MNK Publication

International Journal of Latest Research in Science and Technology **ISSN (Online):2278-5299**
Volume 5, Issue2: Page No59-64, March-April 2016
https://www.mnkpublication.com/journal/ijlrst/index.php

THE EFFECTIVENESS OF A CONSTRUCTED WETLAND IN REDUCING THE LEVELS OF ESTROGENIC-ENDOCRINE DISRUPTING COMPOUNDS (EEDC) IN AGRICULTURAL RUNOFF

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Abstract- Observations have shown the detrimental effects of estrogenic-endocrine disrupting compounds (EEDC) in aquatic ecosystems. These endocrine disruptors remain biologically active at low, environmentally relevant doses, can then be absorbed in vertebrates, and interfere with the normal function of the endocrine system of humans and animals. Two of the major sources of EEDCs in the aquatic environment are Publically Owned Water Treatment Plants (POWTP) and agricultural activity. The objective of this study was to determine the effectiveness of a constructed wetland in ameliorating agricultural runoff containing EEDCs. Water samples were collected from a constructed wetland, a POWTP, and a public water supply lake. The water samples were prepared by solid phase extraction, and the concentrations of the estrogenic steroid hormones estrone, 17β-estradiol, estriol, progesterone, and 17α-ethynylestradiol were determined by HPLC. The results showed that the constructed wetland reduced the concentrations of each of the EEDCs entering the wetland and there was an average reduction of 70% in EEDC concentration before the runoff water was released into the environment. The concentration of EEDCs entering the watershed from the constructed wetland was about almost ten times lower than the concentration entering the watershed from the POWTP.

Key Words: wetland, estrogens, progesterone, endocrine disrupting compounds, manure fertilizer, HPLC, watershed

1. INTRODUCTION

Starting in the 1990s, researchers began to observe the detrimental effects of certain residues on aquatic ecosystems. It was hypothesized that, when brought into direct contact, some of these contaminants may interfere with the normal function of the endocrine system of humans and animals.This was confirmed to be the case in fish populations located around effluent discharges [1]. The term endocrine disrupting compounds (EDCs) was given to this class of chemicals. EDCs are defined as any chemical present in the environment which adversely alters the normal function of an organism's endocrine system. This change in the normal function of an organism's endocrine system can lead to a variety of complicationsranging from alterations to the reproductive system to defects in the growth and development [2]. Once they enter an aquatic ecosystem, the lifespan of EDCs can range from a matter of minutes to a permanent presence [3]. If not dealt with in a proper and timely manner, EDCs have the potential to cause significant ecological problems [4].

Currently there is estimated to be over 200 plant and animal species known or suspected to be affected by EDCs [5];however, not all EDCs have the same effect on all species. For example, steroidal estrogens have been shown to have a potent effect on fish but little effect on invertebrates such as copepods [6]. Because fish are one of the most thoroughly studied groups of wildlife, in terms of the effect of chemicals on developmental and reproductive processes, they are the ideal biological indicator for determining if contaminants are present in an aquatic environment [7]). Reproductive disorders in fish due to endocrine disruption have been observed in several recent studies [8]. Work by Jobling *et al.* [9]with wild populations of a fishcalled theriver roach (*Rutilusrutilus)*demonstrated that small amounts of testosterone blocking chemicals from wastewater effluent led to feminization of the male species of river roach. Research conducted by Alvarez *et al.*[10] found that populations of smallmouth bass (*Micropterusdolomieu*) and largemouth bass (*Micropterussalmoides*) in the Potomac River showed similar signs of intersex crossover, along with the production of oocytes in male testes as observed by Guy *et al.*[11]. Since then, a large amount of research has been devoted to looking at the occurrence of intersex crossover and other developmental issues concerning aquatic species located in US watersheds receiving direct sources of EDCs.

In many classes of EDCs, the critical concentration of the contaminant has to be fairly high to elicit a change in the endocrine system of an organism. The class of compounds which are of primary concern are the compounds which cause changes to organisms at very low concentration$\geq$10 ng/L[12].Government and independent researchers have identified the estrogenic-endocrine disrupting class of compounds (EEDC) as the largest known group of endocrine disruptors that remain biologically active at low, environmentally relevant doses [13]. Although there are

Publication History
Manuscript Received : 15 April 2016
Manuscript Accepted : 22 April 2016
Revision Received : 26 April 2016
Manuscript Published : 30 April 2016

numerous compounds within this class, scientists have found that the estrogenic steroids estrone (E1), 17β-estradiol (E2), and estriol (E3) are the primary contaminants that affect a wide range of species at low doses. In addition, a number of studies have shown that, the synthetic steroid, 17α-ethynylestradiol (EE2), and another natural steroid, progesterone (P4), regularly occur along with E1, E2, and E3 [14]. These estrogenic compounds can then be absorbed in vertebrates and cause disruption through the targeting of endocrine receptors [15].

Research conducted by Jobling *et al*.[9] and Guy *et al*,[11] has indicated that the highest known incidences of EEDCs in waterways were from effluents from publicly owned water treatment plants (POWTP), but agricultural activityalso contributes significantly to theEEDC load because a large amount of material is swept into drainage ditches and estuaries during rain events. In this runoff material, there is an array of EEDCchemicals ranging from pesticides such as tributyltin (TBT) to fungicides and herbicides that include atrazine, diazinon and permethrin [16].Although these compounds are known for their endocrine disrupting effects, it is a side note to the focus of this study. The concern of this study was the use of chicken litter as well as the manure from the cattle and swine industries as fertilizer [17]. This nitrogen rich manure is a good substitute for the higher pricedcommercial nitrogen fertilizers, but these substances are also rich in estrogenic compounds, which after a rain event,can be washed into nearby watersheds where they can have an impact on aquatic organisms such as fish [18]. E1, E2, E3, and P4 are the estrogenic compounds most often found in this naturally produced nitrogen rich fertilizer [19]. The compounds are synthesized in the animals during ovulation and, like many other steroids, are excreted in the wastes. Poultry litter is very high in nitrogen content and is therefore the preferred fertilizer by farmers [20]. However, research performed by Shemesh and Shore [21] showed that while E2 concentration ranged from 14 to 533 ng/g in dry poultry litter, the averagewas 44 ng/g. The other major agricultural source of hormone steroids is livestock waste.Erb*et al.*[22] determined that the average level of E2 in the urine of cattle was 13ng/l; although this is much less than poultry litter, it is constantly produced as the animals graze in pastures. Estrogenexcretion by livestock in the United States was estimated at 45 tons annually [23],several-fold higher than was estimated for humans[24].These steroidal compounds degrade slowly in the environment which allows for sufficient time to become absorbed by other organisms in the area [25]. Once a rain eventor crop irrigation occurs, the compounds are concentrated into field drainage ditches and eventually make it to estuaries, lakes, and rivers [1].

Typically, wetlands have been shown to successfully ameliorate water contaminated with phosphorus, nitrogen, hydrocarbons, animal waste, and heavy metals,and constructed wetlands have been found to be an effective option for on-site wastewater treatment when properly designed, installed, and maintained. Constructed wetlands are wetlands created from non-wetland sites for the purpose of treating wastewater [26]. Constructed wetlands consist of saturated substrates, emergent and submergent vegetation, invertebrates and vertebrates, aerobic and anaerobic microbial populations, and a water column [26]. The objective of this study was to determine the effectiveness of a constructed wetland in ameliorating agricultural runoff containing EEDCs. The Louisiana State University Agricultural Center Red River Research Station provided an ideal location forthis study. The Red River Research Station (RRRS) consists of 162 ha of agricultural land in the Red River Basin of northwest Louisiana [27]. At this research station,cotton, soybean and sweet peas are the major crops and field testing of the effect of different pesticides and fertilizers, including the application of poultry litter, on a variety of crops has been conducted in multiple field plot locations. Since 1998, the Red River Research Station has been conducting research to identify practices that minimize the impact of agricultural production on the quality of runoff water[27] utilizing a constructed wetlands located in the southeastern corner. Approximately 80% of the runoff water from the RRRS flows through this constructed wetland before eventually draining into a nearby river. Studies have shown that a constructed wetland on the site improves the water quality from runoff from the nearby farmland[28], but there have been no studies on the effectiveness of the constructed wetland on reducing the discharge of EEDCs.

11. METHODS AND MATERIALS

Sampling Sites: The RRRS consists of cultivated fields and pasture land. The cultivated fields are intersected by three drainage canals to control excessive rainfall.One of these canals also drains pasture land that houses a small cow/calf herd (less than 100 animals), and it was chosen to be the first sampling site (Figure 1). Discharge water from the cultivated acreage and most of the pasture land on the station flows through these canals to the southeastern corner where they enter the first of two man-made ponds as shown in Figure 1[28].Thedrainage canals enter a shallow pond which then feeds into a larger, deeper pond. The deeper pond serves as a holding tank to contain the field runoff before entering the Flat River which is less than a third of a mile away. These two locations were the second (shallow pond) and third (deep pond) sampling sites (Figure 1).

For comparison purposes, water samples were also collected from two other sites. The fourth sample site was immediately downstream to the Lucas Water Treatment Plant (a POWTP)located on the Red River in Southeastern Shreveport, LA. The Lucas Water Treatment Plantis responsible for the treatment of a large portion of the City of Shreveport's sewage. the city of Shreveport, LA operates two wastewater treatment plants at the Lucas station with a combined capacity of about 51.4 million gallons per day with a peak hydraulic flow of 132 million gallons per day. Wastewater collection is provided for about two hundred thousand people through 1,024 miles of city sewer mains and 115 lift stations. Since POWTPs are reported to have some of the highest known incidences of EEDCs in waterways[9, 11], it was of interest to compare the results from the constructed wetland at RRRS with this facility. The fifth sampling site was Cross Lake located in Shreveport. It is a 3,470 hectare man-made lake built in 1926 [29]. The lake is the primary source of potable water for the City of Shreveport. This lake receives no effluents from industry or sewage and very little agricultural runoff; however, it is heavily used for recreation such as fishing, boating, and hunting. Hence, it was expected to have low levels of EEDCs as compared to RRRS or the POWTP.

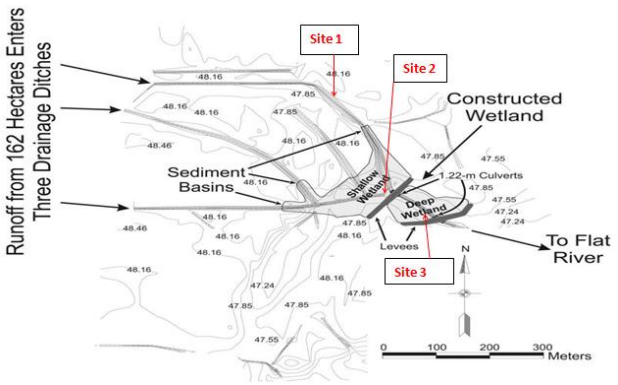

Figure 1: Location of construction wetlands and the pathway of discharge to Flat River [28]

Sampling and Sample Preparation:

All samples were collected after rain events because prior to rain events, the water levels at some of the sites were too low to permit accurate sampling. Samples were obtained each month starting in September 2011 and continued to June 2012. Although the rain events may have not occurred on the same day of each month, the majority of the samples were obtained within the first two weeks of each month. Collection of the sample was accomplished by means of an alpha water sampler unit made by Wildco, model 1130-G45. The sample was transferred from the alpha jar to a 1000 mL amber glass bottle and stored for 4^{o}C for transfer to the lab [30], and the sample was prepared for analysis within 24 hours of collection [31]. In preparation for solid phase extraction (SPE), the samples were first passed through a series of filters to remove undesired debris and organisms from the samples [32]. After the desired clarity was achieved using a Buckner funnel with Whatman47mm 541 hardened ashless paper, a Millipore 0.45 micron pore size 47mm diameter filter attached to a vacuum [33] served to remove fine-contaminant material removal filter for the sample.

Chemicals:

High pressure liquid chromatography reagents were purchased from Fisher Scientific (USA). These consisted of HPLC grade methanol(≥99.9% purity) and HPLC grade acetonitrile (≥99.9% purity). These two reagents were used in the preparation of the SPEcartridges as well as the mobile phase of the HPLC. The standards which were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) consisted of the compounds estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-Ethynylestradiol (EE2), and progesterone (P4). In the preparation of these analytes as standards for analysis, multiple dilutions were made from a stock prepared by dissolving microgram amounts of the compounds in theHPLC grade water used for calibrating the equipment [33].

Solid Phase Extraction (SPE):

The primary means of collecting EEDCs from water samples was achieved through the use of silica bed cartridges [34]. Concentration of the samples was performed using custom built semi-automated equipment in conjunction with the SupelcleanENVI-18 Environmental Cartridges with a 500 mg bed weight produced by Supelco Analytical USA. Conditioning of a clean cartridge was performed by the application of 5 mL of methanol followed by 5 mL of deionized water at a flow rate of 3 mL/min [33] under a vacuum of 15 mm Hg. Conditioned cartridges were loaded into a custom built semi-automatic vacuum manifold and processed simultaneously. 500 mL of the sample was passed though the ENVI-18 cartridge at a flow rate of 3 mL/min under a 20 mm Hg vacuum. The cartridge was then dried under a vacuum an additional two hours [35]. The dried cartridges were removed from the vacuum manifold, and the extracts were eluted with 8.0 mL HPLC grade methanoldispensed in two steps of 4mL each with a 5 minute delay between steps [33]. As the eluent exited the cartridge,it was collected and transferred to a conical drying tubewhere nitrogen gas was then injected to drive off 98% of the methanol and any residual water.The dried extracts were brought up to a final volume of 100µL with a mixture of HPLC grade acetonitrile/methanol/deionized water in a 15/40/45% ratio and stored at -18^{o}C until ready for HPLC analysis.

Chromatographic and Analysis Conditions:

The DionexHPLCUltiMate 3000system was used for separation and identification of the various EEDCs. The system was equipped with a 4.6X150mm 3µm particle size Dionex Acclaim Phenyl-1 column with a surface area of 300 m^2/g and a pore size of 120 angstroms [36].An isocratic mobile phase of acetonitrile/methanol/and deionized water in a 15/40/45% ratio was run at 5300 PSI with s flow rate of 1.2 mL/min and a temperature of 40^{o}C.The sample injection volume was 10µL, and the run time was 20 minutes. The UV detector wavelengths were set at 210nm for E2, 220nm for EE2, 240nm for P4, and 281nm for E1 and E3.

Peak Identification andQuantification and Data Analysis:

Peaks were integrated using Dionex Corporation's Chromeleon 7 (version 1.7.3). Sample peaks were identified according to retention times and quantified according to integrated peak areas based on standard curves generated from serial dilutions of crystalline standards of E1, E2, E3, P4, and EE2 ranging from 0.05 – 50 ng/L. All data points are based on a mean of the measurements taken from a minimum of three samples. All data were subjected to a one-way analysis of variance, and significance was determined at the 95% confidence limits. Samples were collected and analyzed monthly over a ten-month period (September 2011 – June 2012), and there was some variation in the steroid levels from month to month, presumably due to differences in rainfall; however, the trend in the changes in steroid concentrations remained constant throughout the sampling period. Hence, in order to simplify the presentation of the data, the average of the steroid concentrations in the water samples collected over a three-month period (April, May, and June, 2013) will be presented in the results as representative of all the measurements made during the ten-month study.

III. RESULTS AND DISCUSSION

The concentrations of E1, E2, E3, P4, and EE2 associated with the RRRSconstructed wetlands are shown in Figures 2 and 3, and the concentrations of these hormones in the samples collected at the Lucas Water Treatment Plant are presented in Table 1.

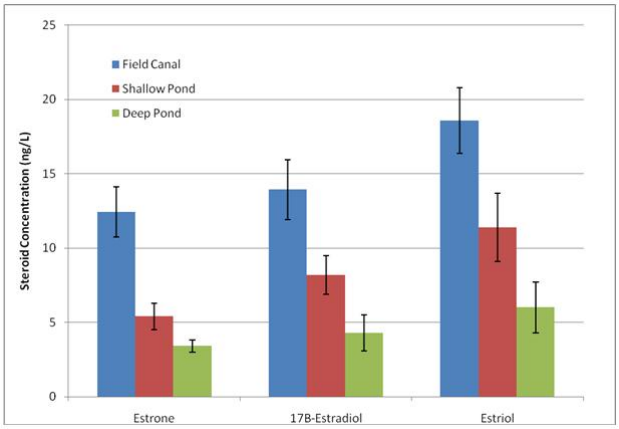

Figure 2: Average Estrone (E1), 17β-Estradiol (E2), and Estriol (E3) concentrations (ng//L ±SE)

in the filed canal, shallow pond, and deep pond of the constructed wetland locatedat LSU Agricultural Center Red River Research Station in Bossier Parish, Louisiana

Table 1: Average Estrone (E1), 17β-Estradiol (E2), Estriol (E3), Progesterone (P4), and 17α-Ethynyl-estradiol concentrations (ng//L ±SE) at the Lucas Water Treatment Plant.

Location	Estrone	17β-Estradiol	Estriol	Progesterone	17α-Ethynylestradiol
Lucas Water Treatment Plant	6.3±1.3	27.5±3.6	31.4±4.1	14.6±2.7	21.2±3.5

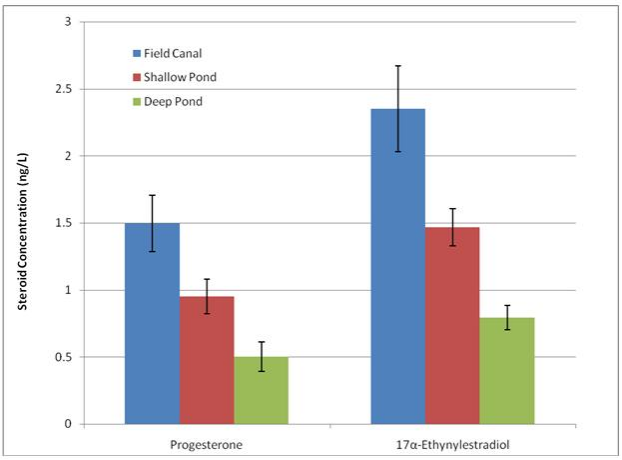

Figure 3: Average Progesterone (P4) and 17α-Ethynylestradiol (EE2) concentrations (ng//L ±SE)

in the filed canal, shallow pond, and deep pond of the constructed wetland located at LSU Agricultural Center Red River Research Station in Bossier Parish, Louisiana

The steroid concentrations in all the samples collected at Cross Lake were below the detectable limits.

At the constructed wetlands, P4 was the least abundant (ranging from 0.5 – 1.5 ng/L) hormone present, and E3 was the most abundant (ranging from 6.0 – 18.6 ng/L). In all instances, the hormone concentrations decreased significantly as the water traveled through the constructed wetland. The average concentration for all five hormones was 57.8% higher (ranging from 43.5% for E1 to 63.7% for P4) in the drainage canal than the shallow pond. On the other hand, the average concentration for all five hormones was 45.6% lower (ranging from 38.1% for E1 to 47.6% for E2) in the deep pond than in the shallow pond. Overall, this represented an average 69.2% decrease (ranging from 67.7% for P4 to 69.2% for E1) in the total concentration of theses steroidal hormones in the runoff water leaving the RRRS.

When compared to the hormone concentrations for the samples collected at the Lucas Water Treatment Plant, E1 was the only steroid found at concentrations comparable to those observed at the RRRS. The other four hormones were present in significantly higher concentrations in the Lucas Water Treatment Plant samples. In fact the levels of these four hormones at the treatment plant were significantly higher than the levels measured in the runoff water in the drainage canal at the RRRS. The concentrations of P4 and EE2 were 9 – 10 times higher at the Lucas Treatment Plant than in the drainage canal at the RRRS.

In humans, progestagens and androgensserve as the precursors for the production of all estrogens in the female body.The androgenic hormones androstenedione and testosterone are the precursors from which estrone (E1) and 17β-estradiol (E2), respectively. The production of E3 occurs only in the liver and placenta and is a metabolite of E1 and E2 [37]. The progestagenic hormone pregnenoloneis converted to progesterone. During a female's reproductive years, E2,which is responsible for development of the tissues of the reproductive organs, is the predominant estrogen in terms of absolute serum levels (10 to 29% of the circulating estrogens) and estrogenic activity in the endocrine system[38]. At equal concentrations, E2 has been found to be ten times as powerful as E1 and about eighty times as powerful as E3 in its estrogenic effects [37]. However, during pregnancy E3 is synthesized in large quantities by the liver and placenta, accounting for 60 to 80% of circulating estrogens in the body [39]. Once menopause occurs, E1 concentrations are the highest of the three estrogenic steroids circulating throughout the female body[40]. P4, which is produced in the ovary, maintains the endometrium and secrete proteins to support a fertilized egg once implanted. If no implantation takes place, the production of P4 drops and causes the shedding of the endometrium [41]. However, if an egg does become implanted, P4 production continues in the developing placenta and remains up-regulated throughout the pregnancy.Both naturally occurring and synthetic estrogens are widely used as medicinal drugs [42]. In many oral contraceptives, the active ingredients are a combination of a progestin, such as P4, coupled with 100-300 micrograms of the synthetic hormone, EE2 [43]. The prevention of ovulation is accomplished by EE2 mimicking the role of the natural steroidal estrogen E2

Just as in humans, the physiological development of many vertebrates is controlled by the production of E1, E2, E3, and P4 at the correct times and concentrations. These compounds, both natural and synthetic, elicit a specific response in an organism by acting upon a cell's endocrine receptor [15]. In each instance, the binding of a compound to its target

receptor causes a cellular response. Since the same endocrine compounds and receptors that are found in humans are also present in other vertebrates, the ability of compounds produced in one species to affect the receptors of another species is the mode of action of EDCs [5]. The physiological impact that E1, E2, E3, P4, and EE2 have on the developmental stages of fish has been comprehensively studied in the fathead minnow *(Pimephalespromelas)*, the largemouth bass *(Micropterussalmoides)*, and smallmouth bass *(Micropterusdolomieu)*[11,44]. In one largemouth bass study, researchers found large concentrations of egg yolk proteins, primarily vitellogenin (VTG), in the blood of males[45]. Under normal conditions, these VTG proteins are only produced by the liver of sexually maturing female oviparous animals when their eggs are maturing. This lead to the hypothesis, and later conclusion, that male fish were being exposed to EEDCs in their environment[46].

In the present study, the data indicate that significant levels of EEDCs are being released into the Red River Watershed by POWTPs and agricultural activities. Policies such as the Clean Water Act in America has demonstratedthat the implementation of new filtration and detection methodshave reduced contamination of aquatic environments by estrogenic steroids and other endocrine disruptors,but it has not been eliminated. While this study has done nothing to address the problem of EEDC release from POWTPs, it has shown that a constructed wetland, designed and installed to control agricultural field runoff of pesticides and fertilizers, also reduced the levels of endocrine disrupting hormones. The shallow holding pond reduced the hormone concentrations coming in from the drainage canal by an average of more than 40%. A further decrease in the concentration of steroids was observed in the deep pond due to the larger volume of water as well as a larger mass of aquatic vegetation. In the deep pond,the concentration of some of the estrogens such as P4 and EE2 dropped to almost undetectable levels. Before exiting the deep pond the average total concentration of all estrogenic steroids was reduced to 15.01 ng/L, as compared to the average combined concentrations of 48.75 ng/L in the water of the canal emptying into the constructed wetland. This equated to almost a 70% reduction in the concentration of the EEDCs entering the watershed from the RRRS and amounted to almost ten time lower than the levels entering the watershed from the Lucas Water Treatment Plant.

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