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The Quantification of Phthalates in Wastewater Treatment Influent and Effluent

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Abstract

The French Broad River (FBR) serves as the home to several endangered species, raising concern due to the presence of endocrine-active compounds (EAC). Research shows that exposure to EACs in wastewater treatment plant (WWTP) effluent, namely phthalates, induces complications in reproductive physiology of fish and animals. Quantification of EAC concentration in WWTP effluent was conducted in Asheville, NC. Multiple phthalate monoester concentrations have been successfully detected in WWTP effluent above the LOD and LOQ, through the use of solid-phase extraction (SPE) and a Shimadzu Liquid Chromatograph Mass Spectrometer-8040 (LCMS). Moving forward, metabolite concentrations acquired in WWTP influent will be compared to concentrations located in the effluent. This comparison will describe the effectiveness of WWTP removal of EACs, and increase awareness in the community for environmental sustainability. Results indicate successful removal of phthalate monoesters from the wastewater, moving many of the phthalate monoesters below the limit of quantitation (LOQ). Detectable concentrations were found in ng L^{-1}, considerable lower than concentrations found in various other WWTPs detecting concentrations in ug L^{-1} and even mg L^{-1}.

1. Introduction

Phthalates are considered ubiquitous chemicals due to their extensive use in industrial products such as plasticizers, lubricants, solvents, personal care products, children's toys, medical devices, and food containers.[1, 2, 3] Although phthalates are added to provide beneficial properties to the materials in production, they are not covalently bound to polymers making phthalates easily removable from the matrix they are in.[3,4] This gives phthalates the ability to break free from their matrix, exposing humans and the environment to these compounds during use or disposal of the materials. Exposure to phthalates has been reported to induce activity in the endocrine system causing complications with sexual development. Phthalates are exposed to biological systems through several pathways including the atmosphere, indoor dust, food, contact with contaminated water, and direct exposure to the products, making research on phthalates essential.

Compounds that are known to induce activity in the endocrine system include metabolites of: di-(2-ethlyhexyl) phthalate (DEHP), dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP), and benzylbutyl phthalate (BBP).[5] These compounds have been studied for occurrence in WWTP waterways around the world, making the goal of this project to quantify EACs in WWTP influent and effluent in Asheville, NC.[6] Analysis at these sites will better explain the efficiency of EAC removal at the local WWTP. The source of all these chemicals originates from multiple arenas, including industrial waste and phthalate metabolite release coming from human excretion. Therefore, phthalate removal at the WWTP is essential for the protection of the environment.[7]

Phthalates were chosen to study due to their history of being an EAC as well as their major production volume in

industry. Phthalate production in 2006 more than doubled the amount of phthalates produced in 1975 going from 1.8 million tons to over 4.3 million tons.[4] Several compounds of phthalates have been associated with reproductive disruptions, especially in the case of male exposure.[8] Sperm malformations as well as a decrease in semen volume have both been seen with an increased exposure to phthalates.[8]

Considering phthalates are introduced into products that are in direct contact with the body, exposure to phthalates is omnipresent. Guo et al. conducted a study reporting on the frequency of finding phthalate compounds in several "leave-on" products. In the case of fragrances, a 100% detection frequency was discovered; alongside a 90% detection frequency for skin toners.[9] The highest concentrations of phthalates reported were at levels of 1000 ug/g of product. Respectively, adult females are exposed to 0.37 ug/kg body weight when taking into account the median concentrations of phthalate compounds in the products.[9] Epidemiological studies report that prenatal exposure to phthalates can lead to health risks including sex hormone changes, decreased anogenital distance, changes in sex-specific behavioral patterns and reduced sperm count.[10] Not only are phthalates health concerns for humans, phthalates have also shown to be hazardous to aquatic species through the release of waste into the environment.

Aquatic species come into contact with phthalates originating from various sources, one being phthalate metabolites excreted from humans. Specific phthalates have tested positive in human blood (15 to 83.2 μg L^{-1}) along with their metabolites in human urine (2.1 to 44.5 μg L^{-1}).[4] Considering that these compounds have been found in humans, it is essential to determine their effects as well as how they are being transferred into the environment.

When humans are exposed to phthalates, the body follows a two-step metabolic pathway to release the chemical from the body: phase 1 biotransformation and phase 2 conjugation.[10] In phase I biotransformation, the phthalate compound is hydrolyzed from a diester to a monoester. Phase 1 biotransformation is executed by lipases and esterases.[11] This step allows for the compound to become more reactive for further action in phase 2 conjugation. Monoesters then undergo phase 2 conjugation by adding a hydrophilic glucuronide, allowing the conjugated phthalate to be excreted from the body. Phase 2 conjugation is catalyzed by uridine 5′-diphospho- glucuronyl transferase (UGT). Lower molecular weight phthalates can be excreted from the body as free or conjugated monoesters, due to higher hydrophilic properties, whereas higher molecular weight phthalates have the ability to undergo further biotransformation prior to conjugation. In both cases, excretion can occur anytime after the hydrolysis of the ester. This is exemplified in figures 1 and 2.

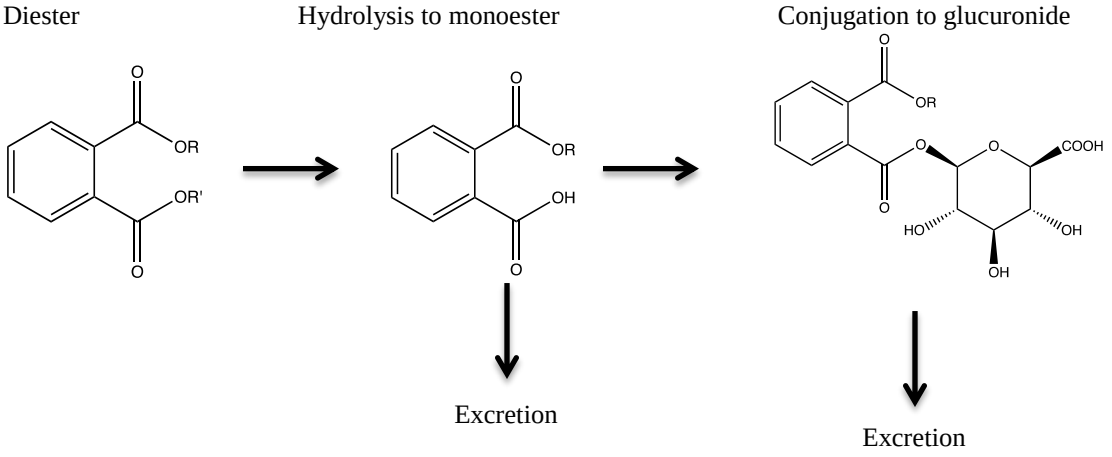


Figure 1 Lower Molecular Weight Pathway

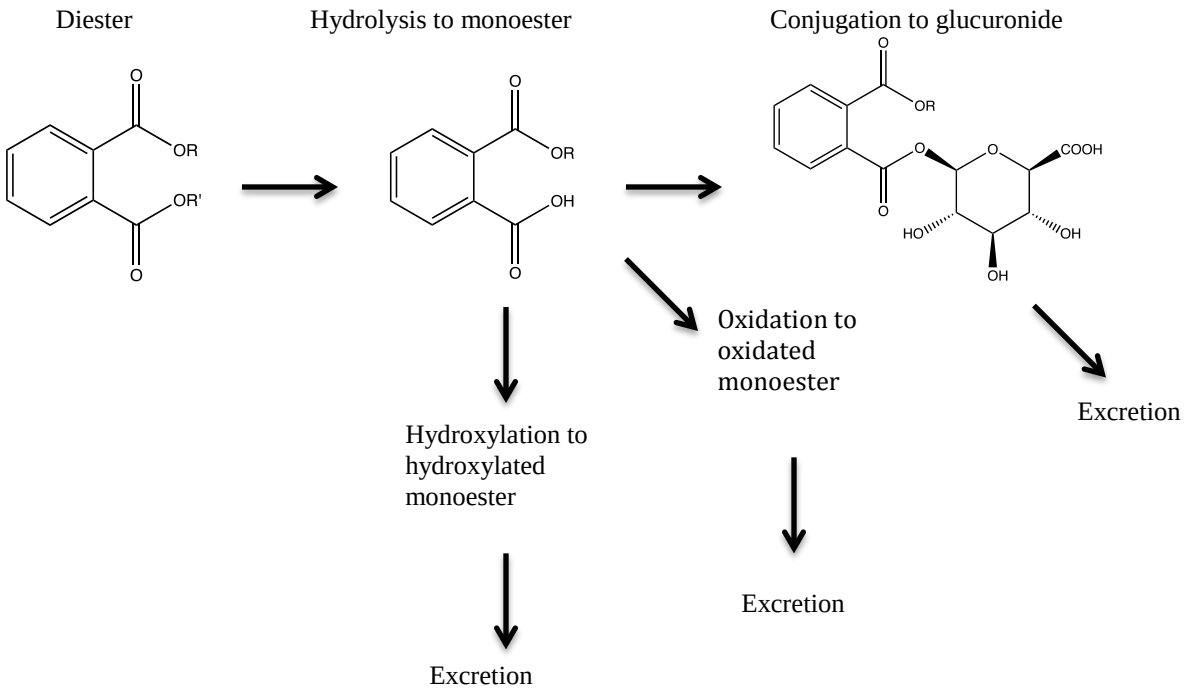


Figure 2 Higher Molecular Weight Pathway

Once phthalates are excreted from the body, they are transported to the wastewater treatment plant along with industrial and commercial waste, where the phthalates are processed and released into a nearby body of water, namely, The French Broad River. Any contaminants found in the water are then capable of acting on aquatic species located in the river.

Research at locations around the world has shown that WWTP effluent can cause physiological effects to the surrounding aquatic species due to the release of phthalate compounds: even at low concentrations.[12] A study from Barse *et al.* reports evidence of vitellogenin production in male carp exposed to concentrations as low as 1 mg DEP L^{-1}.[13] This data is consistent with Jobling et al. where they report phthalates binding to estrogen receptors *in vitro.* In the case of female fish, exposure to phthalates has shown anti-estrogenic effects presumably due to competition for the binding site with endogenous estrogens.[14] Concurrent with the evidence of estrogenic and anti-estrogenic properties, phthalates have shown to be anti-androgenic.[15] Research has also shown that simultaneous exposure to multiple phthalate compounds can produce additive anti-androgenic effects, increasing the amount of harm that can be done.[16]

The Asheville, NC WWTP, being a conventional biological treatment system, goes through a series of steps to reduce EACs from the water. The first step in the clarification is the removal of larger material such as sticks and other solids entering the sewage, by a bar screen filtering system. This material can then be disposed of by means of landfill dumping. Following this step is the primary clarification process. In this step, a large percentage of the suspended matter will settle to the bottom of the tank, which will then be removed. The wastewater will then go through rotating biological contractors where bacteria will execute anaerobic bacterial degradation. This step removes the majority of the organic matter, which is then followed by the settlement of suspended matter. Once this step has been completed, the wastewater will continue on to the clarifier, which removes the bacterial organic matter. The water is then navigated to a chlorine contact chamber containing sodium hypochlorite to kill bacteria and pathogens. Finally, the processed water is released downstream of the WWTP.

Dargnat et al. conducted research on the removal of various phthalates at a WWTP in France. In their study, they found that the concentrations of DEHP were consistently the highest with an average concentration of 5.02 PPB. Following the DEHP concentrations were the concentrations of DEP resulting in 0.78 PPB. The remaining phthalate

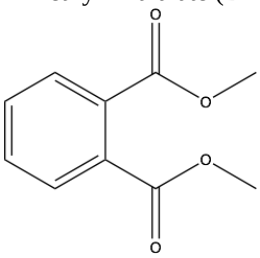
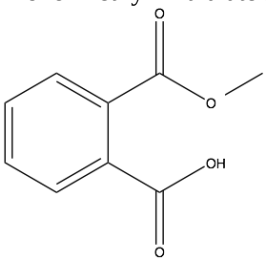
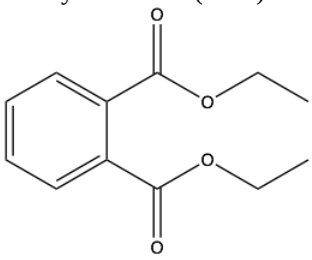
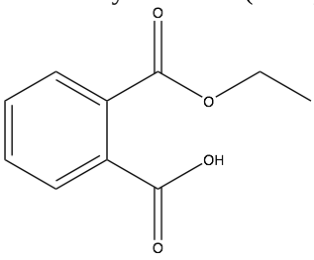
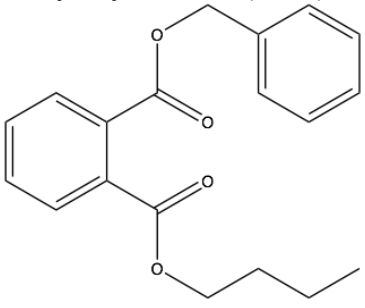
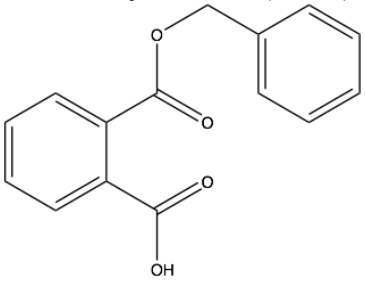
concentrations were much lower: all averaging out to below 0.30 PPB. The removal rate for DEHP was shown to be 78% while the concentrations of DEP were removed at 90%. This WWTP had multiple clarification steps, including an activated sludge process.

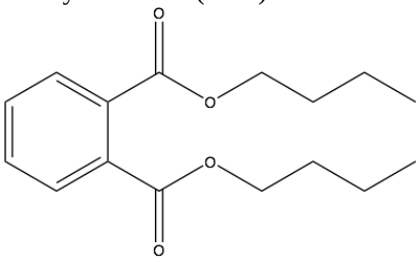
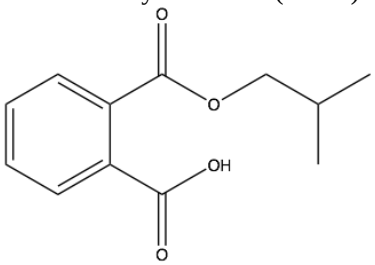
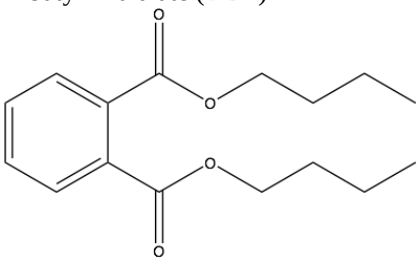
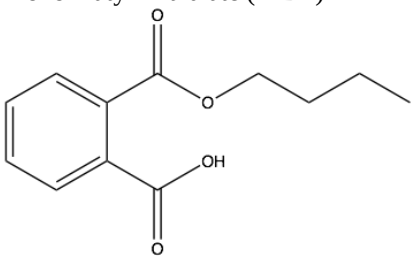
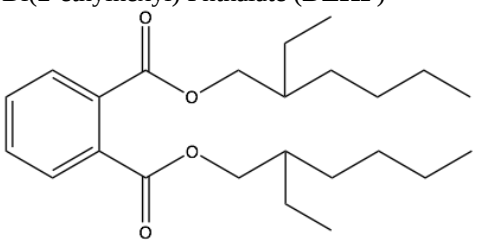
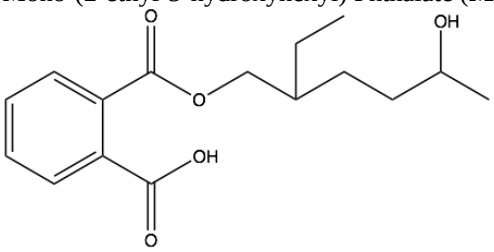
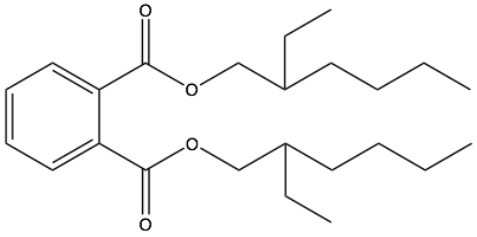
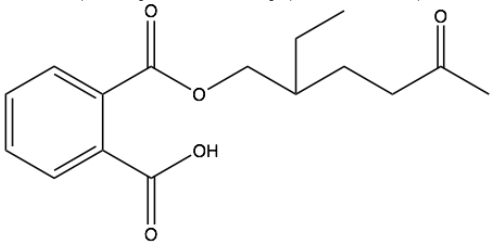
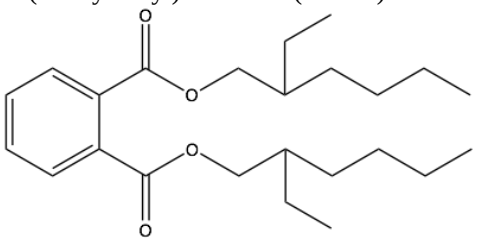
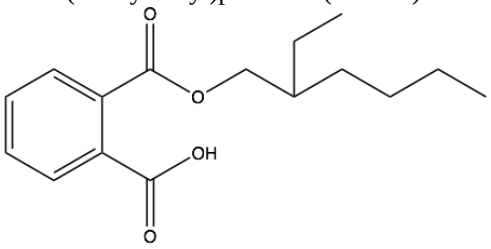
This project will utilize solid-phase extraction and the use of high performance liquid chromatography-mass spectrometry for specific and sensitive analysis of the chosen compounds. Upon completion of EAC detection, the measured values can then be statistically analyzed to calculate concentrations of EACs in all of the samples.

2. Methods

Previous research identified that the toxic effects of phthalate compounds arise when they are metabolized into their monoester form.[17] It is likely due to their higher reactivity, but the mechanism of their toxicity is still under discussion. Due to this, the compounds of interest were the phthalate metabolites (monoester). Table 1 shows the diester phthalates and their subsequent monoester metabolites.

Table 1 Diester and Monoester Phthalates

Phthalate	**Metabolite**
Dimethyl Phthalate (**DMP**) 	Mono-Methyl Phthalate (**MMP**) 
Diethyl Phthalate (**DEP**) 	Mono-Ethyl Phthalate (**MEP**) 
Benzylbutyl Phthalate (**BzBP**) 	Mono-Benzyl Phthalate (**MBzP**) 

Dibutyl Phthalate (**DBP**) 	Mono-Isobutyl Phthalate (**MiBP**) 
Dibutyl Phthalate (**DBP**) 	Mono-Butyl Phthalate (**MBP**) 
Di(2-ethylhexyl) Phthalate (**DEHP**) 	Mono-(2-ethyl-5-hydroxyhexyl) Phthalate (**MEHHP**) 
Di(2-ethylhexyl) Phthalate (**DEHP**) 	Mono-(2-ethyl-5-oxohexyl) Phthalate (**MEOHP**) 
Di(2-ethylhexyl) Phthalate (**DEHP**) 	Mono(2-ethylhexyl)phthalate (**MEHP**) 

2.1 Sampling Procedure

Influent samples were retrieved followed by effluent samples taken 5 hours later. The clarification of wastewater takes approximately 5 hours; so sampling the effluent 5 hours later would correspond to the sample taken at the influent site. Sample sizes were 150 mL for influent and 300 mL for effluent. Influent and effluent samples were collected in plastic Nalgene containers and frozen to reduce the effects of biological degradation.

2.2 Enzymatic Deglucuronidation of Phthalate Samples

Samples were deglucuronidated by taking 5 uL of 200 ng/mL 4-methylumbelliferone glucuronide and adding it to 5 mL of sample. This was followed by adding 50 uL of internal standard spiking solution to each sample followed by 5 uL of beta-glucuronidase/ammonium acetate solution and incubated for 90 minutes at 37 C.

2.3 Phthalate Extraction Procedure

Solid-phase extraction was utilized in the extraction of analytes from solution, using Oasis HLB 6cc (200 mg) Extraction Cartridges. Initially, 1 mL of acetonitrile followed by 1 mL of an acidic buffer was added to condition the cartridges prior to addition of the samples. Next, 1 mL of the acidic buffer was added to the deconjugated samples, then loaded into the cartridges. Once the entire sample was pulled through the cartridge, the analytes were eluted with 2 mL of acetonitrile followed by 2 mL of ethyl acetate. These samples were evaporated to dryness, under nitrogen, using a Zymark Turbovap at 55 C. Samples were then resuspended to 200 uL with HPLC grade water to be used in the LCMS.

2.4 HPLC-MS Analysis

Analysis was preformed using a Shimadzu Liquid Chromatograph Mass Spectrometer-8040 using a C-13 column. The settings were ran on electrospray ionization-multi reaction monitoring-negative (ESI-MRM(-)) mode to yield the highest sensitivity. The results were then analyzed using Microsoft Excel.

3. Results and Discussion

Analyzed samples confirm the presence of phthalates in influent and effluent, with the concentrations shown in Table 2. Although most of the samples are shown to be below the limit of detection (LOD), MEHP resulted with concentrations that were above the LOD and limit of quantitation (LOQ) in both the influent samples. The free effluent sample for MEHP was detected above the LOD, but below the LOQ.

The step in clarifying dissolved organic solids at the WWTP makes use of a rotating biological contactor. This step uses bacteria/organisms to take up the nutrients and organic pollutants in the water removing possible contaminants. Considering the glucuronide that is attached to the phthalate compounds is a derivative of sugar, it is possible that microbes located on the biofilm of the RBC are cleaving the glucuronide bond. This cleaving would revert the compound back to the more toxic monoester. Research shows that esterases have been reported on the outer layer of biofilms at various WWTP locations; possibly leading to glucuronide cleavage.[18] Base catalyzed hydrolysis could also be a likely candidate for glucuronide cleaving considering sodium hypochlorite is added to the effluent.

Although this was originally suggested, the data shows otherwise. In all cases shown in Table 2, effluent concentrations of phthalate monoesters have been reduced in comparison to phthalate concentrations found in the influent. This is indicative of successful removal of phthalate monoesters from the wastewater. Another observation is that many of the free phthalate monoesters are more concentrated than the total phthalate monoesters. This could possibly be explained by esterases in the water that were activated when total phthalate samples were incubated. This activation of the enzyme could result in the cleavage of the remaining ester make the phthalate compounds undetectable in the mass spectrophotometer. Future experimentation will be undergone, with the addition of an autoclaving step, to denature any enzymes prior to incubation of the total phthalate samples. MEHHP and MEOHP were detected at levels much lower than that of MEHP indicating that the glucuronidation pathway for DEHP excretion

is preferred over hydroxylation and oxidation. Another observation indicates ion suppression of MMP. Peaks for the C-13 isotopic labeled MMP were suppressed indicating interference. This could be due to the lack of excess charge available during electrospray ionization. If more easily ionizable compounds are present in the sample matrix, then those compounds will be ionized in place of the MMP, making it impossible to detect MMP.[19]

Table 2 Concentrations of MEHP, MBP, MBzP, MBP, and MiBP.

Compound	**Influent Concentration**		**Effluent Concentration**	
MMP	**Total/Free:** Ion Suppression		**Total/Free:** Ion Suppression	
MEP	**Total:** 270 ppt **LOD:** 1.49 ppt	**Free:** 346 ppt **LOQ:** 4.54	**Total:** 146 ppt **LOD:** 0.749 ppt	**Free:** 103 ppt **LOQ:** 2.27 ppt
MEOHP	**Total:** 3.93 ppt **LOD:** 1.23 ppt	**Free:** Below LOQ **LOQ:** 3.73 ppt	**Total:** Below LOQ **LOD:** 0.616 ppt	**Free:** Below LOQ **LOQ:** 1.87 ppt
MiBP	**Total:** 9.27 ppt **LOD:** 2.34 ppt	**Free:** 10.8 ppt **LOQ:** 7.08 ppt	**Total:** Below LOQ **LOD:** 1.17 ppt	**Free:** Below LOQ **LOQ:** 3.54 ppt
MBP	**Total:** 184 ppt **LOD:** 23.6 ppt	**Free:** 197 ppt **LOQ:** 71.4 ppt	**Total:** Below LOQ **LOD:** 11.8 ppt	**Free:** Below LOQ **LOQ:** 35.8 ppt
MEHHP	**Total:** ND	**Free:** ND	**Total:** ND	**Free:** ND
MBzP	**Total:** 20.6 ppt **LOD:** 1.52 ppt	**Free:** 24.8 ppt **LOQ:** 4.59 ppt	**Total:** 3.11 ppt **LOD:** 0.758 ppt	**Free:** 3.19 ppt **LOQ:** 2.30 ppt
MEHP	**Total:** 67.2 ppt **LOD:** 7.10 ppt	**Free:** 89.6 ppt **LOQ:** 21.5 ppt	**Total:** 29.9 ppt **LOD:** 3.55 ppt	**Free:** 20.7 ppt **LOQ:** 10.8 ppt

4. Conclusion

The French Broad River is the home to several endangered species making it crucial to keep the waterways clean and free of threatening chemical compounds. One of the contaminants of concern is phthalates, comprised of an aromatic ring with two esters of varying length R-groups. These compounds are known to be endocrine-active, causing havoc on the sexual development of several different species. One avenue for these contaminants to reach the environment is through the effluent of a wastewater treatment plant. For this reason, we have analyzed and quantified the concentrations of several phthalate compounds in the wastewater treatment effluent. Results show that phthalate compounds were present in the influent and effluent, but were greatly reduced during the WWTP process.

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