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## Research Article

# Occurrence of Steroid Sex Hormone Progesterone in Influent and Effluent of Oxidation Pond and the River Outlet of Waste Water Treatment Case Study

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## Abstract

**Background and Objective:** This studied about the occurrence of Endocrine Disrupting Compound (EDC) in interest of steroid sex hormone progesterone excretion in the environment, that focusing on water resource in terms of effluent and influent of the waste water treatment into the outlet river and environment. **Materials and Methods:** A method called dispersive liquid-liquid micro extraction with solidification of floating organic drop followed by High Performance Liquid Chromatography (HPLC) was used for determination of progesterone. The water sample was obtained through grab sample from the influent and effluent of waste water treatment-oxidation pond and the outlet river (Kalansanan river) where the effluent was discharged. **Results:** Based on the result obtained it is found that there were few detections and occurrences of steroid sex hormone progesterone in the study with detection range from lowest concentration of  $4.278 \pm 7.411 \text{ ng mL}^{-1}$  and the highest concentration recorded at  $16.687 \pm 6.233 \text{ ng mL}^{-1}$  and the average concentration of progesterone is at  $6.356 \pm 3.112 \text{ ng mL}^{-1}$ . The highest detection of the progesterone was recorded in the effluent site sampling that indicates that the conventional treatment plant was not able to remove steroid sex hormone progesterone effectively and efficiently. **Conclusion:** From that point of the study, monitoring on the progesterone presence and status should be done due to the presence of progesterone even in nanogram per milliliter in environment bring and posed a life threat towards the living organism in the environment.

**Key words:** Endocrine disrupting compound, steroid hormone, progesterone, high performance liquid chromatography, oxidation pond, waste water treatment

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The key amongst the emergence of this environmental concern are the Endocrine Disrupting Compound (EDCs). The presence of the endocrine disrupting compound in the ecosystem has become an alarming issue towards the world community now due to the presence of EDC in the environment<sup>1</sup>. In waste water treatment system the techniques that being used using membrane filtration which based on reverse osmosis technique has limited performance<sup>2-4</sup>. This membrane technique, which has large pore size will allow the EDCs to pass through and present even in the treated waste water<sup>5</sup>. Another kind of treatment in waste water that is Advanced Oxidation Process (AOPs) which include the ozonation and non-thermal plasma also has few limitation<sup>6,7</sup> which it required high energy consumption and at the same time there is interference of the radical scavengers and the effects of the oxidation product that have not being understood yet that made the treatment system to less effective and understandable in removing EDCs in waste water<sup>8-10</sup>. According to United States Environmental Protection Agency<sup>11</sup>, EDC is defined as an exogenous agent that interfering with the production, release, transport, metabolism, binding, action or elimination of natural hormone responsible for the maintenance of homeostasis and the regulation of developmental processes<sup>12</sup>.

The EDCs interact with the endocrine system by mimicking or blocking the natural hormones and it will influence the abnormalities of production, secretion or metabolism of the endogenous hormones and their nuclear receptor towards the body function and system<sup>13</sup>. According to Molina-Molina *et al.*<sup>14</sup> the interference with the nuclear receptor-mediated hormone signaling that will cause the health effects. Generally, EDC is the compound that is associated towards the cases of infertility, breast cancer, stunts of growth patterns in human (development dysfunction) and the immune and neurological disorder<sup>15,16</sup>. The EDC is being released from the chemical factory, pharmaceutical industry, animal farming, agricultural activity, waste water treatment plant and natural human waste, such as menstrual excretion, urine and feces discharge. According to Kannel *et al.*<sup>17</sup> steroid hormone is a very wide group of biologically active endocrine disruptor compounds that control many functions of the endocrine systems and all of it cyclopentane-perhydro-phenanthrene structure was being derived from the cholesterol<sup>9,18</sup>.

On the other hand, steroid sex hormone is part of EDCs compounds that interfere with reproduction and development, induce hermaphroditism of aquatic organisms<sup>19</sup>

and increase the possibility of breast and testicular cancers in humans<sup>20</sup>. Steroids hormone and their metabolites are constantly discharged into environment due to their incomplete removal in waste water treatment plants or direct excretion and discharge. The presence steroid sex hormone of the human in the surface water was firstly being reported in 1965, which indicating that the steroid compound is not being completely remove and eliminated from waste water treatment system<sup>21</sup>, thus into the last extent of waste water discharge point that will reached into the surface water outlet, such as streams and rivers. Progesterone known as the gestagens is among one of the main steroid hormone compound that regularly presence in the environment system<sup>22</sup> derived from pharmaceuticals, steroids and hormones consumptions and its excretions during the menstrual and oestrous cycle and acts as progestins to maintain pregnancy<sup>23</sup>.

The waste water treatment process consists of 3 stages, which are preliminary, primary and secondary treatment<sup>11</sup>. During the preliminary treatment, raw waste water influent passes through bar screens which are metal rods immersed in the incoming flow to separate the large objects. The liquid enters a large settling tank where heavy solids will settle at the bottom of the basin<sup>24</sup>. For the primary treatment a skimming process continues by slowly skimming the top of the water in order to remove fats, oils and grease. Next the remaining water flows over the weirs and into an aeration basin. Air is added into the aeration basin to create an environment for microorganisms to grow and continue treat the pollutants in the waste water. For the secondary treatment, chlorination of the waste water reduces the number of bacteria to a safe level followed by dechlorination so as to minimize any potential toxic effect of the disinfectant<sup>25</sup>. Filtration and purification are also carried out. These steps are conducted in order to remove harmful constituents, such as fecal coliform and EDC steroid hormones. However, few studies showed that there could not be a complete removal of EDC steroid hormones foremost despite these few processes in the system. According to Lei *et al.*<sup>26</sup>, the average removal efficiencies for targeted EDCs for the waste water treatment system varied from 30-82%. This indicates that environmental endocrine disrupting compounds including steroid hormone are not completely removed during reclaimed water treatment and may be carried over into the general aquatic environment.

Over the last half century, the sewerage industry of waste water treatment system in most part of countries have started to improve especially after the 20th century as before that there was no proper sewage treatment due to a small

number of populations and lacked of the urbanization development<sup>27</sup>. Small number of population during the period has caused lower and lacked of awareness regarding the need of proper sanitation system in their country<sup>28</sup>. The lack of concerned and awareness eventually lead to lack of facilities improvement to their sanitation quality and sewerage system in their country. Now a days, in modern era for all countries that it is a need and mandatory for their township and urban areas to have their waste water to be treated before being discharge into the surface water. However, substantial amounts of medication, such as steroids sex hormone have been found excreted unmodified and travel via urine and feces into waste water course as the compounds are commonly detected at elevated level in waste water influents<sup>29</sup>. A wide range of waste water treatment practices are employed worldwide to remove these compounds, nevertheless steroid hormone presence still has often been reported in waste water plant effluents and outlet rivers<sup>30,31</sup> and it becomes such a big concern as surface water is the main drinking water source for human consumption in a watercourse cycle that will bring a serious health effect to the living organism, thus to the environment<sup>21,32</sup>. Therefore, the aim of the study is to determine the concentrations of the sex steroid hormone progesterone in waste water influent, effluent and river outlet.

## **MATERIALS AND METHODS**

The oxidation pond of waste water treatment plant for 3 main parts the influent (for the untreated waste water), effluent (treated) and the outlet river (Kalansanan river that was discharged into the river) for the after treatment product of the plant system were analysed for the occurrence steroid sex hormone progesterone in this study. Kalansanan river was the surface water where the effluent was discharged. Next to Kalansanan river with 500 m distance is Kalansanan village that located very near to the oxidation pond. This study area involved the Northern part of Borneo island in Malaysia. Twelve sampling locations were chosen for each influent, effluent and outlet river. Oxidation pond was chosen for the study as it was the last stage of the waste water treatment system that will produce the final effluent product into the watercourse cycle.

**List of chemicals, standards and apparatus for progesterone analysis:** The chemicals and standard that were used in the analytical method for determination of

progesterone concentration in waste water and river water sample are Dimethyl Sulfoxide (DMSO) at >99.99% concentrations, sodium thiosulfate pentahydrate ( $\text{Na}_2\text{O}_3\text{S}_2 \cdot 5\text{H}_2\text{O}$ ), 1-undecanol, methanol, acetonitrile and eionized water purified in milli-Q water purification, sodium chloride (NaCl) and progesterone (98%). Instrument that been used in this study is HPLC.

### **Instrumentation and optimization for analysis of progesterone in waste water sample:**

The HPLC system was equipped with the photo iodide array detector (4 August, 2016 CQuity HPLC photodiode array), 50 mL injection loop and auto sampler. Analytes were separated on HPLC phenyl column (1.7  $\mu\text{m}$ , 2.1  $\times$  100 mm). The column temperature was adjusted at 30°C and the wavelength was adjusted to 280 nm (UV detector with fixed wavelength can be used). The separation gradient for the acetonitrile-water mobile phase started with 5% acetonitrile. Secondly, the mobile phase was slowly being increased to 40% over 2 min and followed by the linear increase to 50% over 4 min and increased to 60% over 1 min. Separation gradient method was not the isocratic method because separation gradient method was able to detect more than one peaks of the interest compound at one time. The flow rate was kept at 0.4 mL min<sup>-1</sup>. During the actual analysis the flow rate was adjusted to 1.5 mL min<sup>-1</sup> so that more amounts of sample and mobile phase can be injected and analysed. The mobile phases were pre-treated by filtering through the 0.22  $\mu\text{m}$  membrane of nylon filter media and vial was used to place the sample<sup>33</sup>.

### **Analysis for progesterone in waste water and river water sample:**

There will be a total of 3 replications sample taken from every sampling location including waste water effluents sites by using the pyrex bottle glass. Grab sampling method will be used to collect the water sample. The volume taken for each of the sample will be 1000 mL. The bottle is directly wrapped with aluminum foil and kept in cooler box. All of the samples will be placed in the cooler box with an icepack (~4°C) after the samples being taken from the field. The samples are refrigerated at 4°C once reach to the laboratory. Solvents are added as the preservatives. The sample pH will be adjusted to the values between 2 and 3 with hydrochloric acid (HCL). This step is taken in order to prevent the samples from spoil and reduced the activity of the bacteria<sup>11</sup>. For the determination of progesterone concentration in waste water sample (influent and effluent) and river water sample the steps.

**Standard stock solution preparation:** The standard stock solution (progesterone) was prepared at 2000 mg L<sup>-1</sup> in acetonitrile. Each of the solution need to be further diluted to the appropriate concentration using acetonitrile. The solution was maintained at 4°C. For standard calibration curve, the concentrations that were chosen were 10, 100 and 200 µg mL<sup>-1</sup> since it gave the R<sup>2</sup> which was nearly 1. The retention time for the detection of progesterone was at 3.83±0.05 min.

**Sample preparation:** About 251.1 mg Na<sub>2</sub>O<sub>3</sub>S<sub>2</sub>·5HO was added into the waste water and river water sample and the resulting solution was filtered. The waste water sample was filtered through the 0.45 µm membrane prior to extraction.

**Sample extraction:** The water samples were filtered by using normal filtration before being filtered with microfiltration with the membrane filters of 0.45 µm. Dispersive Liquid-liquid Micro Extraction Solid Floating Organic Drop (DLLME-SFO) was performed by injecting a mixture (10 mL extraction solvent which is 1-undecanol and 200 mL of dispersive solvent which is methanol) into the 5 mL water samples spiked with 50 µg L<sup>-1</sup> of analyte (progesterone). After formation of the cloudy solution, liquid organic drop was formed and floated on the surface. The glass screw-capped test tube was cooled in an ice bath for a few min. The liquid organic drop was frozen to solid phase before to the end of the step. The solid organic drop was easily being sucked up from the surface of water sample by a syringe. At room temperature the solid organic drop melted quickly in the microtube. In order to separate water and organic solvent the whole drop was transferred to a microtube by a syringe. About 2 mL of the organic solvent (which was on the upper portion of the solvent in the microtube) need to be transferred into an auto-sampler vial and was mixed with the 35 mL DMSO by a syringe and 25 µL of this mixture was injected into the HPLC system for the water sample analysis. The extraction of the samples can be summarized by the flow chart as shown in Fig. 1.

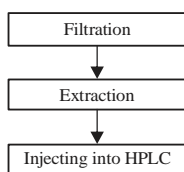


Fig. 1: Summary of sample extraction

## RESULTS AND DISCUSSIONS

Before the sample analysis was done it was very important to do the data validation in order to obtain more precise result especially dealing with lower value of concentrations such as steroid hormones compound. Thus, in order to obtain that, calibration curve for the standard solution (progesterone), the Limit of Detection (LOD), Limit of Quantification (LOQ) and accuracy were calculated and were stated as in Fig. 2 and Table 1 and 2, respectively. Figure 1 shows the concentration of progesterone form station 1 until station 12.

**Steroid sex hormone progesterone occurrences in influent, effluent and outlet river:** Based on Fig. 3, the range of detection was from the range 4.278-16.687 ng mL<sup>-1</sup>. The lowest detection of progesterone was at influent station 2 with detection 4.278±7.411 ng mL<sup>-1</sup> and the highest detection was at effluent station 6 with detection of

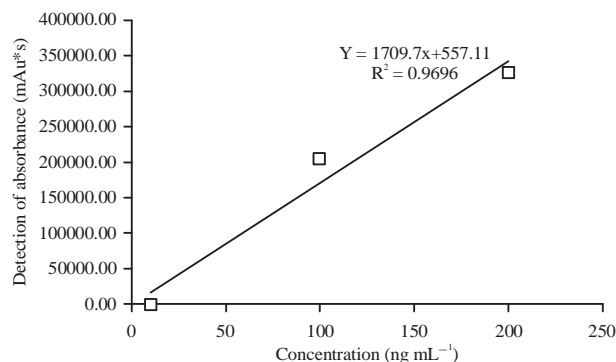


Fig. 2: Calibration curve for the standard solution

Table 1: Recovery of HPLC machine

Concentration (ng mL <sup>-1</sup> )	Retention time (min)	Area (mAU*s)	Found	Recovery
10.00	3.83	188.43000	-0.215640171	-2.156402
100.00	3.84	204713.90000	119.410885	119.410880
200.00	3.82	326781.20000	190.807796	95.403898
Average	3.83	177227.84333	103.334347	70.886127
Standard deviation	0.01	165022.18589	96.521136	64.385496

Table 2: Validation of data

Accuracy	70.89±52.57%
Slope	1709.7
Intercept	557.11
Linearity	Y = 1709.7x+557.1
Correlation coefficient (R <sup>2</sup> )	0.969
Number	3
Standard error of Intercept	39.60
LOD	0.08 ng mL <sup>-1</sup>
LOQ	0.23 ng mL <sup>-1</sup>

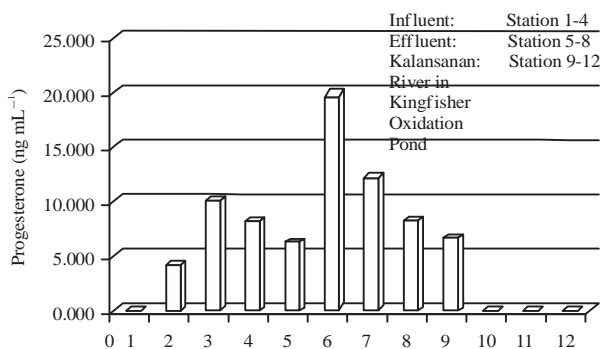


Fig. 3: Progesterone concentration levels from each station

16.687 ± 6.233 ng mL<sup>-1</sup>. The total average concentration of progesterone for all stations is 6.356 ± 3.112 ng mL<sup>-1</sup>. There was non-detection of progesterone in station 1 influent, while station 3 of influent recorded at 10.148 ± 17.577 ng mL<sup>-1</sup> and station 4 influent recorded 8.294 ± 7.407 ng mL<sup>-1</sup>. Station 5 of effluent recorded 6.263 ± 4.791 ng mL<sup>-1</sup>, station 7 of effluent recorded at 12.217 ± 21.160 ng mL<sup>-1</sup> and station 8 of effluent was 8.310 ± 9.261 ng mL<sup>-1</sup>. The detection of progesterone in station 9 of Kalansanan river was 6.741 ± 6.693 ng mL<sup>-1</sup>. The other stations, which were station 10-12 showed non-detection of progesterone. The non-detection of the progesterone in station 1 of influent and station 10, 11 and 12 of Kalansanan river was due to below and lower detection limit (Limit of Detection-LOD) as stated in Table 2. Based on the result obtained the difference of progesterone across the stations were significant with value of  $p = 0.042$  ( $p < 0.05$ ). There was no standard being set up and provided by Malaysian Department of Environment and also WHO regarding the progesterone concentration in waste water and river. Previous study done in overseas however may support the result obtained from this study as for comparison. However, from this study it is found that all of the progesterone concentrations were detected higher compared to the previous literatures or study.

Progesterone is one the steroid hormone that is being divided into 2 which are naturally produced and synthetically progesterone<sup>34</sup>. Example of naturally produced progesterone was in the corpus luteum which present normally in menstrual and oestrus cycle to suppress the ovulation<sup>35</sup> and synthetically produced in oral contraceptives<sup>22</sup>. According to Sneader<sup>22</sup>, the progesterone is being excreted from our body through urine and faeces. Thus, based on this fact a correlation analysis was done to investigate the relationship between the progesterone presences with faeces in this study. From the result it was found that correlation between progesterone

and faecal coliform showed a moderately strong negative relationship with coefficient value of  $R = -0.498$  ( $p = 0.046$ ). Negative relationship between both parameters indicates that if there was a higher amount of faecal coliform then it will be less amounts of progesterone concentrations detected. This was due to the fact that faecal coliform can degrade progesterone. There were few considerations that need to be taken regarding the correlation between faecal coliform and progesterone. Firstly, even there might be higher amount of faecal coliform need to understand that the presence of this progesterone was from the women who consumed contraceptive pill or originally discharge from the women body through menstrual and oestrus cycle. Thus, logically if the theory by Sneader<sup>22</sup> was taken into consideration a man faeces will not contained the progesterone as men did not consume and produced progesterone naturally.

Based on the previous study, the progesterone concentration is normally higher in influent and lowered in effluent. However, if the amount of progesterone was higher in the effluent compared to the influent, biological conversion was said to happen<sup>1</sup>. This indicates that the treatment plant cannot remove the progesterone effectively. In another cases, the progesterone might undergoing sorption and biodegradation process during the waste water treatment<sup>36</sup>. Biological conversion might explain the highest detection of progesterone in station 6 and 7 of effluent. In the cases of this study, biological conversion could happen during the retention time of the waste water in the maturation pond before it was discharged as the effluent. Maturation pond was the last stage for the waste water treatment before it was discharge as effluent into the outlet river. The retention time for waste water in the oxidation pond was about 2.5 days. Supposedly, the amount of progesterone should be lower in the effluent after treatment. Station 8 of effluent was the end point for the effluent before it was discharge into Kalansanan river. Station 9 was the first station in Kalansanan river and was very near to the outfall of discharge point. Station 9 recorded almost the same amount of progesterone as station 8. According to Cao *et al.*<sup>37</sup>, the amount of progesterone detected in the river was at 9 ng L<sup>-1</sup>. It was found that the result obtained in Kalansanan river was lower compared to the previous study. Based on the result obtained in Kalansanan river especially station 9 might be due to 5 possibilities. Firstly was due to sorption<sup>38-40</sup> secondly, there was the presence of faeces originated from the improper drainage of sanitary system<sup>41-43</sup> originated from Kalansanan village residents, thirdly was the discharge of the mixture of treated waste

water and naturally attenuated untreated waste water<sup>44</sup>, fourthly was the discharge of other sources, such as the untreated sewage in the upstream of the river<sup>45</sup> and lastly was de-conjugation of the glucuronide and sulphate conjugated steroids that occurred in the waste water treatment processes<sup>46,47</sup>.

## CONCLUSIONS

In conclusion from the result obtained the waste water treatment plant of oxidation pond was not able to remove the steroid sex hormone progesterone efficiently since there was still a presence and detection value in the effluent and was even higher than the influent. Future study in monitoring the occurrence of progesterone should be done more often in the future. Monitoring in terms of waste water quality status was needed as the discharged effluents which was not complying the standard can affecting the aquatic ecosystem and the watercourse cycle. Monitoring for progesterone concentration or other steroid sex hormones in the environment is very much in need because progesterone itself can undergo sorption and biological conversion that its concentration even in trace level could affect badly on the water ecosystem mainly.

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