

**Puerto Rico Water Resources & Environmental
Research Institute
Annual Technical Report
FY 2014**

Introduction

The Puerto Rico Water Resources and Environmental Research Institute (PRWRERI) is located at the Mayagüez Campus of the University of Puerto Rico (UPRM). The Institute is one of 54 water research centers established throughout the United States and its territories by Act of Congress in 1964 (P.L. 88-379) and presently operating under Section 104 of the Water Research and Development Act of 1984 (P.L. 98-42), as amended. The Puerto Rico Water Resources Research Institute was established in April 22, 1965, as an integral division of the School of Engineering at the College of Agricultural and Mechanic Arts, the official name of the UPRM at that time. An agreement between the Director of the Office of the Water Resources Research Institute of the Department of the Interior and the University of Puerto Rico at Mayagüez was signed in May 25, 1965. This agreement allowed the Institute to receive funds as part of the Water Resources Act of 1964. In June 1, 1965, the Chancellor of the Mayagüez Campus appointed Dr. Antonio Santiago-Vázquez as the first director. The first annual allotment of funds for fiscal year 1965 was \$52,297.29.

Since its inception, the Institute has had eight directors in nine appointment periods as shown in the list below.

- 1 - Dr. Antonio Santiago-Vázquez - 1965 - 1968
- 2 - Eng. Ernesto F. Colón-Cordero - 1968 - 1972
- 3 - Eng. Felix H. Prieto-Hernández - 1972 - 1974
- 4 - Dr. Roberto Vázquez (acting director) - 1974 - 1975
- 5 - Dr. Rafael Ríos-Dávila - 1975 - 1980
- 6 - Dr. Rafael Muñoz-Candelario - 1980 - 1986
- 7 - Eng. Luis A. Del Valle - 1987 - 1989
- 8 - Dr. Rafael Muñoz-Candelario - 1989 - 1994
- 9 - Dr. Jorge Rivera-Santos – 1995 - Present

The official name of the Institute was changed in 2005 to Puerto Rico Water Resources and Environmental Research Institute.

The general objectives of the Puerto Rico Water Resources and Environmental Research Institute are (1) to conduct research aimed at resolving local and national water resources and environmental problems, (2) to train scientists and engineers through hands-on participation in research, and (3) to facilitate the incorporation of research results in the knowledge base of water resources professionals in Puerto Rico and the U.S. To accomplish these objectives, the Institute identifies Puerto Rico's most important water resources research needs, funds the most relevant and meritorious research projects proposed by faculty from island high level education institutions, encourages and supports the participation of students in funded projects, and disseminates research results to scientists, engineers, and the general public.

Since its creation, the Institute has sponsored a substantial number of research projects, supported jointly by federal, state, private, and University of Puerto Rico's funds. Through its website, the Institute's work is more widely known to the Puerto Rican and world communities and, at the same time, provides means of

information transfer with regard to the reports produced through the institute's research activities.

The Institute is advised by an External Advisory Committee (EAC) composed of members from water resources related government agencies, both federal and state levels. This committee virtually convenes annually to established research priorities and to evaluate and recommend proposal for funding under the 104-B program. The EAC has representation from the private sector as well. During EAC meetings, members are supported by the Institute's Director and Associate Director.

Due to recent retirement of some of the members and continues changes in government directorate officials, the Institute's Director is engaged in recruiting new members for next fiscal year. New agencies that may participate in the EAC include the PR Department of Natural and Environmental Resources (PRDNER), Federal Emergency Management Agency (FEMA), US Fish and Wildlife Service (FWS), and US Army Corps of Engineers (CoE).

Research Program Introduction

The Institute functions as a highly recognized advisor to the industry and government sectors on water resources and environmental issues. This role translates into multidisciplinary functions and activities that add relevance and impact to the research program the Institute supports. By virtue of the local relevance of its research and the prestige and leadership of the investigators it has supported, the Institute has become the focal point for water - related research in Puerto Rico.

FY-2014 104-B base grant supported two new research projects. A continuing project was active but no new funds added. FY2013 proposal included three projects, but one of them had to be cancelled after the original budget was substantially reduced by the USGS Water Resources Research Institutes Program. The project “An Integrated Approach for the Detection of Estrogenic Activity in a Tropical Urban Watershed: phase III,” submitted by Dr. Jorge Ortiz, a PI from the Río Piedras Campus of the University of Puerto Rico system, was resubmitted and approved in FY2014. This project aimed to assess the presence of estrogenic activity in freshwater environments in Puerto Rico affected by human activities. The PI resubmitted the proposal with some modifications to incorporate some work performed during FY2013, and was approved for continuation (it is tagged as a new project since the 104b Program did not fund the project in FY2013).

The other projects are continuing projects by PIs from our home campus. One of them, “Microbial Source Tracking: The hunt for *E. faecalis* the dominant Enterococci among non-pigmented environmental Enterococci in the water systems of Puerto Rico,” by Dr. Luis Rios from the Department of Biology, had difficulties in achieving substantial progress due to budgetary restrictions. The PI estimated that the allocated budget, after the budgetary adjustment requested by the USGS in FY2013, was not enough to complete the project and decided to wait for additional fund allocation. Nevertheless, the PI has been granted a no cost time extension for the second time to complete the project. The PI desisted the search for additional funds and made some modification to the project’s goal to adjust to the approved budget.

The second active project named “Feasibility of sintered recycled glass functionalized with micro- and nanosized TiO₂ particles for the degradation of trihalomethane precursors from surface waters,” was aimed to develop and implement a low cost pre-oxidation alternative made of sintered recycled glass functionalized with anhydrous titanium (IV) oxide powder (TiO₂) for the degradation of NOM in raw waters for the control of THM’s. To accomplish such goal, the project was divided in three main parts: (1) structural evaluation of the glass/TiO₂ composite in order to determine porosity and percolation rates; (2) mechanical analysis to determine compression characteristics of the composite; and (3) to evaluate the efficacy of the composite in degrading NOM under the influence of UV light for the photo-catalytic reaction. The project has produced essential data and information that set the foundations for a novel process in the degradation of THM’s precursors, and as a consequence, minimize THM’s formation in drinking water.

During FY2014, a new relationship was initiated with the PR Planning Board. This government agency among other responsibilities, is in charge of receiving, analyzing and resubmitting all hydrologic-hydraulic studies for flood plain delineations and modifications to FEMA. A new contract is being prepared to hire the Institute to provide expertize advice for the analysis of such study reports and submit comments on the correctness and appropriateness of such reports. Continuing collaboration with other federal and state agencies has resulted in various externally funded projects. The Puerto Rico Department of Natural and Environmental Resources (PRDNER) provided funds for the development of “Hydrologic and Hydraulic (H-H) Studies Guidelines” for urban development siting and design data, water body channelization and other engineering projects requiring an H-H study. This project consists in the evaluation of current engineering practices for conducting hydrologic and hydraulic studies in the Island. The new guidelines will include two manuals, namely, “Hydrologic and Hydraulic Study Guidelines: Technical Manual” and “Hydrologic and Hydraulic Study Guidelines: Practice Handbook.” In FY2014 this project was expanded to include the development of

Research Program Introduction

guidelines for the hydrologic and hydraulic studies required for sand and gravel extraction operations in rivers in Puerto Rico. The products of this project will influence the DNER's decision making process related to the approval of new housing developments and other type of projects that affect natural water bodies.

The Natural Resources Conservation Service (NRCS) of the Department of Agriculture supported the project named "Establishment of a demonstration field in salt tolerant vegetative materials as conservation buffers in salt flats: started in October 2011 with a duration of three years. In FY2014, the project was granted a NCTE and was finished in March, 2015. This project compared the effectiveness of natural salt flat vegetative species as sediment detention barriers in coastal areas assisting Caribbean NRCS in the development of technical information needed for implementation of USDA conservation programs. The objectives of this project were: 1) To create a demonstration and technology transfer program at the project site for farmers, government personnel and visitors to the Boquerón Wildlife Refuge (BWR) in Cabo Rojo, Puerto Rico, 2) To apply, implement and monitor a demonstration project using recommended salt flat species for Puerto Rico as vegetative erosion protection method at the coastal area of BWR, 3) To generate guidelines and recommendations for application of this conservation technology in coastal areas based on the plant effectiveness as soil erosion protection and plants adaptation, and 4) To reduce the impact of nutrients coming from the Boquerón Wildlife Refuge and the Lajas Valley Irrigation canal on the coastal zone during flood conditions. The project was a Conservation Innovation Grant.

The Total Microbial Community Structure and Enterococci Population Dynamics During a “recent fecal contamination event” in Seawater samples of Puerto Rico

Basic Information

Title:	The Total Microbial Community Structure and Enterococci Population Dynamics During a “recent fecal contamination event” in Seawater samples of Puerto Rico
Project Number:	2013PR160B
Start Date:	3/1/2013
End Date:	2/28/2016
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	Water Quality, Non Point Pollution, Ecology
Descriptors:	Enterococci, fecal pollution
Principal Investigators:	Luis A Rios-Hernandez

Publications

There are no publications.

This project has been granted another No-Cost-Time Extension (NCTE) from March 01, 2015 to February 29, 2016. The project did not achieve the expected performance in FY2013 due to budgetary restrictions. The original budget approved by March 1, 2013, was substantially reduced after the USGS announced a budget cut of more than 40%. The PI decided by himself that the reduced budget was not enough to complete and achieve the objectives of the project in the original proposal. The PI failed to communicate his conclusion and decision to the Director on a timely manner to evaluate possible alternatives to the budgetary issue. After an explanatory letter submitted by the PI in May, 2014 and a request for additional time, the Director approved a NCTE while additional funding alternatives were considered. After an analysis of various funding alternatives, the PI decided he could continue the project without student assistantship.

On February 23, 2015, the PI submitted a new time extension request (letter included herein). He sustained his request on the fact that he has been limited of students and has been doing the actual lab work by himself. The PI stated that currently he has made extensive progress but now the project is entering in the phase of the research project that depends on the services of the sequencing facility and because of the their work policies, this will take a few more weeks. After the sequencing work is done, the PI needs to do the sequencing analysis on the results. He also stated that all other work was done and it is finished with certain modifications due to the budget constraints. The NCTE was granted by the Director and the project is extended to February 29, 2016.

February 23, 2015

Dr. Jorge Rivera Santos
Director PRWRERI

Dear Dr. Rivera Santos:

I would like to ask for an extension on my research project; **“The Microbial Population dynamics and total community structure during a “recent fecal contamination event” in seawater samples of Puerto Rico”** as you know I have being limited of students and I have been doing the actual lab work myself. Currently I have made extensive progress but I am entering in the portion of the research project that depends on the services of the sequencing facility. They are doing all they can to process our samples but they typically run the samples in the order that they are submitted. This will take a few weeks and after that I will need to start the sequencing analysis. All other work was done and it is finished with certain modifications due to the budget constraints. I will appreciate if you could consider this petition favorably.

Thanks,

A handwritten signature in black ink, appearing to be 'Luis A. Rios Hernandez', written in a cursive style.

Luis A. Rios Hernandez
Associate Professor

Progress Report

Task	Not started	In progress	Completed
Enumeration of Enterococci			X
Artificially contaminated samples			X
Isolation of DNA		X	
NGS barcoding		X	
NGS Sequencing		X	
NGS data analysis	X		

AN INTEGRATED APPROACH FOR THE DETECTION OF ESTROGENIC ACTIVITY IN A TROPICAL URBAN WATERSHED: PHASE III

Basic Information

Title:	AN INTEGRATED APPROACH FOR THE DETECTION OF ESTROGENIC ACTIVITY IN A TROPICAL URBAN WATERSHED: PHASE III
Project Number:	2014PR162B
Start Date:	3/1/2014
End Date:	2/28/2015
Funding Source:	104B
Congressional District:	N/A
Research Category:	Water Quality
Focus Category:	Surface Water, Water Quality, Toxic Substances
Descriptors:	None
Principal Investigators:	Jorge R Ortiz-Zayas

Publications

There are no publications.

Estrogenic Activity of Contaminants of Concern in Tropical Urban Waters

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2015
Rio Piedras, Puerto Rico

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Summary

Modernity has arrived with important advances but has also introduced new environmental challenges, especially those related to water pollution control. Particularly, urban rivers are receiving increasing inputs of treated and untreated industrial and domestic wastewater. Concern exists that synthetic chemicals present in commercial products used in the everyday life may interfere with the endocrine system of both humans and wildlife. These chemicals are named endocrine disrupting compounds (EDCs). There has not been any formal screening of EDCs in tropical urban rivers. In this study, the application of a chemiluminescent yeast assay as a rapid monitoring tool allowed assessing estrogenic activity in an urban river in Puerto Rico. The Rio Piedras, an urban river in San Juan, Puerto Rico was intensively monitored for estrogenic activity revealing levels of estrogenic activity at the picomolar range (pM). Our results show an average estrogenic activity in the watershed of 2.24 pmol/L Eeq, although not site-specific ($\chi^2(7) = 4.82, p = 0.68$). For the temporal variation, a significant difference in estrogenic activity was observed between sampling dates ($\chi^2(3) = 8.81, p = 0.03$). No correlation between estrogenic activity and precipitation and discharge was observed. The physicochemical parameters measured showed a weak significant relationship between estrogenic activity and temperature ($r = 0.35, p = 0.051$) but not with conductivity or pH. To quantify the potential sources of the estrogenic activity, solid phase extraction (SPE) and GC-MS techniques were used to characterize potential estrogenic compounds, specifically, phthalate esters (dimethyl, diethyl, dibutyl, benzyl butyl and bis-2-ethylhexyl phthalates or DMP, DEP, DBP, BBP, and DEHP, respectively) in water samples collected in the Rio Piedras. For the phthalates detected,

variation was not site-specific. Significant differences in temporal variation of phthalate concentrations between sampling dates were observed for the phthalate esters DMP and DBP ($F(3,28)=15.5, p<0.01$; $F(3,28)=8.68, p<0.01$), respectively. There was a positive, significant correlation between precipitation and the phthalates DMP ($r = 0.823, p < 0.01$), DBP ($r = 0.608, p<0.01$) and BBP ($r = 0.421, p =0.02$). A positive weak significant correlation relation was observed between discharge and DBP ($r = 0.354 p = 0.0467$). Among all physicochemical parameters, two significant relationships were observed. Temperature was only significantly related to BBP ($r = -0.38, p = 0.03$) and conductivity was only related to DMP ($r = -0.45, p = 0.01$). No significant relationships were observed between pH and any of the phthalate esters. Overall, low levels of estrogenic activity and phthalate esters are occurring at the Rio Piedras Watershed, potentially degrading this aquatic ecosystem.

Finally, the removal efficiency of EDCs and the possible impact of wastewater treatment plants (WWTPs) discharges were assessed by sampling two regional WWTPs with different treatment technologies. A primary and a secondary advanced wastewater treatment facility located in the San Juan Metropolitan Area (SJMA) were monitored. The recombinant yeast bioassay was used to measure estrogenic activity in the influents and outflows. Estrogenic activity removal was significantly different between inflow and outflow in the secondary advanced RWWTP (two sample $t(22) = 5.062, p = <0.001$), whereas the primary regional WWTP showed no significant differences between inflow and outflow (two sample $t(22)= - 0.617, p = 0.543$). Comparing the outflows of both levels of treatment, statistically significant differences were obtained (two sample $t(21) = -4.498, p = <0.001$). Analytical determinations involving SPE and GC-MS to screen for

phthalate esters revealed variation in removal efficiency of the phthalates in the regional WWTP with primary treatment. A % decrease in concentrations were observed for DBP, DMP and DEP (-87.2, -12.8, and -0.88 %, respectively) while a percent change increase of 472.4% and 70.61% was observed for BBP and DEHP, respectively. At the secondary advanced facility, a percent change decrease between inflow and outflow ranged from 42.9 to 92.4 % for all the phthalates. Our data reveals an unknown angle in urban pollution control efforts and represent an initial framework upon which to expand and add more information in relation to EDCs or other contaminants in inland water in the urban tropics.

Chapter 1: Introduction

I. Background

Overview of Endocrine Disruption

The term endocrine-disrupting compound was defined by the U.S. Environmental Protection Agency (EPA) as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process” (Kavlock *et al.* 1996). They are also called “xenobiotic” chemicals. Endocrine-disrupting chemicals (EDCs) were originally thought to exert actions primarily through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors, progesterone receptors, thyroid receptors, and retinoid receptors, among others. Today, basic scientific research shows that the mechanisms are much broader than originally recognized. While it is well-established that EDCs that activate the nuclear estrogen receptors alpha and beta (ER α , ER β) can have deleterious effects on humans and wildlife, an important recent advance in endocrinology has been the realization that estrogens can have non-genomic effects on gene expression via membrane bound receptors and second messenger pathways (Watson *et al.* 2011). They can act directly on gene expression via the nuclear ER α and ER β , or indirectly via multiple cellular signaling pathways, some of which can be activated quickly and at very low EDC’s concentrations (Blumberg *et al.* 2010). Thus, EDCs can act via nuclear receptors, non-nuclear steroid hormone receptors (*e.g.*, membrane ERs), non-steroid receptors (*e.g.*, neurotransmitter receptors such as the serotonin receptor, dopamine

receptor, norepinephrine receptor), orphan receptors (*e.g.*, aryl hydrocarbon receptor (AhR), enzymatic pathways involved in steroid biosynthesis and/or metabolism, and many other mechanisms that act together upon endocrine and reproductive systems (Diamanti-Kandarakis *et al.* 2009). The effects of these chemicals are believed to be due to their ability to: (1) mimic the effect of endogenous hormones, (2) antagonize the effect of endogenous hormones, (3) disrupt the synthesis and metabolism of endogenous hormones, and (4) disrupt the synthesis and metabolism of hormone receptors (Sonnenschein *et al.* 1998). Thus, from a physiological perspective, an endocrine disrupting substance is a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that allow the organism to communicate with and respond to its environment (Diamanti-Kandarakis *et al.* 2009). In this scenario, EDCs have the potential to interfere with various animal internal systems and are related to a number of health effects in both humans and wildlife (Colborn *et al.* 1994, Hotchkiss *et al.* 2008, Shved *et al.* 2008).

EDCs include both natural and synthetic estrogens. Specific examples of EDCs include: pesticides like atrazine, dieldrin, and toxaphene; surfactants such as alkylphenol-ethoxalates; natural hormones; the pharmaceutical estrogens 17 β -estradiol and 17 α -ethynylestradiol; phytoestrogens including isoflavonoids and coumestrol; industrial compounds like bisphenol A and the very widely used phthalates (Campbell *et al.* 2006).

Impacts to Wildlife and Human Health

Starting in 1947, awareness about chemical pollutants affecting wildlife was raised in the United States when the first linkage was observed between chemical contamination of the natural environment and the onset of disease, reproductive failure, and death of wildlife species (Hotchkiss *et al.* 2008). Wildlife biologists began to document crashes of top predator fish populations in the Great Lakes (Colborn *et al.* 1992). In the 1950's, a decline in the lake trout populations was reported (Colborn *et al.* 1992). In the 1960's and 70's, biologists began to report severe losses among colonial nesting water birds (Colborn *et al.* 2000). It has also been found that even when the concentrations of those chemicals have decreased in the lakes because of regulatory actions, they can persist in the fish tissue (Colborn *et al.* 2000). Dioxin concentrations lethal for the eggs and hatchlings of lake trout (*Salvelinus namaycush*) and other species, such as herring (*Larus argentatus*) were found in Lakes Ontario and Michigan (Colborn *et al.* 2000). A variety of reproductive anomalies and other adverse health effects have been reported in the American alligators from Lake Apopka, Florida, after exposure to a spill of DDT (Guillette *et al.* 1994). Anomalies include reduced hatching success, small phallus size, poor survivorship, and decreased clutch viability.

Many more findings of this nature can be found in the literature as well as associations with human health conditions. Epidemiological studies have revealed associations between health conditions in children from mothers with prenatal, chemical exposure to EDCs (Fein *et al.* 1984, Hatch *et al.* 1998, Hatch *et al.* 2001, Palmer *et al.* 2001, Focazio *et al.* 2008). Fein *et al.* (1984) reported that babies from mothers who ate

2-3 meals/mo of Lake Michigan fish for at least six years prior to their pregnancies were behind in neurodevelopmental maturity when compared to a control group. This deficit was associated with levels of PCBs in umbilical cord blood, which provided an estimate of prenatal exposure (Fein *et al.* 1984). Other mother-infant studies (Fein, *et al.*, 1984; Focazio, *et al.*, 2008; E. Hatch, *et al.*, 2001, Palmer, *et al.*, 2001) have also shown neuromotor delays in babies when exposed to PCBs and/or dioxins. Other research shows that daughters of women who were prescribed with the synthetic hormone diethylstilbestrol (DES) are at increased risk of developing clear cell adenocarcinoma (CCA), experience vaginal epithelial changes, uterine leiomyomas (fibroids), fertility problems, pregnancy complications, and having structural differences in the anatomy of the reproductive tract (Hatch *et al.* 1998, Hatch *et al.* 2001, Palmer *et al.* 2001, Baird *et al.* 2005, Titus-Ernstoff *et al.* 2006). DES is a synthetic form of the hormone estrogen that was prescribed to pregnant women between 1940 and 1971 to prevent miscarriage, premature labor, and other related pregnancy complications. All these studies show the possible transgenerational effects of xenobiotics in the organisms exposed. Other impacts on human health function includes compromised immune response and cancer, primarily those of breast, endometrial, or testicular tissues (Lathers 2002).

An important modern concern is the increasing number of xenobiotic chemicals introduced into the market and their disposal into the environment. This calls for a specific research agenda on the fate and transport of new synthetic endocrine-disrupting chemicals. With such a large number of compounds already known, or suspected to be EDCs, it is difficult to understand the sanitary impacts and environmental fates of individual compounds. Long-term (chronic) effects related to continuous low-level

exposure present another concerning issue, together with the risks associated with repeated exposure to mixtures of different byproducts or with the introduction of new commercial compounds in different areas (Ankley *et al.* 2007). In this context, market forces seem to be more overriding environment and safety considerations.

Urban Water Pollution with EDCs

The rapid and ever changing technological era has brought to the world important advances but has also introduced new environmental challenges, especially those related to the pollution of aquatic resources. Rivers, and particularly urban rivers, are receiving increasing inputs of treated and untreated industrial and domestic wastewaters. Urban rivers have an important part to play in urban ecosystems because their position in the landscape makes these ecosystems particularly vulnerable to impacts associated with the urban environment. The Urban Stream Syndrome (USS) is a term used to describe a consistent pattern of hydrological, physical, and biological conditions seen in aquatic ecosystems downstream of urban inputs (Walsh *et al.* 2005). Research suggests that the two primary causes of the USS are storm water runoff and wastewater treatment plant effluents (Paul *et al.* 2001, Walsh *et al.* 2005, Grimm *et al.* 2008). Compared to pristine streams, urban-impacted streams tend to receive larger loading rates of man-made organic chemicals such as PCB's and pharmaceuticals (Kolpin *et al.* 2002). Increased concentrations and loads of several chemical pollutants appear universal in urban streams, often occurring even at low levels of catchment urbanization (Hatt *et al.* 2004).

Currently, more than 80,000 chemicals are in use and 2,000 are introduced each year in the United States (NIEH, 2010) with little or no data on their toxicological effects.

Data from the most recent report of the Toxics Release Inventory (TRI) Program under the US Environmental Protection Agency (EPA), revealed that in 2012, 3.6 billion pounds of chemicals were released to the environment in the United States and its territories, including Puerto Rico, who showed a contribution of approximately 4.3 million pounds of the total amount registered nationally (<http://www2.epa.gov/toxics-release-inventory-tri-program/2012-tri-national-analysis>). The EPA in Puerto Rico has included approximately 225 sites in the Comprehensive Environmental Response Compensation and Liability Information System (CERCLIS), and 20 of these are included in the National Priority List (NPL) for immediate cleaning and monitoring (http://www.epa.gov/region2/cleanup/sites/prtoc_sitename.htm). The main contaminants found in the NPL for Puerto Rico include heavy metals, volatile organic compounds, and pesticides known to cause effects to different organ systems in both humans and aquatic wildlife. Among the reported contaminants there are known endocrine disruptors showing steroid-like properties such as bisphenol-A (BPA) (Sonnenschein *et al.* 1998, Wagner *et al.* 2009) and trichloroethylene (Goh *et al.* 1998, Xu *et al.* 2004, Wu *et al.* 2008).

EDCs have been found to be present in surface waters and often at mixtures of high concentrations of low-potency disruptors and low amounts of very powerful ones (Focazio *et al.* 2008), making both wildlife and humans at risk of exposure. They are capable of interacting with the estrogen receptor in cells causing effects even at trace-level concentrations (Kortenkamp 2008, Diamanti-Kandarakis *et al.* 2009). Effects include alteration of the normal biological signaling that control development (Colborn *et*

al. 1994), and reproduction (Cooper *et al.* 1997, Anway *et al.* 2005, Barber *et al.* 2007), among other internal functions controlled by the endocrine system.

EDCs are found in many of the products that people use everyday such as pharmaceutical drugs, plastics, cosmetics, detergents, pesticides, and many other chemicals that are present in the environment or are in widespread use. Their ubiquitous nature in cities causes EDCs to often emerge in municipal wastewater effluents. For example, many pharmaceutical drugs excreted by people end up in the municipal wastewater system. Both human and domestic animal excretion of hormones may be an important source of endocrine-active chemicals in the environment and have yet to be thoroughly investigated (Lathers 2002). Detergents used for washing clothes also drain into the same systems. In addition, EDCs can be transported through runoff from lawns, farmland or feedlots and industrial wastewater. The failure of wastewater treatment plants (WTP) to filter out EDCs leave them free to interact with fish, humans and other organisms that may ingest them downstream.

In 2002, the USGS published results from its first national reconnaissance of pharmaceuticals, hormones, and other organic wastewater contaminants in streams from the contiguous United States (Kolpin *et al.* 2002). The study revealed that a wide range of chemicals are present in most streams, and that substantial levels of hormones, detergent metabolites (APEOs), plasticizers such as phthalates, and nonprescription drugs are common (Kolpin *et al.* 2002, Focazio *et al.* 2008).

EDCs enter aquatic ecosystems mainly through water treatment plant (WTP) effluents. Natural and synthetic hormones and certain industrial chemicals capable of estrogenic effects have been identified in sewage effluents (Desbrow *et al.* 1998, Solé *et al.* 2000). Another potential source of EDCs to aquatic environments is the runoff from impervious surfaces in urbanized catchments. Urbanization and changes in land use can have profound impacts on runoff characteristics increasing concentrations of contaminants (Meyer *et al.* 2005). Conversion of land use from rural to urban can also affect stream characteristics via multiple pathways, including altered hydrology and water chemistry, channel geomorphology, and trophic resources (Paul *et al.* 2001). Hydrological alterations include increased total runoff and shorter duration and higher volume peak runoff (Chadwick *et al.* 2006). Water quality is degraded in urbanized streams by both point and non-point sources of contaminants (Paul *et al.* 2001, Kolpin *et al.* 2002, Grimm *et al.* 2008). Clearly, effects of catchment urbanization on stream ecosystems arise from many interrelated sources but catchment impervious cover, however, has been shown to be a powerful driver that accounts for many of the factors mentioned earlier (Chadwick *et al.* 2006).

Areas with high population density such as cities are typically linked with pollution, due to greater volumes of material and energy throughput arising from human activities (Eriksson *et al.* 2005). Generation and transport of pollutants are well studied because of requirements for compliance with national quality standards (see www.epa.gov/safewater/html). However, we do not know how the effects of water

pollution with EDCs from different human activities vary within the urban spatial context.

The hydrologic connectivity framework (Pringle 2001) provides a useful context for understanding how pollution-generating activities affect nearby water bodies and how much risk this pose to humans and wildlife. Hydrologic connectivity is referred to water mediated transfer of matter, energy or organisms within or between elements of the hydrologic cycle (Pringle 2001). In the urban environment, human perturbations including the load of pollutants to surface waters can alter the hydrologic connectivity. Some elements such as ditches or road network can enhance the runoff movement from upstream to downstream areas while increasing loading of pollutants coming from impervious surfaces. The magnitude and extent of human impacts have altered the nature of hydrologic connectivity on local, regional, and now global spatial scales. Many impacts such as loadings with pollutants have complex and interrelated cumulative effects. Moreover, because of the continual transport that characterizes hydrologic systems, an effect originating in one part of the landscape may be expressed at a distant geographic location, often with a significant time lag (Pringle 2001). As summarized by (Colborn *et al.* 1996), ocean currents are capable of transporting biota that have sequestered polychlorinated biphenyls (PCBs) into the arctic food web, where they undergo further biological magnification within the top-predators. PCB levels in seals and predatory polar bears are, respectively, 384 million and 3 billion times the PCB concentration in ocean water, potentially affecting the long-term reproductive capacity of these animals and the humans that eat them. Possible reproductive and immune function disturbances have also been reported in polar bears showing high levels of PCBs and

brominated flame retardants (Goksoyr 2006). This reflects the global distribution and diffuse action of persistent chemicals that have the ability to biomagnify in the food chain.

1.2. Statement of the Problem

Stream degradation caused by urbanization is large and evident in the tropics but is poorly documented (Dudgeon 2000, Ramirez *et al.* 2008). Water management, including wastewater and drinking water, are frequently major issues for new city sectors and have important consequences on human health and ecosystem integrity (Cohen 2008). Managers in tropical regions often must rely on information from non-tropical regions for guidance. Internationally, efforts are being made to develop official screening programs for EDCs. However, no program has been established yet. Although many chemicals have been widely considered EDCs, numerous chemicals still remain unidentified. A challenge to the field of endocrine disruption is that these substances are diverse and may not appear to share any structural similarity other than usually being small molecular mass compounds (Diamanti-Kandarakis *et al.* 2009). Thus, it is difficult to predict whether a compound may or may not exert endocrine-disrupting actions. For the tropical Rio Piedras Watershed in Puerto Rico, no data are available to address pollution with estrogenic compounds. Because of the potential global scope of the EDC problem, the possibility of serious harms in humans and wildlife and the persistence of some suspected EDC's in tropical fast growing cities, research on EDCs in the tropical environment should be a high priority in order to protect the rich biodiversity of tropical waters and human health. Chemical information (presence and concentration level of

EDCs) combined with molecular tools for gene expression will allow governmental agencies to assess the exposures of aquatic life and humans to EDCs and to evaluate their impact at these levels. For the Rio Piedras Watershed, this study represents the first assessment of estrogenic activity and can be potentially useful for water pollution control programs.

Puerto Rico has one of the highest population densities in the World (340 inhabitants/km² ; <http://factfinder.census.gov>) and has experienced rapid rates of urbanization. Land use changed dramatically in the Island during the transition from an agriculture-based economy in the early 1900s to an industry-based economy around the 1940s and 1950s (Grau *et al.* 2003). Industrialization led to rapid population growth and expansion of metropolitan areas, especially San Juan (Pares-Ramos *et al.* 2008). San Juan represents a hot-spot of energy and material consumption which contributes to the load of pollutant discharges to the surface waters, most likely including estrogenic compounds. The Rio Piedras drains parts of the SJMA, the most intensely urbanized area in Puerto Rico (3,500 inhabitants/km²) (Ramirez *et al.* 2009, De Jesus-Crespo *et al.* 2011). Streams in the Rio Piedras are degraded, with high nutrient concentrations, channelization in some areas, and even illegal discharges of waste water. Houses in rural areas commonly rely on septic tanks for sewage treatment, but gray waters from showers and sinks are often discharged untreated into streams. In addition, the concern exists that Puerto Rico's climate differs in comparison to the United States and Europe, and the behavior of these pollutants can be different from these countries because of the higher and less variable tropical temperatures.

1.3. Objectives, Hypotheses and Rationales

The high population density of Puerto Rico makes it a unique site to study anthropogenic influences on the tropical biosphere on a small spatial scale. This research is the first effort to identify the presence of EDCs in Puerto Rico water resources. *The main goals of this project are: (1) to understand the potential estrogenic activity in tropical surface waters, specifically in an urban watershed and wastewater treatment plants effluents in large cities in Puerto Rico, and (2) to establish the possible impact to human health and to aquatic ecosystems in the urban surface waters of the San Juan Metropolitan Area of Puerto Rico.* EDCs can potentially persist during conventional water treatment and are known to induce estrogenic activity (Basile *et al.* 2011). Steroid hormones are known to elicit measurable ecological change at concentrations below 1 nM (McLachlan 2001, Kolodziej *et al.* 2003). The phthalates, a group of compounds widely used in many products, among other group of compounds known to exert EDCs effects, have been found in most streams in the United States in both environmental and biological samples (Colon *et al.* 2000) Focazio, *et al.*, 2008; Fromme, *et al.*, 2002; Kolpin, *et al.*, 2002) at the nM range of concentration. Phthalates with high molecular weights (eg, di-2-ethylhexyl phthalate (DEHP) and butylbenzyl phthalate (BBP)) are used as plasticizers in the production of vinyl, flooring, wall coverings, food packaging and medical devices, among other products (Hauser *et al.* 2006) . Low-molecular-weight phthalates (eg, diethyl phthalate (DEP) and dibutyl phthalate (DBP)) in are used in personal care products (e.g., perfumes, lotions, cosmetics), as solvents and plasticizers for cellulose acetate, and in making lacquers, varnishes, and coatings, including those used to provide timed release in some pharmaceuticals (Hauser *et al.* 2006). The present study

focused on four phthalates: butylbenzyl phthalate (BBP), dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), and dimethyl phthalate (DMP).

Specifically, the following objectives were addressed in this study:

Objective 1: To assess the potential estrogenic activity in the urban surface waters of the SJMA of Puerto Rico.

Hypothesis 1.1: Estrogenic activity will be present in the surface waters of the Rio Piedras particularly downstream of the more populated areas.

Rationale: The Rio Piedras Watershed is a heavily urbanized tropical river. It drains part of the SJMA, the most highly urbanized area in Puerto Rico. The SJMA is home to 68% of the Island's population, has one of the largest economies in the Caribbean and is often seen as a model for the development of Caribbean or Latin American economies. The 67 km² Rio Piedras watershed is 47% urban and only 25% is covered by forest-like vegetation (A. Ramirez, personal communication). The Rio Piedras presents rapid stage increases after intense rainfall events and flashy streamflows (De Jesus-Crespo *et al.* 2011). A factor that contributes to this is the large fraction of impermeable surfaces in the city which contributes to storm runoff. Limited information exists on urban impacts to the Rio Piedras ecosystem, but known sources of pollution include urban runoff and discharges of untreated wastewater, particularly in the upper parts of the watershed (De Jesus-Crespo *et al.* 2011). Because EDCs can be found in many of the products that people use everyday such as pharmaceutical drugs, plastics, cosmetics, detergents, and pesticides, estrogenic activity is expected to be found in this heavily urbanized watershed.

Hypothesis 1.2: Given the common use of plastics in many commercial products, phthalate esters are expected to be present in the urban surface waters of the SJMA of Puerto Rico.

Rationale: Phthalates are widely used as plasticizers since their introduction to the market in the 1920's (Graham, 1973) in poly(vinyl chloride), poly(vinyl acetate)s, cellulose, and polyurethanes, and as non-plasticizers in products such as lubricating oils, automobile parts, paints, glues, insect repellents, photographic films, perfumes, and food packaging (e.g., paperboard and cardboard) (Mackintosh, et al., 2004). These compounds are not chemically bound to the plastic matrix, making them capable of leaching out of products and be released into the environment (Latini, et al., 2003; Thompson et al., 2009). These esters have been found in all types of environmental and biological samples (Fromme, et al., 2002; Halgberg, et al., 2008; Vethaak, et al., 2005). The vast volume of plastics in consumer and industrial products, its persistence, and its routine disposal to the environment help to explain why human exposure to phthalate esters is nearly ubiquitous. Specifically, Bi(2-ethylhexyl)phthalate (DEHP) has been found in human blood, seminal fluid, amniotic fluid, breast milk, and saliva. In an epidemiologic study, 75% of the 289 human subjects tested were positive for the presence of four different types of phthalates in their urine samples (Blount, et al., 2000).

Recent developments in the analytical methods used for emerging and newly identified contaminants in water and wastewater, published in an *Analytical Chemistry* biennial (2007–2008) review (Richardson 2009), indicate the increasing and continuing health concerns associated with the presence of trace organics in the environment. In cities such as San Juan, the rapid growth experienced has outpaced planning efforts and

the waters of the Rio Piedras river have been classified as highly polluted by the Puerto Rico Environmental Quality Board (EQB) and the EPA (Lugo *et al.* 2011). However, no published data exist about the estrogenic loads into the Rio Piedras. Phthalate esters represent a group of EDCs used on a daily basis and are found in many commercial products. Typical detected concentrations reported in wastewater are in levels of micrograms per liter (ug/L) (Fromme *et al.* 2002, Clara *et al.* 2010). A study conducted by Andrade *et al.* (2006) showed biological impacts in rats within the range of common human exposure of the phthalate DEHP at environmentally-relevant levels. Thus, the detectable presence of many EDCs in various water environments including surface and wastewaters causes significant concern. In an urban area with high population density, it is expected that higher concentrations and abundance will be detected. In order to evaluate the potential risk of the endocrine disruption, the occurrence of individual compounds needs to be documented and characterization of EDC's associated with the estrogenic activity detected in selected water samples will be conducted.

Objective 2: To determine the efficiency of wastewater treatment technologies in removing EDCs.

Hypothesis 2.1: The removal efficiency of estrogenic compounds will increase with the level of wastewater technology treatment.

Rationale: Conventional wastewater treatment is divided into primary, secondary, and tertiary treatment depending on the level of pollutant removal. Each of the levels of treatment progressively increases the removal efficiency of pollutants. Primary treatment of wastewater only removes heavy solids and floating materials. Secondary treatment

also removes dissolved and suspended biological matter while tertiary treatment includes the removal of nutrients (nitrogen and phosphorus). Typically, all treatment levels include a final disinfection stage to eliminate pathogenic microorganisms. Since tertiary treatment usually includes activated carbon filtration, it is expected that this treatment will assist in removing some of the organic compounds present, potential lowering estrogenic activity of the effluent waters.

Today, using water only once is a luxury, and sustainable reuse from alternative sources, including municipal wastewater, is reasonable from both technical and economic points of view (Postel 2000). To make wastewater reuse beneficial, conventional wastewater treatment plants (WWTPs) must be designed in such a way that they can achieve removal not only of biodegradable organic matter and pathogens but also of persistent organic (and inorganic) pollutants present at trace levels to minimize their sanitary and environmental impacts (Basile *et al.* 2011). WWTPs receive a large spectrum of molecules from domestic and/or industrial waste which is not completely eliminated during the treatment process (Ternes *et al.* 1999). In this context, WWTP discharges are considered a major source of EDCs and important contributors to water pollution. When treated effluents are released to natural water bodies, a wide range of bio-persistent compounds, incompletely degraded or formed as byproducts during conventional biological oxidation, enter aquatic ecosystems (Campbell *et al.* 2006, Nelson *et al.* 2007). The increasing number of xenobiotic chemicals introduced into the market and their disposal into the environment makes necessary research focused on the fate and transport of new synthetic endocrine-disrupting chemicals. With the vast

number of compounds already known or suspected to be EDCs, it is difficult to understand the sanitary impacts and environmental fates of individual compounds. Because most of these chemicals are not readily biodegradable, they become ubiquitous contaminants in wastewater effluents and, sooner or later, end up in surface and sea waters (Ying *et al.* 2003, Ternes *et al.* 2006).

Hypothesis 2.2: The wastewater treatment plants in Puerto Rico do not totally eliminate EDCs; thus, phthalate esters will be detected in wastewater effluents.

Rationale: Chemicals used in homes, industry, and agriculture are collected by the sanitary collection system and transported to the WWTP where a variety of engineering processes are used to treat the sewage. The collection system serves as a concentrator of pollutants disposed by humans. However, not all WWTP are designed to remove all types of pollutants. To date, it is known that municipal WWTPs reduce EDC's to some extent; although frequently not to levels lower than the known effective concentrations for aquatic wildlife (Metcalf *et al.* 2001, Kolodziej *et al.* 2003). Thus, it is expected that higher concentrations of potential estrogenic compounds, specifically phthalate esters, will be present in the effluent of the WWTPs. This makes the WWTP a very important potential point-source of pollution of EDCs to the environment because the treated water is discharged to a receiving water body where humans and aquatic organisms will be exposed to.

1.4. Study Site

According to Lugo et al. 2011 , the Río Piedras Watershed is an urban watershed fully contained within the SJMA (Fig. 1). The Río Piedras Watershed has an area of 67 km² (De Jesus-Crespo *et al.* 2011). It originates at about 150 m elevation and flows north for 16 km in the Caimito sector of San Juan and as it flows towards Río Puerto Nuevo (where it enters Bahía de San Juan), the river passes through the Río Piedras, Hato Rey, and Puerto Nuevo sectors of San Juan.

Human activities in San Juan have altered the Río Piedras and even its watershed area has changed over time due to flow alterations (Lugo *et al.* 2011). Haire (1971) describes the Río Piedras as having a watershed area of 49 km² in the 1970s, contrasting with the latest measurements of 67 km² published by de Jesús-Crespo and Ramírez (2011). The authors attribute the difference in area to the large number of modifications that the river has undergone, in particular at the lowest reaches where the Río Puerto Nuevo joins the main stem of the Río Piedras (Lugo et al. 2011). In addition, the watershed has been affected by channelization and concrete lining in numerous tributaries, paving over ephemeral and intermittent streams, and considerable land movements that have reshaped the local topography (Lugo et al. 2011). Impervious surfaces now reach 49 percent of the total area (de Jesús-Crespo and Ramírez 2011), which results in high runoff rates (e.g., 72 percent of rainfall becomes stream flow; Osterkamp, 2001). Urbanization in the SJMA started in the coastal areas, thus the Río Piedras Watershed is more heavily urbanized in lowland areas and more rural at its headwaters. Currently, most of the watershed is serviced by a municipal water system for

drinking water. Although sewage collection is provided for a large percent of the area, some areas in the headwaters rely on septic systems.

Eight sites were selected for sampling in the Río Piedras Watershed (Figure 1). Each sampling site is associated with focal study areas of the San Juan ULTRA Program (<http://sanjuanultra.com>). Six of the selected sites are small tributaries with clearly delineated subwatersheds, whereas two are located in the main stem of the river. The two main stem sites were selected in close proximity of a United States Geological Survey (USGS) gaging stations. The first main stem sampling site, La Sierra, is located near the USGS 50048770 Río Piedras at El Señorial station, which is located 60 m.a.s.l., drains 19.4 km² and reports an average yearly discharge of 0.61m³/sec (18-year record). The second main stem sampling site, Avenida Central, is located at Avenida Central near the USGS 50049100 Río Piedras at Hato Rey station, which is located just above sea level (1 m.a.s.l), drains 39.4 km² and reports an average yearly discharge of 1.6 m³/sec (23-year record).

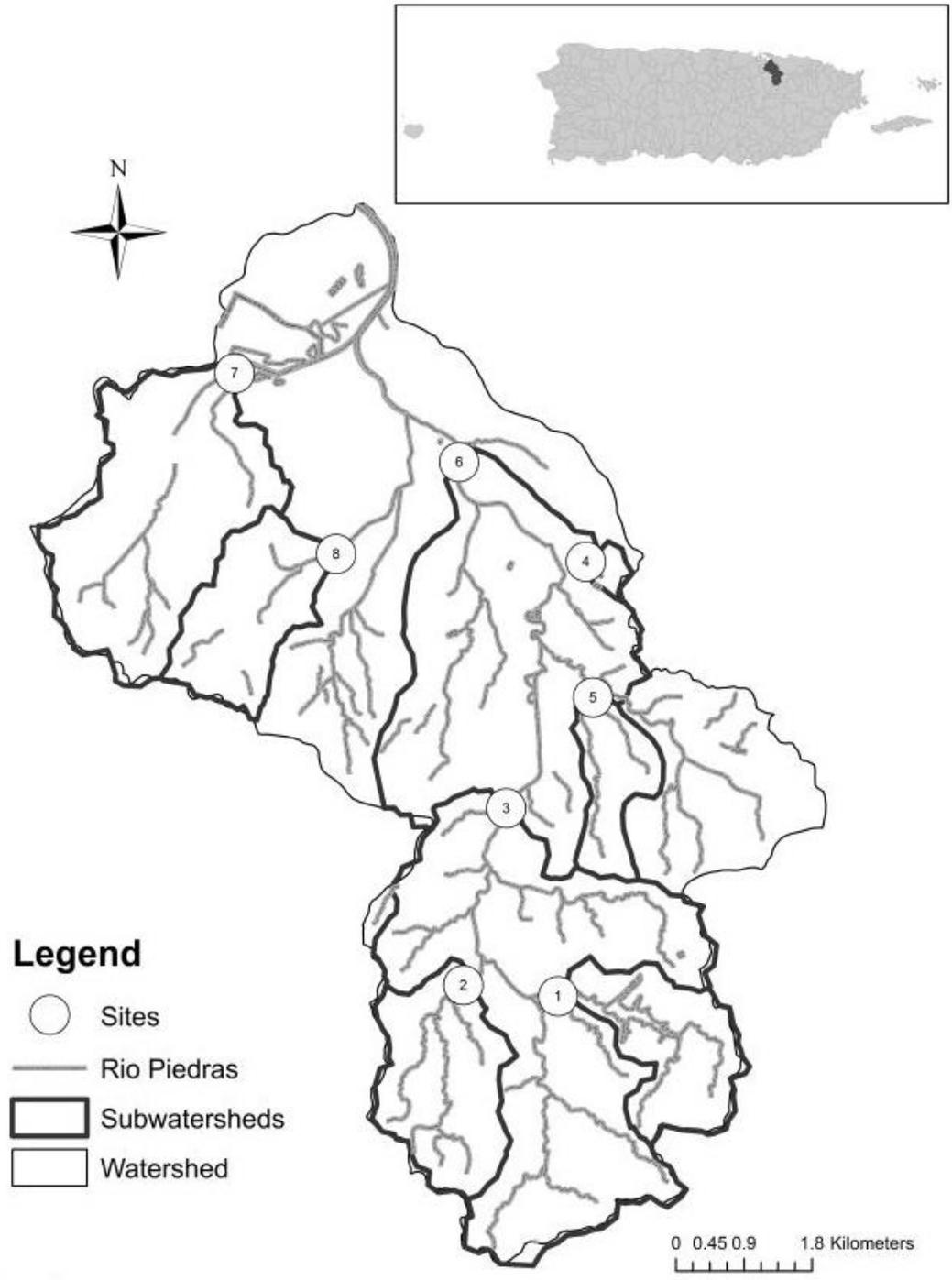


Figure 1. The Rio Piedras Watershed and the subwatersheds sampled (from Ramírez *et al.*, 2014). Site numbers are described in Table 1.

1.5. Organization of the Dissertation

This introduction has established the theoretical framework considered to design this project. The second chapter of this dissertation will focus in the determination of estrogenic activity in the Rio Piedras and the concentration of phthalates esters. The third chapter will address the efficiency of wastewater treatment technologies in reducing estrogenic activity and in removing phthalate esters. Finally, a synthesis chapter is presented at the end of the dissertation to integrate the information presented in the preceding chapters.

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Chapter 2: Methodology

2.1 Chemical and Biological Analysis

The natural environment consists of complex mixtures of components and processes that represent a challenge to characterize. Chemical compounds present in the environment are usually in mixtures and at low concentration levels that are difficult for analytical instrumentation to detect and very sensitive methods are required for identification. Currently, there is no way to anticipate the endocrine disrupting effects of a compound solely based on its chemical structure. *Moreover, EDCs are not defined by their chemical nature, but by their biological effect.* This is why recombinant bioassays using estrogen receptor constructs are a useful tool to establish if estrogenic activity exists in the water samples. These assays are capable of responding to *substances with receptor-mediated estrogenic activity regardless of the chemical structure.* It has been demonstrated that a number of the environmental contaminants, also known as xenoestrogens (XE), are able to function in a manner similar to estradiol (E2), the endogenous hormone in the body (Colborn *et al.* 1992, Kavlock *et al.* 1996, Sonnenschein *et al.* 1998). These include compounds such as bisphenol A (Suzuki *et al.* 2004), alkylphenols (White *et al.* 1994), PCBs (Roy *et al.* 2009), and pesticides (Du *et al.* 2010). When the estrogen receptor (ER) is bound by its endogenous hormone, subsequent activation of the ER results in conformational changes, protein interactions, and gene transcription (Blair *et al.* 2000). Therefore, xenoestrogen-induced alterations in normal endocrine function may result in adverse effects at the cellular level. Although bioassays are capable to respond to estrogenic activity they do not differentiate between

compounds present in complex mixtures. Analytical-chemical analyses are needed to identify the individual estrogenic compound in environmental samples. In this study, a recombinant yeast assay followed by Gas Chromatography/Mass Spectrometry (GC/MS) was used for biological and chemical analyses, respectively.

Studies combining chemical and biological analysis have demonstrated a relationship between concentration of EDCs in water samples and the estrogenic activity (Focazio *et al.* 2008, Baldigo *et al.* 2014). Bioassay-directed fractionation of WWTP effluents revealed the natural and synthetic estrogens as being the compounds mainly responsible for the estrogenic activity measured in a yeast reporter gene assay (Metcalf *et al.* 2001).

2.2 Analytical determination in liquid matrices

A. Sample Preparation

Environmental samples cannot be analyzed without some preliminary sample preparation, mainly because pollutants are diluted and the matrix is rather complex. As a result, detection in the chromatographic systems becomes difficult. To overcome this, purification and concentration of the analytes is highly recommended before the analysis. When an extraction technique is chosen for routine analyses, attention must be paid to various elements to develop a technique that provides reproducible results and extracts without impurities that can cause interferences. Solid Phase Extraction (SPE) is a very useful sample preparation technique to achieve this goal. It is excellent for sample extraction, concentration and cleanup. It consists of the extraction of the analytes onto a

solid immobilized phase. Analytes will be recovered from the solid phase depending on its equilibria with the extraction solvent. Thus, careful selection of the extraction solvent must be taken to ensure that recovery percentages are acceptable. SPE represents a rapid and reliable sample treatment procedure compared to conventional liquid-liquid extraction (LLE), which present various disadvantages such as laborious and time-consuming extraction steps, expensive, has a tendency to form emulsions, it requires the evaporation of large volumes of solvents and the disposal of toxic or flammable chemicals (Pico *et al.* 2007). Alternative solid-phase-based extraction techniques, which reduce or eliminate the use of large volumes of solvents, can be employed to prepare samples for chromatographic analysis. These include SPE, solid phase micro-extraction (SPME), and stir-bar sorptive extraction (SBSE) (Sabik *et al.* 2000, Balsiger *et al.* 2010). SPE presents several benefits such as high recoveries of analytes, purified extract, and low consumption of organic solvents. SPE can be used to isolate semi-volatile or nonvolatile analytes from a wide range of matrices: blood, urine, water, beverages, soil, animal tissue, and consumer products. In the following section, the SPE technique will be described.

B. Solid Phase Extraction (SPE)

SPE is a sample preparation method that concentrates and purifies analytes from solution by sorption onto a solid phase cartridge, disk or syringe barrel, followed by elution of the analyte with a solvent appropriate for instrumental analysis. The principle of SPE is similar to that of liquid-liquid extraction (LLE), involving a partitioning of solutes between two phases. However, instead of two immiscible liquid phases, as in LLE, SPE involves partitioning between a liquid (sample matrix or solvent with analytes)

and a solid (sor bent) phase. This sample treatment technique enables the concentration and purification of analytes from solution by sorption on a solid sor bent and purification of extract after extraction. The general procedure is to load a solution onto the SPE solid phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube.

The SPE technique consists of a four-step process: 1) conditioning, 2) load sample onto conditioned cartridge, 3) wash off weakly retained interferences with weak solvent and 4) eluting compounds with strong solvent. During the conditioning step, a solvent is passed through the sor bent to wet the packing material and to solvate the functional groups of the sorbents. The air is removed from the sor bent material and the empty spaces are filled with the solvent. A cleaning step is a good laboratory practice necessary in the conditioning step to eliminate impurities that may be present in the packing material. Next step consists of loading the sample through the sor bent. Volumes could range between 1mL-1L of sample depending on the sor bent. It is critical not to allow the sor bent to dry during the loading step because the mechanism of sorption will not work effectively and recoveries will be poor for the analyte. The third step is to rinse the sor bent material to remove interferences while retaining the analytes. Finally, step 4 is the elution of the analyte from the sor bent with an appropriate solvent that disrupts the analyte-sor bent interactions.



Figure 1.2. Vacuum manifold for solid-phase extraction. Photo from <http://www.teknokroma.es>

A variety of stationary phases makes the technique broader to separate a wide range of compounds: reverse phase, normal phase, size exclusion and ion exchange. Most of these stationary phases are based on a bonded silica material that is derivatized with a specific functional group. Reverse-phase sorbents are more hydrophobic than the sample matrix and it is excellent for aqueous samples. Reversed phase separations involve a polar or moderately polar stationary phase. The analyte of interest is typically mid-to-nonpolar. The sorbent material has hydrophilic silanol groups at the surface of the raw silica packing. The most common functional groups are: octadecyl (C-18), octyl (C-8), ethyl (C-2), cyclohexyl and phenyl. Retention of organic analytes from polar solutions (e.g. water) onto the material is due primarily to the van der Waals attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. To elute the analytes, a nonpolar solvent is used to disrupt the forces that bind the compounds to the packaging.

Other materials are also used as sorbents in the reversed phase. Polymeric adsorption media such as ENVI-Chrom P, the sorbent used for this study, is a styrene/divinylbenzene material that is used for retaining hydrophobic compounds which contain some hydrophilic functionality. Elution steps are done with mid-to- nonpolar solvents, because the polymeric packing is stable in almost all matrices.

Normal phase sorbents involves a polar analyte, a mid-to non polar matrix (e.g. acetone, chlorinated solvents, and hexane), and a polar stationary phase. This sorbent is generally used when the sample is an organic solvent containing an analyte of interest. Polar interactions, such as hydrogen bonding and dipole-dipole interactions are the primary mechanism for solute retention. The most common functional groups used in normal-phase are cyanopropyl (CN), aminopropyl (NH₂), and diol (containing two –OH groups). They are chemically bonded to silica gel. The analytes adhere to the functional groups by interactions between the polar functional groups of the analyte and polar groups on the sorbent surface. The elution solvent must be more polar than the sample's original matrix in order to disrupt the binding mechanism.

Ion exchange SPE can be used for compounds that are charged when in a solution (usually aqueous, but sometimes organic). The primary retention mechanism of the compound is based mainly on the electrostatic attraction of the charged functional group on the compound to the charged group that is bonded to the silica surface. In order for a compound to retain by ion exchange from an aqueous solution, the pH of the sample matrix must be one at which both the compound of interest and the functional group on

the bonded silica are charged. Strong cation exchange sorbents contain ion-exchange sites consisting of sulfonic acid groups, and weak cation-exchange sorbents contain sites consisting of carboxylic groups. Strong-anion-exchange sites are quaternary amines, and weak anions-exchange site are primary, secondary and tertiary amines.

Size-exclusion sorbents utilize a separation mechanism based on the molecular size of the analyte. For this sorbent a silica gel matrix with a large pore size, between 275-300Å are used, compared with the 60Å pore of most bonded-phase silica (Quinones 2011).

C. Experimental Section:

a. Procedure

Pre-concentration of the water samples was performed using SPE. A 6mL glass cartridge custom packed with EnvichromP was placed in an SPE vacuum manifold. The cartridges were conditioned with 6mL of ethyl acetate followed by 6mL methanol and 6 mL nanopure water in sequence. Ethyl acetate is used to remove impurities that could interfere with the analysis and methanol was used to wet the surface and penetrate the sorbent phase, allowing water to efficiently wet the sorbent surface. After conditioning, 100 mL of the sample were loaded into the cartridge. After extraction, the cartridge was washed with 3mL of nanopure water to eliminate possible polar interferences from the matrix. Is important not to allow the cartridge to dry during the conditioning or sample loading steps to avoid damage to the

retention capacity of the solid phase. After all the sample has passed through the cartridge, the cartridge was dried by centrifuging for 15 minutes at 2000 rpm and placed under a flow of N₂ until the solid phase is visibly dry and loose. The analytes are then extracted with 6mL ethyl acetate. The extracts from the SPE were evaporated to 0.5mL with a gentle stream of N₂ and reconstituted to a final volume of 1mL in ethyl acetate.

The analysis of all standard solutions and extracts was performed by GC/MS. A Bruker Scion TQ Gas Chromatographer Mass/Spectrometer was used. One microliter (1μL) of sample was injected to the system. The detector was equipped with a DB-5 capillary column 30m x 0.25 mm I.D. with a 0.25 μm film thickness. Injections were done in splitless mode at 250 °C with a solvent delay of 5 minutes. Oven temperature program was an initial temperature of 50 °C for 1 minute, then programmed at 8 °C/min until a final temperature of 260 °C, with helium as the carrier gas. MS spectra were generated by fragmenting the analyte separated on the column with an electron impact source (70 eV), followed by acceleration of the fragments into a quadrupole analyzer and electron multiplier detection at 280 °C. Data acquisition was performed in the full scan mode measuring from *m/z* 50 to 550.

b. Data analysis

The MS workstation software was used to acquire, integrate and identify analyte peaks. To characterize the separated components in the GC/MS data file, the Automatic Mass Spectral Deconvolution and Identification System (ADMIS 2.6) was used. The mass spectrum for each component was compared and identified using the NIST Mass Spectral database 2008. To establish the identification, a correlation factor higher than 80% was selected. Confirmation was done by comparing with the standard retention time. For quantification, calibration curves for each target compound were run in parallel with the samples. Standard points ranged from 1-100 ppm. A calibration curve was constructed with peak area plotted against concentration. Analyte concentration was determined by constructing a regression line equation and used to solve for concentration of analyte in the sample.

D. Materials

1. Solvents

- a. Ethyl Acetate, 99.9%, Fluka, Sigma-Aldrich Co.
- b. Methanol, 99.9%, Omnisolv®, VWR
- c. Nanopure Water, 17.5 MΩ-cm, Barnstead
- d. Ultra Pure Water, 18.2 MΩ-cm, Millipore

2. Standards and Reagents

- a. Dibutyl Phthalate 99%, Sigma Aldrich Co.

- b. Benzyl Butyl Phthalate, 98%, Sigma Aldrich Co.
- c. Bis (2-ethylhexyl) phthalate, 98%+, Sigma Aldrich Co.
- d. Dimethyl Phthalate, 99%, Sigma Aldrich Co.
- e. Hydrochloric Acid, Certified ACS, Fisher Scientific

3. Cleaning Materials and Solutions

- a. Synthetic and natural fiber brushes
- b. Alconox Liquid Cleaner

4. Gas

Nitrogen, 99.995% High Purity, Linde

5. Other Materials

- a. GF/F Glass Fiber Filters, Fisherbrand, Fisher Scientific
- b. Sampling Bottles, 1L, Amber, Fisher Scientific
- c. Supelclean™ ENVI-Chrom P glass SPE Tube, 6cc/500mg, 30/box, Sigma-Aldrich Co.
- d. GC vials, Amber, 2mL, Supelco, Sigma-Aldrich

E. Instrumentation

- a. Bruker- Scion Gas Chromatographer-Mass Spectrometer
- b. Analytical Balance
- c. SPE Vacuum Manifold

F. Data Analysis

- a. MS Workstation 8 for SCION

- b. NIST Mass Spectral Database
- c. Excell 2010
- d. JMP 10
- e. PAST software

G. Quality Control

1. Glassware Cleaning

All laboratory and field sampling glassware was cleaned using a rigorous cleaning process to reduce interferences. Glassware was cleaned with Alconox® soap and rinsed with tap water, then soaked in 10% HCL for 5 minutes and rinsed three times with distilled and ultra-pure water and let air dry, upside down, then capped and stored until use.

2. Solvent and Reagents

All solvents used were analytical grade (>99%). Nanopure water was collected in amber glass bottles before analyses. For each analysis, a solvent blank was analyzed to determine interferences and background signals. All stock standards solutions were prepared fresh before analyses.

3. Field Blanks

A field blank was carried out during each sampling event. A 1L glass amber bottle was filled with nanopure water and brought to the sampling site and opened for a period of time equal to the sampling time, then capped.

4. GC/MS System

The GC/MS system was cleaned and conditioned as needed. Connections of the helium supply and gas lines were checked for leaks with a soap solution. The electron multiplier, filament and pump oil was changed as needed. The mass spectrometer was calibrated performing an autotune with PFTBA to verify the sensitivity. System performance was checked by running a system blank and a solution of EPA 8270 GC-MS Tuning Solution. This was used to monitor system sensitivity over time. The system blank consists of running the instrument without injecting any sample. The septum was changed after 25-30 injections of a 10 μ L syringe. Once the analyses were completed, the column was left at 150°C to eliminate or reduce interferences.

5. Nitrogen Flow

Nitrogen gas was used to concentrate extracts and to dry SPE cartridges. To reduce contamination between samples, pipettes used to direct the nitrogen flow were changed. A system of Teflon tubing was used to direct the nitrogen gas and was changed as needed.

2.3 Recombinant Yeast Bioassay

The recombinant yeast cell bioassay is a highly sensitive human estrogen receptor-based screening assay for the rapid detection of estrogens. It has been shown that the use of yeast-based assays may be a highly reliable methodology for a first level screening to assess surface water quality in terms of estrogenic activity (Brix *et al.* 2010). The yeast

assay used for the experiments described in this dissertation is a modification of the recombinant yeast estrogen screen assay (rYES) first described by (Routledge *et al.* 1996). The rYES assay is based on the stimulation of β -galactosidase expression in a recombinant yeast culture exposed to potential estrogenic compounds. In this assay, the human estrogen receptor (hER) sequence was inserted into the yeast genome, which also contained expression plasmids harboring estrogen-responsive sequences (ERE) controlling the expression of the reporter gene *Lac-Z*, which encodes the enzyme β -galactosidase. The enzyme is then produced when exposed to estrogens, causing a color change in the medium from yellow to red in the presence of substrates that binds to the estrogen receptor. This change in color occur when β -galactosidase is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red-b-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance after 3 days of incubation. The color change can then be quantified and calibrated for measurement of estrogenicity. However, the use of CPRG has demonstrated that the degradation products of chlorophenol red act as an estrogenic compound itself, which has led to the modification of the rYES assay (De Boever *et al.* 2001).

A. Sample Preparation

The water samples were filtered with Whatman glass fiber, 0.45 μm pore size filters as soon as delivered to the laboratory and stored in amber glass bottles at 4°C until analyzed, not more than two days after sampling. Water samples aliquots were then analyzed for estrogenic activity.

B. Experimental Section

The yeast (*Saccharomyces cerevisiae*) bioassay used was previously described by (Balsiger *et al.* 2010). Specifically, it is a receptor-mediated β -galactosidase reporter assay for use in the functional analysis of receptor regulatory proteins. The parental yeast strain is W303 α (MAT α leu2-112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2) with a deleted pleiotrophic drug resistance gene (PDR5). The parent strain was co-transformed with a TRP1-marked constitutive human ER α expression plasmid (pG/ER) and a URA3-marked estrogen-inducible β -galactosidase reporter plasmid (pUC Δ SS-ERE) and maintained in synthetic complete media lacking uracil and tryptophan (SC-UW) to select for plasmid retention.

The yeast colonies were grown in SC-UW agar plates. The day prior to the experiment, 5 ml of sterile, SC-UW liquid media were inoculated with several yeast colonies picked from the agar plates using a sterile inoculating loop. The liquid culture was incubated overnight at 30°C with shaking until an optical density of 0.1 at 600 nm is achieved. Then, the culture is diluted back with fresh SC-UW media to an O.D.₆₀₀ of 0.08 and incubated at 30 °C until an O.D.₆₀₀ of 0.1 is determined again. Typically, after 1 hour the culture have exited lag phase and begun to grow. Once the cultures reach an O.D.₆₀₀ of approximately 0.1, 100 μ L of the culture were transferred into an opaque 96 well plate, then 1 μ L of standard or sample was added. A standard calibration curved of 17 β -estradiol (E2 mol/L) was prepared in ethanol and assayed along with the samples. Yeast can tolerate up to 1% ethanol in solution

without any toxic effects. Plates were covered with film to prevent evaporation and incubated for 2 hrs/30 °C . The yeast assay described here benefits of a commercially available chemiluminescent substrate Tropix Gal-Screen reagent for the detection of estrogen induced β -galactosidase expression. The reagent should be prepared according to the instructions provided by the manufacturer and place on ice. After the two hours of incubation, 100 μ l of the Tropix Gal-Screen solution were added to each well and incubated for another 2 hrs/30 °C. The plates were readed in a Tecan Infinite 200Pro luminometer.

1. Materials

a. Standards, reagents and solvents

- i. CSM Ura-trip drop-out powder, Qbiogene
- ii. 17 β -estradiol, \geq 98%, Sima-Aldrich Co.
- iii. Tropix® Gal-Screen® assay for yeast cells, Applied Biosystems
- iv. Ethanol, 99.5%, Fisher Scientific

b. Other materials

- i. Opaque 96 well plates, Fisher Scientific
- ii. Inoculating loops, Fisher Scientific
- iii. Disposable cuvetts, Fisher Scientific
- iv. Culture plates, Fisher Scientific
- v. Whatman 0.45 μ m GF/F filters, VWR
- vi. Amber glass bottles, 1L

2. Instruments

- a. Tecan Infinite 200Pro Luminometer

b. BioRad SmartSpec Plus Spectrophotometer

3. Data Analysis

a. Graphpad Prism trial version 6

b. Excell 2010

c. PAST software

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CHAPTER 3: DETECTION OF ESTROGENIC ACTIVITY AND PHTHALATES IN WASTEWATER EFFLUENTS IN A TROPICAL URBAN SETTING

Abstract

Synthetic chemicals known as endocrine disrupting compounds (EDCs) are capable of interrupting the normal function of the endocrine system of the organisms exposed to them. Low, continuous presence of EDCs in the environment aggravates the problem as exposure remains constant. Because large volumes of material and energy throughput associated to concentrated human activities are common in polluted urban clusters, EDCs are likely to be present in cities. Sources of raw water for public supply are particularly vulnerable as the sewage treatment facilities may not eliminate EDCs. The purpose of this study is to assess estrogenic levels and removal effectiveness of certain EDCs in primary and secondary advanced wastewater treatment facilities of the San Juan Metropolitan Area in the tropical island of Puerto Rico. A recombinant yeast bioassay was used to measure estrogenic activity in the influents and outflows. Of the two wastewater treatment technologies evaluated, secondary advanced treatment showed significantly higher removal capacity of estrogenic activity and phthalate compounds. Significant differences in estrogenic activity resulted from the outflows between the two technologies. However, levels of estrogenic activity at the picomolar range, suggests that low doses of estrogenic compounds are being released to receiving waters. Application of solid phase extraction (SPE) and GC-MS techniques to screen for phthalate esters in the wastewater revealed a reduction in the outflow that ranged from 42.9 to 92.4 % with secondary advanced treatment. More than 90% removal was achieved for BBP, DBP and DEHP. However, concentrations ranging from 0.86 to 1.29 ppm for all the phthalates in

the outflow were detected even at the secondary advanced WWTP effluent implying that EDCs are not completely removed even with the most advanced wastewater technology available. Our data can assist managers in evaluating pollution control technologies to ameliorate the impacts of EDCs in the tropics.

Introduction

Growing evidence associates modern synthetic chemicals with changes in water quality and detrimental biological effects in aquatic ecosystems. Many environmental and industrial chemicals may interfere with the endocrine system of both humans and wildlife. These are referred to as endocrine disrupting compounds (EDCs) (Colborn *et al.* 2000). Substantial levels of hormones, detergent metabolites (APEOs), plasticizers such as phthalates, and nonprescription drugs are common in surface waters at mixtures of high concentrations of low-potency disruptors and low amounts of very powerful ones (Kolpin *et al.* 2002, Focazio *et al.* 2008). Other widespread pollutants with endocrine disrupting effects include metals, pesticides, persistent organohalogens like dioxins, furans and PCBs, food antioxidants and BPA (Iavicoli *et al.* 2009, Roy *et al.* 2009, Pop *et al.* 2014).

Some EDCs are capable of inducing estrogenic activity in animals. The activity occurs when these compounds interact with the estrogen receptor in the cells causing effects even at trace-level concentrations (Kortenkamp 2008, Diamanti-Kandarakis *et al.* 2009). Effects include alteration of the normal biological signaling that control development and reproduction among other internal functions controlled by the endocrine system (Cooper *et al.* 1997, Anway *et al.* 2005, Barber *et al.* 2007) such as reduced fertility, feminization, reproductive organ anomalies and changes in the sexual behavior of various aquatic organisms (Pal *et al.* 2010). Detrimental effects have also been observed in the human population thus posing a risk to the public health. Epidemiological studies report associations between health conditions in children from

mothers with prenatal, chemical exposure to EDCs (Fein *et al.* 1984, Hatch *et al.* 1998, Hatch *et al.* 2001, Palmer *et al.* 2001, Focazio *et al.* 2008). Fein *et al.* (1984) reported that babies from mothers who ate 2-3 meals/month of Lake Michigan fish for at least six years prior to their pregnancies were behind in neurodevelopmental maturity when compared to a control group. This deficit was associated with levels of PCBs in umbilical cord blood, which provided an estimate of prenatal exposure (Fein *et al.* 1984). Other mother-infant studies (Fein, *et al.*, 1984; Focazio, *et al.*, 2008; E. Hatch, *et al.*, 2001, Palmer, *et al.*, 2001) have also shown neuromotor delays when exposed to PCBs and/or dioxins. Other research show that women exposed in uterus to the synthetic hormone diethylstilbestrol (DES), a synthetic form of the hormone estrogen that was prescribed to pregnant women between 1940 and 1971 to prevent miscarriage, premature labor, and related complications of pregnancy, are at increased risk of developing clear cell adenocarcinoma (CCA) (Hatch *et al.* 1998) experience vaginal epithelial changes (Hatch *et al.* 2001) uterine leiomyomas (fibroids) (Baird *et al.* 2005), fertility problems (Titus-Ernstoff *et al.* 2006), pregnancy complications, and having structural differences in the anatomy of the reproductive tract (Palmer JR 2001). These studies show the possible transgenerational effects of EDCs in the organisms exposed. Other impacts on human health function includes compromised immune response and cancer, primarily those of breast, endometrial, or testicular tissues (Lathers 2002).

Among the EDCs, some phthalate esters have been considered as such because of their effects on the estrogen and androgen systems. They can bind to the estrogen receptor (ER) and induce estrogen-like cellular responses as well as to bind weakly to

the androgen receptor (AR), disrupting the normal androgenic mechanisms (Borch *et al.* 2006). Dibutyl phthalate (DBP), benzylbutyl phthalate (BBP) and di-*i*-butyl phthalate (DiBP) have been shown to bind most effectively to the androgen receptor (Fang *et al.* 2003). Nevertheless, a recent study from (Hsieh *et al.* 2012) revealed a novel oncogenic mechanism of phthalates in breast cancer which is independent from their estrogenic effects highlighting that the phthalates are capable of inducing tumorigenesis through several cellular mechanisms. Oehlman *et al.* (2009) summarizes the effect of phthalates in reproduction and impair development in mollusks, crustaceans and amphibians but mollusks and amphibians appear to be particularly sensitive to these compounds and biological effects have been observed in the low ng/L to µg/L range. Besides the acute toxicity, wider biological effects have been shown, including mitotic inhibition, induction of chromosomal aberrations and effects on larval development. These effects observed in the laboratory coincide with measured environmental concentrations, thus there is a very real probability that these chemicals are affecting natural populations (Oehlmann *et al.* 2009).

Phthalates are widely used as plasticizers since their introduction to the market in the 1920's (Graham, 1973) in poly(vinyl chloride), poly(vinyl acetate)s, cellulose, and polyurethanes, and as non-plasticizers in products such as lubricating oils, automobile parts, paints, glues, insect repellents, photographic films, perfumes, and food packaging (e.g., paperboard and cardboard) (Mackintosh, et al., 2004). Annually, more than three million metric tons of phthalates are produced globally (Schettler, 2006). Plasticizers are merged with polymers to increase flexibility and extensibility, changing the polymer

from a glasslike material to a flexible, strong elastomer (Graham, 1973). They are not chemically bound to the plastic matrix, making them capable of leaching out of products and be released into the environment (Latini, et al., 2003; Thompson et al., 2009). These esters have been found in all types of environmental and biological samples (Fromme, et al., 2002; Halgberg, et al., 2008; Vethaak, et al., 2005) and in the effluents of wastewater treatment plants (WWTPs) (Fromme *et al.* 2002, Clara *et al.* 2010). The failure of WWTPs to remove EDC's leave these compounds free to interact with fish, humans, and other organisms that may ingest them downstream. Specifically, in humans Di(2-ethylhexyl)phthalate (DEHP) has been found in blood, seminal fluid, amniotic fluid, breast milk, and saliva. In an epidemiologic study, 75% of the 289 human subjects tested positive for the presence of four different types of phthalates in urine samples (Blount, et al., 2000). The enormous volume of plastics in consumer and industrial products, its persistence, and its routine disposal to the environment help to explain why human exposure to phthalate esters is nearly ubiquitous.

Urbanization and changes in land use can have profound impacts on runoff characteristics increasing concentrations of contaminants (Meyer *et al.* 2005). Urban runoff often contains toxic contaminants that cause deterioration of the water quality entering the sewage systems. Areas with high population density may imply more pollution due to greater volumes of material and energy throughput arising from human activities (Eriksson *et al.* 2005). By their nature, cities dispose their wastes into wastewater collection and treatment systems which, depending on their treatment infrastructure, can eliminate some pollutants from water. Conventional wastewater treatment is divided into

primary, secondary and secondary advanced treatment depending on the level of pollutant removal (Nathanson 2003). Each of them increases the standards of removal starting with removing heavy solids and floating materials (primary treatment). Secondary treatment removes dissolved and suspended biological matter. Secondary advanced treatment usually includes filtration through a granular media to remove solids and organic matter, and chemical precipitation and microbiological processes to remove phosphorus and nitrogen from wastewaters. Given the additional capacity to remove organic matter, it is expected that secondary advanced treatment will remove EDCs present in the wastewaters, thus lowering its estrogenic activity.

Puerto Rico is a tropical US territory with one of the highest population densities in the World (340 inhabitants/km² (<http://factfinder.census.gov>) and has experienced rapid rates of urbanization. Land use changed dramatically on the island during the transition from an agriculture-based economy in the early 1900s to an industry-based economy around the 1940s and 1950s (Grau *et al.* 2003). Industrialization led to rapid population growth and expansion of metropolitan areas, especially San Juan (Pares-Ramos *et al.* 2008). This represents a hot-spot of energy and material consumption contributing to the load of pollutant discharges to the surface waters, most likely including estrogenic compounds. Puerto Rico only treats 50% of its sewage water in water treatment plants and, of that, 75% receive primary treatment in large regional facilities located near the coasts that discharge treated effluents through ocean outfalls (Marrero *et al.* 2006). The effluents from these regional plants are rich in nitrogen, organic matter, and suspended solids (Ortiz-Zayas *et al.* 2006). Given their high

operational costs, advance treatment plants are not common. In Puerto Rico, there are only three wastewater treatment plants that provide advance treatment and with regulated discharges to rivers and creeks. Despite the efforts made to improve the quality of effluents, a recent study from Figueroa *et al*, (2014), revealed that WWTP effluents still affect water quality of receiving streams, particularly for nutrients, accounting for > 40% of stream nutrient loads, with the effects on NO_3^- and PO_4^- loads being the greatest. Inputs from WWTP to streams contribute substantially to changes in water quality, thus potentially affecting downstream ecosystems. Still, EDCs loads in the effluents and its possible effects are unknown.

The main purpose of this study is to summarize levels of estrogenicity and selected phthalate esters in WWTP influents and effluents. The efficacy of the treatments used by WWTPs to remove the compounds from wastewater is also discussed.

Materials and Methods

Study sites

Two regional WWTPs (RWWTP) located in two large cities in Puerto Rico were sampled. These facilities provided either primary or secondary advanced treatment to large urban areas. Specifically, the Puerto Nuevo RWWTP is designed to provide primary treatment to wastewater generated in the municipalities of San Juan, Trujillo Alto, and portions of Bayamón, Guaynabo and Carolina. The Puerto Nuevo RWWTP is designed with an average hydraulic loading of 72 million gallons per day (MGD) and a peak hydraulic loading of 144 MGD wastewater prior to discharge to the Atlantic Ocean. The treated effluent from the Puerto Nuevo RWWTP is combined with the treated effluent from the Bayamón RWWTP and the Bacardí Corporation's WWTP. The combined effluent is then discharged approximately 7,365 ft (2,246 m) from the shoreline into the Atlantic Ocean. This operation is currently regulated by EPA under a special waiver which allows primary treatment under special circumstances as specified in Section 301(h) of the Clean Water Act. The Caguas RWWTP is an advanced secondary treatment facility (max. design capacity of 12 mgd) designed to meet rigorous effluent quality standards including significant nutrient removal. The effluent is discharged into Río Bairoa, a tributary of Río Grande de Loíza and Lago Loíza, one of the most important sources of raw water for the San Juan aqueduct system. A secondary treatment facility was excluded from the study design to focus on extreme treatment processes.

Sample collection

Influent and effluent samples from each plant were assayed for estrogenic activity and the efficiency of removal of estrogenic activity by the WWTPs was determined based on the differences between influent and effluent estrogenic activity. Four sampling events occurred from September to December 2012 in each plant (Table 3.1). Composite of water samples collected at regular time intervals was taken for a 24 hr period in each inflow and outflow stations. The samples were collected by personnel of the Puerto Rico Aqueduct and Sewer Authority and handed to us immediately after collection. The composite sample was later analyzed in triplicate for estrogenic activity and concentration of phthalates. For quality control purposes, all samples were taken in amber glass bottles and stored in iced during transportation. Glassware was previously cleaned using a rigorous cleaning process to reduce interferences and minimize microbial degradation of the analytes. Glassware were cleaned with Alconox® soap and rinsed with tap water, soaked in 10% HCL for 5 minutes and rinsed three times sequentially with tap, distilled and ultra-pure water and let air dry, upside down. In the field, water bottles were rinsed with sample water three times before collecting the sample. Once in the lab, samples were immediately filtered using a 0.45um glass fiber filter (GF/F). Samples were stored at 4°C for no more than two days before estrogenic activity analysis.

Table 3.1. Description of sampling events at each WWTP

Location	Type of treatment	Sampling event	Date
Puerto Nuevo	Primary	1rst	11/6/2012
		2nd	11/8/2012
		3rd	11/13/2012
		4th	11/15/2012
Caguas	Secondary Advanced	1rst	9/21/2012
		2nd	11/27/2012
		3rd	11/29/2012
		4th	12/18/2012

Recombinant yeast assay

A receptor-mediated β -galactosidase reporter yeast assay was used as previously described (Balsiger *et al.* 2010) to detect for estrogenic activity in wastewater samples. A standard calibration curved of 17β -estradiol (E2 mol/L) was prepared in ethanol and assayed along with the samples following the same procedure mentioned. The total estrogenic activity of the unknown samples was determined based on the response of the assay and interpolated to a dose-response curve of the standard compound E2 in mol/L and appropriately converted to ng/L of 17β -estradiol estrogen equivalents (EEq). The plates were read in a Tecan Infinite 200Pro luminometer.

Chemical analyses

Extraction of phthalates compounds: We focused on four phthalates compounds: Dimethylphthalate (DMP); Dibuthyl Phthalate (DBP), Benzylbutyl Phthalate (BBP) and

Bis-2-ethylhexyl Phthalate (DEHP). The concentrations of these phthalates were determined in samples taken in two RWWTPs from September 2012 to January 2013. Water samples were pre-concentrated using solid phase extraction (SPE). Envi-Chrom P 500 mg glass cartridges were conditioned with 6 mL ethyl acetate, 6 mL methanol and 6 mL nanopure water in sequence. Then, 100 mL of sample were loaded to the cartridge. After passing the sample, the cartridge was washed with 3 mL nanopure water to eliminate possible polar interferences from the matrix. Cartridges were dried under vacuum for 15 minutes, centrifuged for 30 minutes and exposed to a N₂ flow for 30 minutes. Analytes were eluted with 6mL ethyl acetate and evaporated to 0.5 mL by a gentle stream of nitrogen gas and reconstituted to a final volume of 1mL in ethyl acetate.

Detection: The concentrated extract (1µL) was injected into the GC/MS system. Samples were heated to an initial temperature of 50°C with a 8°C/ min. ramp to 260°C and held for 40 minutes with ultra pure helium as carrier gas. Target compounds were measured based on the following quantification ions: Dimethylphthalate (DMP): m/z=163; Dibutylphthalate (DBP), Benzylbutylphthalate (BBP) and Bis-2-ethylhexyl Phthalate (DEHP): m/z=149 (Figure 4). Data acquisition was performed in the full scan mode measuring from m/z 50 to 550. Six-point calibration curves were conducted ranging from 1-100 ppm. The linear response of the curves produced correlation coefficients (R²) higher than 0.99 for all compounds.

Statistical analyses

The reported data are the result of four independent experiments with all samples measured in triplicate within each experiment. Graphpad Prism trial version 6 and PAST version 3.01 were used for the Paired t-tests for differences between inflow and outflows and Repeated Measures One Way ANOVA followed by a post-hoc Tukey test for differences between treatment technologies. A p value <0.05 was used to represent a statistically significant difference.

Results

Estrogenic activity

Table 3.1 summarizes the removal capacity of estrogenic activity for both the primary and secondary advanced RWWTPs. Estrogenic activity removal was significantly different between inflow and outflow in the advanced RWWTP (paired sample $t(22) = 5.062$, $p = <0.001$), as opposed to the primary RWWTP where the inflow and outflow showed no significant differences between them (paired sample $t(22) = -0.617$, $p = 0.543$) (Figure 3.1).

Table 3.2. Estrogenic activity (ng/L EEq) in the inflow and outflow of a primary and secondary advanced RWWTPs (n=12).

Treatment	Mean	SE
<i>Inflow</i>		
Primary	0.7823	0.0558
Secondary advanced	0.9133	0.0580
<i>Outflow</i>		
Primary	0.8256	0.0424
Secondary advanced	0.4998	0.0687

Comparing the outflows of both levels of treatment, the t-test shows statistically significant differences between the outflows of the two WWTPs (paired sample $t(21) = -4.498$, $p < 0.001$) (Figure 3.2). Therefore, the advanced RWWTP was more effective in reducing estrogenic activity from the wastewater than the primary wastewater treatment technology.

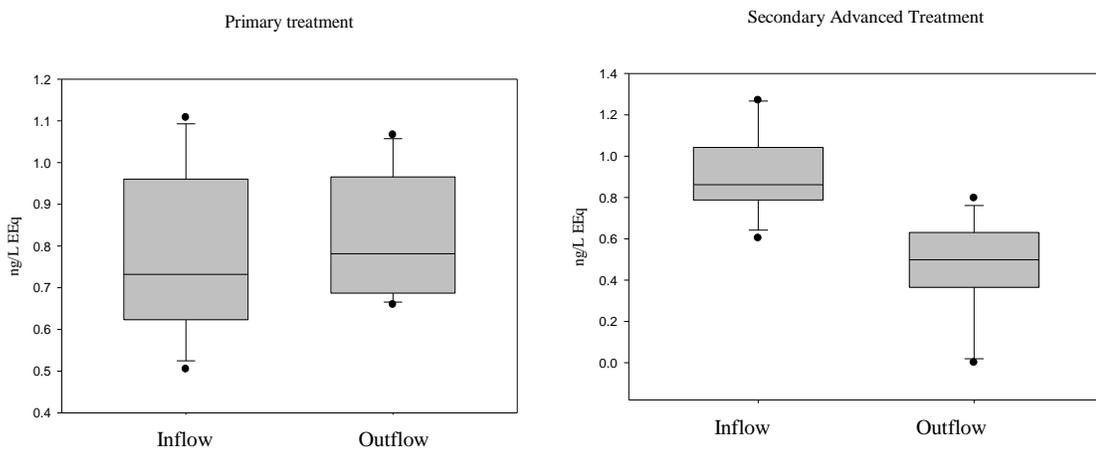


Figure 3.1. Mean comparison for estrogenic activity (n = 12) in the inflow and outflow at each level of treatment technology. The error bars represent the standard error.

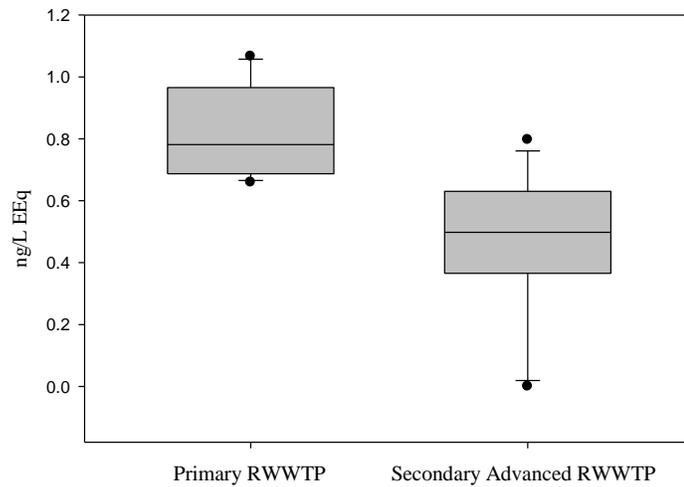


Figure 3.2. Comparison between outflow estrogenic activity by level of treatment (shown are the mean and its standard error, n = 12).

Four sampling events in each RWWT were performed independently. To test for temporal differences between events, a Repeated Measures One Way ANOVA was performed. For the primary RWWT, the test for the inflow showed no statistically significant differences, ($F(3, 6) = 1.757, p = 0.255$), as well as the outflow, ($F(3, 6) = 2.460, p = 0.160$) where there were no significant differences between sampling events. The secondary advanced RWWT Repeated Measures One Way ANOVA showed statistically significant differences between sampling events for the inflow, ($F(3,6) = 7.061, p = 0.021$). Post hoc comparison using the Tukey test revealed that events 2nd vs. 4th and 2nd vs. 1st differed significantly from the other sampling events. The outflow of the secondary advanced WWTP showed no statistically significant differences ($F(3, 6) = 2.201, p = 0.189$).

Phthalates compounds

Measured, percent (%) change, and *p* values in phthalate concentrations in raw and treated wastewater of the primary and secondary advanced WWTPs are listed in Table 3.2. We used a GC/MS instrument operating in the scan mode to analyze the samples for DMP, DEP, DBP, BBP, and DEHP. As confirmed by mass spectral data, four of the peaks in the extracted ion chromatogram corresponded to compounds of the phthalate ester family (Figure 3.3). Phthalate esters were consistently detected at concentration levels ranging from 0.33 to 9.20 ppm in the inflow of the primary WWTP and from 0.29 to 6.89 ppm in the outflow (BBP> DEHP> DBP >DEP> DMP). A paired t- test show significant differences in removal for DMP, DBP and DEHP (*p* values: <0.001, 0.010 and 0.023, respectively).

In the secondary advanced WWTP, the phthalates were detected in concentrations ranging from 0.52 to 16.92 ppm in the inflow (BBP>DBP>DEHP>DEP>DMP) and from 0.09 to 1.29 ppm in the outflow (BBP>DBP>DEHP>DMP>DEP). Between the inflow and the outflow, concentrations were consistently reduced. The percent change ranged from 42.9 to 92.4 % BBP=DEHP>DBP>DEP>DMP). A paired-samples t-test was conducted to compare concentrations between the inflow and outflow. There was a significant difference between the concentrations of all compounds.

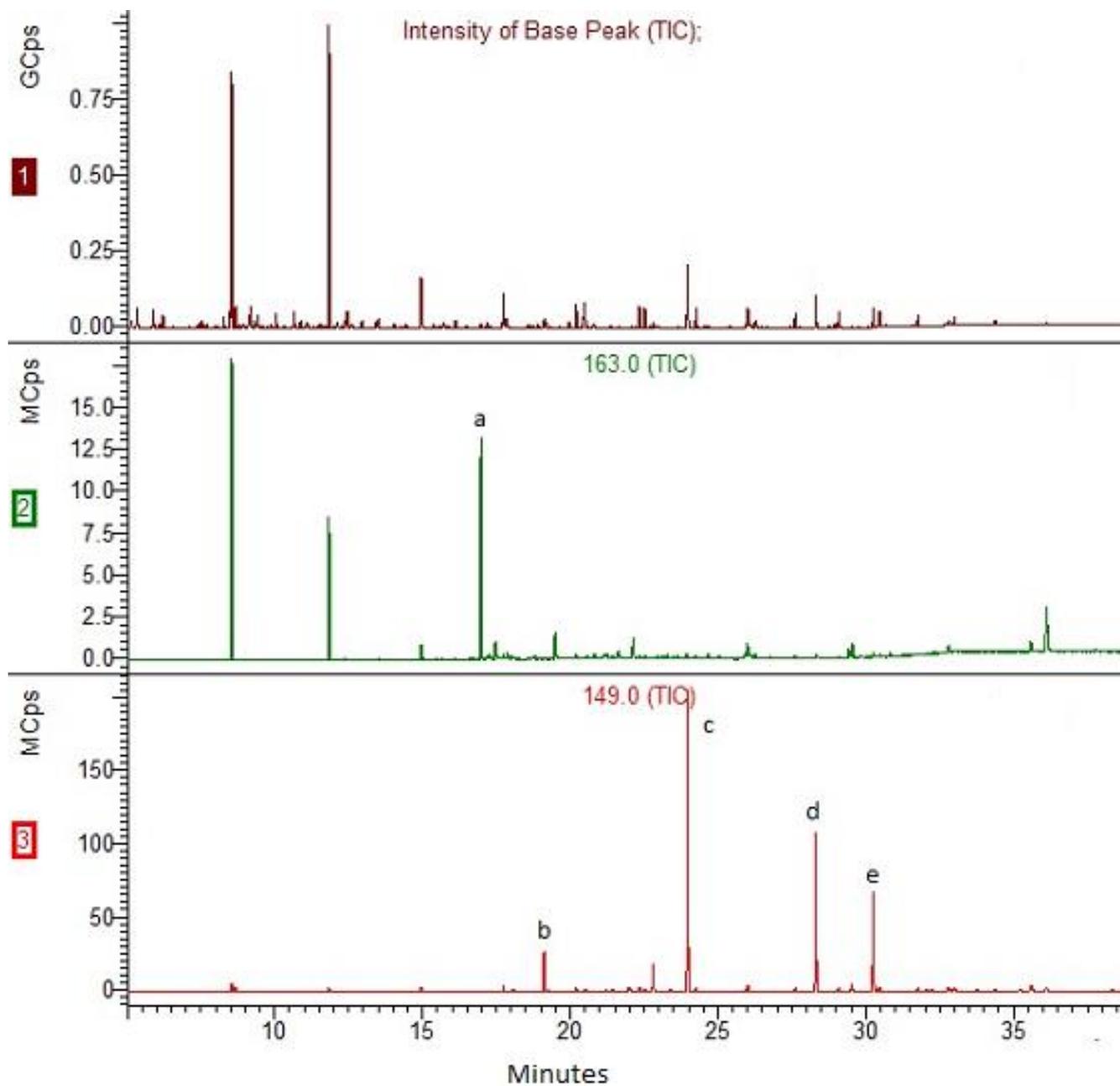


Figure 3.3: 1) Total ion chromatogram (TIC) representative of a water sample from the WWTP; 2) and 3): extracted ion chromatograms for $m/z= 163$ and 149 , respectively. The peaks identified as a, b, c, d and e correspond to compounds of the phthalate ester family (DMP, DEP, DBP, BBP, DEHP, respectively).

Table 3.3. Measured phthalate concentrations (shown are mean and standard error) in raw and treated wastewater of the primary and secondary advanced RWWTPs (n=12).

	Primary						Secondary Advanced					
	Inflow (ppm)		Outflow (ppm)		% change	<i>p</i>	Inflow (ppm)		Outflow (ppm)		% change	<i>p</i>
	Mean	SE	Mean	SE			Mean	SE	Mean	SE		
DMP	0.33	0.08	0.29	0.04	-12.8	<0.01	0.52	0.21	0.29	0.13	42.9	0.02
DEP	0.466	0.03	0.45	0.04	- 0.9	0.79	0.62	0.05	0.09	0.01	86.1	<0.01
DBP	8.07	6.16	5.15	2.88	-36.2	0.01	13.02	5.30	1.17	0.43	92.3	<0.01
BBP	9.20	7.69	6.89	4.50	-25.1	0.21	16.92	7.98	1.29	0.89	92.3	0.01
DEHP	6.25	5.27	5.62	3.01	-10.2	0.02	7.49	3.28	0.65	0.33	91.3	0.01

Discussion

This project aimed to assess the presence of estrogenic activity in WWTPs environments in Puerto Rico affected by human activities. Puerto Rico is a tropical, highly populated island that has undergone rapid land use changes as a consequence of the economic transformation from agricultural to an industrialized economy (Grau *et al.* 2003). Pollution of aquatic resources became an important issue in the Island, especially after the 1950's when industry began to surpass agriculture as the base of the economy, mainly those related to pharmaceuticals, electronics, textiles and clothing, petrochemicals, processed foods, and tourism (Hunter *et al.* 1995). Urban-impacted streams tend to receive larger loading rates of man-made organic chemicals if compared to pristine rivers (Kolpin, et al., 2002). Increased concentrations and loads of several chemical pollutants appear universal in urban streams, often occurring even at low levels of catchment urbanization (Hatt et al. 2004). A wide range of chemicals are present in most streams and substantial levels EDCs are common (Focazio, et al., 2008; Kolpin, et al., 2002). A large contribution of EDC's to aquatic ecosystems is attributed to the discharges of wastewater effluent from sewage treatment facilities (Harries *et al.* 1996, Ternes *et al.* 1999, Sando *et al.* 2005, Kathryn M *et al.* 2012) .

The National Institute of Environmental Health (NIEH) addresses some key points to be highlighted about endocrine disruption: 1) low dose effects, 2) wide range of health effects, 3) persistence of biological effects, and 4) ubiquitous exposure. EDCs present in the environment at very low levels have shown to have adverse effects in wildlife as well as in laboratory experiments. Normal endocrine signaling involves very

small changes in hormone levels. Hormones act at extremely low level concentrations, typically at the picomolar ($1\text{E}-12$) to nanomolar ($1\text{E}-9$) range. A peer-reviewed article (Vandenberg *et al.* 2012) document that EDCs can act at the nanomolar to micromolar range and some show activity at the picomolar range. Our results are in accordance with previous findings that point sources such as WWTPs discharges are contributors of EDCs to the environment (Auriol *et al.* 2006, Kasprzyk-Hordern *et al.* 2009, Basile *et al.* 2011) and that WWTPs can be a significant source of phthalates in receiving waters of tropical streams, as has already been demonstrated in temperate streams (Zolfaghari *et al.* , Dargnat *et al.* 2009, Clara *et al.* 2010). While research on EDCs is necessary globally, tropical studies are particularly needed as tropical cities continue to expand. The impacts of EDCs on coastal aquatic ecosystems may differ from temperate ecosystems as it has been observed in tropical rivers with high nutrient loadings (Figueroa-Nieves *et al.* 2014). As these compounds are ubiquitous in the environment, concerns over EDCs include the high incidence and the increasing trends of many endocrine related disorders in humans, observations of endocrine related effects in wildlife populations and the identification of chemicals with endocrine disrupting properties linked to disease outcomes in laboratory studies (Bergman *et al.* 2013). Although, the EPA has established criteria for compounds that end up being EDCs, there are no formal regulations against their effects in living organisms thus, environmental concentrations are also critical to regulate since exposure to these compounds is constant. Moreover, WWTPs are not designed to remove emerging contaminants that could be persistent and, thus, not metabolized or bioremediated, releasing them back into the environment (Basile *et al.* 2011).

Efficiency of wastewater treatment technologies. This study showed that WWTPs effluents in urban areas in Puerto Rico are contributors of low doses of EDCs to the environment. Even the secondary advanced WWTP showed levels of estrogenic activity in its effluent. The type of technology treatment at the different WWTPs appeared to have a notable effect on the estrogenicity of the effluents. For instance, the Puerto Nuevo RWWTP is a primary treatment facility that includes a pumping station, mechanical bar screen, grit removal mechanism, primary clarifiers, sludge handling facilities and disinfection area (EPA, 2010). The major goal of primary treatment is to eliminate pollutants that float from wastewater leaving soluble pollutants (Davis *et al.* 2008). Our results show that the Puerto Nuevo RWWTP discharges an effluent with estrogenic activity significantly higher than the secondary advanced Caguas RWWTP. For the phthalate esters, DMP, DBP and DEHP were significantly removed by the primary treatment but not DEP and BBP. It should be noted that, although the outflow showed a reduction in concentration in some of the compounds, primary WWTPs are not designed to eliminate chemical/toxic substances in the process. However, adsorption to particulate matter and/or complex or micelle formation and posterior sedimentation of suspended particulate matter could aid in the removal of these compounds during the process. As previously reported, DEHP showed reduction through sorption process (Marttinen *et al.*, 2003, Dargnat *et al.*, 2009). Nevertheless, these compounds will be deposited to the environment through the sludge collected.

Between Puerto Nuevo and Caguas RWWTPs, the latter showed significantly higher removal of estrogenic activity and phthalate compounds. More than 90% removal

was achieved for BBP, DBP and DEHP. The Caguas RWWTP is a secondary advanced facility where treatment consists of primary clarification, biological activated sludge, final clarification, dual media effluent filters, disinfection with chlorine, and effluent re-aeration (Malcom Pirnie, Inc. & Vincenty, Heres & Lauria 2004). The activated sludge process at the Caguas RWWTP makes it more efficient in removing pollutants although soluble organic compounds resistant to biological degradation may persist in the effluent. In addition, microbial species capable of degrading these compounds may not be present in the bioreactor, making them available in the effluent (Basile *et al.* 2011). Dual media effluent filters are efficient in removing particulates and thus, pollutants adsorbed to suspended solids but not soluble compounds. Therefore, soluble compounds and those that are not biologically degraded are prone to persist in the effluent and later discharged into the receiving stream. The disinfection step through chlorination at the secondary advanced facility could also aid in the removal of EDCs via the oxidation process (Schilirò *et al.* 2009). Chlorine has the potential to react with some EDCs, namely antibiotics and estrogens, although performance of chlorine oxidation is better achieved in acidic media as the reaction is pH dependent (Basile *et al.* 2011). However, this oxidation reaction is toxic and causes the formation of carcinogenic byproducts (Davis *et al.* 2008, Schilirò *et al.* 2009). The effluent of the Caguas RWWTP is discharged into Río Bairoa, a tributary of Río Grande de Loíza which feeds Lago Loíza, a major drinking water source for the San Juan Metropolitan Area. Hence, the importance to produce a high water quality effluent.

Managing emerging contaminants in tropical settings. Inputs from WWTPs have been shown to contribute to the estrogenic loads in receiving streams although there is high variation between sites and sampling seasons (Martinovic-Weigelt *et al.* 2013, Baldigo *et al.* 2014). Although EDCs in effluents and receiving surface water are of increasing concern worldwide, it is still poorly understood how these emerging contaminants are persistent in the environment (Deblonde *et al.* 2011). In rapidly developing tropical countries, managing these new contaminants is challenging. For instance, the Caguas RWWTP effluent is discharged into Río Bairoa, a tributary of the Río Grande de Loíza, whose waters provide about 100 million gallons per day to the San Juan Metropolitan Area through the Carraizo-Sergio Cuevas Filtration Water Plant. A wastewater treatment system not efficient in removing these persistent compounds could make them readily available in drinking water systems. However, high rates of river metabolism could have a role in minimizing their persistence in tropical rivers. Tropical streams and rivers differ from temperate regions because of their year-round high temperature (Ortiz-Zayas *et al.* 2005). Furthermore, urbanization increases water temperature and microbial activity in urban tropical streams (Ramirez *et al.* 2009) possibly increasing degradation of EDCs and thus, the respiration (R) rates in the stream. However, higher R can lead to large oxygen fluctuations and oxygen deficits in urban streams (Faulkner *et al.* 2000, Gücker *et al.* 2006). Although R is not always directly related with urbanization, it is often elevated in streams receiving wastewater discharges (Gücker *et al.* 2006, Wenger *et al.* 2009). Most of the flow of the Río Bairoa (71 to 94 %) comes from the effluent discharge from the Caguas RWWTP (Figueroa-Nieves *et al.* 2014). Contributions from WWTPs to streams with low flow could have more substantial effects, not only to stream flow but to

the estrogenic load into the receiving stream as the estrogenic effluent will dominate the natural river flow. As a result, a larger effect is expected in a stream with low flow and a high amount of sewage input as is the case for Río Bairoa. Our results show estrogenic activity in the effluent of the Caguas RWWTP at the picomolar range. Whether this concentration could have negative impacts in the aquatic life in this site is still unexplored. However, research on exposures to WWTP effluents with < 1 ng/L EEq induced estrogenic effects in the organisms exposed (Jobling *et al.* 2004, Liney *et al.* 2006).

Without adequate wastewater treatment, not only pollution of inland water occurs, but also coastal waters are affected. Coastal ecosystems are highly vulnerable to anthropogenic inputs due to high population densities, and rapid transport of pollutants from land to the ocean (Ortiz-Zayas *et al.* 2006). In coastal urban centers, such as those currently present in Puerto Rico, wastewaters receive primary treatment only which is not capable to remove chemical pollutants such as EDCs. The primary treated effluent from the Puerto Nuevo RWWTP is discharged into the Atlantic Ocean. The failure to remove chemical pollutants from the effluent could result in an increase load of EDCs to the ocean. EDCs have been found in seawater and sediments in marine environments worldwide (Atkinson *et al.* 2003, Pinto *et al.* 2005, Gómez-Gutiérrez *et al.* 2007) and in invertebrate and vertebrate marine species (Allen *et al.* 1999, Depledge *et al.* 1999, Andrew-Priestley *et al.* 2012). Given these findings, the presence of EDCs in tropical coastal waters such as those near the ocean outfalls in Puerto Rico should be assessed in the near future.

Associations between environmental pollution and ecosystems and human health is complex and often difficult to characterize (Briggs 2003, Eggen *et al.* 2004). Levels of exposure, for example, are often uncertain or unknown as a result of the lack of detailed monitoring and inevitable variations within any population group (Briggs 2003). However, it is well known that low concentrations of continuous and increasing number of pollutants have chronic effects in the organisms exposed (Eggen *et al.* 2004). Therefore, the need exists to establish rigorous criteria and enforcement for the adequate management of anthropogenic pollutants in effluent discharges and environmental concentrations of these compounds in order to protect the health of the ecosystem and human beings. These criteria must recognize latitudinal differences in degradation rates, particularly in tropical waters.

Conclusions

Although it has been shown worldwide that EDCs are detrimental, the consequences of such impacts to tropical streams and coastal environments have not been fully evaluated. The extent to which tropical receiving waters may be affected by EDCs and the threat that these compounds pose to aquatic life or human consumption remain largely unknown because comprehensive surveys are lacking. Our data are the first to characterize estrogenic levels in effluents from sewage treatment facilities in Puerto Rico and can prove to be helpful for managers. The comparison of the two treatment technologies indicated that, as expected, secondary advanced technology is more efficient than the primary in the removal of estrogenic activity and the phthalate esters studied. Insufficiently treated municipal wastewater discharges could be responsible for surface and coastal water contamination with EDCs. Establishing more efficient technologies in WWTPs could improve the quality of the effluent discharge and in turn the quality of the receiving water bodies. Unfortunately, water quality standards for EDCs in the environment do not exist yet. As a first step, the establishment of criteria for EDCs in receiving waters is needed in order to minimize degradation of downstream ecosystems and human health. Management of sewage effluents is critical for the conservation and restoration of tropical inland and coastal waters. Given the economic importance of clean tropical beaches associated with touristic activities, careful water pollution control strategies must be strengthened in tropical islands if a sustainable economic development is to be achieved.

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CHAPTER 4: DETECTION AND OCCURRENCE OF ESTROGENIC COMPOUNDS IN A TROPICAL URBAN WATERSHED

Abstract

Urban streams are vulnerable to the accumulation of potentially harming contaminants, such as EDCs, from urban stormwater runoff, atmospheric deposition and untreated wastewater discharges. This study reports the estrogenic activity in the surface waters of the urban, tropical Rio Piedras Watershed using a recombinant yeast bioassay to screen for estrogenic activity followed by the characterization of phthalate esters, such as dimethyl, diethyl, dibutyl, benzyl butyl and bis-2-ethylhexyl phthalates (DMP, DEP, DBP, BBP, and DEHP, respectively) by GC/MS analysis. The relationships between physico-chemical properties and estrogenic activity and concentrations were explored. Precipitation and discharge were correlated with the phthalate esters measured. Our results show an average estrogenic activity in the watershed of 2.24 pmol/L Eeq. Sites varied in their estrogenic activity concentrations but the range of that variation was relatively similar among study sites ($\chi^2(7) = 4.82, p = 0.68$). For the temporal variation, a significant difference in estrogenic activity was observed between sampling dates ($\chi^2(3) = 8.81, p = 0.03$). No correlation between estrogenic activity and precipitation and discharge was observed. The physicochemical parameters measured showed a weak significant relationship between estrogenic activity and temperature ($r = 0.35, p = 0.051$) but not with conductivity or pH. For the phthalates detected, variation was not site-specific. Significant differences in temporal variation of phthalate concentrations between sampling dates were observed for the phthalate esters DMP and DBP ($F(3,28)=15.5, p<0.01$; $F(3,28,)=8.68, p<0.01$), respectively. There was a positive, significant correlation between precipitation and the phthalates DMP ($r = 0.823, p < 0.01$), DBP ($r = 0.608, p<0.01$) and BBP ($r = 0.421, p = 0.02$). A positive weakly significant correlation was observed between discharge with DBP ($r = 0.354, p = 0.0467$). Among all physico-chemical parameters, two significant relationships were observed. Temperature was only significantly related to BBP ($r = -0.38, p = 0.03$) and conductivity was only related to DMP ($r = -0.45, p = 0.01$). No significant relationships were observed between pH and any of the phthalate esters. Overall, low levels of estrogenic activity and phthalate esters are occurring at the Rio Piedras Watershed, potentially degrading this aquatic ecosystem. The results from this study can serve as framework upon which to expand and add more information related to EDCs or other emerging contaminants in tropical urban watersheds.

Introduction

Currently, more than 80,000 chemicals are in use in the United States and 2,000 are introduced each year with little or no data on their estrogenic effects (NIEH, 2014). Data from the most recent US EPA Toxics Release Inventory (TRI) revealed that during 2012, 3.6 billion pounds of chemicals were released to the environment in the United States and its territories, including Puerto Rico. Puerto Rico alone reported a contribution of approximately 4.3 million pounds (US EPA, 2014). The US EPA in Puerto Rico has included approximately 225 sites in the Comprehensive Environmental Response Compensation and Liability Information System (CERCLIS), and 20 of these are included in the National Priority List (NPL) for immediate cleaning and monitoring based on risk assessment studies (US EPA, 2014). A national reconnaissance of pharmaceuticals, hormones, and other organic wastewater contaminants in streams from the United States showed that a wide range of chemicals are present in most streams, and that substantial levels of hormones, detergent metabolites (APEOs), plasticizers such as phthalates, and nonprescription drugs are common (Kolpin *et al.* 2002, Focazio *et al.* 2008).

Concern exists that many environmental and industrial chemicals may interfere with the endocrine system of both humans and wildlife. These chemicals are called endocrine disrupting compounds (EDCs) (Colborn *et al.* 2000). EDCs have been found in surface waters and often at mixtures of high concentrations of low-potency disruptors and low amounts of very powerful ones (Focazio *et al.* 2008), making both wildlife and humans at risk of exposure. EDCs are capable of interacting with the estrogen receptor in the cell causing effects even at trace-level concentrations, at the nanomolar to micromolar range and some show activity at the picomolar range (Kortenkamp 2008, Diamanti-

Kandarakis *et al.* 2009, Vandenberg *et al.* 2012).

EDCs are found in many of the products that people use every day such as pharmaceutical drugs, plastics, cosmetics, detergents, and pesticides. They emerge in municipal wastewater supplies and can be transported through runoff from lawns, farmland or feedlots and industrial wastewater. A large contribution of EDC's to aquatic ecosystems come from wastewater effluents from sewage treatment facilities. These discharges contain high concentration of chemicals of pharmaceuticals origin (Harris *et al.* 1997, Ternes *et al.* 1999). The failure of wastewater treatment plants (WWTP) to filter out EDC's leave them free to interact with fish, humans, and other organisms that may ingest them downstream.

Among the EDCs, phthalate esters have been found in most streams in the United States (Kolpin *et al.* 2002, Focazio *et al.* 2008). Phthalates are widely used as plasticizers since their introduction to the market in the 1920's (Graham, 1973) in poly(vinyl chloride), poly(vinyl acetate)s, cellulose, and polyurethanes, and as non-plasticizers in products such as lubricating oils, automobile parts, paints, glues, insect repellents, photographic films, perfumes, and food packaging (e.g., paperboard and cardboard) (Mackintosh, et al., 2004). Annually, more than three million metric tons of phthalates are produced globally (Schettler, 2006). Plasticizers are interfused with high polymers to increase flexibility, extensibility, and workability, changing the polymer from a hard glasslike solid to a flexible, tough elastomer (Graham, 1973). These compounds are not chemically bound to the plastic matrix, making them capable of

leaching out of products and be released into the environment (Latini, et al., 2003; Thompson et al., 2009). These esters have been found in all types of environmental and biological samples (Fromme, et al., 2002; Halgberg, et al., 2008; Vethaak, et al., 2005). The vast volume of plastics in consumer and industrial products, their persistence, and their routine disposal into the environment explain why human exposure to phthalate esters is nearly ubiquitous. Phthalates are readily absorbed through the skin (Janjua, 2008) and can also enter the body through inhalation or medical injection procedures (Schettler, 2005). Specifically, Di(2-ethylhexyl)phthalate (DEHP) has been found in human blood, seminal fluid, amniotic fluid, breast milk, and saliva. In an epidemiologic study, 75% of the 289 human subjects tested were positive for the presence of four different types of phthalates in their urine samples (Blount *et al.* 2000).

Due to their potential health and environmental risks, phthalate esters have become a matter of worldwide concern. Six phthalate esters (DMP, DEP, DnBP, BBzP, DEHP and DnOP) have been included as priority pollutants of US-EPA, the European Union (EU) and Chinese waters list (Net *et al.* 2015). A review by Net *et al.* 2015 summarizes the concentration range of several contaminants occurring all through the worldwide environment, including atmospheric aerosols and air (Xie *et al.* 2005, Wang *et al.* 2014), municipal solid waste compost (Dargnat *et al.* 2009), sludge (Dargnat *et al.* 2009, Reid *et al.* 2009), river and marine waters/sediments (Xie et al., 2007; Blair et al., 2009), wastewater (Gao *et al.* 2014) and drinking water (Gao *et al.* 2014, Liou *et al.* 2014). The review reports individual concentrations ranging from lower than the limit of quantification (bLOQ) to tens of $\mu\text{g/L}$ but can be up to 500 mg/L in surface water; from

bLOQ to tens mg/kg dw in sediment; from bLOQ to few hundred mg/kg in sludge; from bLOQ to few thousands ng/m³ in air and from bLOQ to few thousands mg/kg in air dust according to the location and the activities nearby (Net et al., 2015).

In urbanized streams, water quality is degraded by both point and non-point sources of contaminants (Paul *et al.* 2001, Kolpin *et al.* 2002, Grimm *et al.* 2008). Another significant source of EDCs to aquatic environments is the runoff from impervious surfaces in urban areas that arrives to streams. Urbanization can have profound impacts on runoff characteristics increasing concentrations of contaminants (Meyer *et al.* 2005). Urbanized streams show altered hydrology, water chemistry, channel geomorphology, and trophic resources (Paul *et al.* 2001). Hydrological alterations include increased total runoff, shorter storm flow duration, higher volume of peak runoff, and changes in base flows (Chadwick *et al.* 2006).

Clearly, the effects of urbanization on stream ecosystems arise from many interrelated sources but catchment impervious cover, however, has been shown to be a powerful proxy indicator that accounts for many of the factors mentioned earlier (Chadwick *et al.* 2006). Impervious surfaces do not allow precipitation to infiltrate into the ground increasing stormwater runoff. Surface runoff collects oils, gasoline, pesticides and all kinds of materials and substances present in the impervious surfaces as it flows through streets, parking lots and lawns. These pollutants are eventually transported into creeks and streams potentially degrading aquatic ecosystems (Pal *et al.* 2014).

Puerto Rico has one of the highest population densities in the World (340 persons/km²; <http://factfinder.census.gov>) and has experienced rapid rates of urbanization. Industrialization in the Island led to rapid population growth and expansion of metropolitan areas, especially San Juan (Pares-Ramos *et al.* 2008). In the year 2011, 40% of the potable water produced by the Puerto Rico Aqueduct and Sewage Authority (PRASA) was processed as sewage and of that 58% received only primary treatment (Lugo *et al.* 2011). An unknown proportion of the total potable water produced by PRASA becomes sewage that does not received treatment and is discharged untreated to streams and septic tanks(Lugo *et al.* 2011). Houses in rural areas commonly rely on septic tanks for sewage treatment, but gray waters from showers and sinks are often discharged untreated into streams (Ramirez *et al.* 2009). In addition, behavior of these pollutants can be different in Puerto Rico in comparison to the U.S. and Europe due to differences in climate and temperature. Managers in tropical regions, however, often rely on information from non-tropical regions for guidance.

Puerto Rico provides a unique opportunity to study anthropogenic influences on the tropical biosphere on a small spatial scale. The Rio Piedras watershed is part of the San Juan metropolitan area, the most intensely urbanized area in Puerto Rico (3,500 inhabitants/km²) (Ramirez *et al.* 2009). This region represents a hot-spot of energy and material consumption which may be contributing important loads of pollutants, including estrogenic compounds, to surface waters and eventually to the San Juan Bay Estuary.

This study will assess estrogenic activity in the Rio Piedras by testing, in a tropical setting, a recently published bioassay protocol. These data can be valuable in future pollution control and restoration efforts in tropical urban rivers.

The main purpose of this study is to understand the potential estrogenic activity in tropical urban watershed and to explore the possible impacts to human health and to aquatic ecosystems in the urban waters of the San Juan Metropolitan Area of Puerto Rico.

Materials and Methods

Study site

Eight sampling stations located along a topographic gradient (highland, medium, lowland) in the Rio Piedras watershed, Puerto Rico were selected (Figure 1.1) as part of the San Juan ULTRA Project (<http://sanjuanultra.org/>). The sampling locations were established based on demographic gradients from census block level data from the 2000 Census (Seguinot and Hernandez, 2010). The San Juan ULTRA Project sampling stations are part of a long-term water quality sampling program in which water samples are analyzed for nutrients (N+P), organic carbon, common ions, and physicochemical parameters (discharge, temperature, pH, and specific conductivity) (Ramírez *et al.* 2014).

We have used the existing sampling framework of San Juan ULTRA to test for estrogenic activity. Sampling for analysis of estrogenic activity was done on 3/20/2012, 6/19/2012, 9/18/2012, and 1/15/2013. This sampling scheme allowed the development of correlations between physico-chemical variables and estrogenic activity and to explore spatio-temporal variations in these variables.

Streamflow data collected at a USGS gaging station located in the watershed (Rio Piedras at Hato Rey, PR; USGS 50049100) were used to estimate flow conditions at each sampling site to account for the effects of discharge fluctuations on estrogenic activity. For ungaged sites, an estimation of streamflow was performed based on the ratio of drainage areas between an ungaged site and the gaged site

(<http://water.usgs.gov/osw/streamstats/ungaged2.html>). The drainage-area ratio (A_u/A_g) is assumed to be equal to the ratio of mean daily streamflow between the two stations (Q_u/Q_g) raised to an empirical exponent (b) as follows:

$$Q_u/Q_g = (A_u/A_g)^b \quad \text{Equation 1}$$

where Q_u is the estimated flow statistic for the ungaged site, A_u is the drainage area for the ungaged site, A_g is the drainage area for the stream gaging station, Q_g is the flow statistic for the stream gaging station, and b is the exponent of drainage (Heisel *et al.* 1983). The b exponent was initially estimated from the ratio of drainage areas of the two gaging stations at the mainstream (Rio Piedras at El Señorial; USGS 50048770 named here as La Sierra and the Rio Piedras at Hato Rey,; USGS 50049100 named here Avenida Central). A historic median ratio of mean monthly streamflows from the Rio Piedras at El Señorial and the Rio Piedras at Hato Rey, PR was calculated based on published USGS records (1990 to 2008 water years). To calculate the empirical exponent b , the ratio of drainage areas of these two stations was raised to a value so that the result will match the ratio of historic mean monthly flows between the two gaged stations. The empirically calculated b exponent was 1.28. This exponent was applied to Equation 1 to estimate streamflow at the seven ungaged San Juan Ultra Project stations in the basin (Figure 1.1). To accomplish this, Equation 1 was solved for Q_u as follows:

$$Q_u = (A_u/A_g)^{1.28} Q_g \quad \text{Equation 2}$$

This equation provides a reasonable estimate of mean daily streamflow at ungaged sites in the Rio Piedras basin. Streamflows estimated from Equation 2 were

within 23% of the mean monthly streamflow values reported by the USGS. Because Equation 2 has not been field tested yet, its validation is recommended with streamflow data measured at each of the ungaged sites of the San Juan Ultra Project so that the b exponent can be further adjusted if necessary.

Sample collection

Strict quality control measures were followed before, during, and after sample collection. Glassware was previously cleaned using a rigorous cleaning process to reduce interferences and minimize microbial degradation of the analytes. Glassware were cleaned with Alconox® soap and rinsed with tap water, soaked in 10% HCL for 5 minutes and rinsed three times sequentially with tap, distilled and ultra-pure water and let air dry, upside down. Finally, once in the field, water bottles were rinsed with sample water three times before collecting a final sample. To maintain their integrity, all samples were taken in amber glass bottles and stored in iced during transportation. Once in the lab, samples were immediately filtered using a 0.7µm glass fiber filter (GF/F). Samples were stored at 4°C for no more than two days before estrogenic activity analysis.

Recombinant yeast assay

A receptor-mediated β -galactosidase reporter yeast assay was used as previously described (Balsiger *et al.* 2010) to detect estrogenic activity in the water samples. A standard calibration curve of 17 β -estradiol (E2 mol/L) was prepared in ethanol and assayed along with the samples following the mentioned procedure. The total estrogenic activity of the unknown samples was determined based on the response of the assay and interpolated to a dose-response curve of the standard compound E2 in mol/L and appropriately converted to ng/L of 17 β -estradiol estrogen equivalents (EEq). The plates were read in a Tecan Infinite 200Pro Luminometer reader.

Chemical analyses

Extraction of phthalates compounds: We focused on four representative phthalates compounds of common use and array of molecular weights: Dimethylphthalate (DMP); Dibutyl Phthalate (DBP), Benzylbutyl Phthalate (BBP), and Bis-2-ethylhexyl Phthalate (DEHP). Water samples were pre-concentrated using solid phase extraction (SPE). Envi-Chrom P 500 mg glass cartridges were conditioned with 6 mL ethyl acetate, 6 mL methanol, and 6 mL nanopure water in sequence. Then, 100 mL of sample were loaded to the cartridge. After passing the sample, the cartridge was washed with 3 mL nanopure water to eliminate possible polar interferences from the matrix. Cartridges were dried under a vacuum for 15 minutes, centrifuged for 30 minutes, and exposed to a N₂ flow for 30 minutes. Analytes were eluted with 6mL ethyl acetate and evaporated to 0.5 mL by a gentle stream of nitrogen and reconstituted to a final volume of 1mL in ethyl acetate.

Detection: The concentrated extract (1 μ L) was injected into the GC/MS system. Samples were heated to an initial temperature of 50°C with a 8°C/ min. ramp to 260°C and held for 40 minutes with ultra pure helium as carrier gas. Target compounds were measured based on the following quantification ions: Dimethylphthalate (DMP): $m/z=163$; Dibutylphthalate (DBP), Benzylbutylphthalate (BBP) and Bis-2-ethylhexyl Phthalate (DEHP): $m/z=149$. Data acquisition was performed in the full scan mode measuring from m/z 50 to 550. Six-point calibration curves were conducted ranging from 1-100 ppm. The linear response of the curves produced correlation coefficients (R^2) higher than 0.99 for all compounds.

Statistical analyses

The reported data are the result of four independent experiments with all samples measured in triplicate within each experiment. Graphpad Prism trial version 6 was used to analyze estrogenic activity data. The MS Workstation 8 and the NIST Mass spectral database along with standard calibration curves for each phthalate ester were used to identify and quantify the phthalate esters. After verifying the data for normality and homocedasticity, JMP version 10 was used for either Analysis on Variance (ANOVA) for normally distributed data or Kruskal Wallis Analysis of Variance on Ranks for non-normally distributed data. To identify which grouped variables were significantly different, post-hoc Tukey test and Wilcoxon method for pairs were used for multiple comparisons. When data failed the normality tests even after transformations, Spearman Rank Order Correlations were done to explore relationships between variables. A p value <0.05 was set to represent a statistically significant difference. Non-detectable limits for estrogenic activity or phthalate concentrations were converted to one-half of the lower

calibration value in order to test for significant differences across seasons and sampling sites.

Results

Estrogenic activity

Table 4.1 summarizes the sampling dates (n=4), estrogenic activity and discharge along the Rio Piedras Watershed. A Kruskal-Wallis Analysis of Variance on Ranks was performed to explore the spatio-temporal variation of estrogenic activity along the watershed. Spatially, there was not a statistically significant difference ($\chi^2(7) = 4.82, p = 0.68$) in estrogenic activity along the watershed during the sampling period (Figure 4.1). For the temporal variation, a significant difference in estrogenic activity was observed between sampling dates, $\chi^2(3) = 8.81, p = 0.03$ (Figure 4.2). A nonparametric comparison for each pair using Wilcoxon method revealed significant differences between the dates 3/20/2012 and 6/19/2012 ($p < 0.001$). All other paired comparisons were not significant.

In San Juan, Puerto Rico, rain events are common to occur year round and, for this reason, seasonality in rainfall and discharge is difficult to define, although a dry season is commonly accounted from January to May (Ramírez *et al.* 2014). In this study, sampling dates on 3/20/2012, 6/19/2012, 9/18/2012, and 1/15/2013 recorded rainfall daily totals of 0, 5.8, 0.25 and 23.9 mm, respectively. A correlation matrix was constructed in order to explore relationships between estrogenic activity, discharge, and precipitation using Spearman's *rho*. However, a significant correlation between

estrogenic activity and discharge ($r = -0.0367$, $p = 0.840$) or precipitation ($r = 0.256$, $p = 0.156$) was not present.

Table 4.1. Description of sampling sites, sampling dates, estrogenic activity, and discharge for the Rio Piedras Watershed.

Sampling site number and name	Sampling date	Estrogenic activity (ng/L EEq)	Mean daily discharge (Q, 1000*m3/sec)
1. Quebrada Las Curias	3/20/ 2012	5.0E-18	41.15
	6/19/2012	2.2E-12	0.38
	9/18/2012	2.5E-16	89.91
	1/15/2013	5E-16	48.77
2. Montehiedra	3/20/ 2012	4.1E-14	37.62
	6/19/2012	1.0E-12	0.34
	9/18/2012	2.5E-16	82.21
	1/15/2013	5E-16	44.59
3. La Sierra	3/20/ 2012	3.4E-14	328.20
	6/19/2012	1.4E-12	5.47
	9/18/2012	2.5E-16	717.17
	1/15/2013	5E-16	388.97
4. Rio Piedras	3/20/ 2012	5.3E-14	0.79
	6/19/2012	3.7E-12	0.00

	9/18/2012	1.82E-11	1.72
	1/15/2013	5E-16	0.93
5. Correo de Cupey	3/20/ 2012	4.0E-14	13.13
	6/19/2012	2.5E-13	0.09
	9/18/2012	2.5E-16	28.69
	1/15/2013	5E-16	15.56
6. Ave. Central	3/20/ 2012	7.6E-14	764.64
	6/19/2012	8.6E-13	16.15
	9/18/2012	1.9E-11	1670.88
	1/15/2013	5E-16	906.24
7. San Patricio	3/20/ 2012	5E-18	29.36
	6/19/2012	1.3E-12	0.25
	9/18/2012	2.5E-16	64.15
	1/15/2013	8.95E-12	34.79
8. Las Lomas	3/20/ 2012	2.2E-14	95.43
	6/19/2012	6.1E-13	95.43
	9/18/2012	2.5E-16	208.54
	1/15/2013	5.3E-11	113.11

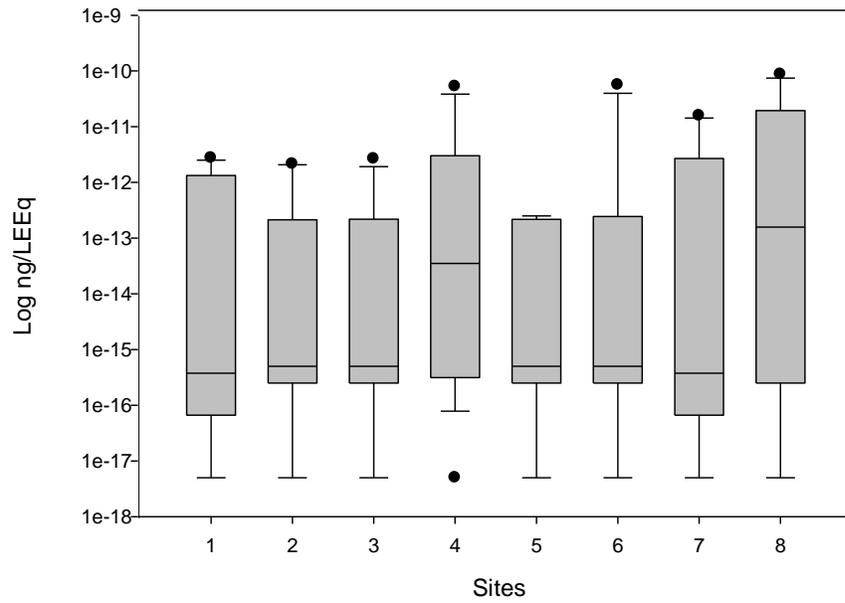


Figure 4.1. Whisker box plots of estrogenic activity along the Rio Piedras Watershed at each sampling site. See Table 1 for a description of sampling sites.

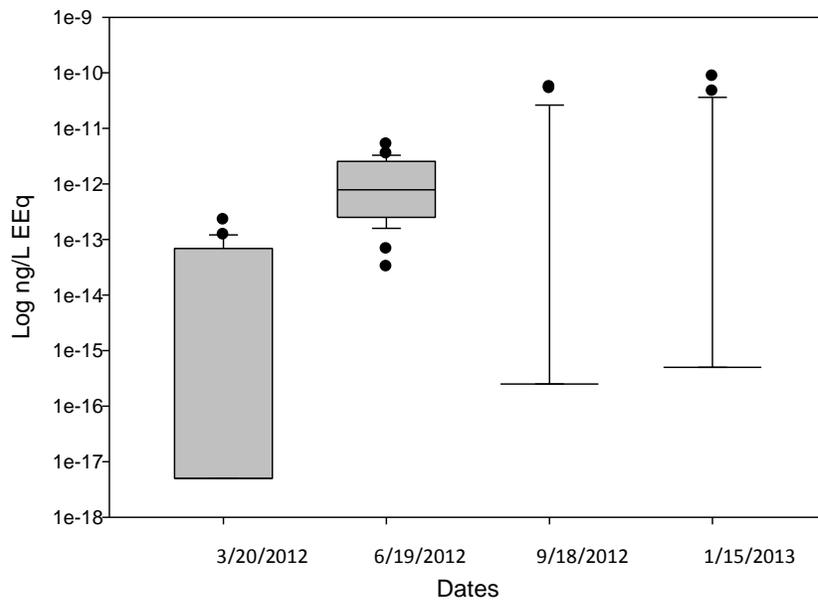


Figure 4.2. Whisker box plots of estrogenic activity for the four sampling dates.

Phthalates esters

The concentration for the five phthalate esters measured at all sampling sites along the Rio Piedras Watershed is presented in Table 4.2 and Figure 4.3.

Table 4.2. Mean concentration (ppm) and standard error (SE) for each phthalate ester at each sampling site along the watershed during the sampling period.

Site	DMP	SE	DEP	SE	DBP	SE	BBP	SE	DEHP	SE
Quebrada Las Curias	0.12	0.06	0.09	0.02	0.19	0.07	0.07	0.05	0.15	0.07
Montehiedra	0.09	0.03	0.06	0.02	0.19	0.08	0.19	0.19	0.15	0.11
La Sierra	0.14	0.07	0.05	0.01	0.19	0.06	0.12	0.11	0.07	0.01
Correo de Cupey	0.08	0.03	0.04	0.00	0.12	0.03	0.01	0.01	0.09	0.02
Rio Piedras	0.06	0.03	0.11	0.04	0.15	0.08	3.95	3.95	8.86	8.82
Ave. Central	0.09	0.02	0.05	0.00	0.19	0.07	0.01	0.01	0.05	0.03
Las Lomas	0.10	0.02	0.06	0.01	0.19	0.07	0.01	0.01	0.05	0.02
San Patricio	0.14	0.05	0.29	0.21	0.32	0.17	0.12	0.11	0.23	0.16

Of the five phthalates measured, DBP appears with the highest concentration along the watershed except for Montehiedra, where it is equal to BBP at 0.19 ppm. At Rio Piedras, BBP and DEHP are with the highest concentrations, 3.95 ppm and 8.86 ppm, respectively. The concentrations of phthalates per sampling dates are described in Table 4.3.

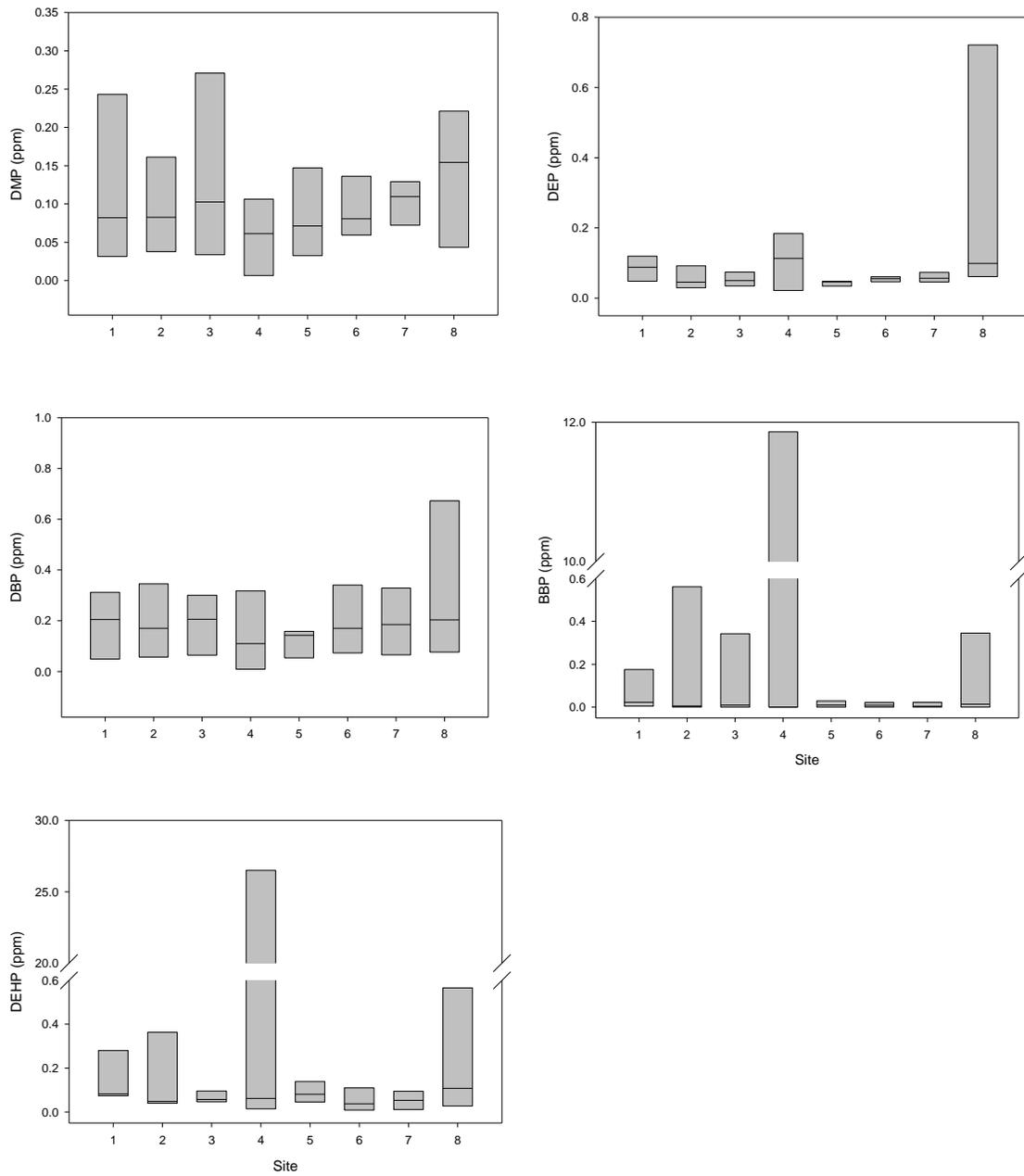


Figure 4.3. Box plots of the representative phthalate esters concentration at sampling sites along the Rio Piedras Watershed.

Table 4.3. Concentrations of each phthalate specie at each sampling date per sampling site along the Rio Piedras Watershed.

Sampling dates per site	Phthalates concentration (ppm) *1000				
	DMP	DEP	DBP	BBP	DEHP
Quebrada Las Curias					
3/20/ 2012	14.60	128.40	28.40	0.00	85.50
6/19/2012	81.90	36.90	111.60	18.00	79.20
9/18/2012	82.20	82.70	297.80	224.60	345.30
1/15/2013	296.70	93.30	316.90	27.70	71.40
Montehiedra					
3/20/ 2012	23.50	25.20	30.30	0.001	53.80
6/19/2012	80.50	42.50	137.30	0.001	42.30
9/18/2012	84.60	106.30	203.10	11.40	38.80
1/15/2013	186.70	48.80	392.70	744.80	466.30
La Sierra					
3/20/ 2012	15.30	30.10	30.80	18.50	56.00
6/19/2012	116.40	51.30	165.40	0.001	43.10
9/18/2012	88.80	48.50	246.30	0.001	108.00
1/15/2013	322.30	81.80	318.60	450.10	58.30
Correo de Cupey					
3/20/ 2012	22.80	30.30	26.60	32.60	155.80
6/19/2012	61.70	46.50	160.60	18.50	88.90
9/18/2012	80.90	46.10	135.20	0.001	72.60
1/15/2013	169.00	48.50	149.90	0.001	36.50
Rio Piedras					
3/20/ 2012	26.00	139.10	36.80	0.001	66.70
6/19/2012	96.50	87.30	183.00	0.001	56.60
9/18/2012	0.001	0.001	0.001	0.001	0.001
1/15/2013	109.50	198.90	362.40	15816.40	35312.70

Ave. Central					
3/20/ 2012	59.20	44.10	40.90	0.001	34.50
6/19/2012	101.30	53.40	397.30	0.001	39.80
9/18/2012	60.00	62.00	170.60	23.40	132.80
1/15/2013	147.60	57.10	169.70	16.50	0.001
San Patricio					
3/20/ 2012	62.10	43.30	33.00	0.001	61.60
6/19/2012	116.80	54.50	163.00	0.001	0.001
9/18/2012	102.30	78.50	369.70	7.67	44.30
1/15/2013	133.20	58.80	206.60	27.30	105.50
Las Lomas					
3/20/ 2012	19.70	918.30	45.70	0.001	144.00
6/19/2012	114.30	58.80	168.00	0.001	12.30
9/18/2012	194.10	128.70	817.20	26.90	71.80
1/15/2013	230.30	69.40	238.90	451.50	705.80

The spatio-temporal variation between phthalates concentration along the watershed during the sampling period was analyzed by One Way Analysis of Variance (ANOVA). For the spatial variation in phthalate concentrations along the watershed, no significant differences were found. These results are presented in Table 4.4.

Table 4.4. Analysis of variance table investigating differences in the concentration of each phthalate per sampling site along the Rio Piedras Watershed.

Phthalate	DF	Mean Square	F	P
DMP	7	0.008	0.467	0.849
	24	0.178		
DEP	7	469.12	0.688	0.682
	24	682.4		
DBP	7	0.014	0.352	0.921
	24	0.041		
BBP	7	7.541	0.961	0.481
	24	7.848		
DEHP	7	286.4	0.693	0.678
	24	413.4		

The analysis of temporal variation of phthalate concentrations between sampling dates resulted in significant differences for the phthalate esters DMP and DBP. These results are shown in Table 4.5.

Table 4.5. Analysis of variance table investigating differences in the concentration of each phthalate per sampling date along the Rio Piedras Watershed.

Phthalate	DF	Mean Square	F	P
DMP	3	0.101	15.463	<0.0001
	28	0.006		
DEP	3	1127.03	1.938	0.146
	28	581.43		
DBP	3	0.174	8.677	0.0003
	28	0.020		
BBP	3	9.471	1.247	0.312
	28	7.598		
DEHP	3	177.5	0.436	0.729
	28	406.9		

The Tukey-Kramer post-hoc comparison revealed that sampling dates 3/20/2012, 6/19/2012 and 1/15/2013 are significantly different in the concentration of DMP while the concentration of this phthalate on 9/18/2012 overlapped with those reported on 3/20/2012 and 6/19/2012. The concentrations for DEP, BBP and DEHP did not result in significant differences per sampling date. Post-Hoc Tukey-Kramer means comparisons

and connecting letters report are presented in Table 4.6. The connecting letters report shows the traditional letter-coded report where means that are not sharing a letter are significantly different.

Table 4.6. Tukey-Kramer HSD means comparison for phthalates concentrations.

Phthalate	Dates	Mean	Comparison Groups
DMP	3/20/ 2012	0.168	C
	6/19/2012	0.308	B
	9/18/2012	0.270	BC
	1/15/2013	0.439	A
DEP	3/20/ 2012	48.25	A
	6/19/2012	57.85	A
	9/18/2012	41.11	A
	1/15/2013	29.70	A
DBP	3/20/ 2012	0.18	B
	6/19/2012	0.42	A
	9/18/2012	0.47	A
	1/15/2013	0.51	A
BBP	3/20/ 2012	0.01	A
	6/19/2012	0.004	A
	9/18/2012	0.04	A
	1/15/2013	2.19	A
DEHP	3/20/ 2012	47.68	A
	6/19/2012	38.96	A
	9/18/2012	45.95	A
	1/15/2013	38.54	A

A Spearman's rank-order correlation was run to determine the relationship between the concentrations of phthalates, discharge and the daily total precipitation recorded during the sampling event. There was a positive, statistically significant correlation between precipitation and the phthalates DMP ($r = 0.823, p < 0.01$), DBP ($r = 0.608, p < 0.01$) and BBP ($r = 0.421, p = 0.02$). In relation to discharge, a positive weakly significant correlation was only obtained between discharge and DBP ($r = 0.354, p = 0.0467$).

In order to explore relationships between the physicochemical parameters temperature, pH, and conductivity with estrogenic activity and the phthalate esters detected in this study, Spearman correlations were done resulting in a weak significant relationship between estrogenic activity and temperature ($r = 0.35, p = 0.051$). Results of this test did not show a relationship between estrogenic activity and pH or conductivity. For the phthalates detected, there were only two significant relationships. Temperature was only significantly related to BBP ($r = -0.38, p = 0.03$) and conductivity was only related to DMP ($r = -0.45, p = 0.01$). The parameter pH was not significantly related to any of the phthalate esters.

Discussion

The present study investigated estrogenic activity occurrence in one of the largest urban areas in the tropical island of Puerto Rico, the Rio Piedras Watershed. Surface water quality of the Rio Piedras Watershed has been described as having higher dissolved solids, conductivity, ammonia (NH₄), alkalinity, and hardness and lower phosphorus (P), nitrate (NO₃), and nitrite (NO₂) when compared to 46 insular rivers (Lugo *et al.* 2011). The Puerto Rico Environmental Quality Board and the U.S. Environmental Protection Agency classify the waters of the Río Piedras River as highly polluted (Lugo *et al.* 2011). However, estrogenic activity at this watershed has not been described.

Aquatic environments in urban areas are especially vulnerable to receive higher loads of contaminants such as EDC's because urban areas reunite a large human population and, as a result, serve as concentration points for these compounds. EDC's are present in synthetic products used in the everyday life and often are coming from human biological waste, personal care products, pharmaceuticals and industrial wastes (Diamanti-Kandarakis *et al.* 2009). Additionally, urban areas have large impermeable surfaces carrying water inputs through surface runoff and stormwater. A multiple regression analysis to explore relationships between the levels of estrogenic activity and the concentration of all the phthalates detected with road density, structure density, impermeable surface density, drinking and wastewater pipe densities and % of green cover did not return any relationship between the variables (multiple regression analyses, $p > 0.05$). Based on these results, urbanization does not appear to be a major driver

influencing estrogenic activity in this watershed, at least, based on the sampled dates in this study.

In this study, the sampling sites were chosen based on demographic gradients along the watershed. However, the estrogenic activity detected did not vary significantly between sampling sites. Similar results were obtained by Matinovic *et al*, 2013 where estrogenicity of the water samples did not follow predictable spatial patterns. In that study,, many of the upstream heavily urbanized sites had levels of estrogenic activity comparable to those found in downstream locations. However, an average concentration of 2.24 pmol/L Eeq along the Rio Piedras watershed was found for the sampling period. This implies that an overall estrogenic activity at the picomolar range was detected. Others have found estrogenic activity in streams and rivers ranging from 0.25 to 1.72 pmol/L Eeq in the Netherlands (Murk *et al*. 2002); <1 to 21 ng/L Eeq in Luxemburg where the concentrations increased during spring and summer; <10 ng/L Eeq in streams from the US (Alvarez *et al*. 2013); from 0.23 to 324 ng/L Eeq in three rivers from China, and 2.75 up to 81.4 ng/L Eeq in Flemish rivers. Endogenous hormones act at extremely low level of concentrations, typically at the picomolar (1E-12) to nanomolar (1E-9) range and it has been shown that very low levels of exposure to EDCs cause endocrine or reproductive abnormalities, particularly if exposure occurs during critical stages of embryologic development (Sheehan *et al*. 1999, Chang *et al*. 2008, Rubin 2011). Our results are higher to those detected in the Netherlands and are below those from China, US, Luxemburg and Flemish rivers. It should be noted that results depends on the experimental methods used. Further studies in the Rio Piedras watershed investigating

the state of aquatic biota in relation to abnormalities caused by EDCs could reflect if this concentration is an actual risk for aquatic systems exposed to this level of estrogenicity.

Statistically significant variation in the temporal occurrence of estrogenic activity suggests environmental factors influencing the presence and/or concentrations of estrogenic compounds. The 6/19/2012 sampling event showed the highest median concentration at 0.787 pmoles/L Eeq, while the 3/20/2012, 9/19/2012 and 1/15/2013 events showed median concentrations at 5.0E-018 moles/L Eeq, 2.5E-016 moles/L Eeq and 5.0E-016 moles/L Eeq, respectively. The concentration of estrogenic activity was evaluated together with discharge or precipitation to elucidate if any association exists between the variables. A significant correlation between estrogenic activity and discharge or precipitation was not obtained, suggesting that rainfall/runoff at the specific sampling dates are not related to the levels of estrogenic activity found in the watershed. Point discharges are possible explanations of the presence of estrogenic activity in the surface waters analyzed that deserve attention for future research.

Some phthalate esters are an example of synthetic chemical compounds commonly found in the aquatic environment worldwide (Bhatia *et al.* 2014) and have shown to have estrogenic effects (Jobling *et al.* 1995, Harris *et al.* 1997, Chen *et al.* 2014). These compounds are widely used in both industrial processes and consumer products, mainly plastics. The global production of plastics has reached a level of ~150 million tons year⁻¹ and its widespread use have made them ubiquitous in urban environmental matrices (Zeng *et al.* 2009). Phthalate species did not vary among

sampling sites, suggesting continuous use of products containing these compounds in the urban environment surrounding the watershed. At all sites, during the sampling period, the phthalates found at highest concentration along the watershed is DMP, with a concentration of 0.19 ppm at all sites except for Montehiedra where it is equal to BBP at 0.19 ppm. At Rio Piedras, BBP and DEHP are with the highest concentrations, 3.95 ppm and 8.86 ppm, respectively. This site occurs in a heavily urbanized area where illegal, raw sewage discharges are evident (personal observation). There is one specific sampling date where the concentration of these two phthalates was detected at 15.8 and 35.3 ppm, respectively, suggesting high inputs at that specific date. Previous research around the world have found the most abundant phthalate in surface water was DEHP, as this is a high-production volume plasticizer that is regularly detected in aquatic ecosystems due to its continuous release into the environment (Fromme *et al.* 2002, Oehlmann *et al.* 2008, Zeng *et al.* 2009, Murray *et al.* 2010). A temporal trend was observed for DMP and DBP. Concentrations for DMP differed at events on 3/20/2012, 6/19/2012 and 1/15/2013 while DBP only differed on 3/20/2012. The positive and significant correlation with precipitation can be probably explained by urban runoff inputs as previously reported by (Gasperi *et al.* 2008). In this watershed, impervious surfaces reach approximately 49% of the total area (de Jesús-Crespo and Ramírez 2011), which results in high runoff rates, e.g., 72% of rainfall becomes stream flow.

Overall, the lack of correlation between estrogenic activity and physicochemical parameters are in accordance with previous studies reporting that water quality parameter

measurements are not predictive of estrogenic activity (Martinovic *et al.*, 2013; Montuori *et al.* 2008, Zheng *et al.* 2014).

The Rio Piedras Watershed has a sewage infrastructure serving a highly urbanized area where sewage overflows during storm events are common and still there are areas relying on poorly designed or old septic tanks. All of this discharge wastewater containing not only stormwater but also untreated human and industrial waste, toxic materials, and debris directly to nearby streams.

Conclusions

This work has served to assess levels of estrogenic activity along the Rio Piedras Watershed. Inputs from the surrounding urban environment could be affecting estrogenic activity levels. The estrogenic activity varied between sampling events, but was not related to discharge or precipitation. This suggests possible point sources, such as direct discharge of raw wastewater to the stream. In addition to estrogenic activity, phthalate esters were also identified along the watershed. Of the phthalates detected, DMP, DBP and BBP were indeed related to precipitation suggesting runoff inputs could be contributing to the presence of these contaminants in the surface water of the San Juan Metropolitan Area. Further research to identify specific sources of contamination with EDCs in this watershed could include 1) atmospheric inputs, 2) runoff from several urban areas along the watershed, 3) septic tanks not connected to the sewer system, and 4) gray waters from showers and sinks that in some areas are discharged untreated into streams. Rio Piedras flows into Rio Puerto Nuevo meaning low doses of estrogenic compounds flowing into this water body and eventually to the San Juan Bay.

The information collected in this study is a new assessment of estrogenic activity since no data are available to address pollution with estrogenic compounds in this watershed. It should help policymakers and natural resources managers across the tropics to minimize potential future degradation of downstream ecosystems and to protect the public health in urban areas. Furthermore, these data can be valuable in future pollution control and restoration efforts in tropical urban rivers.

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CHAPTER 5: THE OCCURRENCE OF EMERGING CONTAMINANTS IN A TROPICAL URBAN SETTING

Endocrine disrupting compounds (EDCs) were analyzed in this study by integrating chemical and biological analyses. This approach allowed the determination of not only the concentration of specific analytes in the samples, but also for screening the estrogenic activity of the samples. The application of this technique to samples taken in the framework of an urban tropical watershed permitted to have an idea of the potential contamination of this watershed with EDCs. This scheme allowed a perspective of how significant are the levels found in the surface waters of the watershed and of wastewater effluents, and the capability of the plant to eliminate them. The use of a bioassay to test water samples directly without concentration of the samples represent a quicker and simple alternative in comparison to other yeast based assays currently in use.

In this study, we have assessed the occurrence of estrogenic activity and EDCs in a highly urbanized watershed and we have assessed the occurrence and removal of EDCs and estrogenic activity in two RWWTPs in the San Juan Metropolitan Area, with a focus on phthalate esters as an example of EDCs. At the watershed, the estrogenic activity detected did not varied significantly between sampling sites, although sites were chosen based on a demographic gradient. However, an average concentration of 2.239 pmol/L EEq along the Rio Piedras watershed was found for the sampling period. This implies that an overall estrogenic activity at the picomolar range was detected. Further studies in this watershed investigating the state of aquatic biota in relation to abnormalities caused by EDCs could reflect if this concentration is an actual risk for aquatic systems exposed to this level of estrogenicity. A temporal variation in estrogenic activity was observed

between sampling dates although not related to discharge or precipitation. This suggests other environmental factors such as point discharges influencing the presence and/or concentrations of estrogenic compounds. Phthalate esters did not vary among sampling sites but a temporal trend was observed for DMP and DBP specifically. A correlation of these compounds and precipitation can be probably explained by urban runoff inputs contributing to the presence of these contaminants in the surface water of the San Juan Metropolitan Area. Previous studies in the Rio Piedras Watershed report that impervious surfaces now reach 49% of the total area which results in high runoff rates, e.g., 72% of rainfall becomes stream flow.

When evaluating the efficiency of two wastewater treatment technologies in removing EDCs, the concentrations of EDCs and estrogenic activity in effluent were higher in the primary RWWTP at Puerto Nuevo in comparison to the secondary advanced RWWTP in Caguas, mainly because primary RWWTPs plants do not apply any secondary treatment (e.g. activated sludge) to the wastewater, and most of the process is centered in eliminating solids. Between Puerto Nuevo and Caguas RWWTPs, the latter showed significantly higher removal of estrogenic activity and phthalate compounds. More than 90% removal was achieved for BBP, DBP and DEHP. The Puerto Nuevo WWTP discharges its effluent to the Atlantic Ocean. The failure to remove chemical pollutants from the effluent could result in an increase load of EDCs to the ocean as EDCs have been found in seawater and sediments in marine environments worldwide. Further research is needed to account for potential impacts of estrogenic compounds released. The Caguas RWWTP effluent is discharged into Río Bairoa, a tributary of the

Río Grande de Loíza, whose waters provide about 100 million gallons per day to the San Juan Metropolitan Area through the Carraizo-Sergio Cuevas Filtration Water Plant. Our results show estrogenic activity in the effluent of the Caguas RWWTP at the picomolar range. Whether this concentration could have negative impacts in the aquatic life in this site is still unexplored. However, research on exposures to WWTP effluents with < 1 ng/L EEq induced estrogenic effects in the organisms exposed. Analysis of effluent, surface water and sediment could be the first approach for determining possible impact to the wildlife.

Estrogenic activity removal was significantly different between inflow and outflow in the advanced RWWTP as opposed to the primary RWWTP where the inflow and outflow showed no significant differences in removal. Comparing the outflows of both levels of treatment, statistically significant differences between the outflows of the two WWTPs were determined. Therefore, the advanced RWWTP was effective in reducing estrogenic activity from the wastewater. For the phthalates detected, at the primary RWWTP some of the compounds were found at higher concentrations in the outflow than at the inflow suggesting accumulation of these compounds during the treatment process. A technical difficulty of this study was the failure in obtaining samples for each step of the treatment process. This could have aid in determining the validity of the aforesaid hypothesis. It should be noted that primary WWTPs are not designed to eliminate chemical/toxic substances in the process. At the secondary advanced RWWTP, a reduction in concentration at the outflow was detected for both estrogenic activity and the phthalates.

Taking into account the information obtained from both chemical and biological analyses the use of at least secondary treatment removes EDCs such as phthalates in the wastewater with a reduction that ranged from 42.9 to 92.4 %. However, a concentration from 0.86 to 1.29 ppm for all the phthalates in the outflow of the secondary advanced WWTP was detected.

Because of the estrogenic potency of these compounds in the environment, further studies are needed to determine if the discharge of the outflow in the receiving environment could affect the biota. In the Rio Bairoa, the effluent contributes a large volume of the flow of this river and it is possible that aquatic organisms may be exposed to estrogenic chemicals at levels sufficient to produce biological responses. This needs to be investigated by conducting additional assessments of the receiving aquatic environment.

As shown by the experiments presented in this study, I suggest that it is of great importance that primary WWTPs in Puerto Rico expand their facilities and upgrade their system to ensure that 100% of the wastewater can be treated by at least, secondary treatment. More studies concerning the fate of EDCs in WWTPs will help to find answers that justify that large economic investments required upgrading wastewater treatments in these facilities. The results presented in the previous experimental chapters will undoubtedly serve as the initial framework upon which to expand and add more information in relation to EDCs or other contaminants in water resources in Puerto Rico.

Inactivation of Bacillus subtilis with a Modified Solar-Powered Engineered Experimental Drum Filtration and Disinfection (SEED) Unit

Basic Information

Title:	Inactivation of Bacillus subtilis with a Modified Solar-Powered Engineered Experimental Drum Filtration and Disinfection (SEED) Unit
Project Number:	2014PR163B
Start Date:	3/1/2014
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Focus Category:	Water Quality, Water Supply, Treatment
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Publication

1. S. Hwang, 2014. "Flask to field toward Non-PRASA water sustainability" in EPA Region 2 Caribbean Science Workshop, San Juan, PR.

Final Report

SEED Demonstration of *Bacillus subtilis* Removal

The SEED unit was assessed in three different drum combinations that were constructed with the same hydraulic characteristics. Physicochemical and bacteriological parameters were monitored for each sampling time, port and drum configuration.

Physicochemical and Biological Water Quality Parameters

During the demonstration, five different physicochemical parameters were measured at each port and sampling times (i.e. 0, 2, 4, 6 and 7 hours) except at 6 hours for TOC. The first parameter measured was temperature (Figure 1) and showed no significant variations among the ports. Minor differences between the sampling times at the first two days and the third day were noticed. However, these differences might be due to the sampling times, i.e. experiments were performed at different times during the day and hence highest temperature were observed at ~15:00 pm.

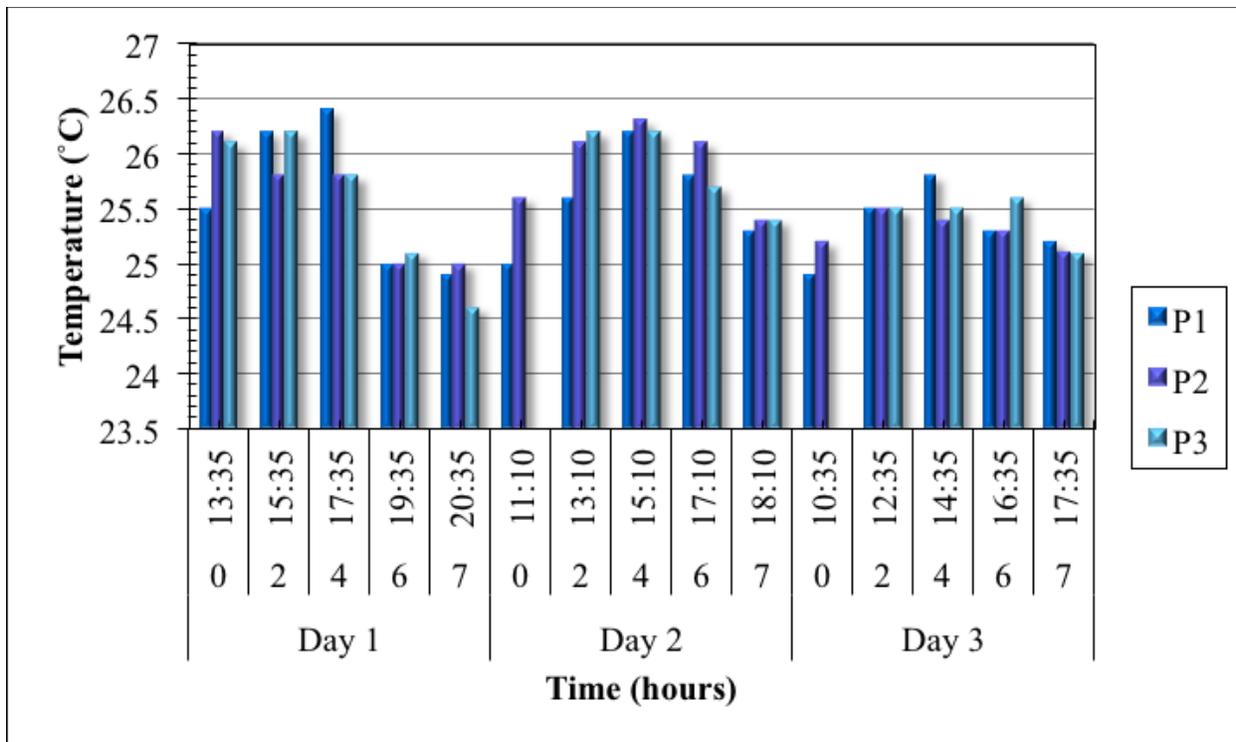


Figure 1 Temperature measurements at field-scale.

For pHs (Figure 2), difference was significant between the ports at the same sampling times. In general, a reduction in pH was noticed after filtration (P2) but then increased after chlorination (P3), except at the first three samples of Day 1. Despite a decrease in pH, the final measurement of the treatment train stayed between 8.4 and 8.5, which was in the range of the recommended Secondary Standards of pH (USEPA, 2009) but was slightly higher than the preferred pH value (< 8.0) suggested by WHO (2011).

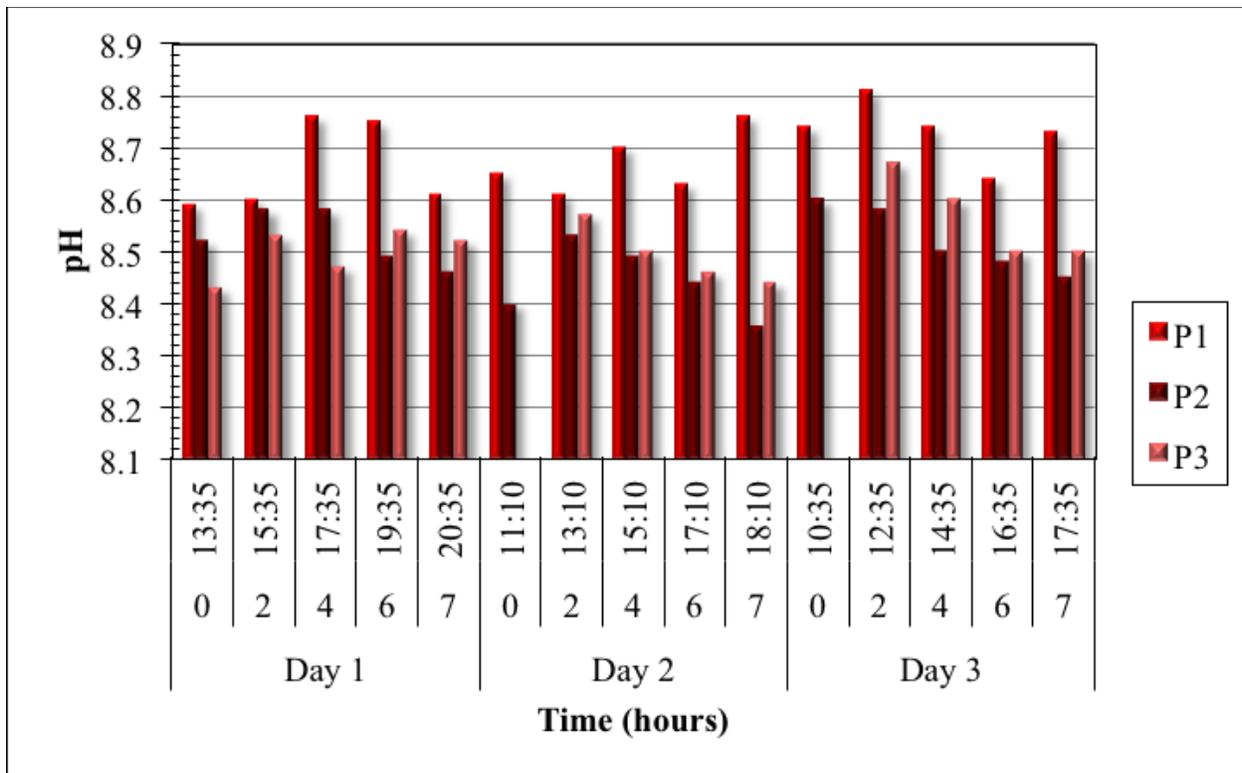


Figure 2 pH measurements at field-scale.

Conductivity was observed to increase at P3 in all samples at the three experiments (Figure 3). Although no specific trend was noticed at P2. At P1 and P2 during Day 1, the values were in the range of 365 - 370 $\mu\text{S}/\text{cm}$. At Day 2, the values at P1 and P2 were slightly lower (350 - 360 $\mu\text{S}/\text{cm}$). A greater variation was noticed during Day 3, measurements fluctuated from approximately 345 to 370 $\mu\text{S}/\text{cm}$.

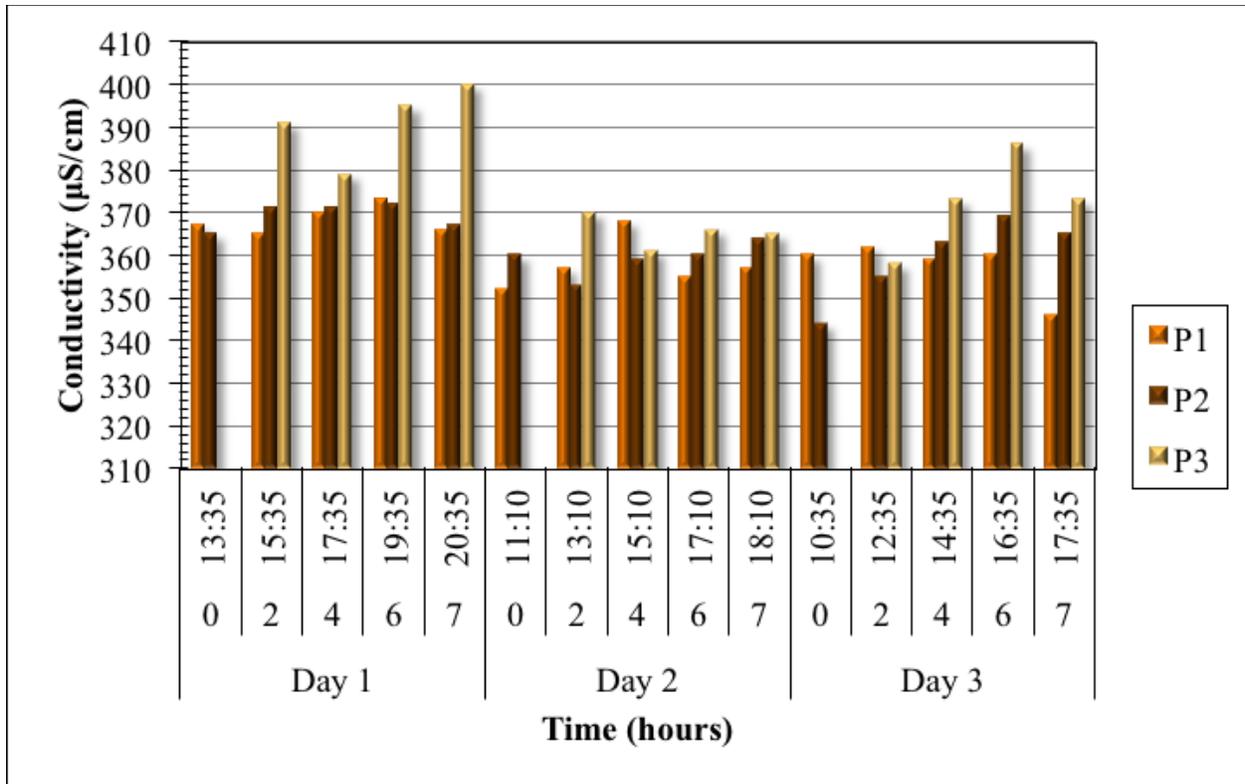


Figure 3 Conductivity measurements at field-scale.

Turbidity measurements were increased through sampling times during the experiments (Figure 4). All turbidity measurements were lower than 1 NTU at P1 but increased at P2 and P3. Increase in turbidity at P2 might be due to escape of colloidal sand particles from the filters. An increase in turbidity at P3 might be due to the type of chlorination technique used. It was observed that the chlorine tablet did not completely dissolve in the water, resulting in suspended debris in the system. This high abrupt increase was only noticed during the first day of experiment. Hence, further field adjustments were performed at the subsequent experiments to solve this issue. However, the final turbidity values were still slightly higher than 1 NTU and hence did not comply with the USEPA MCL regulation (USEPA, 2009).

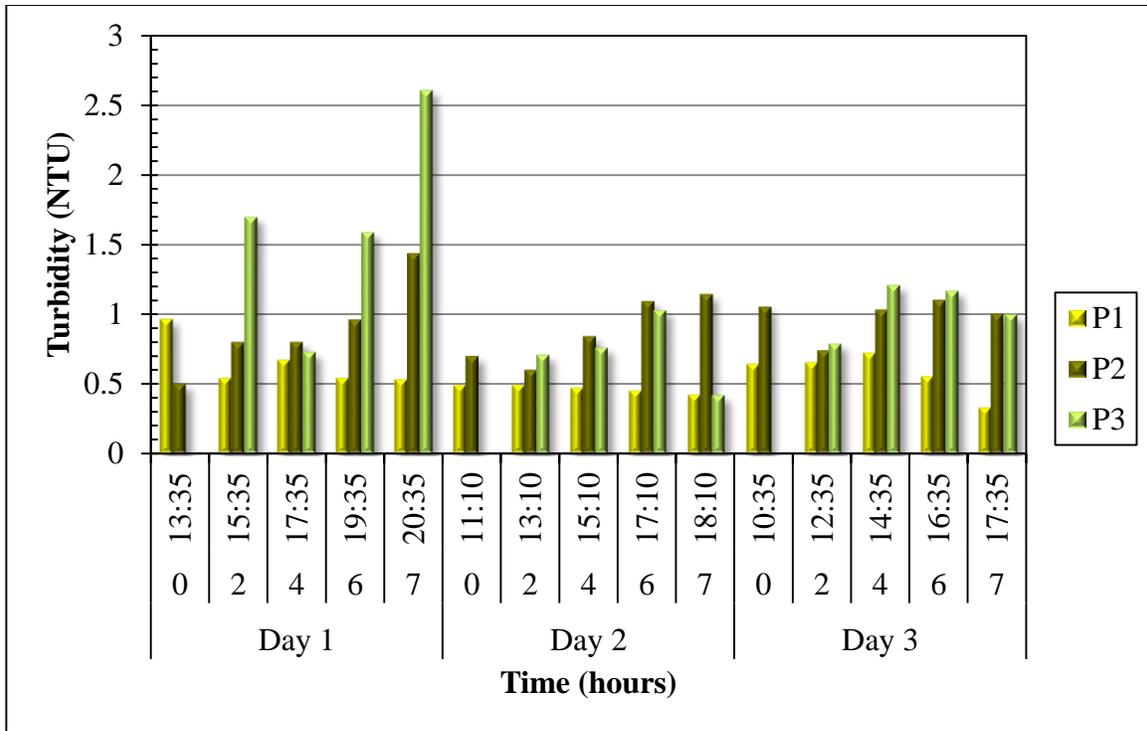


Figure 4 Turbidity measurements at field-scale.

TOC measurements varied significantly through the three days (Figure 5). At Day 1, slight increases in TOC measurements were observed through sampling times and in some instances through ports, ranging from ~13 to ~20 mg/L. In contrast, variations of measurements at Days 2 and 3 were abrupt with a lowest value of ~3 mg/L and a maximum value of ~30 mg/L. However, no specific trends within the days were observed. Dissimilarities in TOC concentrations at the influents among the three days were found. Yet these variations were present still no similar reductions or increases neither by port nor by contact time were seen.

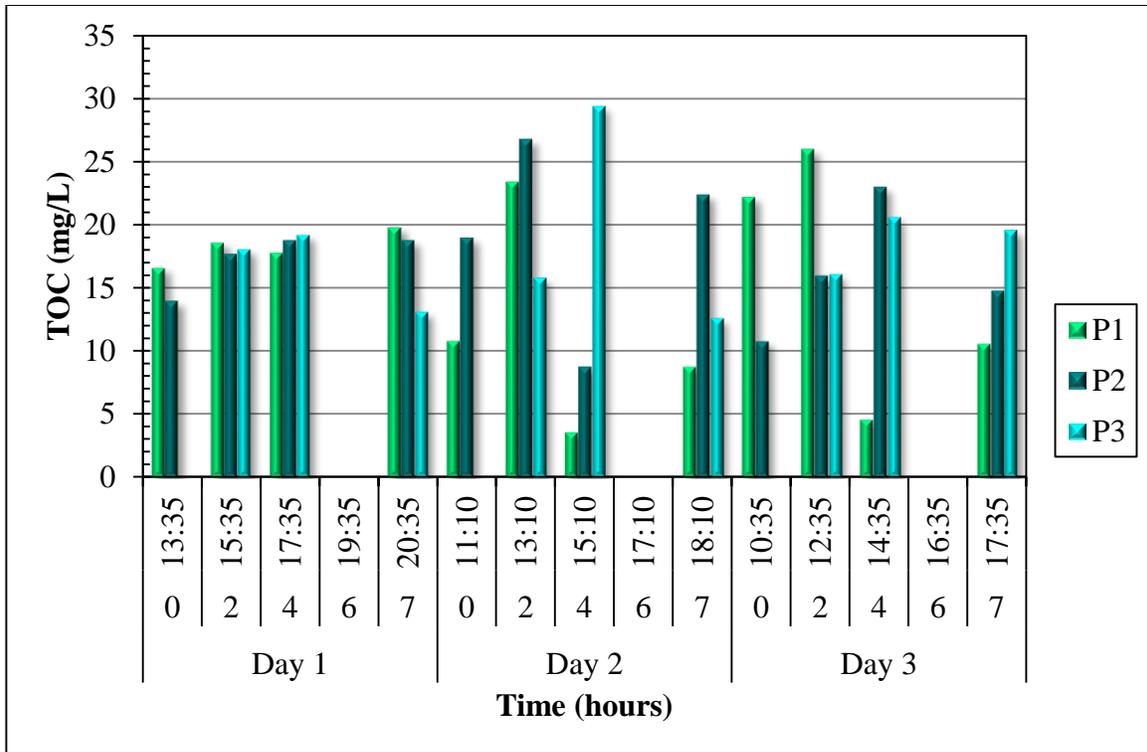


Figure 5 TOC measurements at field-scale.

The initial numbers of *B. subtilis* (P1) were lower than 500 CFU/100 mL through the three days ranging from 75 to 214 CFU/100 mL at Days 2 and 3 (Figure 6). Increase in the numbers at P2 and P3 were reported in almost all samples and days. Particularly, at Day 3 a steeper increase was observed with maximum values of 16,400 CFU/100 mL at P2 and 27,000 CFU/100 mL at P3. These high numbers might indicate saturation of the system during the last day of demonstration.

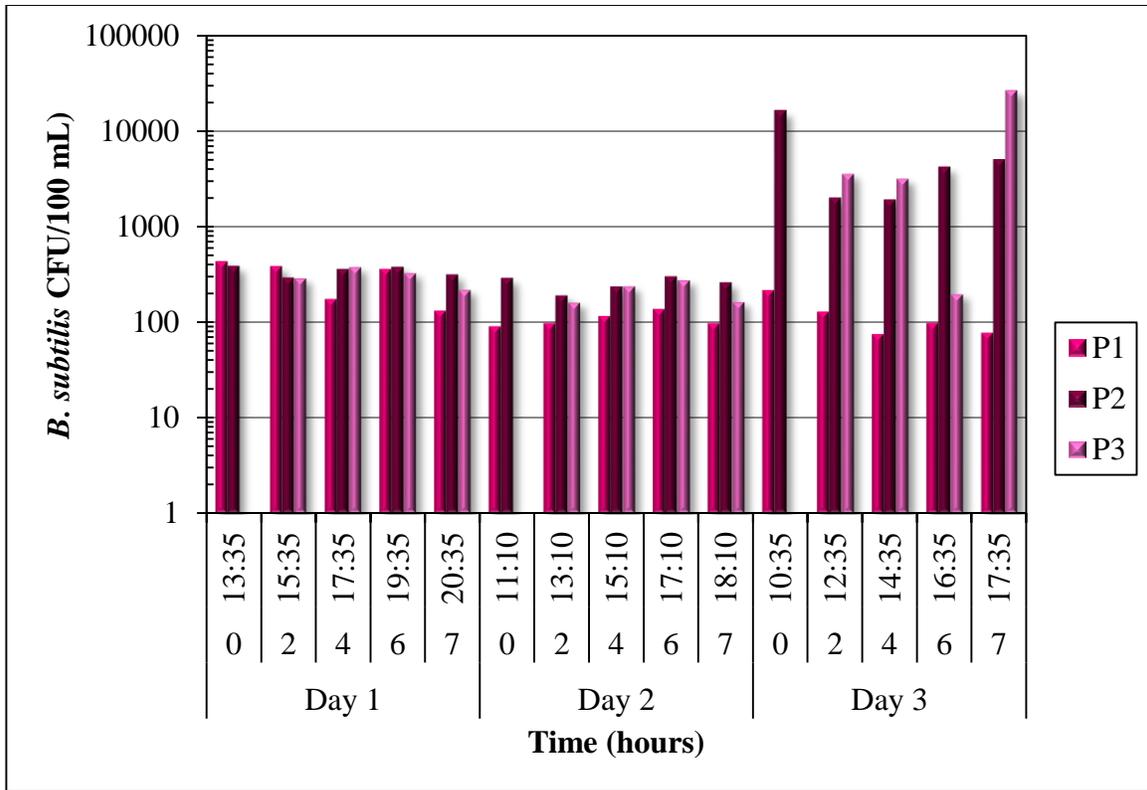


Figure 6 *B. subtilis* numbers at field-scale.

Statistical Analysis

Statistical analyses were performed to the physicochemical and bacteriological parameters, including matrix plots, Pearson correlation coefficients and P-values. These analyses were done for each port and day using the statistical program Minitab 17. In general, a clear relationship between parameters could not be identified since none of the combinations perceived the consistent relations during the three days.

The first set of statistical analyses was applied to P1 at Day 1. The matrix plot (Figure 7) suggested a positive relation between pH and conductivity and a negative relation between TOC and turbidity. Pearson correlation coefficients and P-values (Table 1) evidenced the highly positive relation between pH and conductivity with the values of 0.895 and 0.4, respectively. The high

inverse relation between TOC and turbidity was sustained by the Pearson correlation coefficient of -0.914 and P-value of 0.086. In addition, the Pearson correlation coefficient (-0.654) and P-value (0.346) suggested that TOC might be negatively correlated to *B. subtilis*. Finally, other Pearson correlation coefficient and P-value (0.432 and 0.467, respectively) suggested that turbidity might also be correlated to *B. subtilis*, although P-values were found to be >0.05.

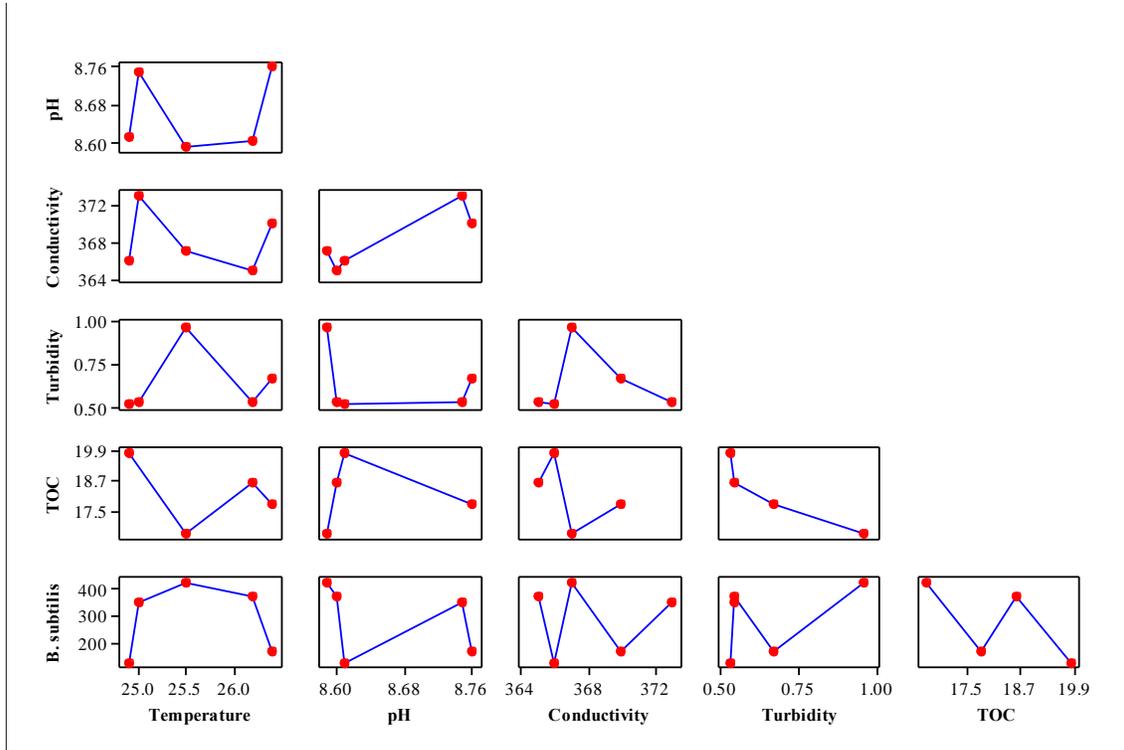


Figure 7 Matrix plot of P1 at Day 1 of SEED unit.

Table 1 Pearson correlation and P-values for P1 at Day 1 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.138				

	0.825				
Conductivity	-0.191	0.895			
	0.759	0.04			
Turbidity	0.138	-0.271	-0.103		
	0.825	0.659	0.869		
TOC	-0.376	-0.099	-0.413	-0.914	
	0.624	0.901	0.587	0.086	
<i>B. subtilis</i>	0.05	-0.288	-0.001	0.432	-0.654
	0.937	0.639	0.998	0.467	0.346

The matrix plot of samples from P1 at Day 2 is shown in Figure 8. The plot suggests a correlation between conductivity and temperature, turbidity and pH, TOC and pH, *B. subtilis* and TOC, and *B. subtilis* and temperature. Moreover, for the proper analysis, Pearson correlation coefficients and P-values were calculated and thus supported the matrix plot suggestions (Table 2). Outcomes from these calculations proved that conductivity was highly positively related to temperature with respective coefficients of 0.832 and 0.081 of Pearson correlation and P-value. In addition, it was shown that TOC was highly negatively related to pH with a Pearson correlation coefficient of -0.727. However, P-value (0.273) was high and hence and did not support the relation between the parameters. Moreover, Pearson correlation (-0.739) indicated that turbidity was highly negatively related to pH but P-value (0.153) did not provide strong evidence of the relation. In terms of bacteriological parameters, *B. subtilis* might be negatively related to TOC as the Pearson correlation implied (-0.518). However, P-value (0.482) was higher than 0.05. In contrast, *B. subtilis* might be positively related to temperature with respective coefficients of Pearson correlation coefficient and P-value 0.692 and 0.195. Likewise, P-value was greater than 0.05 and thus provided weak evidence.

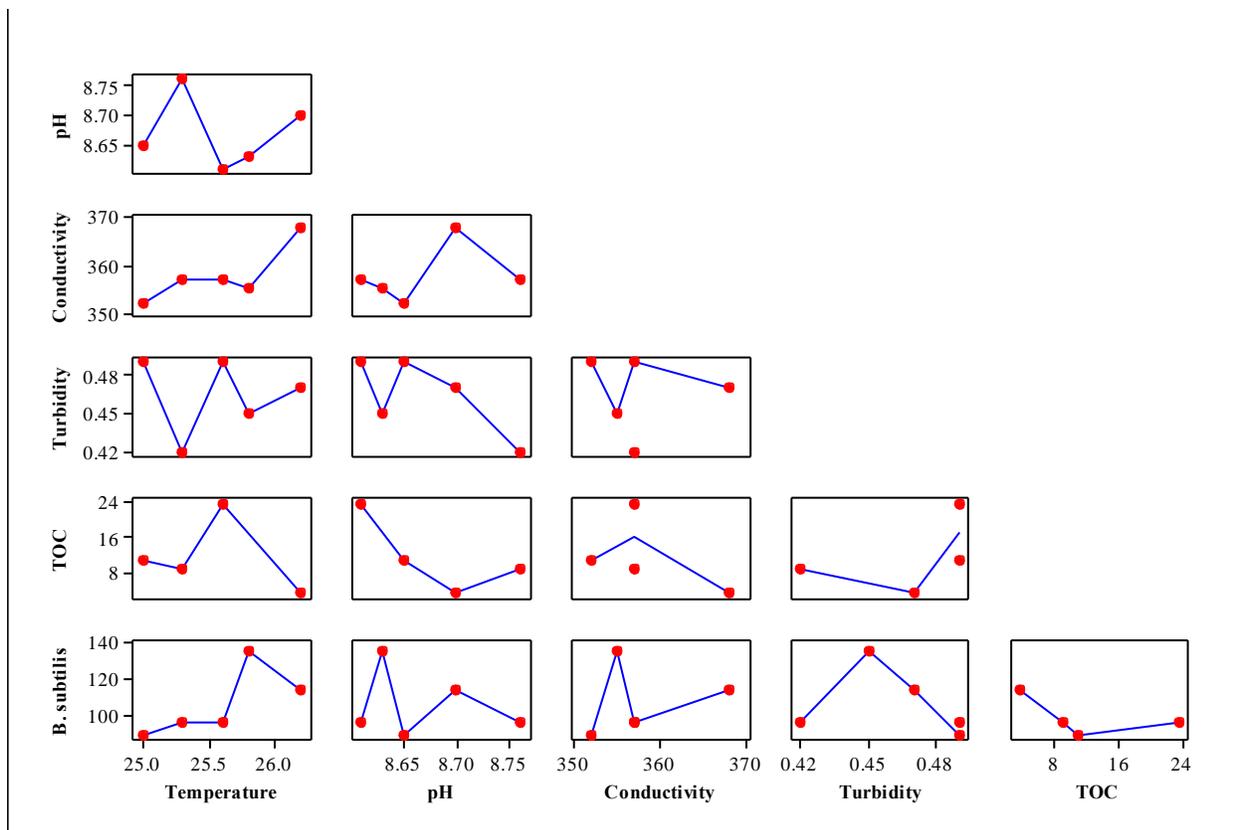


Figure 8 Matrix plot of P1 at Day 2 of SEED unit.

Table 2 Pearson correlation and P-values for P1 at Day 2 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	-0.045				
	0.943				
Conductivity	0.832	0.348			
	0.081	0.566			
Turbidity	-0.029	-0.739	-0.05		
	0.963	0.153	0.936		
TOC	-0.27	-0.727	-0.497	0.434	
	0.73	0.273	0.503	0.566	
B. subtilis	0.692	-0.195	0.254	-0.28	-0.518
	0.195	0.753	0.68	0.648	0.482

The matrix plot analysis for P1 at Day 3 suggested relations between turbidity and conductivity, and *B. subtilis* and TOC (Figure 9). Table 3 gave the Pearson correlation coefficients and P-values for the parameters assessed. The coefficients of Pearson correlation, 0.888, proved that turbidity and conductivity were highly positively related. The P-value, 0.044, of the analysis strongly evidenced the relation. Likewise, other correlation was found between physicochemical parameters, pH might be positively related to TOC with a Pearson correlation coefficient of 0.691. In terms of bacteriological parameters, statistical analysis showed that *B. subtilis* was highly positively related to TOC with a Pearson correlation coefficient of 0.725. Furthermore, *B. subtilis* might also be negatively related to temperature with a Pearson correlation coefficient of -0.696. Although, the Pearson correlation coefficient indicated correlation between the above parameters still P-values were greater than 0.05 indicating weak evidence of the relations.

It was expected that all three days would have had the same correlations between parameters since P1 was the influent to the SEED unit. However, a wide variation was achieved between days. One reason of this variation could be the diverse condition of the lines. Yet one trend was found during Day 1 and 2: the negative relation between *B. subtilis* and TOC.

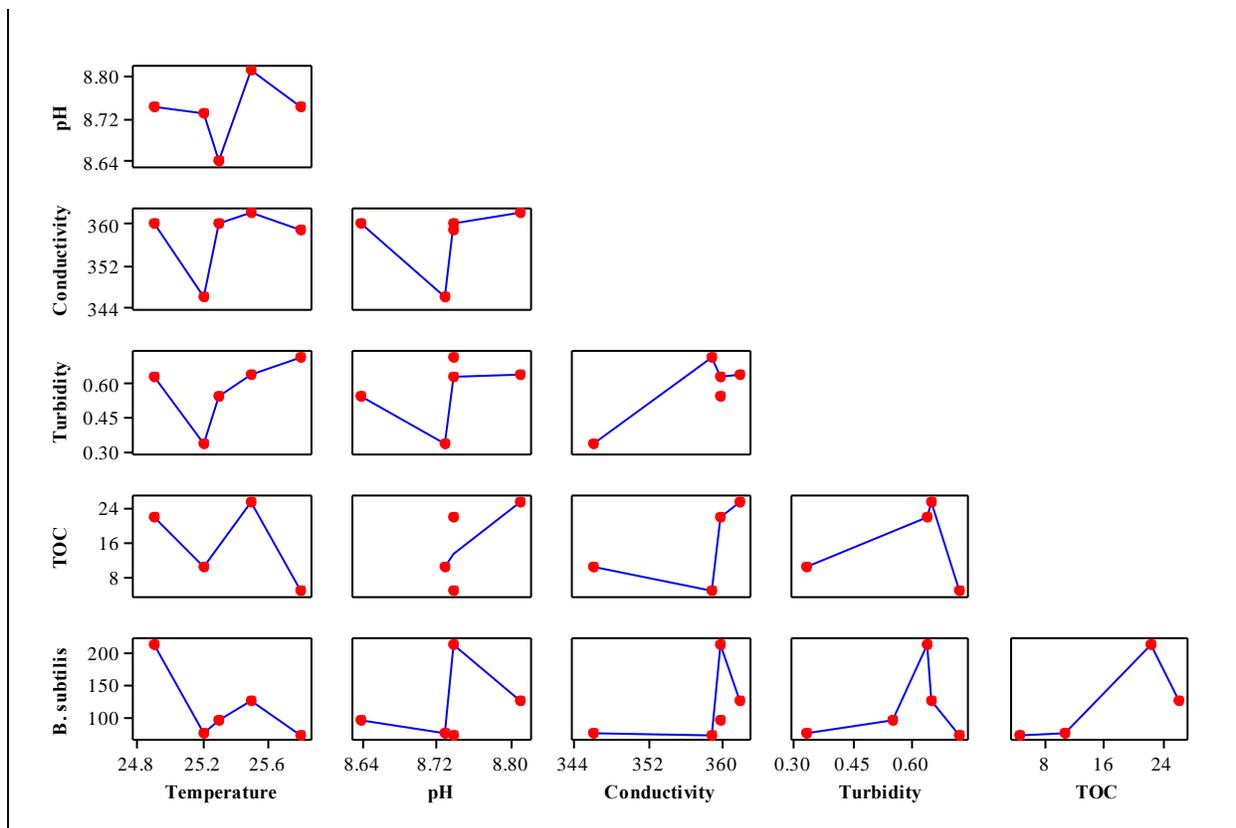


Figure 9 Matrix plot of P1 at Day 3 of SEED unit.

Table 3 Pearson correlation and P-values for P1 at Day 3 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.204				
	0.742				
Conductivity	0.209	0.112			
	0.735	0.857			
Turbidity	0.42	0.282	0.888		
	0.481	0.646	0.044		
TOC	-0.485	0.691	0.483	0.16	
	0.515	0.309	0.517	0.84	
<i>B. subtilis</i>	-0.696	0.224	0.431	0.324	0.725
	0.191	0.717	0.468	0.594	0.275

Figure 10 shows the matrix plot of P2 at Day 1. This analysis suggested relations between conductivity and temperature, *B. subtilis* and TOC, turbidity and conductivity, turbidity and pH, TOC and turbidity and *B. subtilis* and turbidity. The Minitab was used to calculate the Pearson correlation coefficients and P-values for a proper analysis (Table 4). Pearson correlation indicated that turbidity might be negatively related to: TOC with a coefficient of -0.62; and to *B. subtilis* with a coefficient of -0.621. Moreover, conductivity might be negatively related to temperature (Pearson correlation = -0.623). In contrast, turbidity might be positively related to: pH (Pearson correlation = 0.686) and to conductivity (Pearson correlation = 0.721). The only relation found in terms of bacteriological parameters was the highly positive relation between *B. subtilis* and TOC (Pearson correlation = 1). However, the correlations of the parameters had P-values greater than 0.05 (except for the correlation between *B. subtilis* and TOC).

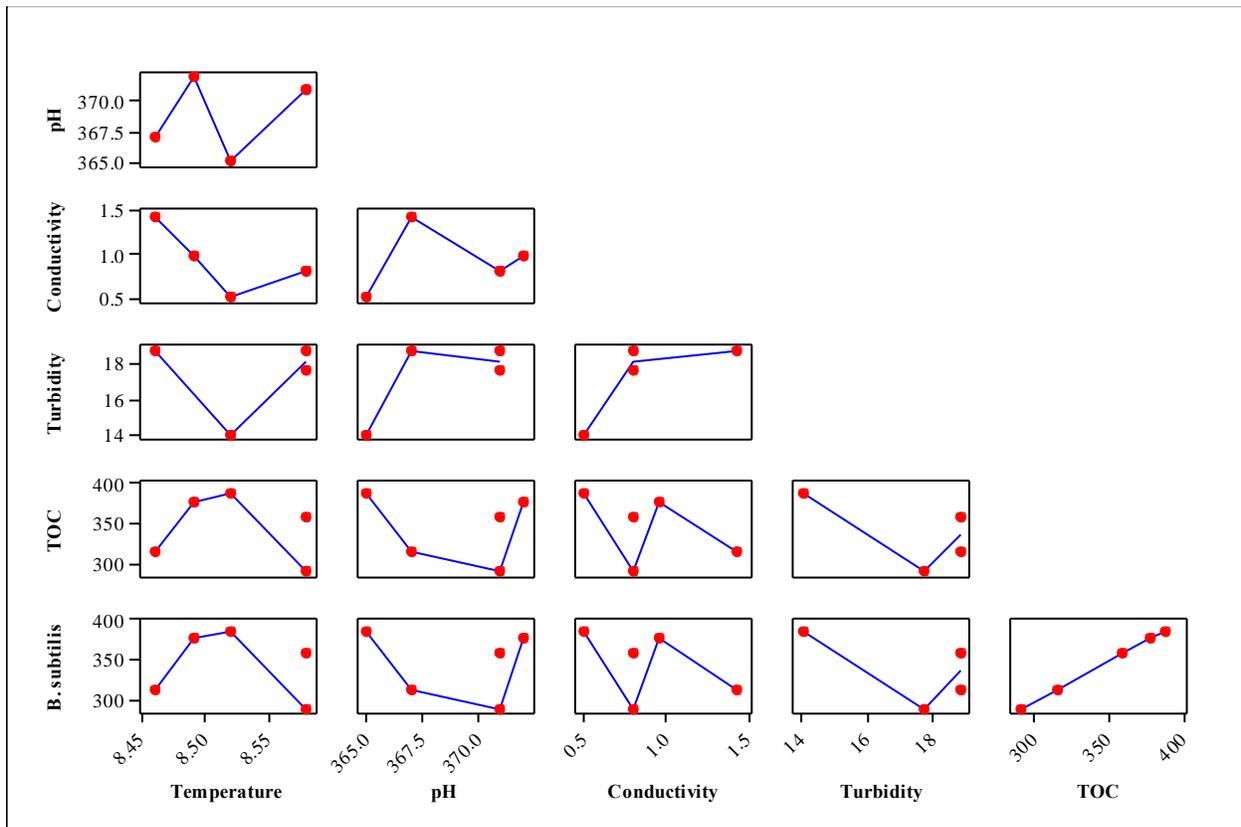


Figure 10 Matrix plot of P2 at Day 1 of SEED unit.

Table 4 Pearson correlation and P-values for P2 at Day 1 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.405				
	0.498				
Conductivity	-0.623	0.078			
	0.262	0.901			
Turbidity	0.057	0.686	0.721		
	0.943	0.314	0.279		
TOC	-0.175	-0.182	-0.478	-0.621	
	0.778	0.769	0.415	0.379	
<i>B. subtilis</i>	-0.175	-0.182	-0.478	-0.621	1
	0.778	0.769	0.415	0.379	*

Samples of P2 at Day 2 were also statistically analyzed. The matrix plot of such samples suggested correlation between pH and temperature, conductivity and temperature, conductivity and pH, turbidity and conductivity, *B. subtilis* and conductivity and *B. subtilis* and turbidity (Figure 11). The Pearson correlation coefficients confirmed those relations and proved two more relations: *B. subtilis* and pH and pH and turbidity (Table 5). The Pearson correlation coefficients, and P-value showed that conductivity and: temperature might be negatively related (-0.63 and 0.255), pH were highly negatively related (-0.912 and 0.031), turbidity were highly positively related (0.804 and 0.101) and *B. subtilis* were positively related (0.7 and 0.188). Additionally, coefficients indicated that pH and: turbidity might be negatively related (-0.632 and 0.253), *B. subtilis* were highly negatively related (-0.711 and 0.178), temperature were highly positively related (0.868 and 0.056). The last relation found was between *B. subtilis* and turbidity. Statistics results indicated that they might be positively related (0.559 and 0.327).

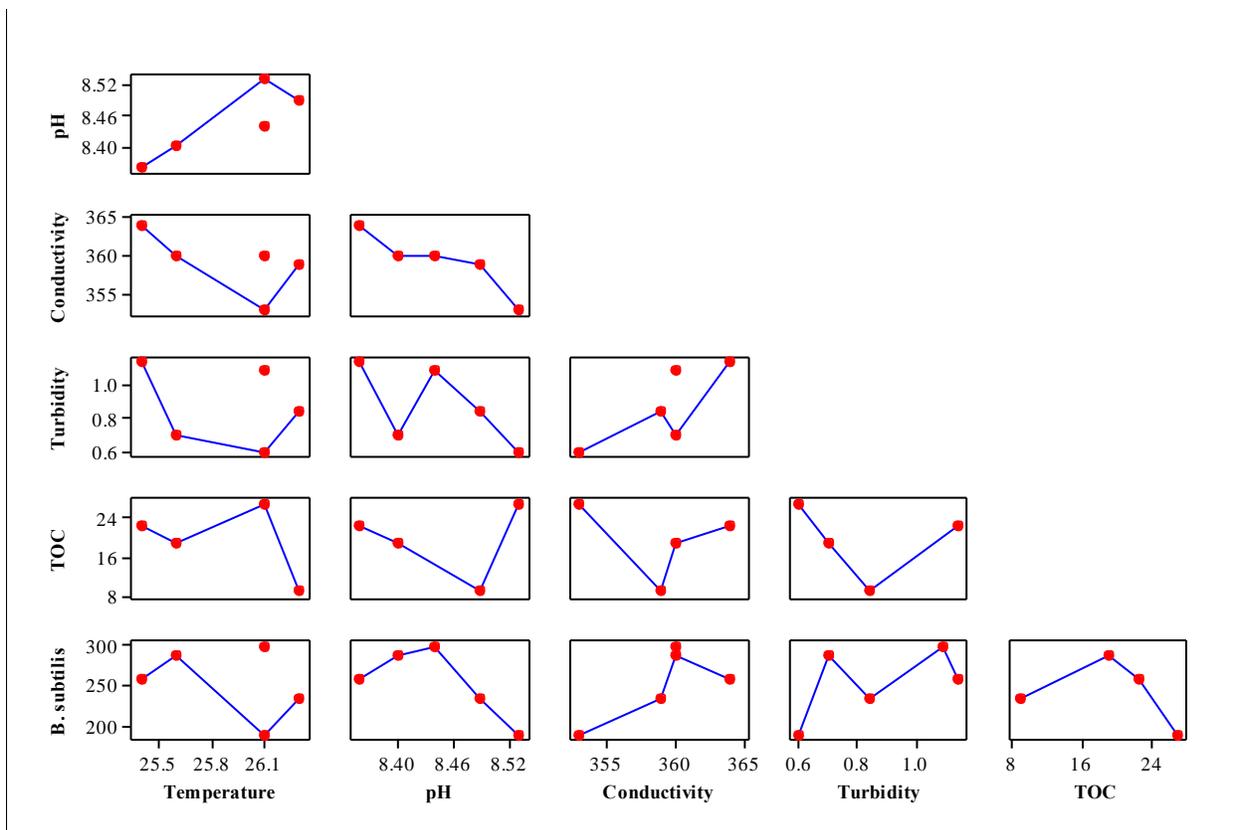


Figure 11 Matrix plot of P2 at Day 2 of SEED unit.

Table 5 Pearson correlation and P-values for P2 at Day 2 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.868				
	0.056				
Conductivity	-0.63	-0.912			
	0.255	0.031			
Turbidity	-0.294	-0.632	0.804		
	0.631	0.253	0.101		
TOC	-0.432	-0.047	-0.285	-0.154	
	0.568	0.953	0.715	0.846	
<i>B. subtilis</i>	-0.364	-0.711	0.7	0.559	-0.282
	0.547	0.178	0.188	0.327	0.718

The matrix plot of P2 at Day 3 suggested correlations between conductivity and pH, turbidity and temperature, TOC and *B. subtilis*, conductivity and *B. subtilis*, *B. subtilis* and pH (Figure 12). Table 6 shows the Pearson correlation numbers and P-values of the parameters measured. High Pearson correlation coefficients (>0.7) were found for pH and conductivity (negative relation, -0.91), *B. subtilis* and conductivity (negative relation, -0.755) and *B. subtilis* and TOC (negative relation, -0.781). Medium Pearson correlation coefficients (in the range of ± 0.5 to ± 0.7) were obtained for turbidity and temperature (negative relation, -0.615), TOC and temperature (positive relation, 0.53), *B. subtilis* and temperature (-0.551), *B. subtilis* and pH (positive relation, 0.522), and TOC and conductivity (positive relation, 0.689).

Similar to P1, it was expected to find similar trends between P2 measurements within the three days. Although the drums were constructed in such a way that it could be assumed to have the same hydraulic properties, varied conditions of the media and lines caused dissimilar behavior within drums. However, two trends were found during Day 1 and 2: the negative relation between conductivity and temperature and the positive relation between turbidity and conductivity. Additionally, one trend was found during Day 2 and 3: the negative relation between conductivity and pH.

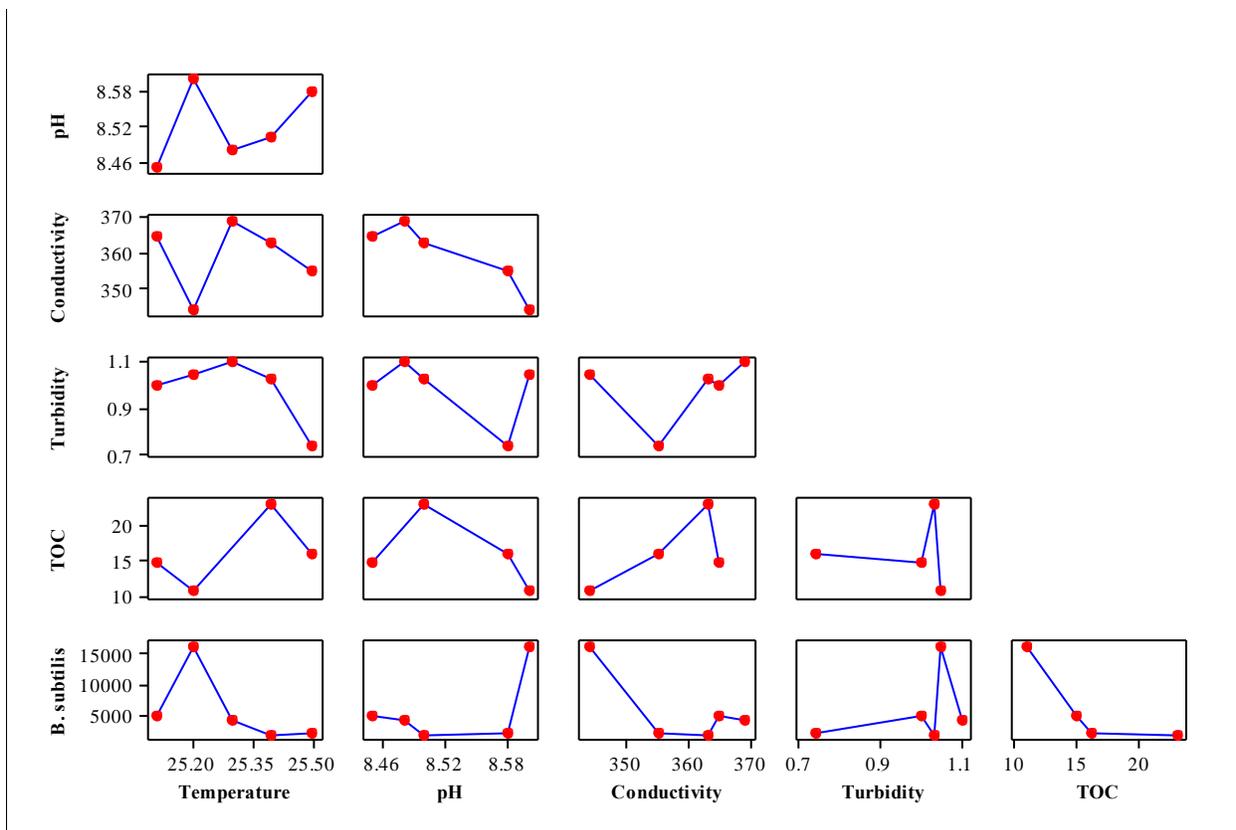


Figure 12 Matrix plot of P2 at Day 3 of SEED unit.

Table 6 Pearson correlation and P-values for P2 at Day 3 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.379				
Conductivity	0.529	-0.91			
Turbidity	0.991	0.032	0.255		
TOC	-0.615	-0.437	0.255		
<i>B. subtilis</i>	0.269	0.462	0.679	-0.01	
	0.53	-0.45	0.689	0.99	
	0.47	0.55	0.311	0.99	
	-0.551	0.522	-0.755	0.367	-0.781
	0.335	0.367	0.14	0.543	0.219

Correlations were found between almost all parameters except for pH and temperature and pH and TOC of P3 at Day 1. These relations were suggested by the matrix plot (Figure 13) and were proved by the Pearson correlation coefficients and P-values (Table 7). Further, *B. subtilis* was highly correlated to conductivity (negative relation, Pearson correlation = -0.866), turbidity (negative relation, Pearson correlation = -0.983), and TOC (positive relation, Pearson correlation = 0.917). These statistical coefficients also indicated that *B. subtilis* might be related to temperature (positive relation, Pearson correlation = 0.552) and to pH (negatively, Pearson correlation = -0.543). In terms of physicochemical parameters, TOC was highly correlated to temperature (positively, 0.913), conductivity (negatively, Pearson correlation = -0.91) and to turbidity (negative relation, Pearson correlation = -0.935). Moreover, turbidity was highly positively related to conductivity (Pearson correlation = 0.94) and might be negatively related to temperature and pH with Pearson correlation coefficients of -0.655 and 0.634 respectively. Lastly, conductivity was highly positively related to pH (Pearson correlation = 0.82) and might be negatively related to temperature (Pearson correlation = -0.69). However, P-values were greater than 0.05 except for the relation between *B. subtilis* and turbidity (0.017).

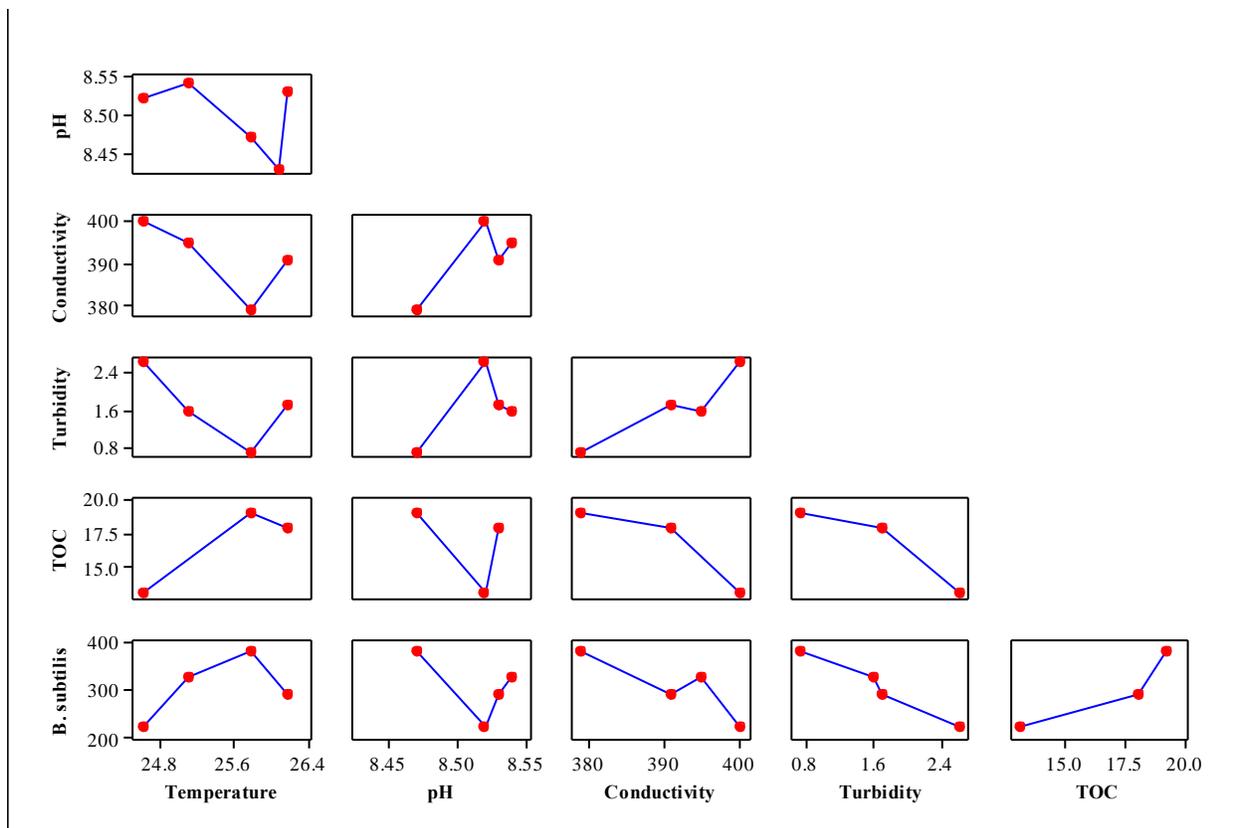


Figure 13 Matrix plot of P3 at Day 1 of SEED unit.

Table 7 Pearson correlation and P-values for P3 at Day 1 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	-0.495 0.397				
Conductivity	-0.69 0.31	0.82 0.18			
Turbidity	-0.655 0.345	0.634 0.366	0.94 0.06		
TOC	0.913 0.268	-0.19 0.653	-0.91 0.272	-0.935 0.232	
<i>B. subtilis</i>	0.552 0.448	-0.543 0.457	-0.866 0.134	-0.983 0.017	0.917 0.261

The statistical analysis of P3 at Day 2 showed relation among pH and temperature, TOC and temperature, pH and conductivity, conductivity and TOC, turbidity and TOC, turbidity and *B. subtilis*, TOC and *B. subtilis* (Figure 14 and Table 8). The correlations found were positive, except for conductivity and TOC. Although Pearson correlations were high (> 0.7 for most of the above mentioned relations) and > 0.5 for others, P-values were > 0.05 for all of the correlations found, which indicated a weak evidence of the relations.

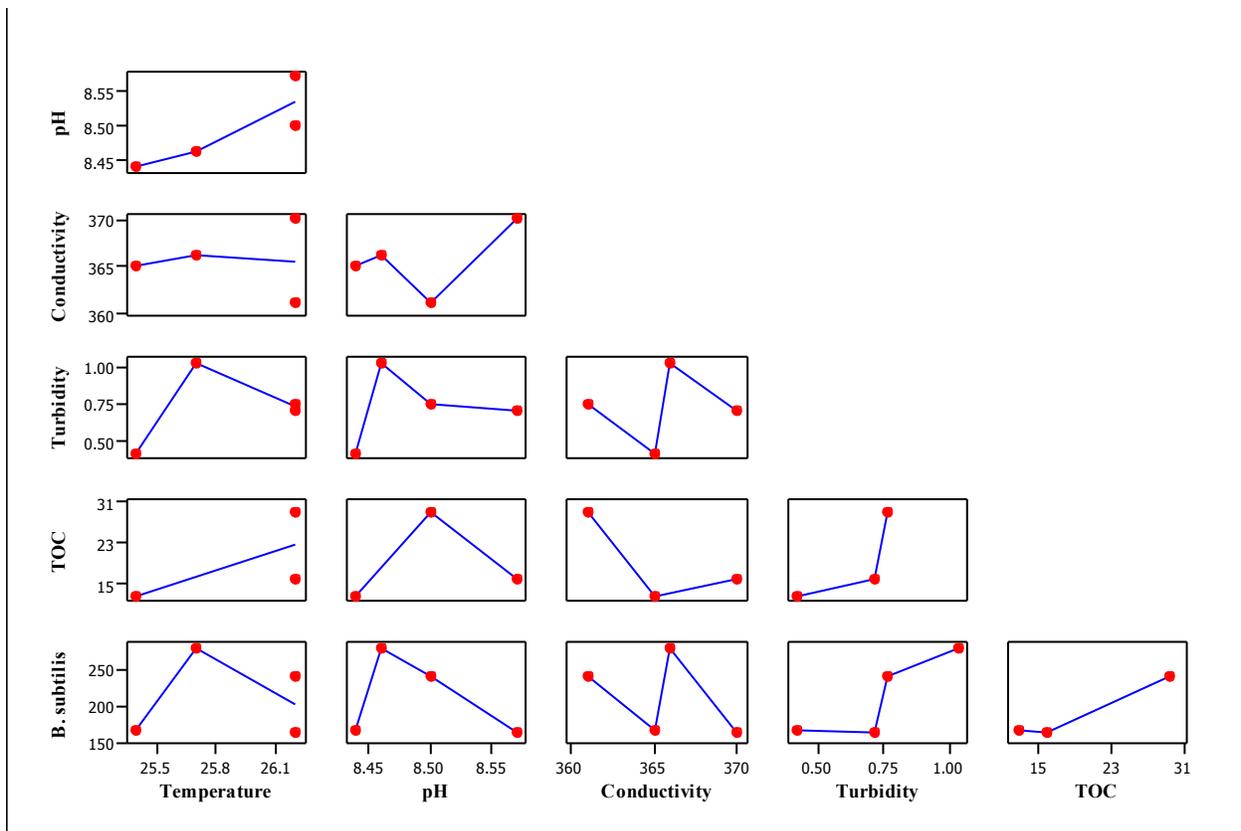


Figure 14 Matrix plot of P3 at Day 2 of SEED unit.

Table 8 Pearson correlation and P-values for P3 at Day 2 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.857				
	0.143				
Conductivity	0.034	0.511			
	0.966	0.489			
Turbidity	0.331	0.121	0.029		
	0.669	0.879	0.971		
TOC	0.647	0.136	-0.719	0.745	
	0.552	0.913	0.489	0.465	
<i>B. subtilis</i>	0.059	-0.333	-0.459	0.847	0.979
	0.941	0.667	0.541	0.153	0.129

In the case of the last samples assessed (P3 at Day 3), the matrix plot in Figure 15 implied correlations among pH and conductivity, conductivity and turbidity, turbidity and pH, TOC and conductivity, TOC and turbidity, TOC and pH and *B. subtilis* and temperature. The Pearson correlation coefficients (Table 9) proved that pH was highly negatively related to conductivity with a value of -0.847. However, P-value of such relation was > 0.05 (0.153) and thus indicated weak evidence. Additionally, conductivity was highly positively related to turbidity with a Pearson correlation number of 0.829. Furthermore, turbidity might be negatively related to pH, having a Pearson correlation number of -0.549. In terms of TOC, this parameter was highly positively related to conductivity, turbidity and might be negatively related to pH with a respective Pearson correlation coefficient of 0.977, 0.952 and -0.669, respectively. Finally, *B. subtilis* was highly negatively related to temperature with a Pearson correlation number of -0.996 and P-value of 0.004. The P-values in all correlations found were higher than 0.05 (except for *B. subtilis* and temperature), and thus indicated weak evidence of the correlations.

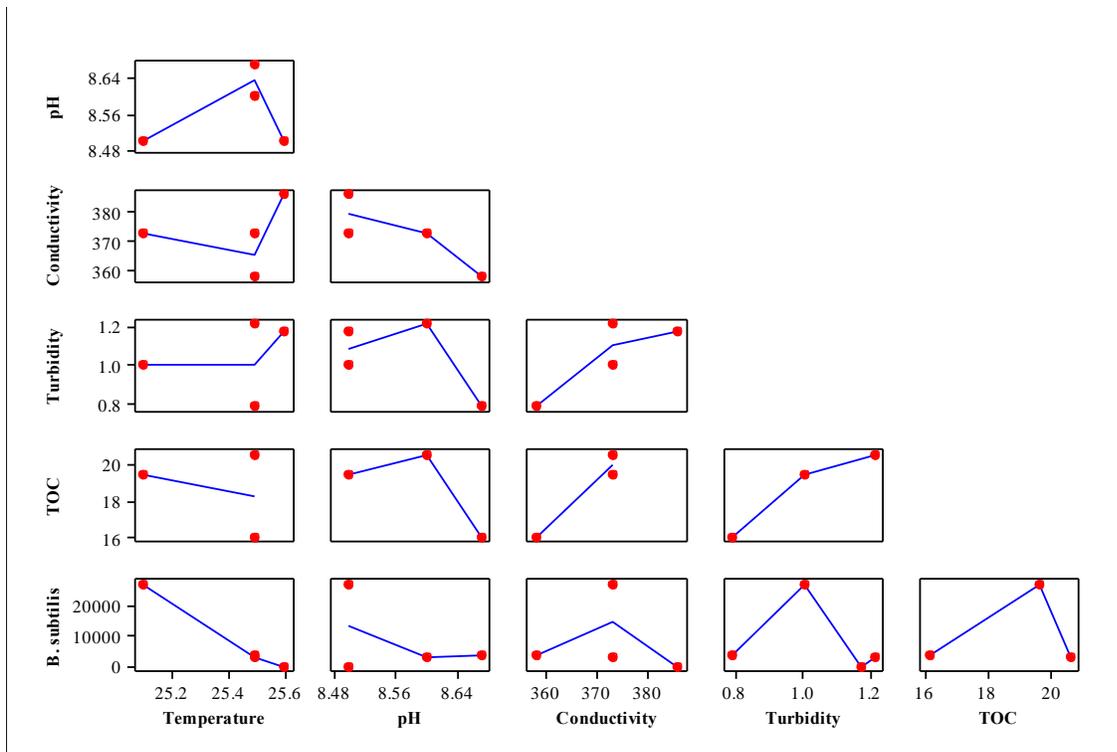


Figure 15 Matrix plot of P3 at Day 3 of SEED unit.

Table 9 Pearson correlation and P-values for P3 at Day 3 of SEED unit.

	Temperature	pH	Conductivity	Turbidity	TOC
pH	0.367				
	0.633				
Conductivity	0.151	-0.847			
	0.849	0.153			
Turbidity	0.234	-0.549	0.829		
	0.766	0.451	0.171		
TOC	-0.305	-0.669	0.977	0.952	
	0.802	0.534	0.136	0.198	
<i>B. subtilis</i>	-0.996	-0.44	-0.081	-0.21	0.291
	0.004	0.56	0.919	0.79	0.812

No similar trends were found between parameters through the three days. However, other trends were found during Day 1 and 2. Such relations were the positive relations between TOC

and conductivity, conductivity and pH and the negative relations between and conductivity and between *B. subtilis* and conductivity. Through Day 2 and 3, one trend was found: the highly positive relation between TOC and turbidity. Likewise, through Day 1 and 3 the only trend found was the positive relation between turbidity and conductivity.

Removal of *B. subtilis* by SEED Unit

The percent removal of *B. subtilis* was calculated to assess the performance of the SEED unit. Each phase, i.e. filtration and chlorination, and treatment train for each day of experiment were assessed in this study. *B. subtilis* removal by filtration varied significantly through the three experiments with ranges of -140% to 24%, -95% to -167% and -1,463% to -6,394% at Day 1, 2 and 3, respectively (Figure 16). It was expected that removals were similar due to the same hydraulic properties of the drums. Even though ranges were not similar, yet they showed low or no removal.

In contrast to this study, Swertfeger (1999) demonstrated a 2.4-log removal of endospores, >4-log removal of *Giardia* and a >2.5-log removal of *Cryptosporidium* using sand filters. Although both studies were performed using a rapid filtration, a difference existed in that Swertfeger (1999)'s studies were performed with influent water already treated by coagulation, flocculation and sedimentation processes and our study used influent water from the effluent of the gravel prefilter. However, initial turbidity measurements at both studies were approximately the same (≤ 2 NTU). Likewise, both studies were challenged with more than one microorganism and hence competition between them inside the filters was not seemed to cause the abrupt difference in filter effectiveness. Yet one major difference was found, which consisted in filter's depth. Swertfeger (1999)'s filters were 10 ft long while our study used ~3 ft long filters.

Configuration of drums in our study (filters were in series) was designed to overcome this issue but apparently more contact time was indispensable for a better performance of the filters.

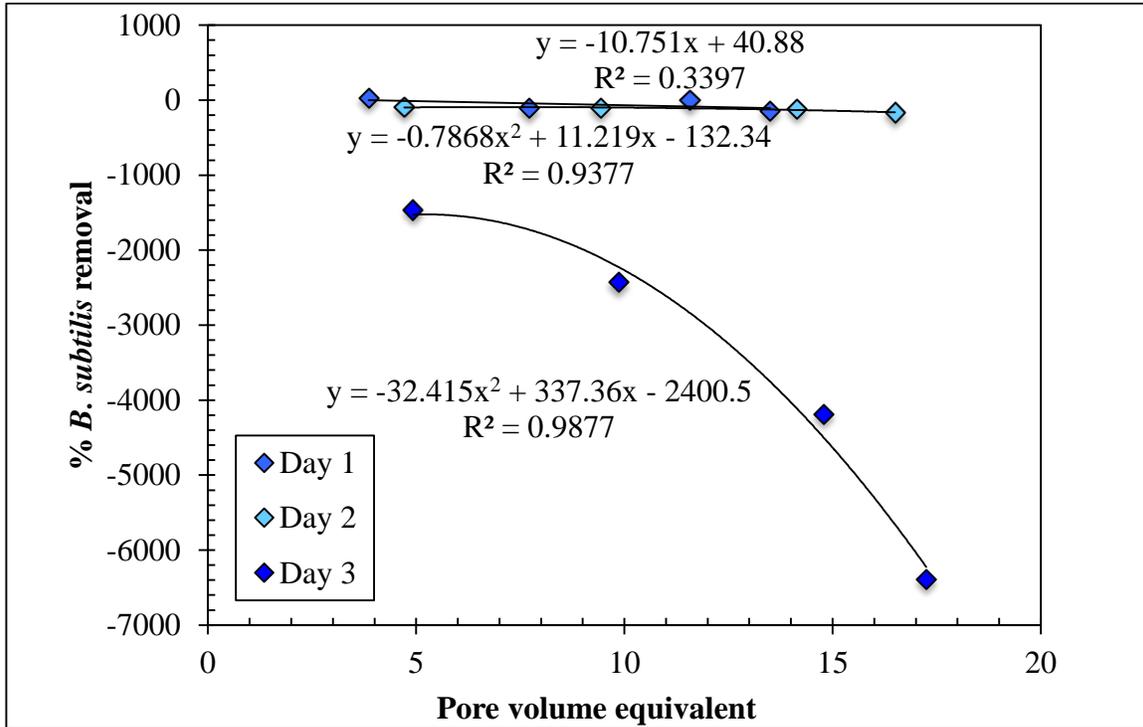


Figure 16 *B. subtilis* percent removal by filtration in the SEED unit.

Renovation of SEED Unit

The SEED unit was initially assembled in January 2008 (Figure 17). Due to lack of maintenance, they got corroded and nonfunctional (Figure 18). Hence, it was decided to replace the metal drums to polyethylene open head drums. Replacement was performed by taking out the sand from the metal drums, washed by hand the sand and lines (which were full of debris), transferred the full amount of sand to the new drums and assembled the equipment with the same previous configuration (Figure 19 and Figure 20). Even though sand was carefully washed, one of the drums (B) still seemed not being completely clean. During the SEED unit demonstration, the

actual treatment system of the Río Piedras community was dealing with a clogging problem that did not allow the residents to have the normal quantity of water and kept the community with a water rationing. Therefore, a backwash could not be performed during the tests of this study. The lack of a backwash was detrimental for the assessment of the unit, which yielded non-favorable results, especially at Day 2 and 3 were drums combinations included the drum B.



Figure 17 SEED unit in 2008.



Figure 18 SEED unit in 2012.



Figure 19 SEED unit replacement process.



Figure 20 SEED unit in 2014.

A similar trend was achieved during the three days in the second phase of the treatment train, chlorination. The trend behaved as a polynomial of second degree (Figure 21). During the first two days, chlorination inactivated *B. subtilis* in the same range (-3% to 36 % *B. subtilis* removal). In contrast, at Day 3 removal trend varied significantly through treated water volume with a lowest *B. subtilis* removal of -440 % at ~480 mL of treated water volumes. The other

samples also achieved negative removals except at 414 mL of the treated water volume that reached 95% removal.

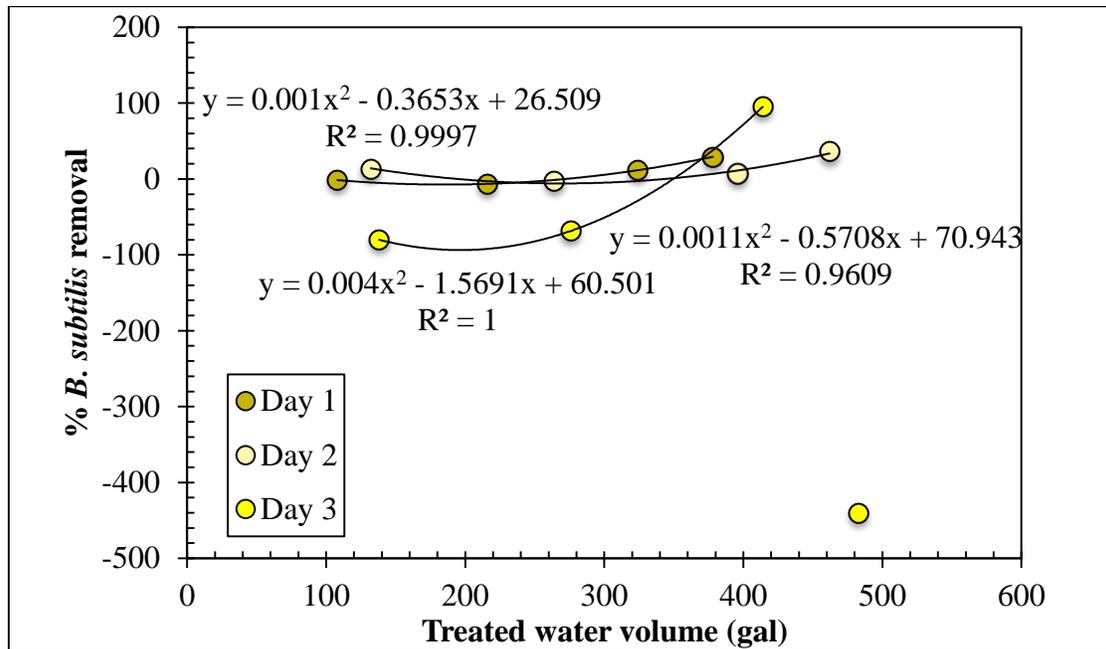


Figure 21 *B. subtilis* removal by disinfection in the SEED unit.

Difference in removals might be due to different contact of water with the chlorine tablet since no constant chlorine concentration was achieved. However, Rice et al. (1996) demonstrated a 2-log reduction of aerobic spores of *Bacillus* genre after 65 minutes and CT of 114 mg-min/L and 3-log after 180 minutes and CT of 315 mg-min/L. The chlorination technique used in Rice et al. (1996)' study was different from our study and hence, difference in removals could have been expected.

Residual free Cl₂ concentrations through the three experiments showed a second polynomial degree trend (Figure 22). At Day 1, 2 and 3, residual free chlorine concentration decreased, having its minimum of 1.75 mg/L at 270 gal, 0.4 mg/L at 395 gal and 0.2 mg/L at 300 gal of treated water volume, respectively, and then increased. It is important to recall that during testing, chlorine tablets in some instances were in more contact with water than in other samples.

This was the reason of the high initial concentration (>4 mg/L) at Day 1 and 2 and of increased residual free chlorine concentration in some samples. Moreover, final concentrations measured, i.e. 3.35, 3.7 and 3 mg/L at Day 1, 2 and 3 respectively were within the WHO Guideline of Drinking Water Quality (>0.5mg/L) (WHO, 2011) and the USEPA MRDL (4 mg/L) (US EPA, 2009).

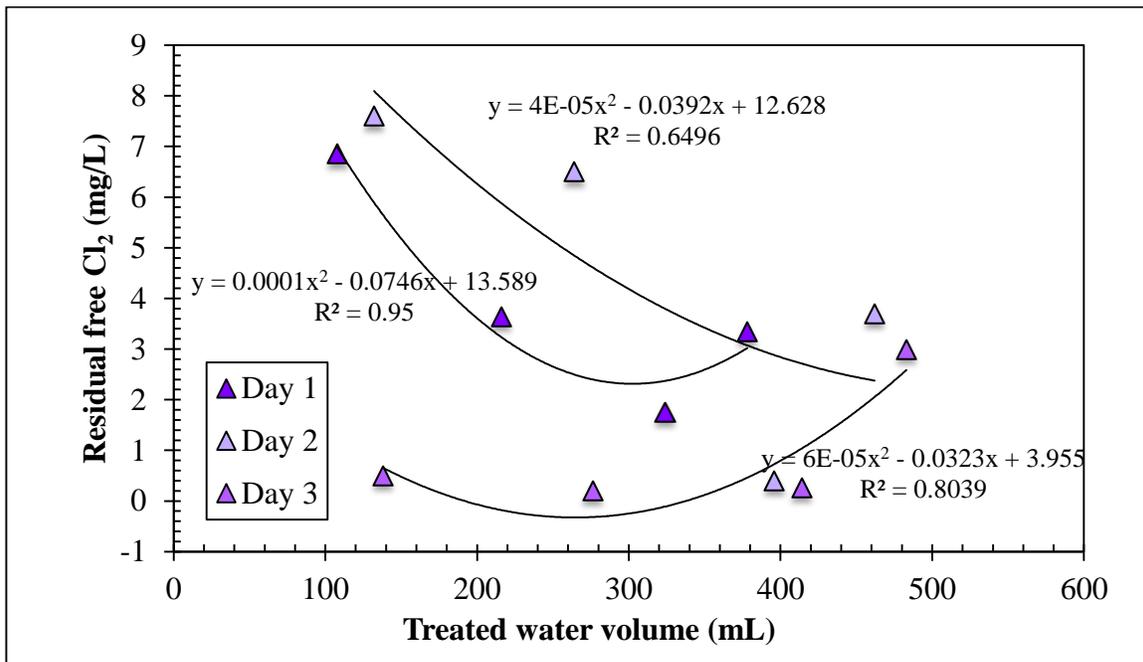


Figure 22 Residual free Cl₂ from field-scale system at Day 1, 2 and 3.

The performance of the SEED unit for *B. subtilis* is summarized in Figure 23 and

Figure 24. Outcomes showed that the SEED unit in this configuration was not efficient for *B. subtilis* removal, since the removals were hardly achieved and only a maximum of 23 % removal (0.11 log removal) was reached. Hence, assessment of the SEED unit, in this configuration, for the efficiency in removal and inactivation of *B. subtilis* did not serve for the prediction of occurrence of *Giardia* and *Cryptosporidium*, contrary to the suggestions of the study of Nieminski et al. (2000). Furthermore, these results showed no compliance with the SWTR. Moreover, several

studies have proved the critically use of chemical pretreatment such as coagulation prior to filtration (Harrington et al., 2003; Logsdon et al., 1985).

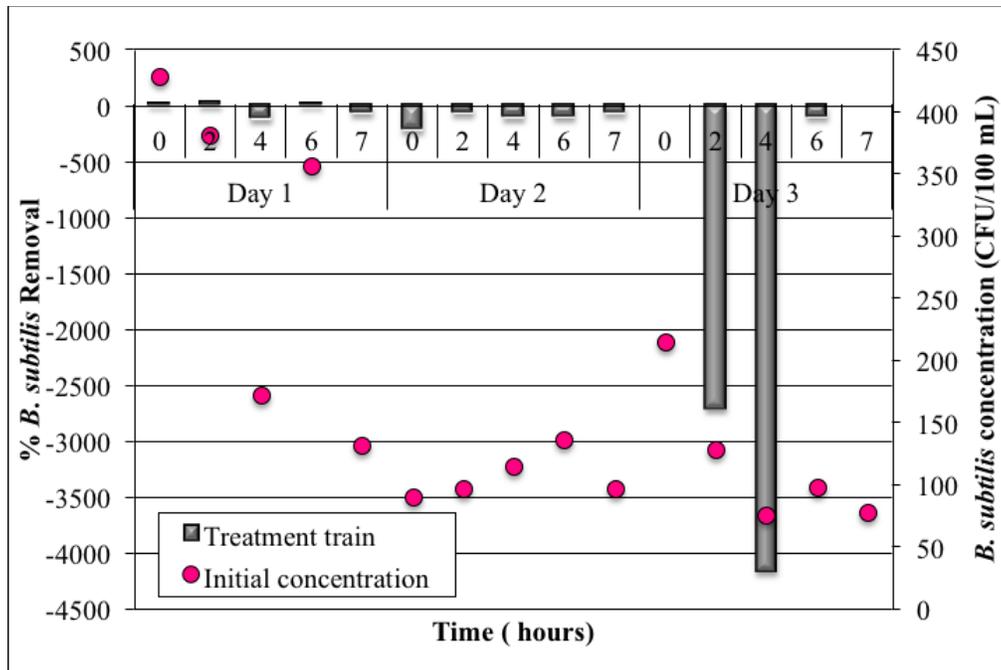


Figure 23 *B. subtilis* percent removal and initial concentration of treatment train (SEED unit).

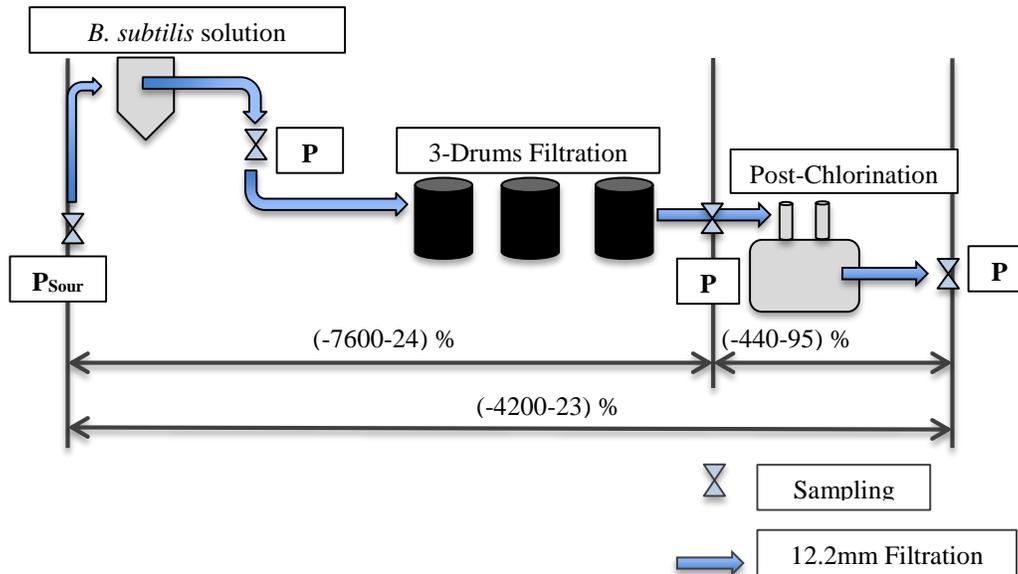


Figure 24 SEED unit schematic with percent removals by phase.

Acknowledgements

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Feasibility of sintered recycled glass functionalized with micro- and nanosized TiO₂ particles for the degradation of trihalomethane precursors from surface waters

Basic Information

Title:	Feasibility of sintered recycled glass functionalized with micro- and nanosized TiO ₂ particles for the degradation of trihalomethane precursors from surface waters
Project Number:	2014PR164B
Start Date:	3/1/2014
End Date:	2/28/2016
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	Treatment, Surface Water, Water Supply
Descriptors:	None
Principal Investigators:	Pedro Javier Tarafa, OMarcelo Suarez

Publications

There are no publications.

Progress Report (As of April 2015)

PI: Pedro J. Tarafa, PhD
Project Number: 2014PR164B
Title: Feasibility of sintered recycled glass functionalized with micro- and nanosized TiO₂ particles for the degradation of trihalomethane precursors from surface waters
Submission Date: April 30, 2015

Brief Project Summary

The presence of organic compounds in water body supplies are causing adverse effects to human health and environment. This research focus in the development and evaluation of a filter-like composite made of crushed, recycled glass and TiO₂ nanoparticles for the degradation of organic compounds through a photo-catalytic reaction. The composite material is obtained by sintering the recycled glass powder (MG-30 grade) with TiO₂ nanoparticles at a specific temperature and time. The composite's hydraulic characteristics, such as porosity and percolation, are first evaluated within a range of sintering parameters to find the best filtering properties. The polymorph structure of TiO₂ phase is also evaluated to determine whether the TiO₂ experienced any phase transformation after sintering. Preliminary data show that percolation rates decrease as sintering temperature and time increase. X-ray diffraction (XRD) analyses were conducted on the TiO₂ and TiO₂-glass composite to evaluate the polymorph structure of TiO₂ phase. The results confirm that the TiO₂ anatase phase remained despite the high sintering temperature used. Ongoing work is intended to study the potential of the glass-TiO₂ composite for the degradation of natural organic matter (NOM) under the influence of UV light to avoid the formation of trihalomethanes when water is chlorinated. This will be key in the viability and further implementation of this technology for polluted water filtering.

Student Training

1. Sheila Arias, MS student from Civil Engineering
Sheila is the lead student for this endeavor and the one sponsored by the project. She is in charge of the design and executing the experiments for photodegradability. She has been mentoring a group of four undergraduate students that are helping in the development and execution of few components for this research. Among other tasks, Sheila is getting trained in the use and operation of the following key instruments: total organic carbon analyzer (TOC), radiometers (light intensity sensors) for UV irradiation, X-ray diffraction analysis (XRD), infrared spectroscopy (FTIR), HACH spectrophotometer for TOC readings.
2. Amarillys Avilés, MS student from Civil Engineering
Amarillys is a graduate student that works in Dr. Tarafa's research group. She is currently sponsored by the Center for Education and Training in Agriculture and Related Sciences (CETARS) and has been giving support to this endeavor. She has been involved in the manufacturing of the glass/TiO₂ composite, studying the mechanical properties of the glass composite and devising alternatives to optimize the deposition of TiO₂ particles onto the glass surface. As a CETARS student, she is looking for potential applications in

the remediation of polluted soils. In addition, along with Sheila, she also mentors and provides guidance for undergraduate students.

3. Rey Cabán, MS student from Civil Engineering
Rey is not currently working in the project, but was the one that helped in conducting the structural and mechanical analyses to the glass composites along with Wesley Cuadrado, a former graduate student from Dr. Marcelo Suarez' group.
4. Liliana Hernandez, Undergraduate student from Civil Engineering
Liliana worked with the surface characterization of the sintered recycled glass by observing the polymorph structure of TiO₂ phase before and after the composite sintering. For conducting such characterization, Liliana was trained in the XRD instrument.
5. Jorge de Jesús, Undergraduate student from Mechanical Engineering
Jorge worked in the study of the behavior of light intensity (specifically, UV light) in the glass/ TiO₂ composite in function of the glass composite thickness and diameter. The irradiation will be carried out using 4W PL series compact ultraviolet lamps. This UV lamp emit radiation wavelength of 365 nm. The intensity is 1,000 $\mu\text{w}/\text{cm}^2$.
6. Leroy Goñez, Undergraduate student from Chemical Engineering
Leroy joined Dr. Tarafa's research group during spring 2015. He has been involved in the sintering of the new glass and studying filtration characteristics such as percolation rate and surface porosity.
7. Erika Montalvo, Undergraduate student from Civil Engineering
Erika also joined Dr. Tarafa's research group during spring 2015. She has been involved in the standards and solution preparation of humic acid, as well, as the detection of total organic carbon by the HACH chemical oxidation method.

Results Dissemination

1. Feasibility of Sintered Recycled Glass Functionalized With TiO₂ Nanoparticles For Degradation of Organic Pollutants. S. Arias, A. Avilés, L. Goñez, J. de Jesús, O.M. Suárez and P. Tarafa. Poster presented at the 2015 Sigma Xi Poster Day.

Work Performed

1. Ceramic molds for the fabrication of the glass/TiO₂ composites

In order for sintering the recycled glass/TiO₂ composite, special ceramic molds were first constructed. The ceramic molds were fabricated by mixing ceramic plaster (powder) with a liquid activator, which are then placed in a pre-constructed container and dried overnight at room temperature. Figure 1 depicts the steps taken for this procedure. As shown in Figure 1(a) the ceramic mix is poured into a plastic, cylindrical container previously impregnated with a lubricant. A cylindrical piece of wax is placed at the bottom and in the center of the container and the ceramic mix is then scattered over the piece of wax. After a 24 h period, the plastic container is removed and the residual

ceramic mold is heated up to melt out the wax. Then the mold is placed inside an oven at high temperatures to remove the residual lubricant in the external walls. The final result is a 2” diameter cylindrical ceramic mold as illustrated in the Figure 1(b).

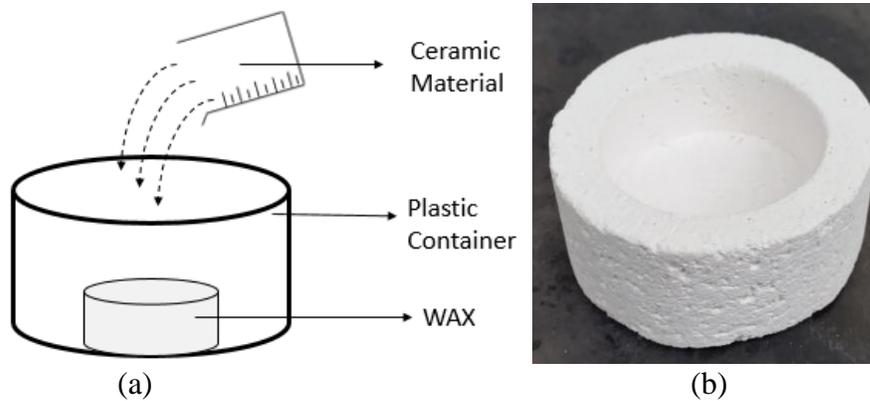


Figure 1. Illustration of the ceramic mold preparation procedure (a) and final ceramic mold (b).

2. Preparation of glass-TiO₂ composites

The glass/TiO₂ composites are obtained by mixing 30 grams of recycled, crushed glass with different amounts of TiO₂ particles that ranges from 0.5-1 wt% of the whole composite. Figure 2 depicts the steps taken to prepare the glass + TiO₂ mixture that is then sintered in the oven. Initially 30.0g of glass powder is mixed with the equivalent amount of TiO₂ particles in an ethanol/water solution for 10 minutes. The solution is stirred and heated. Then, the sample is sintered at the desired temperature and time inside an oven. Figure 3 shows a picture of a sintered glass/TiO₂ composite sample.

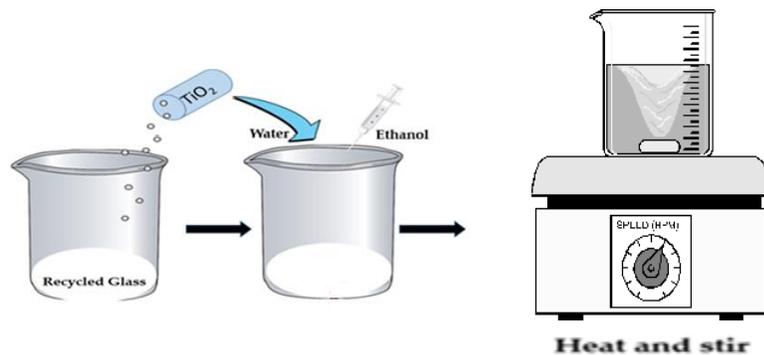


Figure 2. Glass + TiO₂ blend preparation.



Figure 3. Final sample of a sintered TiO₂/glass composite.

3. Structural analysis

All the components/tasks involved in the structural analysis were previously performed; however, due to a recent change in the glass type that is being received (glass properties changed), we had to re-evaluate once again the structural analysis.

a. *Particle size distribution-*

The particle size distribution is determined according to the Standard Test Method for Particle-Size Analysis of the ASTM. This test consists of a sieve analysis in which a pre-weighted sample of the material is placed upon the top of a group of sieves. The sieve with the largest screen opening is placed at the top and the screen opening size decrease with each sieve down to the bottom sieve. Once the sieves are placed in the appropriate order the sample is shaker for a period of time in order to promote that the particles are either retained on a sieve or passed through. After shaking the sample, the material retained on each of the sieve is weighed. Table 1 shows the raw data obtained from the test and Figure 4 is the plot showing the particle size distribution of the glass.

Table 1. Sieve analysis data for the powder glass

Sieve No.	Sieve Size of Opening (mm)	Mass of Glass Retained (g)	Percent of Glass Retained	Cummulative Percent of Glass Retained	Percent Passing
4	4.75	0.00	0.00	0.00	100.00
10	2.00	0.00	0.00	0.00	100.00
20	0.85	140.20	24.32	24.32	75.68
40	0.43	423.90	73.53	97.85	2.15
60	0.25	11.70	2.03	99.88	0.12
100	0.15	0.00	0.00	99.88	0.12
200	0.07	0.00	0.00	99.88	0.12
		0.70	0.12	100.00	0.00

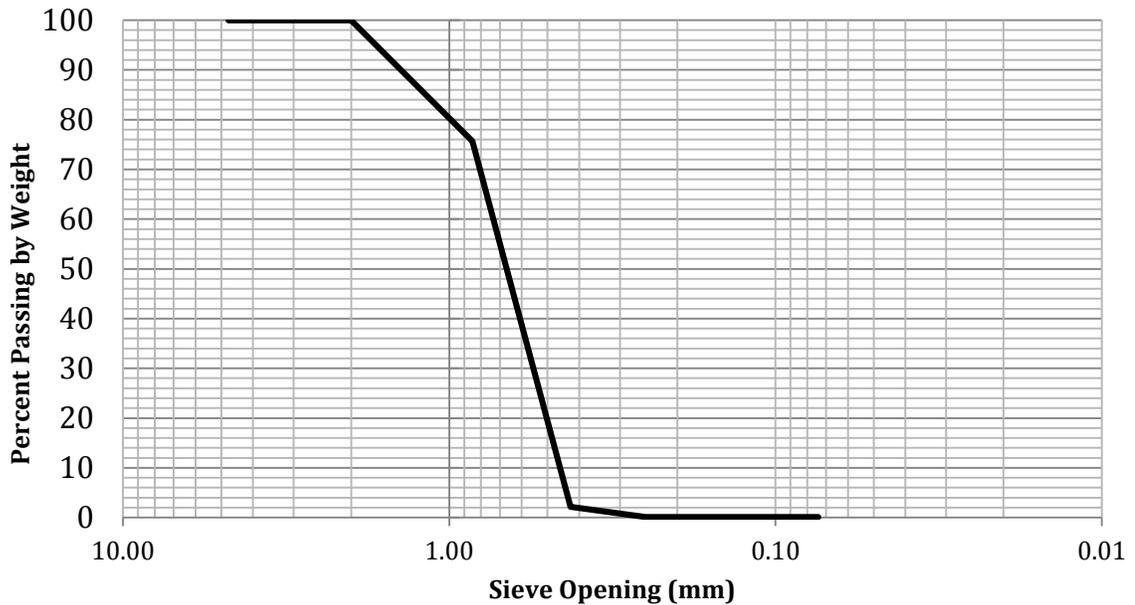


Figure 4. Particle size distribution of the powder recycled glass.

According to the data and from the plot, the powder glass have the following characteristics:

- Effective Size (d_{10}): 0.47 mm
- Finer Size (d_{60}): 0.74 mm
- Uniformity Coefficient (UC): 1.57

b. *Surface porosity-*

Surface porosity is a parameter that will be used in the evaluation of the filtration characteristics for the glass composite. The analysis is carried out for various glass composite samples that are obtained by different sintering conditions (i.e., different temperatures and time). The study is performed through the analysis of micrographs of the sintered specimens using an optical microscope (Nikon Epiphot 200). These micrographs (obtained at 5x magnification) are examined using Image J to assess the percentage of porosity of the samples. The study is still in progress and no data is yet available.

c. *Percolation-*

Percolation is defined as the ratio of water volume change over time through the porous media. Porous glass composites were obtained by sintering thirty grams of recycled glass at the furnace from 950 to 1000 °C at intervals of 25 °C for 45 to 75 minutes at 15 minutes intervals. After sintering, samples were cooled at room temperature. Percolation was determined by recording the elapsed time (in seconds) to obtain a given volume of water.

The analysis was carried out by a hand-made apparatus built by our team. It consisted in a PVC pipe with an adapter to store a glass composite sample and a funnel (in the top of the pipe) to keep a constant hydraulic head over the tested sample (refer to Figure 5). The system is sealed with clear silicon and once dried; water is fed into the system.

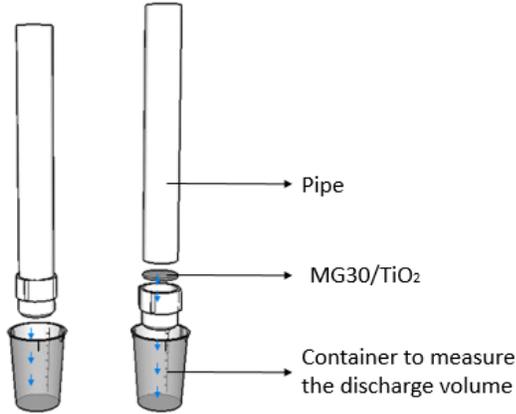


Figure 5. Apparatus to measure percolation.

The highest percolation rate was 16.05 gpm/ft^2 and the lowest 0.095 gpm/ft^2 for glass composites sintered at 975 °C and 45 minutes and 1000 °C and 75 minutes, respectively. Figure 6 shows a graphical representation of the obtained percolation data for two different TiO_2 mass deposited into the glass composite.

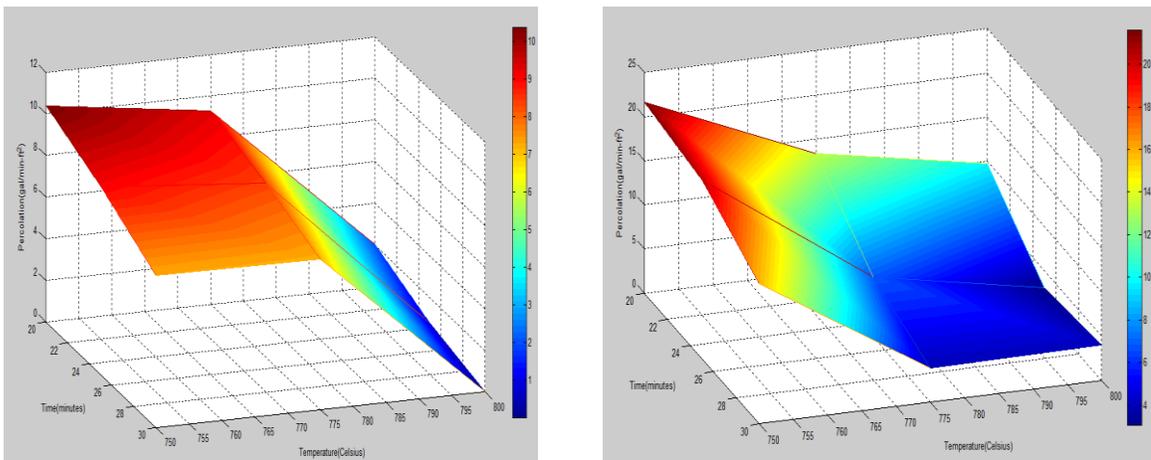


Figure 6. Glass/ TiO_2 composites percolation rate (gpm/ft^2) for 0.5% TiO_2 (right) and 1.0% TiO_2 (left) as function of temperature and time.

4. Polymorph Structure of Titanium (IV) Oxide (TiO₂) Phase

TiO₂ is a photocatalytic material that exhibits three polymorphs phases: rutile, anatase and brookite. Each of these polymorphs presents different properties that impact the photocatalytic performance of the material. Literature states that the anatase phase has the best photocatalytic performance. The filters will be obtained by sintering recycled glass with TiO₂ powder at temperatures beyond 700 °C. The phase of TiO₂ depends on many parameters such as the particle size and shape, surface area, atmosphere, volume of sample, impurities among others. Although this process is influenced by these parameters it is necessary to determine if the sintering time and temperature will impact the TiO₂ phase. An X-ray diffraction (XRD) instrument is used to determine the polymorph structure of the TiO₂ phase. So far, analyses for the pure TiO₂ and sintered glass/TiO₂ composite have been completed. However, additional analyses need to be conducted to determine if phase transformation occurs and, unfortunately, the instrument we have been using (located at the General Engineering dept under Dr. Marcelo Suarez custody) is out of order. Currently, we are scheduling an appointment to use the XRD under the custody of the Geology department. Figure 7 shows the spectrum intensity for an XRD analysis for pure TiO₂, glass powder, and TiO₂ sintered in glass.

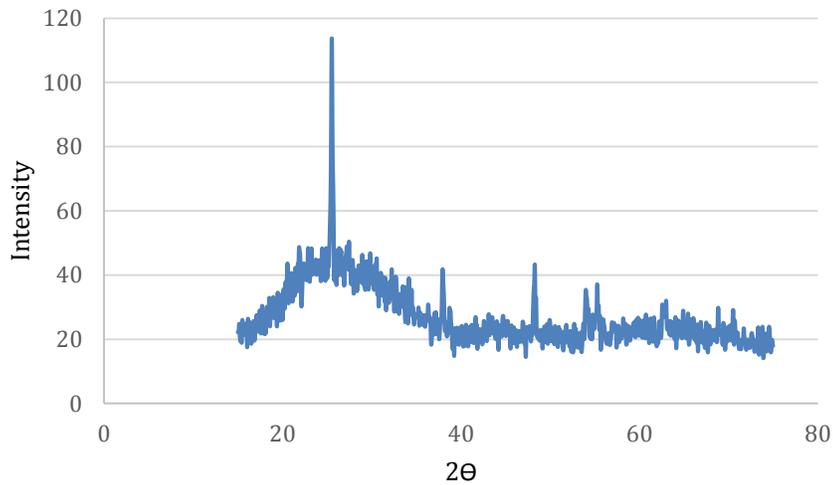
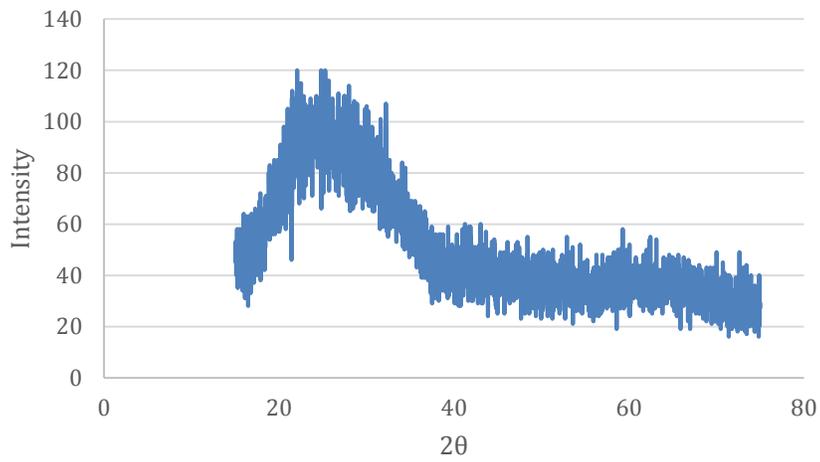
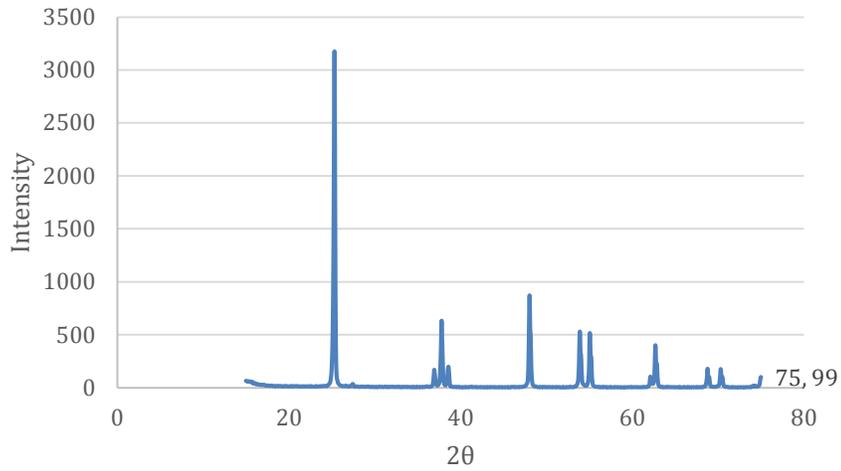


Figure 7. XRD analysis for: (a) pure TiO₂, (b) glass powder, and (c) 1% of TiO₂ sintered in 30 g of glass at 775 °C for 25 minutes.

5. Deposition of TiO₂ onto the glass surface

TiO₂ particles are being immobilized within the recycled glass powder by sintering the glass. The sintering is performed by placing a mixture of glass powder and TiO₂ in the oven at temperatures above 700 °C for times between 15 to 45 minutes. Figure 8 present a scanning electron microscope (SEM) analysis performed to a glass/TiO₂ specimen sintered at 750 °C for 25 minutes.

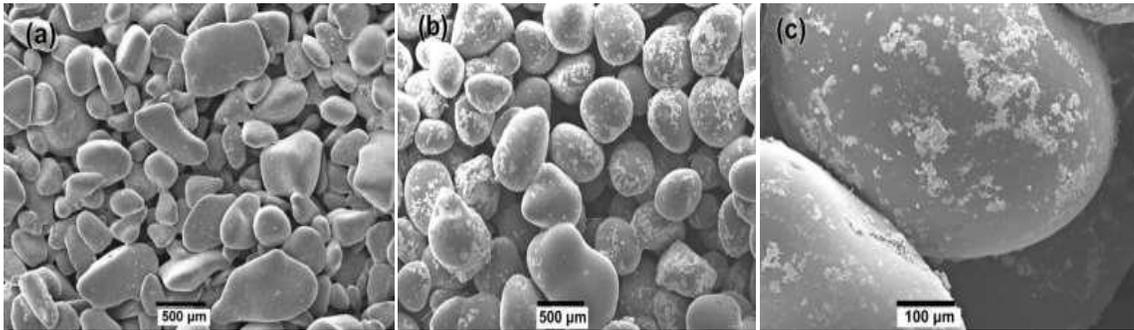


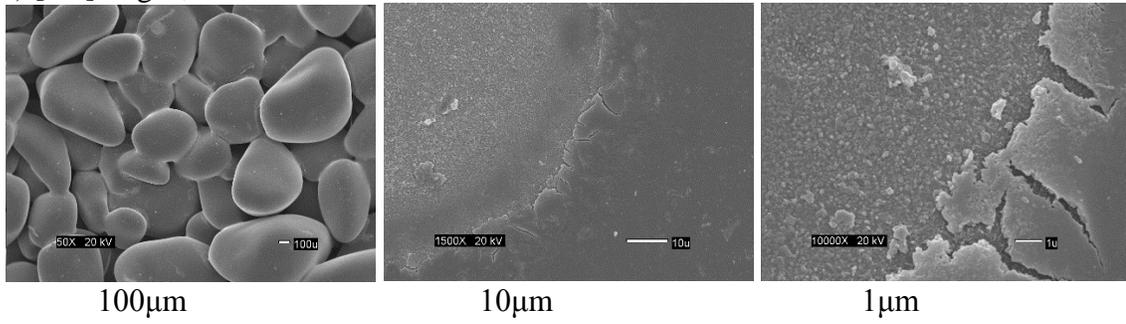
Figure 8. TiO₂ deposition into the glass powder sintered at 750°C for 25min. (a) 0% of TiO₂ at 500 μm (b) 1.0% of TiO₂ at 500μm (c) 1.0% of TiO₂ at 100μm

Although a good adhesion of TiO₂ into the glass powder is observed, alternatives methods to optimize the deposition of TiO₂ are being evaluated, specifically, for those not involving high temperatures, to avoid any potential phase change in the TiO₂, as previously discussed. One of these methods is called *sputtering*, which intends to deposit the TiO₂ with the aid of a coating through a sophisticated machine located in the CID building. The other method is called *heterogenic nucleation* and consists in the development of a TiO₂ film through a chemical reaction performed on the glass surface

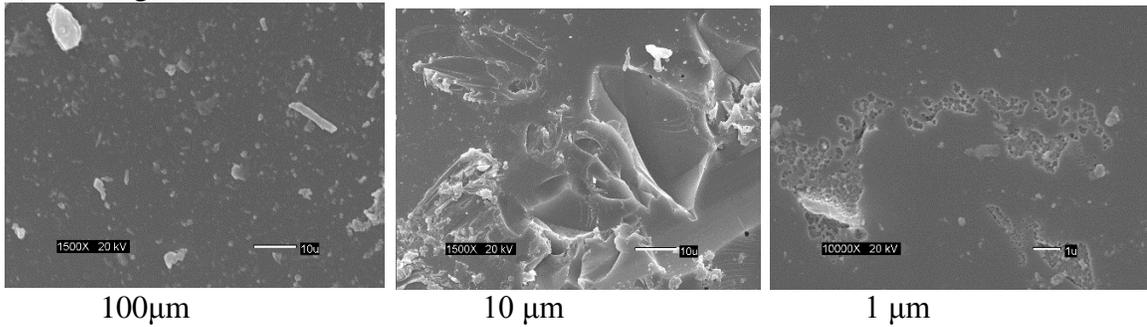
It is also believed that by increasing the glass surface roughness, better adhesion of TiO₂ particles onto the glass will be acquired. With this in mind, a study was conducted to determine the effect of the glass surface when exposed to different concentration of phytic acid (PA).

A sintered glass specimen sintered at 750°C for 25 min was exposed to a solution of PA having concentrations of 3 and 9 g/L and different times. A scanning electron microscope (SEM) analysis was performed to visualize the effect of the PA on the glass surface. Figure 9 shows the SEM images for the glass been exposed to PA.

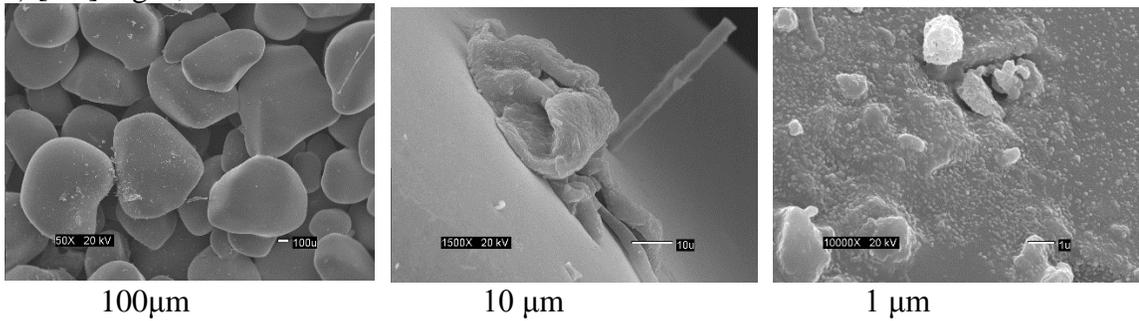
a) [PA]: 3 g/L; time: 5 h



b) [PA]: 3g/L; time: 72 h



c) [PA]: 9g/L; time: 5 h



d) [PA]: 9g/L; time: 72 h

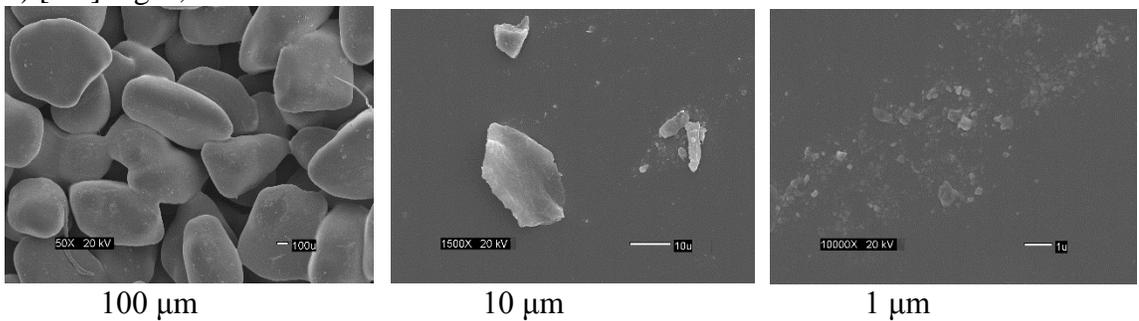


Figure 9. SEM images for the sintered glass composite surface exposed to 3 and 9 g/L of PA at different times.

From the SEM images, a clear effect on the glass surface is not identified. However, it is observed an increase in roughness for specimens exposed for 5 hours in any of the two PA concentrations evaluated. For periods of 72 hours, no roughness is perceived. Apparently, longer exposure decreases the roughness.

6. Degradation Studies

The experiments to perform the proposed treatments for the degradation of the NOM will be conducted in photocatalytic reactors. The reactor consists of a closed, sealed box made out of cardboard to avoid the passing of light from its surroundings. The box will house a UV lamp and a container (beaker) to hold both the glass-TiO₂ composite and the NOM solution sample. The reactor is designed to allow the UV irradiation at controlled conditions. The boxes were already designed and their dimensions are 1.5 ft in height, 1 ft wide and 1 ft long.

The degradation studies will consist of the evaluation of the sintered glass/TiO₂ composite in degrading NOM (THM's precursors) by means of total organic carbon (TOC) levels. The set-up for these experiments requires a total organic carbon analyzer, which was finally obtained from Dr. Sangchul Hwang.

Currently, we have been preparing the standards solutions of humic acid, a common organic compound in natural waters, in order to construct a calibration curve for the total organic carbon. In order to validate our data, we are also working with the method of chemical oxidation (HACH Method), which consist of digesting the sample in an acidic solution with strong oxidant reagents and then read them spectrophotometrically.

7. UV light absorption on the glass/TiO₂ composite

The photocatalytic reaction occurs when the TiO₂ is activated by absorbing a photon with energy higher than 3.2 eV. This results in the generation of excited high energy states of hole and electron pairs. In order for this process to occur, the TiO₂ surface needs to be illuminated to photon energy higher than its band gap. Hence, it is necessary to evaluate if the Glass/TiO₂ composite has the capacity of absorbing the UV light in order to activate the photocatalyst.

The purpose of this component was to evaluate the incident light behavior onto the glass/TiO₂ filter to obtain the optimal distance to place the UV lamp, and to evaluate the efficacy of the Glass/TiO₂ composite to absorb the UV light.

The experiments were carried out using a sintered glass/TiO₂ composite sample placed inside the reactor box in which a UV lamp was installed in one side and a radiometer in the opposite side. The light intensity was measured at different lamp distances from the specimen. The UV source was a 4W PL series compact ultraviolet lamp with a radiation wavelength of 365 nm, which resembles the UV-A region. The measured intensity for the light was 1,000 μw/cm². It was expected to obtain relevant data; however, the performance was ineffective since the intensity of UV light absorbed by the composite could not be determined.

Information Transfer Program Introduction

Meetings, seminars, technical reports, and a web site are used by the Institute to keep the water resources community and general public informed about advances in research. Approximately once every three or four years, the Institute organizes a major conference on water-related research in Puerto Rico and the Caribbean Islands, in collaboration with US Virgin Islands Water Resources Research Institute, Caribbean office of the USGS, and professional organizations in the region. All these activities facilitate the translation of research sponsored by the Institute into practical applications of direct benefit to industry, government, and the general public. In 2011, the last conference held, the Puerto Rico Water Resources and Environmental Research Institute joined the Hawaii Water Resources Research Center, the Virgin Islands Water Resources Research Center, and the Environmental Research Institute of the Western Pacific in Guam to organize the conference titled “Water Resource Sustainability Issues on Tropical Islands.” Next conference is being planned for December, 2015. Other seminar and workshops have been offered as part of the various educational and technology transfer projects in collaboration with Jobos Bay National Estuary Research Reserve under NOAA.

During FY2014 none information transfer projects were funded from 104B program.

USGS Summer Intern Program

None.

Student Support					
Category	Section 104 Base Grant	Section 104 NCGP Award	NIWR-USGS Internship	Supplemental Awards	Total
Undergraduate	6	0	0	0	6
Masters	6	0	0	0	6
Ph.D.	2	0	0	0	2
Post-Doc.	0	0	0	0	0
Total	14	0	0	0	14

Notable Awards and Achievements

None during this reporting period.