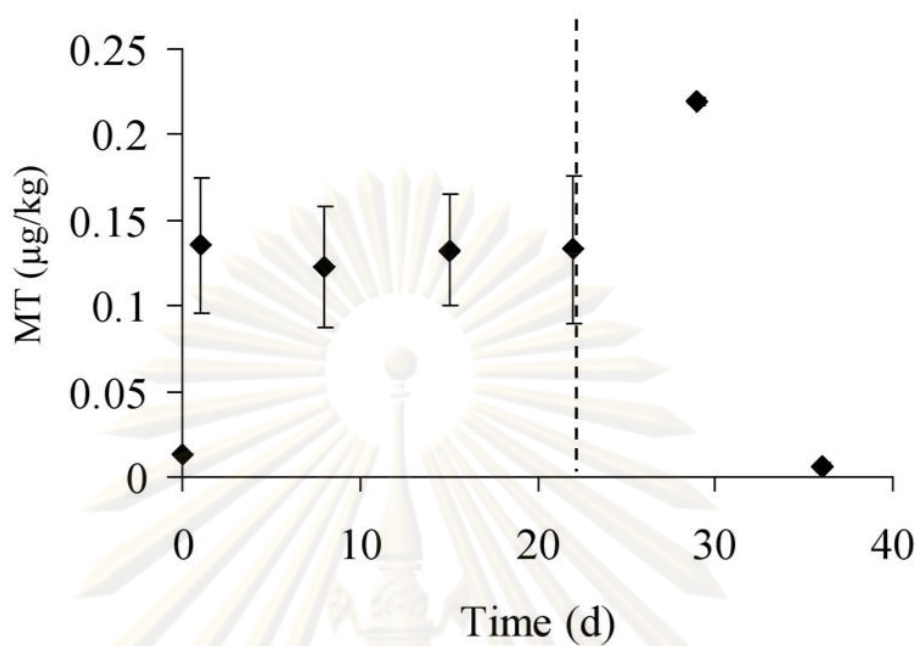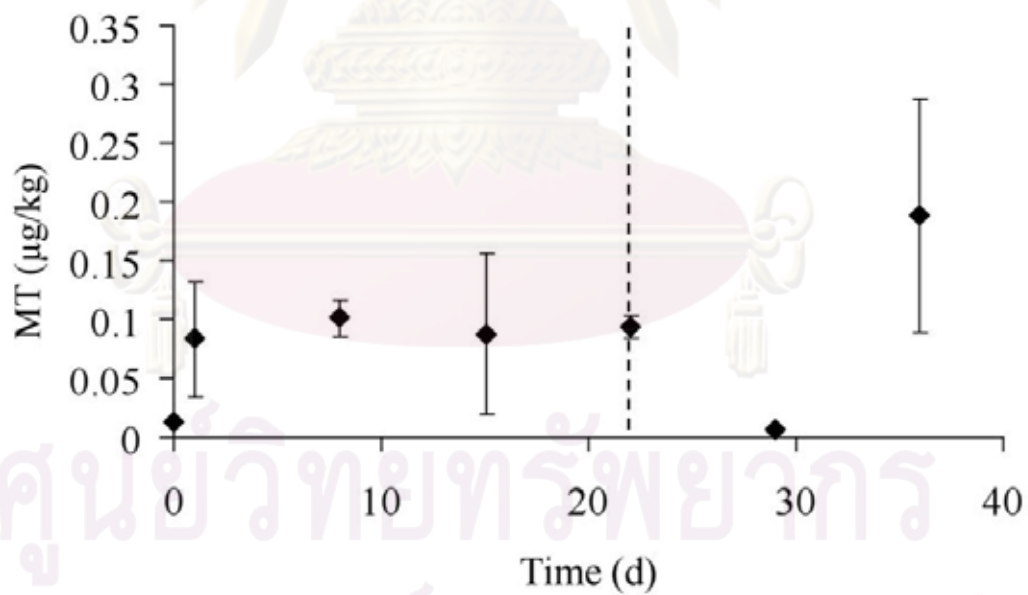**Figure 15**: Concentration of MT determined in sediment from cage 15 in pond B during hormone treatment period.
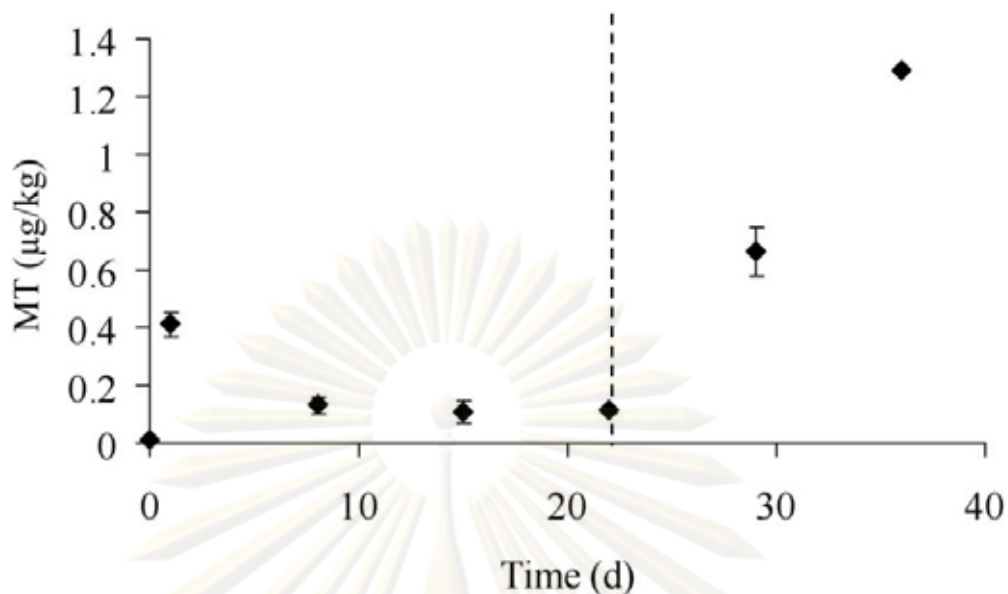
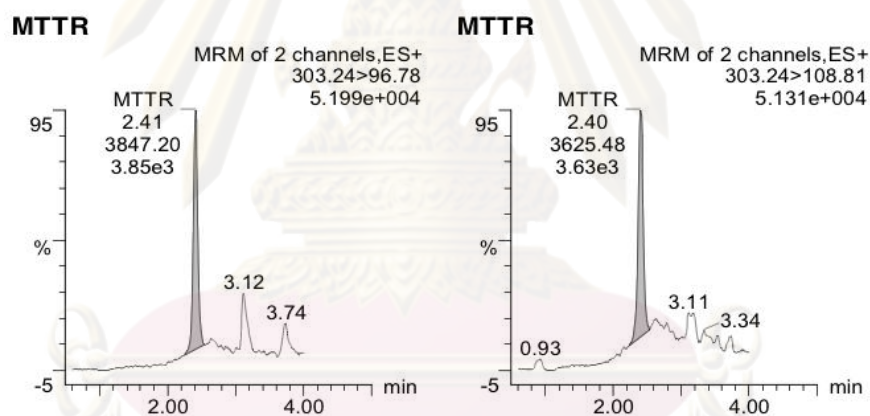**Figure 16**: Concentration of MT determined in sediment from cage 30 in pond B during hormone treatment period.

Figure 17: Exemplarily chromatogram of MT in sediment from pond B for two MRM.
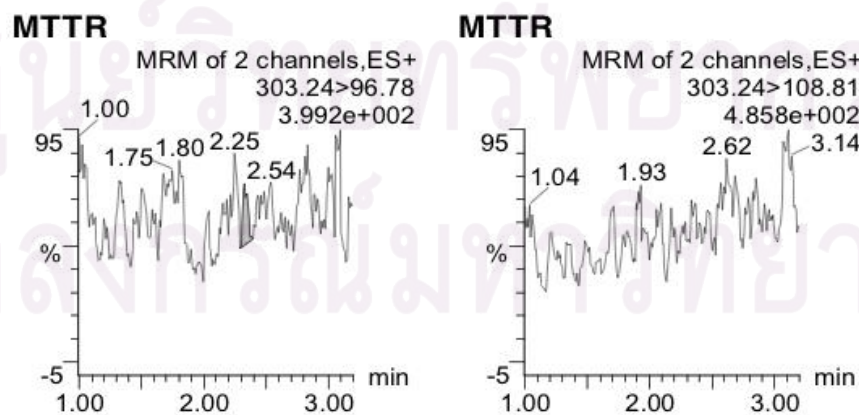

Figure 18: Chromatogram of background from pond B for two MRM.

During the sampling procedure, the water quality rapidly decreased in pond B. Eutrophication was observed from day 8 of sampling and continued throughout the sampling. Due to the fact that Nile tilapia is a fish tolerant to low water quality, this had no effect on the masculinization procedure, and mortality among the fish fry was not observable. With regard to the concentrations of MT measured in all cages during the hormone treatment period, this means that another factor had to be taken into account.

Biosorption of organic compounds onto algal material is a field of study that only recently has been focused on. As of the large quantity of algal waste produced, it may be used as low cost adsorbent. However, the adsorption of organic compound on such material has not extensively been studied. Adsorption of methylene blue on some algal material has been shown to be almost as high as on activated carbon (Vilar et al., 2007). It must be stressed that comparison of the potential adsorption of MT on algae with the adsorption of methylene blue is only valid to some extent as not only structural differences between the two adsorbents are obvious. However, as there is no information available on biosorption of EDC, this may be used as an indication for possible MT sorption on algae and, therefore, explain the low MT concentrations found in all net cages investigated.

MT induced imposex at the lowest nominal test concentrations of 100 ng/L and displayed significant inhibitory effects on spermatogenesis and egg production of *M. cornuarietis* (prosobranch snails) were found by Schulte-Oehlmann et al. (2004). These findings were confirmed by Albanis et al. (2006) finding EC_{10} values of MT for imposex induction and reduced egg production of 36.4 and 1.73 ng/L. With the concentrations of MT found in sediment in this study and therefore resulting water concentrations of MT, possible ecological effects of MT are more likely to occur as reduced egg production rather than induced imposex if this organism is present in the ecosystem.

More recently, the effects of MT on the immunity of dwarf chicks against *Salmonella Pullorum* were examined. In vivo experiments found that after a 19-day treatment of MT at a concentration of 10^{-7} M the susceptibility to *Salmonella Pullorum* infection was enhanced, and cellular immunity against *Salmonella Pullorum* was depressed. It was proposed that MT affected the immune response in dwarf chicks by changing monocytes-macrophages mediated reactive oxygen intermediate-dependent killing (Li et al., 2009). The results showed that MT may not

only affect the sex development, but also the immune system of different organism at low concentrations.

In general, research is focused more on ecological effects caused by other EDCs such as bisphenol A, tributyltin, or ethinyloestradiol. Therefore, necessary information for a detailed discussion of the possible ecological effects of MT found in this study is not possible. In addition, data on the fate of water infiltrating into the subsurface would be necessary. However, due to the low concentration of MT in sediment and its preferable sorption on sediment, it is unlikely for MT to reach the groundwater and therefore cause further harm. Nevertheless, this is only true for the concentrations found here, whereas further application of MT in masculinization of earthen Nile tilapia nursery pond is likely lead to an increased concentration of MT in sediment.

Moreover, sediments may act as a sink for MT and provide a continuous chronic source to sediment-dwelling organisms including invertebrates (Drewes et al., 2002; Heberer et al., 2002; Holthaus et al., 2002). However, there has been no research on desorption of MT from sediment or soil matrices. In general, the concentrations of MT obtained from long-term exposure results at which chronic toxicity was observable, was lower for invertebrates (Czech et al., 2001; Schulte-Oehlmann et al., 2004) compared to fish (Zerulla et al., 2002).

4.2.3 Water analysis

The original scope of this study incorporated water analysis, but this part was not further pursued. A Fugacity Model Level 1 was constructed with the physical properties of MT from Table 1. Volumes of the compartments air, water, sediment, suspended sediment, and biota was assumed to be 2, 1.68, 0.5, 10^{-6}, and 10^{-7} m^3. It has to be stressed that volumes of suspended sediment and biota were estimated and could not further be confirmed. In order to estimate possible errors of the assumed volumes of suspended sediment and biota, the model was run with $\pm$ 10 m^3 for each compartment. This revealed no significant changes in the obtained results. The total load of MT was calculated as weekly load from the daily amount of fish feed applied for each cage (Table 13).

Table 13: Applied fish feed for masculinization of Nile tilapia.

Day	Fish feed (g/d)
0-5	55-70
6-10	118-150
11-16	250
17-22	400

The Fugacity Model revealed a mass distribution for the compartments air, water, bottom sediment, suspended sediment, and biota of 0.0001, 1.84, 98.16, 0.003, and 0%, respectively. The details of the Fugacity Model are given in Appendix A. The results obtained were independent from the weekly loads. It must be stressed that the Fugacity Model was established assuming that no MT uptake and metabolism occurred because an opposite scenario cannot be established with the model.

As there is not information available on the percentage of MT uptake by fish fry, the expected concentrations of MT in water were estimated with assuming MT loss due to uptake and metabolism of 25, 50, and 75%. Together with the obtained results from the sediment analysis, the expected MT concentrations in water are too low for direct analysis. Additional enrichment of water samples, which would have been required in this case, was beyond the scope of this study, and therefore the originally planned water analysis of MT concentrations was not further pursued.

In general, fugacity can be used to describe the escaping tendency of a chemical from a particular environmental compartment, while each environmental medium has a certain fugacity capacity (Z) that describes the relationship between chemical concentrations and fugacity (from Mackay, 2001).

CHAPTER V

CONCLUSION AND OUTLOOK

The two objectives of this study were to develop a method for the extraction and purification of MT from sediment by liquid-phase extraction (objective 1) and to quantitatively determine the concentrations of MT in sediment from an earthen nursery pond during the hormone treatment period (objective 2). Both objectives were fulfilled and the results can be summarized as follows.

A method for the extraction of MT from sediment based on liquid phase extraction combined with sonication was successfully optimized leading to a recovery rate of 97%. Parameters that were optimized included extraction solvent, as well as extraction and sonication time. With sonication, residues of the target compound could be extracted where conventional liquid phase extraction could only extract easily extractable MT. The results obtained from sediment extraction were in well agreement with other comparable results reported in literature. However, the development method needs further validation with respect to limit of detection (LOD), limit of quantification (LOQ), linear dynamic range (LDR), as well as accuracy and precision (inter- and intra-day precision). This should be emphasized on in further research. In addition, further pre-concentration should be applied to reduce the amount MT detectable by the procedure.

The second part of this research was focused on real sample analysis and monitoring of MT concentrations in the environment. Therefore, two ponds were selected with a different history of MT application as masculinization hormone in Nile tilapia aquaculture. The results indicate that MT could qualitatively and quantitatively be determined throughout the duration of sampling as shown by the chromatograms obtained from UPLC-MS/MS analysis. The concentrations of MT found in sediment were in the low µg/kg-range.

In order to assess the possible risk for the ecosystem posed by MT, a clear and final conclusion from the results obtained from this study is not possible. As MT has been shown to cause adverse effects to organisms at environmentally relevant concentrations at ranges between mg/kg to µg/kg body weight dependent on the studied organism, possible ecological effects greatly depend on the bioavailability of

MT sorbed onto sediment from an earthen nursery pond. More information on the accumulation of MT on sediment, and its bioavailability to organisms of such ecosystems should be focused on in order to gain more insight of possible effects caused by MT in the environment. In addition, further research should include a pilot-scale study to assess MT's fate in an earthen nursery pond.

The masculinization of Nile tilapia can also be achieved in concrete tanks. As such, this technique of hormone treatment with MT seems to be more environmentally friendly, assuming that water in the tanks is circulated and treated in a closed-system.
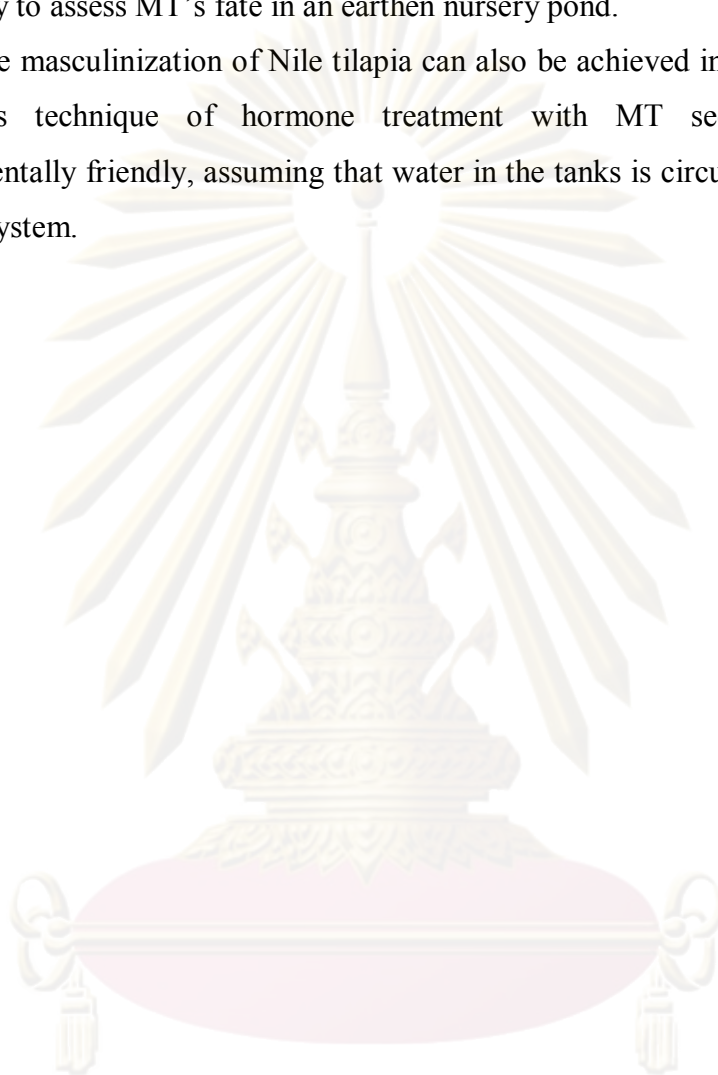
REFERENCES

Abucay, J.S., Mair, G.C. Hormonal sex reversal of tilapias: Implications of hormone treatment application in closed water systems. Aquacult. Res. 28 (1997): 841-845.

Abucay, J.S., Mair, G.C., Skibinski, D.O.F., Beardmore, J.A. The occurrence of incidental sex reversal in *Oreochromis niloticus* L. In: K. Fitzsimmons (Ed), Proceedings from the Fourth International Symposium on Tilapia in Aquaculture, NRAES, Cooperative Extension, Ithaca, New York (1997): 729-738.

Albanis, T., Allera, A., Bachmann, J., Barbaglio, A., Berntsson, P., Dittmann, N., Carnevali, D.C., Ciceri, F., Dagnac, T., Duft, M., Falandysz, J., Galassi, S., Hala, D., Janer, G., Jeannot, R., Jobling, S., King, I., Klingmüller, D., Kloas, W., Kusk, K.O., Lavado, R., Lo, S., Lutz, I., Oehlmann, J., Oredsson, S., Porte, C., Rand-Weaver, M., Sakkas, V., Schmitt, C., Schulte-Oehlmann, U., Sugni, M., Tyler, X., van Aerle, R., van Ballegoy, C., Wollenberger, S. Executive summary on the results of COMPRENDO-Comparative Research on Endocrine Disruptors (EU project no. EVK1-CT-2002-00129) (2006) available online under http://www.comprendo-project.org.

Barry, T.P., Marwah, A., Marwah, P. Stability of 17α-Methyltestosterone in Fish Feed. Aquaculture 271 (2007): 523-529.

Blasberg, M.E., Langan, C.J., Clark, A.S. The Effects of 17α-Methyltestosterone, Methandrostenolone, and Nandrolone Decanoate on the Rat Estrous Cycle. Physiol. Behav. 61 (1996): 265-272.

Beardmore, J.A., Mair, G.C., Lewis, R.I. Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. Aquaculture 197 (2001): 283-301.

Blokland, M.H., van Rossum, H.J., Herbold, H.A., Sterk, S.S., Stephany, R.W., van Ginkel, L.A. Metabolism of methyltestosterone, norethandrolone, and methylboldenone in a heifer. Anal. Chim. Acta 529 (2005): 317-323.

Bovee, T.F.H., Bor, G., Heskamp, H.H., Hoogenboom, L.A.P., Nielen, M.W.F. Validation and application of a robust yeast estrogen bioassay for the

screening of estrogenic activity in animal feed. Food Additives and Contaminants 23 (2006): 556–568.

Bovee, T.F.H., Lommerse, J.S.M., Peijnenburg, A.A.C.M., Fernandes, E.A., Nielen, M.W.F. A new highly androgen specific yeast biosensor, enabling optimisation of (Q)SAR model approaches. J. Steroid Biochem. Molec. Biology 108 (2008): 121-131.

Bowden, J.A., Colosi, D.M., Mora-Montero, D.C., Garrett, T.J., Yost, R.A. Enhancement of chemical derivatization of steroids by gas chromatography/mass spectrometry (GC/MS). J. Chromat. B 877 (2009): 327-3242.

Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E. (Eds.) Merck Index: An Encyclopedia (1989).

Budworth, P.R., Senger, P.L. Fish-to-fish testosterone transfer in a recirculating-water system. Prog. Fish-Cult. 55 (1993): 250-254.

Capelo, J.L., Mota, A.M. Ultrasonication for analytical chemistry. Curr. Anal. Chem. 1 (2005): 193-201.

Chu, P.S., Lopez, M., Serfling, S., Gieseker, C., Reimschuessel, R. Determination of 17α-Methyltestosterone in Muscle Tissues of Tilapia, Rainbow Trout, and Salmon Using Liquid Chromatography−Tandem Mass Spectrometry. J. Agric. Food Chem. 54 (2006): 3193-3198.

Chang, H., Wu, S., Hu, J., Asami, M., Kunikane, S. Trace analysis of androgens and progestogens in environmental waters by ultra-performance liquid chromatography-electrospray tandem mass spectrometry. J. Chromat. A 1195 (2008): 44-51.

Chotisukarn, P. Sorption of methyltestosterone onto sediments and soils. Master Thesis. Chulalongkorn University, 2008.

Clark, A.S., Harrold, E.V., Fast, A.S. Anabolic-androgenic steroid effects on the sexual behavior of intact male rats. Horm. Behav. 31 (1997): 35-46.

Contreras-Sánchez W.M., Fritzpatrick M.S., Schreck C.B. Fate of Methyltestosterone in the Pond Environment: Impact of MT-Contaminated Soil on Tilapia Sex Differentiation In: A. Gupta, K. McElwee, D. Burke, J. Burright, X. Cummings, H. Egna (Eds.) Eightteenth Annual Technical Report. Pond

Dynamics/Aquaculture CRSP, Oregon State University, Corvallis, Oregon, (2001): 83-86.

Contreras-Sánchez W.M., Márquez-Couturier, G., Feist, G.W., Giannico, G., Schreck, C.B Elimination of Methyltestosterone (MT) from Intensive Masculinization Systems: Use of Activated Charcoal in Concrete Tanks In: R. Harris, I. Courter, H. Egna (Eds.), Twenty-First Annual Technical Report. Aquaculture CRSP, Oregon State University, Corvallis, Oregon, (2004): 147-156.

Czech, P., Weber, K., Dietrich D.R. Effects of endocrine modulating substances on reproduction in the hermaphroditic snail Lymnaea stagnalis L. Aquatic Toxicology 53 (2001): 103-114.

Daeseleire, E., de Guesquiere, A., van Peteghem, C. Combined high-performance liquid chromatography and radioimmunoassay for the screening of 19-nortestosterone and methyltestosterone residues in meat samples. J. Chromat. Biomed. Sci. Appl. 564 (1991): 445-449.

Davie, P.S., Thorarensen, H. Heart Growth in Rainbow Trout in Response to Exogenous Testosterone and 17□α-Methyltestosterone. Comp. Biochem. Physiol. 117 (1997): 227-230.

Diaz-Cruz, M.S., de Alda, M.J.L., Lopez, R., Barcelo, D. Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS). J. Mass Spectrom. 38 (2003): 917-923.

Drewes, J.E., Heberer, T., Reddersen, K. Fate of pharmaceuticals during indirect potable reuse. Water Sci. Technol. 46 (2002): 73-80.

Druzhinina, A.V., Andryushina, V.A., Stytsenko, T.S., Voishvillo, N.E. Conversion of 17α-Methyltestosterone to Methandrostenolone by the Bacterium *Pimelobacter simplex* VKPM Ac-1632 with the Presence of Cyclodextrins. Applied Biochemistry and Microbiology 44 (2008): 580-584.

Eding, E.H., Bouwmans, A., Komen, J. Effects of methyltestosterone and 11-keto-androstenedione on sex differentiation in African catfish, *Clarias gariepinus*. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson, S.O. Stefansson (Eds.). Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish. John Grieg, A.S. Bergen, Norway (1999): 258.

Fitzpatrick, MS., Contreras-Sánchez, W.M., Milston, R.H., Hornick, R., Feist, G.W., Schreck, C.B. Detection of MT in aquarium water after treatment with MT

food In: K. McElwee, D. Burke, M. Niles, H. Egna (Eds.), <u>Sixteenth Annual Technical Report. Pond Dynamics/Aqualculture CRSP</u>, Oregon State University, Corvallis, Oregon, USA (1999): 81-84.

Fitzpatrick, M.S., Contreras-Sánchez, W.M. Fate of methyltestosterone in the pond environment: Detection of MT in soil after treatment with MT food. In: McElwee, K., Burke, D., Niles, M., Cummings, X. Egna, H. (Eds.) <u>Seventeenth Annual Technical Report Pond Dynamics/Aquaculture CRSP</u>. Oregon State University, Corvallis, Oregon, USA (2000): 109-112.

Gomelsky, B., Cherfas, N.B., Peretz, Y., Ben-Dom, N., Hulata, G. Hormonal sex inversion in the common carp (*Cyprinus carpio* L.). <u>Aquaculture</u> 126 (1994): 265–270.

Gonzalo-Lumbreras, R., Izquierdo-Hornillos, R. Optimization and validation of conventional and micellar LC methods for the analysis of methyltestosterone in sugar-coated pills. <u>Pharmaceutical and Biochemical Analysis</u> 31 (2003): 201-208.

Heberer, T., Reddersen, K., Mechlinski, A. From municipal sewage to drinking water: fate and removal of pharmaceuticals residues in the aquatic environment in urban areas. <u>Water Sci. Technol.</u> 46 (2002): 81-86.

Hibberd, A., Maskaoui, K., Zhang, Z., Zhou, J.L. An improved method for simultaneous analysis of phenolic and steroidal estrogens in water and sediments. <u>Talanta</u> 77 (2009): 1315-1321.

Holthaus, K.I.E., Johnson, A.C., Jürgens, M.D., Williams R.J., Carter, J.E. The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English river. <u>Environ. Toxicol. Chem.</u> 21 (2002): 2526-2535.

Homkiln, S., Wattanodorn, T., Ong, S.K., Limpiyakorn, T. Biodegradation of 17α-methyltestosterone and isolation of MT-degrading bacterium from sediment of Nile tilapia masculinization pond. <u>Water Sci. Technol.</u> 59 (2009):, 261-265.

Jansen, E.H., van den Berg, R.H., Zomer, G., Stephany, R.W. A chemiluminescent immunoassay for 17 α-methyltestosterone. <u>Food Addit. Contam.</u> 2 (1985): 47-53.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., Moore, R., Nance, M., Brown, A. Production of androgens by microbial transformation of

progesterone *in vitro*: A model for androgen production in rivers receiving paper mill effluent. Environ. Health Perspect. 112 (2004): 1508.

Kamaruzzaman N., Nguyen, N.H., Hamzah, A., Ponzoni, R.W. Growth performance of mixed sex, hormonally sex reversed and progeny of YY male tilapia of the GIFT strain, *Oreochromis niloticus*. Aquaculture Research 40 (2009): 720-728.

Kennel, P.F., Pallen, C.T., Bars, R.G. Evaluation of rodent Hershberger assay using three reference endocrine disrupters (androgen and antiandrogen). Reproductive Toxicology 18 (2004): 63-73.

Laganà, A., Fago, G., Marino, A., Santarelli, D. Liquid-chromatography tandem mass spectrometry applied to the analysis of natural and synthetic steroids in environmental waters. Analytical Letters 34 (2001): 913-926.

Lewis, R.J. Carcinogenically Active Chemicals. Van Nostrand Reinhold, New York, USA (1991): 732.

Li, H., Zhang, Y., Zuo, S.F., Lian, Z.X., Li, N., Effects of methyltestosterone on immunity against Salmonella Pullorum in dwarf chicks. Poultry Science 88 (2009): 2539-2548.

Little, D.C., Bhujel, R.C., Pham ,T.A. Advanced nursing of mixed-sex and mono-sex tilapia (Oreochromis niloticus) fry, and its impact on subsequent growth in fertilised ponds. Aquaculture 221 (2003): 265-276.

Liu, R. Zhou, J.L. Wilding, A. Microwave-assisted extraction followed by gas chromatography-mass spectrometry for the determination of endocrine disrupting chemicals in river sediments. J. Chromat. A 1038 (2004): 19-26.

Lu, H., Conneely, G., Crowe, M.A., Aherne, M., Pravda, M., Guilbault, G.G. Screening for testosterone, methyltestosterone, 19-nortestosterone residues and their metabolites in bovine urine with enzyme-linked immunosorbent assay (ELISA). Anal. Chim. Acta 570 (2006): 116-123.

MacIntosh, D. J., Little, D. C. Nile tilapia Oreochromis niloticus. In: Bromage, N. R., Roberts, R. J. (Eds.) Broodstock management and egg and larval quality. Blackwell, Cambridge, MA, USA (1995): 277-320.

Macintosh, D.J., Singh, T.B., Little, D.C., Edwards, P. Growth and sexual development of 17-methyl-testosterone- and progesterone-treated Nile tilapia (Oreochromis niloticus) reared in earthen ponds. In: The Second International Symposi um on Tilapia in Aquaculture, ICLARM Conference Proceeding 15

(ed. by R.S.V. Pullin, T. Bhukaswan, K. Tonguthai & J.L. Maclean), (1988): 457-463.

Mackay, D. Multimedia Envrionmental Models: The Fugacity Approach. Lewis Publishers, CRC Press, Boca Raton, FL, USA (2001).

Marwah, A., Marwah, P., Lardy, H. Development and validation of a high performance liquid chromatography assay for 17α-methyltestosterone in fish feed. J. Chromat. B 824 (2005): 107-115.

McKinney, A.R., Suann, C.J., Stenhouse, A.M. A stereochemical examination of equine metabolism of 17α-Methyltestosterone. Anal. Chim. Acta 581 (2007): 377-387.

Mosbach, E.H., Shefer, S., Abell, L.L. Identifacation of fecal metabolites of 17α-methyltestosterone in the dog. J. Lipid Res. 9 (1968): 93-98.

Piferrer, F., Donaldson, E.M. Dosage-dependent differences in the effect of aromatizable and nonaromatizable androgens on the resulting phenotype of coho salmon (Oncorhynchus kisutch). Fish Physiol. Biochem. 9 (1991): 145-150.

Piferrer, F., Baker, I.J., Donaldson, E.M. Effects of natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female chinook salmon (Oncorhynchus tshawytscha). Gen. Comp. Endocrinol. 91 (1993): 59-65.

Purdom, C.E., Hardiman, P.A. Bye, V.V., Eno, N.C., Tyler, C.R., Sumpter, J.P. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8 (1994): 275-285.

Ramirez, A.J., Mottaleb, M.A., Brooks, B.W., Chambliss, C.K. Analysis of Pharmaceuticals in Fish Using Liquid Chromatography-Tandem Mass Spectrometry. Anal. Chem. 79 (2007): 3155-3163.

Rijk, J.C.W., Bovee, T.F.H., Wang, S., Van Pouke, C., Van Peteghem, C., Nielen, M.W.F. Detection of anabolic steroids in dietary supplements: The added value of androgen yeast bioassay in parallel with liquid chromatography-tandem mass spectrometry method. Anal. Chim. Acta (2009): 305-314.

Rinchard, J., Dabrowski, K., García-Abiado, M.A., Ottobre, J. Uptake and depletion of plasma 17α-methyltestosterone during induction of masculinization in

muskellunge, *Esox masquinongy*: Effect on plasma steroids and sex reversal. Steroids 64 (1999): 518–525.

Robinson, B.J., Hui, J.P.M., Soo, E.C., Hellou, J. Estrogenic compounds in seawater and sediement from Halifax Harbour, Nova Scotia, Canada. Environ. Toxicol. Chem. 28 (2009): 18-25.

Rojas-Ortiz, Y.A., Rundle-Gonzalez, V., Rivera-Ramos, I., Jorge, J.C. Modulation of elevated plus maze behavior after chronic exposure to the anabolic steroid 17α-methyltestosterone in adult mice. Hormones and Behavior 49 (2006): 123-128.

Rongone, E.L., Segaloff, A. Isolation of urinary metabolites of 17α-methyltestosterone. J. Biol. Chem. 237 (1962): 1066-1067.

Schänzer, W., Donike, M. Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. Anal. Chim. Acta 275 (1993): 23-48.

Schaenzer, W. Metabolism of Anabolic Androgenic Steroids: 5α- and 5β-Reduciton of 3-Keto-4-ene Steroids In: Schaenzer, W., Geyer, H., Gotzmann, A., Mareck-Engelke U. (Eds.) Recent advances in doping analysis (4), Sport und Buch Strauss, Cologne (1997): 185-201.

Schreck, C.B., Giannico, G.R., Feist, G.W., Contreras-Sánchez, W.M., Pascual-Valencia, L.E., Hernández-Vidal, U., Campos-Campos, B. Elimination of Methyltestosterone from Intensive Masculinization Systems: Use of Ultraviolet Irradiation of Water In: J. Burright, C. Flemming, H. Egna (Eds.), Twenty-Second Annual Technical Report. Aquaculture CRSP, Oregon State University, Corvallis, Oregon (2005): 185-194.

Schulte-Oehlmann, U., Oetken, M., Bachmann, J., Oehlmann, J. Effects of Ethinyloestradiol and Methyltestosterone in Prosobranch Snails, In: K. Kümmerer (Ed.) Pharmaceuticals in the Environment. Sources, Fate, Effects and Risks, 2nd Edition, Springer-Verlag, Berlin, Heidelberg (2004): 233-247.

Shareef, A., Angove, M.J., Wells, J.D. Optimization of silylation using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide, *N,O*-bis-(trimethylsilyl)-trifluoroacetamide and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide for the determination of the estrogens estrone and 17α-ethinylestradiol by gas chromatography–mass spectrometry. J. Chromatogr. A 1108 (2006): 121-128.

Shinohara, Y., Isurugi, K., Hashimoto, T. Stable isotope dilution analysis of human urinary metabolites of 17α-methyltestosterone. J. Chromat. B 741 (2000): 271-278.

Stanley, S.M.R., Smith, L., Rogers, J.P. Biotransformation of 17-alkylsteroids in the equine: gas chromatographic-mass spectral identification of ten intermediate metabolites of methyltestosterone. J. Chromat. B 690 (1997): 55-64.

Sumpter, J.P., Johnson, A.C. 10th anniversary perspective: Reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many thing in between). J. Environ. Monit. 10 (2008): 1476-1485.

Sutherland, R.A. Bed Sediment-Associated Trace Metals in an Urban Stream, Oahu, Hawaii. Environmental Geology 39 (2000): 241-265.

Streck G. Chemical and biological analysis of estrogenic, progestagenic, and androgenic steroids in the environment. TrAC 28 (2009): 635-652.

Taylor, W., Snowball, S., Lesna, M. The effects of long-term administration of methyltestosterone on the development of liver lesions in BALB/c mice. J. Pathol. 143 (1984): 211-218.

Urbatzka, R., van Cauwenberge, A., Maggioni, S., Vigano, L., Mandich, A., Benfenati, E., Lutz, I., Kloas, W. Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere 67 (2007): 1080-1087.

Van Peteghem, C., van Look, L., de Guesquiere, A. Chemiluminescence immunoassay for the detection and quantification of methyltestosterone residues in muscle tissue. J. Chromat. B 489 (1989): 219-223.

Vilar, V.J.P., Botelho, C.M.S., Boaventura, R.A.R. Methylene blue adsorption by algal biomass based materials: Biosorbents characterization and process behaviour. J. Hazard. Mat. 147 (2007): 120-132.

Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M.F. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. J. Chromat. A. 1210 (2008): 84-91.

Wason, S., Pohlmeyer-Esch, G., Pallen, C., Palazzi, X., Espuña, G., Bars, R. 17α-Methyltestosterone: 28-day oral toxicity study in the rat based on the

"Enhanced OECD Test Guideline 407" to detect endocrine effects. <u>Toxicology</u> 192 (2003): 119-137.

William, T.M., Kind, A.J., Hyde, W.G., Hill, D.W. Characterization of urinary metabolites of testosterone, methyltestosterone, mibolerone and boldenone in greyhound dogs. <u>J. Vet. Pharmacol. Therap.</u> 23 (2000): 121-129.

Witters, H.E., Vangenechten, C., Berckmans P. Detection of estrogenic activity in Flemish surface waters using an in vitro recombinant assay with yeast cells. <u>Water Sci. Technol.</u> (2001): 117-123.

Xu, C.L., Chu, X.G., Peng, C.F., jin, Z.Y., Wang, L.Y. Development of a faster determination of 10 anabolic steroids residues in animal muscle tissues by liquid chromatography tandem mass spectrometry. <u>J. Pharma. Biomed. Anal.</u> 41 (2006): 616-621.

Xu, J., Wu, L.S., Chen, W.P., Chang, A.C. Simultaneous determination of pharmaceuticals, endocrine disrupting compounds and hormone in soils by gas chromatography-mass spectrometry. <u>J. Chromat. A</u> 1202 (2008): 189-195.

Yamada, M., Aramaki, S., Okayasu, T., Hosoe, T., Kurosawa, M., Kijima-Suda, I., Saito, K., Nakazawa, H. Identification and quatification of metabolites common to 17α-methyltestosterone and mestanolone in horse urine. <u>J. Pharma. Biomed. Anal.</u> 45 (2007): 125-133.

Zerulla, M., Länge, R., Steger-Hartmann, T., Panter, G., Hutchinson, T., Dietrich, D.R., Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). <u>Toxicol. Lett.</u> 131 (2002): 51-63.

Zhang, K., Zuo, Y.G. Pitfalls and solution for simultaneous determination of estrone and 17α-ethinylestradiol by gas chromatography–mass spectrometry after derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide. <u>Anal. Chim. Acta</u> 554 (2005): 190-196.

Zhang, Z., Duan, H., Zhang, L., Chen, X., Liu, W., Chen, G. Direct determination of anabolic steroids in pig urine by a new SPME-GC-MS method. <u>Talanta</u> 78 (2009): 1083-1089.

APPENDICES

APPENDIX A

Fugacity Model (after Mackay, 2001)

Table A 1: General constants of MT for the Fugacity Model.

Property		Value	Unit
MW	Molecular weight	302.45	g/mole
S	Water solubility	3.39	mg/L
VP	Vapor pressure	2.47E-03	Pa
logKow	Octanol/Water partitioning constant	3.36	
Kow		2290.86	
R	Ideal gas constant	8.314	Pa-m3/mole-K
T	Absolute temperature	303	K
mt	Melting point	168	C
Mtotal	Total mass of MT	0.0001	kg
Mtotal	Total mass of MT	0.00033063	moles

Table A 2: Volumes of compartments for the Fugacity Model.

Compartment	Volume (m^3)
Vair	2.0E+00
Vwater	1.2E+00
Vsoil	0.0E+00
Vsed	5.0E-01
Vssed	1.0E-07
Vbiota	1.0E-07

Table A 3: Densities of compartments for the Fugacity Model.

Compartment	Density (g/cm^3)
Dsoil	2.4
Dsed	2.4
Dssed	1.5
Dbiota	1

Table A 4: Organic carbon fractions for the Fugacity Model.

Compartment		OC Fraction
foc_soil	Organic carbon fraction in soil	2%
foc_sed	Organic carbon fraction in sediment	4%
foc_ssed	Organic carbon fraction in suspend. sed	20%
L	Lipid content of fish	0.048

Table A 5: Parameters estimated by the Fugacity Model.

Parameter		Unit	Value
H	Henry's Law Constant	Pa-m^3/mole	0.220
logKoc	Partition Coeffic. organic C /water		2.973
Kp_soil	Partition Coeffic. soil /water	L/Kg-soil	18.785
Kp_sed	Partition Coeffic.sed /water	L/Kg-sed	37.570
Kp_ssed	Partition Coeffic. suspend_sed /water	L/Kg-ssed	187.510
logBCF	Partition Coeffic. biota /water		2.041
BCF		L/Kg-biota	109.962

Table A 6: Fugacity capacity constants (Z) calculated by the Fugacity Model.

Compartment	Z (mol/m^3-Pa)
Zair	4.04E-04
Zwater	4.54E+00
Zsoil	2.05E+02
Zsed	4.10E+02
Zssed	1.28E+03
Zbiota	5.00E+02

APPENDIX B

Extraction Procedure

- Weight 5 g sediment into centrifuge tubes
- Add 10 mL methanol and vortex mix
- Place tubes into ultrasound water bath for 5 min
- Shake tubes on horizontal shaker at 320 rpm for 60 min
- Centrifuge at 8000 rpm for 10 min
- Remove supernatant into new tubes
- Add 10 mL methanol to pellet and vortex mix
- Place tubes into ultrasound water bath for 5 min
- Shake tubes on horizontal shaker at 320 rpm for 60 min
- Centrifuge at 8000 rpm for 10 min
- Remove supernatant, pool with previous
- Add 10 mL methanol to pellet and vortex mix
- Place tubes into ultrasound water bath for 5 min
- Shake tubes on horizontal shaker at 320 rpm for 60 min
- Centrifuge at 8000 rpm for 10 min
- Remove supernatant, pool with previous
- Evaporate solvent in pooled extract at 55 °C
- Reconstitute with 0.5 mL methanol, gently vortex mix
- Transfer sample into Uniprep Filter cup
- Filter to remove particulates
- UPLC-MS/MS analysis

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