PHYTOREMOVAL AND PHYTOTOXICITY OF *TYPHA ANGUSTIFOLIA* L. EXPOSED TO NORETHINDRONE UNDER HYDROPONIC CULTURE

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ABSTRACT

This study was conducted to assess the potential of *Typha angustifolia* L. to tolerate and remove norethindrone. The research objectives were (i) to investigate the phytoremoval efficiency of *T. angustifolia* when exposed to norethindrone; and (ii) to access the phytotoxicity of norethindrone by *T. angustifolia* through phytoremediation. *Typha angustifolia* were collected and acclimatised under hydroponic culture. Young seedlings were harvest and transferred to 0 – 2.0 mg/L of norethindrone for a maximum period of 28 days. Norethindrone in water was analysed using dispersive liquid-liquid microextraction with the solidification of floating organic (DLLME-SFO) and followed with the chromatography analysis by HPLC-UV/Vis. Meanwhile, the plant samples were separated into three parts (root, stem and leaves), air-dried in room temperature and then followed with sea sand matrix disrupting extraction (SSDM), cleaned up with preparative thin layer chromatography (PTLC) and finally analysed by HPLC-UV/Vis. Calculations like BCF, Ti, TF, and RGR were applied, followed with statistical analysis with ANOVA and correlation test. An evaluation showed that norethindrone removal efficiency by plants reached a value of 90% from the initial contents at 28 days. The rate of removal kinetic followed the first order reaction rate with rate constant of 0.5mg/L treatment (-0.131 h^{-1})> 1.0 mg/L (-0.02 h^{-1}) > 2.0 mg/L (-0.003 h^{-1}). The relative growth rate of plant weight and height showed that decreasing order from 0.5 mg/L > 1.0 mg/L > 2.0 mg/L norethindrone exposure treatment. The plants exposed to norethindrone did not experience chlorosis effect on root and necrosis effect on leaves. Norethindrone accumulation was higher in the root (0.012-0.07 mg/g) than stem (0.018-0.049 mg/g) and leaves (0.006-0.041 mg/g). The plants in (i) 1.0 mg/L norethindrone treatment and (ii) 2.0 mg/L norethindrone treatment have similar increasing BCF order: BCF_{leaves} (0.016; 0.017) < BCF_{stem} (0.019;0.02) < BCF_{root} (0.023;0.022), except for the plants in 0.5 mg/L norethindrone treatment with the increasing order BCF_{root} (0.006) < BCF_{stem} (0.026) < BCF_{leaves} (0.027). The root concentration factor was higher in 0.5 mg/L treatment (25.829) than 1.0 mg/L (21.005) and 2.0 mg/L (21.495). The translocation factor (TF) values and tolerance index (Ti) values were >1 but the values were decreasing when exposure of norethindrone concentration increasing. Although the removal performance was greater in 0.5 mg/L norethindrone treatment, *T. angustifolia* still has performed its ability in the removal of norethindrone from aqueous medium. It also proves that *T. angustifolia* able to tolerate the toxicity of norethindrone even they are exposed to high concentration.

ABSTRAK

Fito-penyingkiran dan Fito-ketoksikan Typha angustifolia L. Terdedah kepada Norethindrone secara Kultur Hidroponik

Kajian ini dijalankan untuk menilai potensi Typha angustifolia L. untuk menyingkir dan bertoleransi norethindrone. Objectif penyelidikan ini adalah (i) mengkaji kecekapan T. anustifolia untuk mengyingkir norethindrone; (ii) mengkaji penyesuaian T. angustifolia terhadap norethindrone melalui fito-remediasi. T. angustifolia diambil dan ditumbuh secara hidroponik. Anak tumbuhan telah dituai dan dipindahkan ke 0-2.0 mg/L rawatan norethindrone untuk tempoh maksimum 28 hari. Norethindrone dalam air dianalisa dengan menggunakan dispersive liquid-liquid microextraction with solid floating organic (DLLME-SFO) dan diikuti dengan HPLC-UV/VIS. Sementara itu, sampel tumbuhan dibahagi kepada tiga bahagian (akar, batang dan daun), kering dalam suhu bilik dan kemudian diikuti dengan sea sand matrix disrupting extraction (SSDM), dibersihkan dengan kromatografi lapisan nipis (PTLC) dan akhirnya dianalisis oleh HPLC-UV/VIS. Pengiraan seperti BCF, Ti, TF, dan RGR digunakan, diikuti dengan analisis statistik dengan ujian ANOVA dan korelasi. Penilaian menunjukkan bahawa kecekapan penyingkiran norethindrone oleh tumbuhan telah mencapai nilai 90% dari kandungan pada 28 hari. Kadar kinetik penyingkiran mengikuti kadar tindak balas pesanan pertama dengan kadar tetap rawatan 0.5mg/L (-0.131 h^{-1})> 1.0 mg/L(-0.02 h^{-1})> 2.0 mg/L(-0.003 h^{-1}). Kadar pertumbuhan relatif berat dan ketinggian tumbuhan menurun daripada rawatan norethindrone 0.5 mg/L > 1.0 mg/L > 2.0 mg/L. Tanaman yang terdedah kepada norethindrone tidak mengalami kesan klorosis pada kesan akar dan nekrosis pada daun. Kepekatan norethindrone didapati lebih tinggi pada akar (0.012-0.07 mg/g), batang (0.018-0.049 mg/g) dan daun (0.0061-0.041 mg/g). Tumbuhan dalam rawatan 1.0mg/L norethindrone dan rawatan 2,0 mg/L norethindrone mempunyai sama susunan BCF iaitu BCF_{daun} (0.016; 0.017) < BCF_{batang} (0.019;0.02) < BCF_{akar} (0.023; 0.022) tetapi tumbuhan dalam rawatan 0.5 mg/L norethindrone pula dengan peningkatan BCF_{akar} (0.006) <BCF_{batang} (0.026) <BCF_{daun} (0.027). Manakala, faktor kepekatan akar didapati lebih tinggi dalam rawatan 0.5 mg/L(25.8289) daripada 1.0 mg/L(21.0054) dan 2.0 mg/L(21.4953). Faktor tumpuan akar (RCF) lebih tinggi dalam rawatan 0.5 mg/L (25.829) daripada 1.0 mg/L (21.005) dan 2.0 mg/L (21.495). Nilai translocation factor (TF) dan indeks toleransi (Ti) adalah> 1 tetapi nilai-nilai itu berkurangan apabila pendedahan kepekatan norethindrone meningkat. Sebagai kesimpulan, Walaupun prestasi penyingkiran lebih besar dalam rawatan 0,5 mg/L norethindrone, T. angustifolia berpotensi untuk menyingkirkan norethindrone dalam air. Ia juga membuktikan bahawa T. angustifolia dapat bertoleransi dengan kesan ketoksikan norethindrone walaupun mereka terdedah kepada kepekatan yang tinggi.

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%	-	percent
ANOVA	-	analysis of variance
$CHCl_3$	-	chloroform
et al.	-	and others; latin phrase *exempli gratia*
g	-	gram
GC	-	gas chromatography
h	-	hour(s)
HPLC-UV/VIS	-	high performance liquid chromatography coupled with ultraviolet–visible spectroscopy detector
MeCN	-	acetonitrile
MeOH	-	methanol
mg	-	milligram
mL	-	millilitre
MS	-	mass spectrometry
µL	-	microlitre
n	-	number of samples
ng	-	nanogram
p	-	significant different value
R	-	regression
spp.	-	species
T. angustifolia	-	*Typha angustifolia*
TLC	-	thin layer chromatography

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CHAPTER 1

INTRODUCTION

Phytoremediation is a plant-based bioremediation technique approach in the detoxification or removing pollutants from soil and water environment. Since it is low cost, sustainable, and environmentally sound technology, application of this technique includes passive treatment using vegetation barriers, cleaning of soil and water through pollutant uptake and harvest, and pollutant degradation and volatilization provides a valuable component of integrated solutions for inorganic and organic pollution. Previous research on the plant uptake of pharmaceuticals and steroids, demonstrated their potential to accumulate pollutants (Boxall *et al.* 2006; Dordio *et al.* 2010; Seo *et al.* 2010; Dodgen *et al.* 2013) hydroponically or in nutrient solution has also been reported (Redshaw *et al.* 2008; Herklotz *et al.* 2010). Plants used in phytoremediation are usually evaluated with phytoremoval efficiency and phytotoxicity. Phytoremoval efficiency is evaluated by assessing the ability of plant in removing a compound from water, soil or soil-water in given exposure duration under controllable condition (Dordio *et al.* 2009b). Meanwhile, phytotoxicity or toxicity effect study of a compound to plant is used to determine the sensitivity of plant to xenobiotic action and occurrence of that compound in certain concentration and area (Carvalho *et al.* 2014; Bartikova *et al.* 2016). These two components are important to determine the potential and availability of plants used in phytoremediation.

Typhaceae such as *Typha agustifolia* L. is one of the frequent emergent plants and widely used in a constructed wetland. Various studies have reported Typhaceae ability to withstand and remove pharmaceutical and other trace emerging compounds such as clofibric acid (Dordio *et al.,* 2009b), ibuprofen (Dordio *et al.* 2011a; Li *et al.* 2016), and carbamazepine (Dordio *et al.* 2011b) from water. However, it is interesting that there is lack of research in the application of *Typha* spp. for the accumulation of hormonal pharmaceutical compounds.

Steroids including estrogen, progestin and androgen pose ecotoxicity risk to environment. Norethindrone is a synthetic progestin derived from 19-northestosterone that is used medicinally in the treatment of oral contraceptive with estrogen, hormone replacement therapy as well as menstrual disorders and endometriosis. However, compared to estrogens such as ethinylestradiol, estradiol and estrone, the presence of progesterone and progestin are not frequently reported (Fent 2015). Various studies believed that progestin like norethindrone can converted into estrogeic compounds in animals' metabolism before discharged from body into sewages and wastewater (Kamyab *et al.* 1968; Mayberry *et al.* 1990; Kuhnz *et al.* 1997). Therefore, many studies were focusing on the presence of the estrogenic steroid hormones in environment rather than progestogenic steroid hormones.

But numerous researchers have reported the presence of norethindrone in water even after the water treatments. The concentration have been found in surface water and wastewater from 0.2 ng/L up to 0.8 mg/L in several research such as in Labadie and Budzinski (2005), Vulliet *et al.* (2009), Vulliet and Cren-Olive (2011), and Al-Odaini *et al.* (2012, 2013). Presence of norethindrone in the aquatic environment has recognised norethindrone as an environmental pollutant due to poor efficiency of sewage and wastewater treatment plants. Cormier *et al.* (2015) reported that norethindrone is a non-natural degradable compound. It can persist in water for a long period at normal temperature. In another word, it can pose a risk to environment. Studies by Wibbels and Crews (1995), Maier and Herman (2001), Goto and Hiromi (2003), Gomez *et al.* (2010), Paulos *et al.* (2010), Zucchi *et al.* (2012), Safholm *et al.* (2014) and Wu *et al.* (2016b) have indicated that norethindrone has negative impacts likes decreasing fecundity, leading to population declines and affecting sex determination in aquatic organisms in/or extremely low concentration.

The current sewage treatment plants are not designed to effectively eliminate non-conventional toxic compounds like norethindrone in water. Generally, steroidal hormones have biodegradability, resulting in partial removal from the water phase and less deposited in the sedimentation and biological treatment in sewage and wastewater treatment plants (Ternes *et al.* 2004; Bendz *et al.* 2005; Heidler *et al.* 2006). A study showed that although the occurrence of pharmaceutical compounds in hospital wastewater is consistently higher than urban wastewater, the application of conventional treatments is still less efficiency to eliminate pharmaceutical compounds (Verlicchi *et al.* 2010).

The removal of norethindrone requires advanced technology such as advanced oxidation (Gultekin and Ince 2007; Broseus *et al.* 2009; Fayad *et al.* 2013), chemically removal (Zhang *et al.* 2015; Badejo *et al.* 2017), nano-/ultrafiltration (Verliefde *et al.* 2007; Yoon *et al.* 2007), microbial-based bioremediation (Passatore *et al.* 2014; Rana *et al.* 2014; Gavrilescu *et al.* 2015) and phytoremediation. However, most of the methods are highly cost and non-environmental friendly except for bioremediation and phytoremediation (Abd Manan *et al.* 2015). Most conventional wastewater treatment plants do not have treatment processes such as activated carbon, ozonation or membrane treatment. Therefore, these emerging micro-pollutants are not removed but are easily released to the receiving natural waters (Bolong *et al.* 2009). As a result, considerable amounts of pollutants remain in sewage treatment effluents and sludge. The discharge of the effluents will lead to the contamination of receiving waters (Batt *et al.* 2006).

The usage of phytoremediation technology is widely applied with success to remove some pollutants from wastewater. However, only very few studies have been carried out on the removal of pharmaceuticals by plants. Typhaceae is one of the frequent emergent plants and widely used in a constructed wetland. Various studies have assessed Typhaceae ability to withstand and remove pharmaceutical and other trace emerging compounds. Gray and Sedlak (2005) reported that the overall estradiol and ethinylestradiol removal from water ranged 36 to 41 % by a combination of *Typha* spp. and *Scirpus* spp. Ávila *et al.* (2015) also reported above 80% removal efficiencies for estrogenic compounds such as bisphenol A and ethinyl estradiol by T. angustifolia. Dai *et al.* (2017) reported that T. angustifolia able to remove bisphenol A and esterone about 50% in stacked constructed wetland at 0.5-

2.0 m/d flow rate. Thus, it is possible for *Typha angustifolia* absorbing norethindrone and tolerating to effects of norethindrone.

1.1 Objectives

(i) To investigate the phytoremoval efficiency of *T. angustifolia* when exposed to norethindrone

(ii) To access the phytotoxicity of norethindrone by *T. angustifolia* through phytoremediation.

1.2 Thesis contribution

The increasing pharmaceutical residues such as norethindrone discharged from urban and agricultural areas into natural water resources has alarmed the public concerns. As results, there is a need to develop remediation technique to overcome the problem. Floating and emergent plants such as Typhaceae, mangrove and *Hydra* sp. have the advantage of absorbing these compounds and metabolising them from toxic to non-toxic characteristics. This can decrease the risk of the organisms from directly exposed to high concentration of these compounds. Furthermore, the occurrence of emerging or newly identified contaminants in our water resources is of continued concern for the health and safety of consuming public. This indicates the urgent need for finding cost-effective processes to retain and remove these pollutants before they reach water systems. This information is valuable for evaluating the ability of *Typha angustifolia* in tolerance and removal of norethindrone in the aquatic environment. It will later help in designing effectively constructed wetland to treat pharmaceutical contaminated water.

1.3 Scopes of study

In this context, the research was conducted in a closed environment to prevent the disturbance of weather on the studies of experiments. Besides, the phytoremoval efficiency was evaluated under hydroponic culture. Since the hydroponically research was conducted by using PVC containers, thus adsorption and other abiotic factors of norethindrone in water tank were accounted and performed as a controlled experiment of the research. Meanwhile, phytotoxicity experiment which studied on the toxicity effect of norethindrone in plants was only accounting on the physical characteristics of *T. angustifolia* and accumulation of norethindrone in the plant tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 Pharmaceutical pollution

Pharmaceutical pollution due to uncontrolled agricultural and industrial revolution has resulted in a rapid increase of toxin in the aquatic environment (Ashraf *et al.* 2010). In past, Hignite and Azarnoff (1977) discovered the presence of chlorophenoxyisobutyrate and salicylic acid in the effluent of sewage disposal plant in Kansas City, Missouri. It was alarming to know that drugs, either in form of unchanged or biologically active metabolites, have high potential negative impacts to the ecosystem, especially for biota. Numerous researchers pointed out that the presence of these residues that discharged from urbanization (includes industrial) and agricultural activities are not totally eliminated with current conventional wastewater and sewage treatment plants, although the micro-pollutants remained in low concentration.

However, due to the characteristic of polymorphism and various water solubility in different pH matrix, this might lead to possible biological and toxicity effect to organisms (Caliman and Gavrilescu 2009; Fatta-Kassinos *et al.* 2011). Most of the predominantly water-soluble pharmaceuticals have limited volatility and biodegradability, resulting in partial removal from the water phase and less deposited in the sedimentation and biological treatment in sewage and wastewater treatment plants (Ternes *et al.* 2004; Bendz *et al.* 2005; Heidler *et al.* 2006). Despite their extremely low concentration, they are still capable of negative impacts on the aquatic environment, as well as interrupting the normal functioning of aquatic organisms and human health due to their unpredictable eco-toxicological effects (Stuart *et al.* 2012).

Therefore, even though these compounds usually are not commonly monitored, but they are nowadays a major cause of toxicity in plants and animals.

2.2 Synthetic steroid and hormonally pharmaceutical residues act as emerging micro-pollutants in the environment.

Steroids are originated from lipid compounds (terpenes) with containing or biologically derived from perhydro-1,2-cyclopentanophenanthrene ring system. These varieties are controlled by the type of side-chain substitution at C17, the degree of unsaturation, degree and nature of oxidation and the stereochemical characteristics of the ring junctions (Morgan and Moynihan 2000). Steroidal compounds exist in two categories: natural and synthetic, in which may not only influence in human health but also other organisms' health. Natural steroid such as sterols, bile acids, hormones (estrogen, phytoestrogen, and androgens), corticosteroids, and vitamin D can be found in biotas. On the other hand, synthetic steroid hormones like conjugated and esterified estrogen, and others (de Mes *et al.* 2005) are widely used in various pharmaceutical fields, product care fields, and industrial activities.

However, global demand for consumption has resulted the discharged of hormonal pharmaceutical residues in the overuse of hormones that has resulted with potentially negative impacts to the ecosystem. Severely damaging effects of endocrine disruption can be induced even in low concentration with those units like ng/L that are not commonly monitored (EEA 1996; Kavlock *et al.* 1996; Zoeller *et al.* 2012). The disruption process can be induced through: i) directly influence: mimic (involving agonist response, exhibit estrogenic, ant-androgenic, and androgenic activity); ii) indirectly process that involving hormone pathway disruption (Magnarelli and Fonovich 2013). These residues may not only influence gene expression process but also interrupts the normal biological process like cell proliferation and hormonal secretion. Numerous researches were conducted to study the concentration of these pharmaceutical residues in environment and the consequences of the occurrence to the environment (refer to Appendix A) Figure 2.1