# Potential of *Typha angustifolia* L. in removing norethindrone from water

**Jie-Yinn Lee[1#], Charles S. Vairappan[2], Kartini Saibeh[3]**

1 Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA.
2 Natural Product Laboratory, Institute for Tropical Biology and Conservation, , Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA.
3 Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Mile 10, Sg. Batang, 90000, Sandakan, Sabah, MALAYSIA.
# Corresponding author. E-Mail: silvrie@gmail.com; Tel: +6088-320000; Fax: +6088-435324.

**ABSTRACT** Uncontrollable demand of pharmaceutical especially contraception drugs and poor performance of conventional wastewater treatment plants has resulted in the increasing concentration of pharmaceutical residues in natural environment. Phytotechnology (phytoremediation technology) such as constructed wetland has been introduced as post treatment before the effluent is discharged from wastewater treatment plants to natural water courses. In this context, a study was conducted to assess the potential of the macrophyte, *Typha angustifolia* to remove norethindrone. This evaluation was conducted in hydroponic solutions with 0.5 – 2.0 mg/L of norethindrone for a maximum period of 21 days. The removal efficiency of norethindrone from the water by *T. angustifolia* reached a value of 90% of the initial contents. The range of relative growth rates of *T. angustifolia* in the norethindrone treated assays was 1.821 – 2.589. The result obtained from this study suggests that *T. angustifolia* has high capability to adapt and crop the toxicity of norethindrone when it is applied in phytotreatment.

**KEYWORDS:** Norethindrone, pharmaceutical, phytoremediation, *Typha angustifolia* L.

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## INTRODUCTION

Pharmaceutical pollution has resulted in increasing concentration of toxin in aquatic environment due to the agricultural and industrial revolution in the last few decades. As the result, it has globally acquired the contamination of emerging concern. Numerous researchers have found that the current conventional wastewater and sewage treatment plants are poor to eliminated these pharmaceutical residues that discharged from urbanization and agricultural activities, although the occurrence of these residues are remained in low concentration. They have low polarity compared to conventional pollutants. However, due to the characteristic of polymorphism and various water solubility in different pH matrix, they may lead to potential biological and toxicity effect to organisms (Fatta-Kassinos et al. 2011).

Norethindrone is a progestin that applied in hormone regulation and therapy medications, endometriosis and chronic pelvic treatment (Chawla 2010, Kaser et al. 2012, Simon et al. 2003). It is well-known ubiquitous pharmaceutical as well as synthetic estrogen such as ethinylestradiol, typically found in the ranges from ng/L to ug/L (Al-Odaini et al. 2011, Al-Odaini et al. 2012). Although they are not frequently found in natural environment, however, some studies have revealed the potential of norethindrone converts to ethinylestradiol (Kuhnz et al. 1997). The recurrent of ethinylestradiol as well as other hormonally pharmaceutical residues in natural environment is an evidence of inadequate pollutants removing processes in conventional wastewater treatment plants (Zhou et al. 2016). As result, the distribution of norethindrone and its derivatives has induced toxic effects to organisms (Fent 2015, Goto and Hiromi 2003, Paulos et al. 2010, Safholm et al. 2014, Wu et al. 2016, Zucchi et al. 2012).

Phytoremediation, or also known as phytotechnology, is a plant-based bioremediation technique approach in 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 protection 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. Several studies were conducted looking at the potential of macrophytes to degrade hormonally or phenolic pharmaceutical compounds.

*Typha* is one of the common wetland-adapted macrophytes. They are found in high biomass and tolerant to chemically defined ecosystem (Rogers 2003). They are widely adopted in heavy metal and non-hormonally pharmaceutical residues removal. Previously, a study was conducted on identifying the possibility of phytotechnology in eliminating etrogenic compounds such as estradiol and ethinyl estradiol. Gray and Sedlak (2005) reported that the overall estradiol and ethinyl estradiol removal from water ranged 36 to 41 % by combination of *Typha* and *Scirpus*. Interestingly, there is less literatures on the application of *Typha* in accumulating of hormonally pharmaceutical compounds, especially for progestin such as norethindrone. It is potential to identify the capability of *Typha* in removal of endocrine disrupting compounds, especially synthetic steroid and hormones. Therefore, the objective of this study was to investigate the efficiency of *Typha angustifolia* L. to remove norethindrone from water.

## METHODOLOGY

*Chemical reagent and materials*

The analytical graded chemicals used in this study included the followings: 1-undecanol (Acros Organics, Belgium), hydrochloride acid (Fisher), potassium nitrate (Merck), magnesium sulphate heptahydrate (Systerm), potassium dihydrogen phosphate (Merck), calcium nitrate tetrahydrate (Merck), magnesium sulphate heptahydrate (Systerm), ferric sulphurous chelate EDTA (Aldrich), boric acid (Merck), manganese (II) chloride tetrahydrate (Merck), copper (II) sulphate pentahydrate (Merck) and molybdic (IV) acid (Aldrich) were used in preparation of Hoagland nutrient solution. High purity (>98%) 19-norethindrone was acquired from Sigma-Aldrich. Methanol and acetonitrile with LiChrosol reagent grade were acquired as solvent in extraction and analytical chromatography. Ultra-pure water was obtained from a Milli-Q water purification system (Simplicity® UV, Millipore Corp., France). Modified Hoagland nutrient solution was prepared according to Dordio et al. (2011b).

*Sample Collection*

20 *T. angustifolia* were uprooted from rural area in Sandakan with the height ranged 1.6-2.0 meter. The roots and leaves were washed with distilled water to clean the soil and 0.01M hydrochloric acid for eliminating microorganism. Their above-ground portion were then cut to 30 cm as described by Chong et al. (2009). The plants were then acclimated with hydroponic solution as described in Dordio et al. (2009) for 30 days. After 30 days, plantlets with 50-60 cm height were selected for further experiment.

*Assay set up*

Selected plants, which roots were rinsed with a dilute hydrochloride solution in order to diminish the native microbial population, were transferred to PVC vessels which contained 3.5 L modified Hoagland nutrient solution that spiked with norethindrone at 0.5 mg/L, 1.0 mg/L and 2.0 mg/L in which the norethindrone was prepared in the stock solution 40 mg/L. The hydroponic culture without plants in 1.0 mg/L norethindrone was set up to evaluate photodegradation of norethindrone and the possible effect of norethindrone adsorption on the tank walls. Besides,

hydroponic culture with plant in 0 mg/L norethindrone was also set up as control plants without influence of toxic effect of norethindrone. All assays were done in three replicates. Samples of nutrient solution were collected after 6h, 12h, 18h and 24h of exposure during the first day, and then every 24 h during a period of day-7, day-14 and day-21. Norethindrone removal by the plants will be evaluated along these periods by quantification of remaining norethindrone in the collected samples, following the modified analytical methodology from Chang and Huang (2010) and Payus et al. (2016).

*Quantification of norethindrone in water*

Norethindrone stock standard solution was prepared at 500 mg/L by exact weighing and dissolution in methanol. Calibration standards with the initial mobile phase with the concentration of norethindrone in the range of 0 – 5.0 mg/L were prepared. Hoagland solution was prepared with norethindrone spiked in concentration range 0 – 5.0 mg/L were then analyzed with high-performance liquid chromatography with various wavelength detector.

Dispersive liquid-liquid microextraction with solidification of floating organic (DLLME-SFO) was modified from the methodology of Chang and Huang (2010) and Payus et al. (2016) as follows. 5 mL water sample was mixed with 10 µL undecan-1-ol and 100 µL methanol in the centrifuge tube. The mixture was then vigorously shaken for 90 seconds as described in Xie et al. (2010). After that, the tube was centrifuged for 10 min at 4500rpm. After that, the tube was cooled with ice bath for frozen the floating drop on the surface of water sample. The 5 µL drop was removed and 200 µL methanol was added. Finally, 40 µL was injected for chromatographic analysis.

The chromatographic system was optimized by using the Agilent High-Performance Liquid Chromatography with various wavelength detector (VWD). Separation was carried out at room temperature on a reversed-phase C18 (250 mm×4.6 mm I.D., 5 µm) column. The mobile phase consisted of methanol and distilled water (70:30, v/v). The flow rate was set at 1.0 mL/min, the detection wavelength was set at 254 nm, the temperature of column oven was maintained at 40 °C and the injection volume was 40 µL. The IDL (instrument detection limit) and IQL (instrument quantification limit) were 3.873 µg/L and 11.736 µg/L with limit of detection 0.379 µg/L. The detection with wavelength was applied in this study for the purpose of avoiding the unpredictable susceptible to matrix effects that might cause the signal suppression or enhancement, in which it led to poor performance in the results (Dordio et al. 2011a).

*Statistical Analysis*

In order to characterize the plant growth, relative growth rate (RGR) was applied as an analytical tool (Hoffmann and Poorter 2002). Fresh plant in different exposure times were weighted for each of the different of the different initial concentrations tested. RGR were calculated according to the equation as given as follow:

$$RGR = (\ln W_1 - \ln W_0) / (t_1 - t_0) \quad (1)$$

where $\ln W_0$ and $W_1$ are the initial and final weights of plants and $(t_1 - t_0)$ is the exposure duration of the experiment.

The results were presented as means of the experiments. Each tested concentration was performed in triplicates. Data was analyzed through one-way analysis of variance (ANOVA) for comparisons of effects due to norethindrone exposure on growth performance with those of the

control culture. Tukey's post hoc analyses were used for the evaluation of the significant differences among particular treatments. Comparisons were considered significantly different for $P < 0.05$.

## RESULT AND DISCUSSION

Typhaceae is widely deployed in constructed wetland to detoxify water from the organic compounds. They have good tolerance when exposed to a certain xenobiotic compounds such as pharmaceutical residues (Ávila et al. 2015, Dordio et al. 2011b, Dordio et al. 2009, Li et al. 2016). Therefore, a study was conducted to assess its ability to remove norethindrone from water by spiking 0.5 mg/L norethindrone. This value is selected because it is nearly the range of norethindrone detected in environment and wastewater (Al-Odaini et al. 2011, Al-Odaini et al. 2012, Vulliet et al. 2007, Vulliet et al. 2009).

The percentage of norethindrone removal was the function of the exposure time for initial concentration (1.0 mg/L). From Figure 1, the removal kinetics in the first 72 hours was fast occurring. About 50 % of norethindrone was successfully removed by plants in the water. After 72 hours, the removal kinetics was starting slowly down. Therefore a nearly flatten curve was shown. The removal activities still continued until about 90% of norethindrone was removed from the water within 504 hours (21 days). The concentrations of norethindrone remained in the reactor were maintained in below limit of detection (Figure 1 a.). The removal rate was the first-order reaction with the function:

$$\ln [\text{Norethindrone}]_t = \ln [\text{Norethindrone}]_0 - 0.01994 h^{-1} t \ (R_2 = 0.93) \quad (2)$$

where $[\text{Norethindrone}]_t$ and $[\text{Norethindrone}]_0$ are the concentration of norethindrone (μg/L) at any given time (t) and the beginning, respectively.

In the other hand, the removal kinetic in the treatment without plants was low with the percentage of removal less than 30%. This treatment without plants acted as control treatment for study the other abiotic influences such as adsorption on the vessel's wall, photodegradation and photovolatilation of norethindrone in water. The result in Figure 1 shown that the possibility of norethindrone removal affected by abiotic influences was low. Progestin has more stable chemical properties than progesterone (Fent 2015). Log $K_{ow}$ of norethindrone is 2.97, in which it has potential to tend accumulating in aquatic organisms. According to Okuhata et al. (2010), presence of various kinds of aromatic secondary metabolites such as terpenoids and phenolic on the surface of plant's roots has increased the lipophilic characteristic. This condition allows the plant to uptake low-polar or hydrophobic nature organic contaminants.

The removal of norethindrone in the treatment of plants may be influenced by microbial activities. However, the roots of plants were washed and immersed with dilute hydrochloride acid as suggested by Dordio et al. (2011a) in order to eliminate the presence of microbial on the root surfaces. Therefore the removal reaction is totally depending on the performance of plants.

The heights changes of plants were also recorded as one of the parameter to study the capability of *T. angustifolia* crop with the stress of norethindrone. From Figure 2, the differences of the plant heights in 0.5 mg/L to 2.0 mg/L treatments were significant when compared to control treatment (ANOVA test, $p < 0.05$). The plant grown slowly in first seven days in the norethindrone treatment compared to the control treatment. After that, the plants started to grow rapidly and the heights of plants were approaching to the control treatment in the increasing of exposure time. The maximum

values of the height in norethindrone-treated plants were 109.6 cm in 0.5 mg/L norethindrone and 113.5 cm in 1.0 mg/L norethindrone.

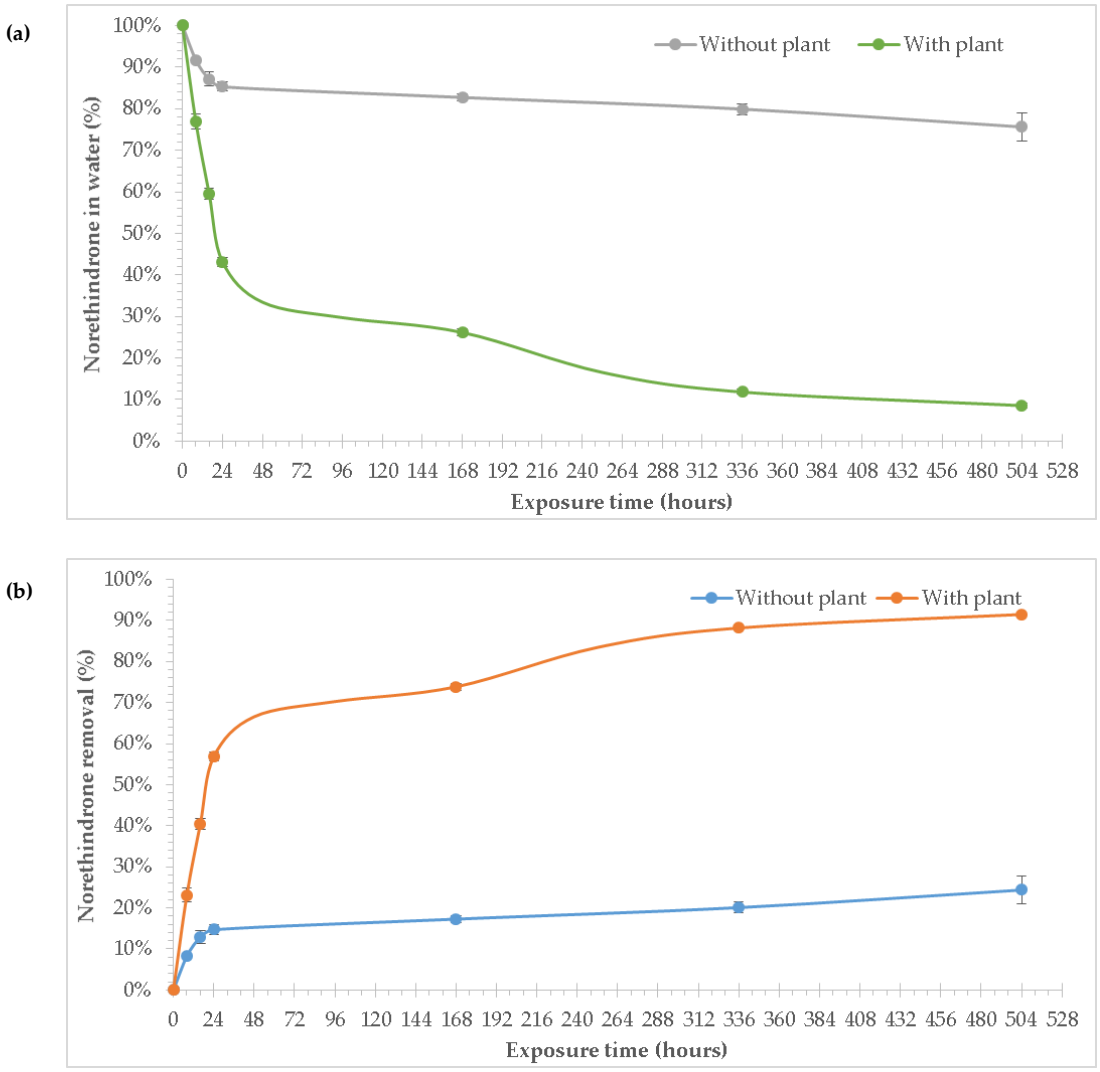


**Figure 1. (a)** The percentage of remaining norethindrone in the water and **(b)** the percentage of norethindrone removal from water as function of the exposure time, for initial concentration 1.0 mg/L and the control without plants (n=9). Error bars represent the standard deviations of the trend.

Table 1 shown the height growth rates of the plants in different concentration treatment to exposure time of experiment. In the control treatment, the growth rate of the height of *T. angustifolia* was ranged 0.1348 – 0.1858 with the mean of growth rate 0.1619. For *T. angustifolia* in norethindrone treatment, their growth rates were lower than the control during the exposure of initial 7 days (less than 0.1). However, the rate of growth was rapidly increasing and the range of rate (0.1448 to 0.2525) was recorded in day-14. However, the rate of the growth started to slow down in the treatments with 0.5 mg/L and 1.0 mg/L norethindrone except for treatment with 2.0 mg/L. The order of the plant height growth rate was as followed: 2.0 mg/L treatment < control treatment < treatment with 0.5 mg/L < treatment with 1.0 mg/L.

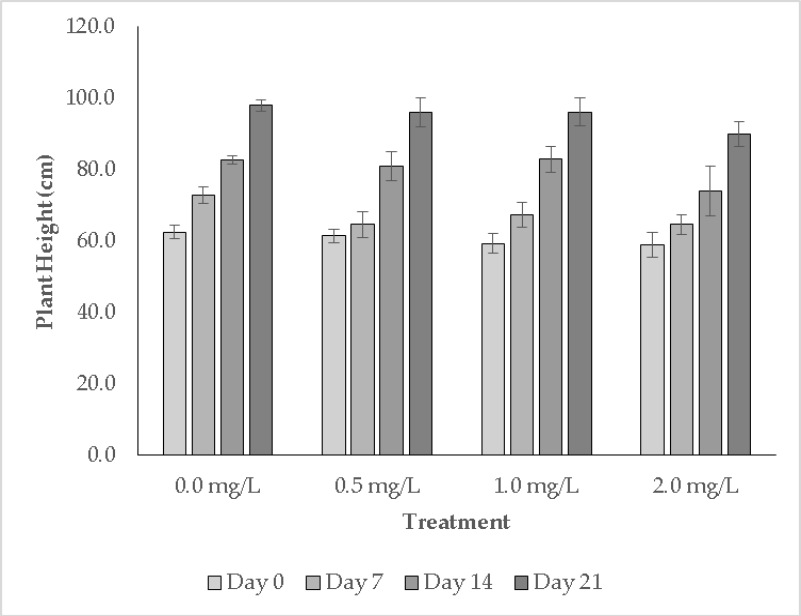


**Figure 2.** The plant heights in the respective treatments to the exposure time with respective error bars as their standard deviation (n=9).

**Table 1:** The mean of growth rate of plants within 21 days in the respective treatments.

| Exposure time | Treatment | | | |
|---|---|---|---|---|
| | **0.0 mg/L (Control)** | **0.5 mg/L** | **1.0 mg/L** | **2.0 mg/L** |
| **Day 7** | 0.1651 | 0.0522 | 0.1339 | 0.0971 |
| **Day 14** | 0.1348 | 0.2525 | 0.2323 | 0.1448 |
| **Day 21** | 0.1858 | 0.1845 | 0.1595 | 0.2160 |

Relative growth rate (RGR) is a typically analytical tool for characterizing the growth rate of a plant. In this study, relative growth rate (RGR) of *T. angustifolia* in norethindrone treatment was shown higher than the control treatment. In **Error! Reference source not found.**, the plant in the treatments with 0.5 mg/L norethindrone has the highest RGR in range of 2.345-2.589 within 21 days. Meanwhile, the plants in treatments with 2.0 mg/L has the lowest RGR in the range of 1.821-2.379. The order of the plant height growth rate was as followed: 2.0 mg/L treatment < control treatment < treatment with 1.0 mg/L < treatment with 0.5 mg/L.

Both relative growth rate and plant height growth rate had shown the good performance of *T. angustifolia* in treatments with concentration of 0.5 mg/L and 1.0 mg/L norethindrone when compared to the control treatments (2.4177) within 21 days. The highest value of RGR obtained in 0.5 mg/L norethindrone treatment and 1.0 mg/L norethindrone treatment were 2.589 and 2.561 respectively. Although the RGR of *T. angustifolia* in treatments with concentration of 2.0 mg/L norethindrone was slightly poor compared to the control treatment (RGR value in 2 mg/L norethindrone treatment = 2.379), the performance trend was still approaching to the performance trend of control treatment (Figure 3).

Presence of hormonal compounds such as estrogen, progesterone and progestin in the plant cultures can influence the plant development such as cell division, root and shoot growth, seed germination and embryo growth, plant reproductive system and callus proliferation(Janeczko and Skoczowski 2005). High concentration of hormonal compounds might negatively result in the growth and development of plants. Meanwhile, low concentration of hormonal compounds has less inhibiting effect of plant growth (Chaoui and El Ferjani 2013, D'Abrosca et al. 2008). The occurrence

of these hormones in plants can increase the rate of photosynthesis, enhance the antioxidant system in plants, and act as biostimulant of plant growth in the low concentration (0.01-10 μM or equivalent to 3μg/L to 3000μg/L). In contrast, the exposure of plants to high concentration that excess 10μM may trigger the transcriptional changes in the plant (Erdal and Dumlupinar 2010, Upadhyay and Maier 2016). For example, D'Abrosca et al. (2008) also reported that *Lactuca sativa, Dacus carota, Lycopersicon esculentum, Amaranthus retroflexus, Avena fatua, Chenopodium album, Lolium perenne,* and *Taraxacum officinale* able to grow in 1 mM concentration of ethinyl estradiol, in which the phytotoxicity effects of ethinyl estradiol on plants were the least and and become stimulant at the low concentration test.

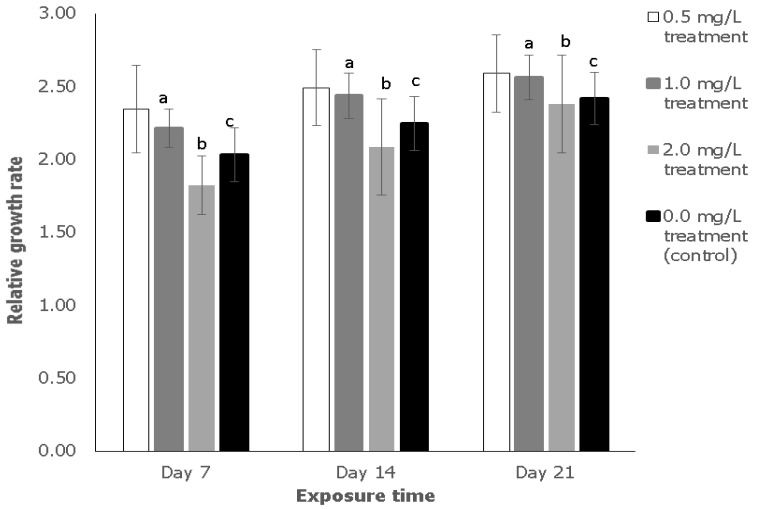


**Figure 3.** The relative growth rate (RGR) as the function with natural logarithm of differences between weights against the exposure time. Value a, b and c represent the significance different between the means of RGR, with the ± standard deviation values. (n=9; ANOVA; a = $p < 0.0001$; b = $p < 0.005$; c = $p < 0.001$).

## CONCLUSION

In this study, the removal efficiency of norethindrone in water by *T. angustifolia* was discovered through the concentration reducing of norethindrone in water. It also discovered that the concentration 0.5 mg/L and 1.0 mg/L of norethindrone in water has less toxicity effects on the *T. angustifolia*. These results are the preliminary test of method to identify the potential of *T. angustifolia* in removing excess progestin compounds in natural environment.

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