# IDENTIFICATION OF ANTIBIOTICS AS TOXIC CONTAMINANTS AT SURFACE WATER AND FILTRATION PLANT IN THE NORTHERN AREA OF PUERTO RICO

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Abstract - Puerto Rico Water Authority (PRWA) complies with regulations and treatments for drinking water, but there are no requisites for the detection of emerging chemical pollutants (ECP). Most Emerging Contaminants (EC's) are unregulated by the National Primary Drinking Water Regulations (NPDWR's or primary standards) under the Safe Drinking Water Act and few agencies have proposed methods to establish a provisional safe level in water treatment plants (WTP). Research has identified pharmaceutical compounds, such as antibiotics, a type of ECP, detected on the surfaces of waterbodies. Conventional analysis methods are not accurate in detecting particles in parts per billion (ppb). The purpose of this research was to analyze the concentrations of penicillin with the risk of active antibiotics metabolite derivatives in waters samples that feed the filtration plant for drinking water at Quebrada Water Treatment Plant in Hatillo, and the Carraízo Reservoir Dam in Trujillo Alto. We developed a method to determine the presence of these ECP using ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS-MS) with a calibration curve correlation coefficient of r = 0.999804 for six different concentrations. Water samples were analyzed, and results indicated ECP detected levels of approximately of 0.7 ppb and 0.9 ppb. Since the detected levels are below the LOD established by the method additional samples are being process. In conclusion, using the green technology of UPLC-MS-MS, we detected the presence of penicillin compounds in surface water of Camuy river, Hatillo. Penicillin G/V antibiotics are unstable in the environment and their concentrations are expected to be low (ng/L) in water. Other types of antibiotics more stable and less biodegradable need to be monitor.
*Key words: emerging chemical pollutants, penicillin, liquid chromatography, mass spectrometry, green chemistry*

Resumen. La Autoridad del agua de Puerto Rico, también conocida como Autoridad de Acueductos y Alcantarillados (PRWA, por sus siglas en inglés) cumple con las regulaciones y tratamientos para el agua potable, pero no existen requisitos para la detección de contaminantes químicos emergentes (CQE). La mayoría de los CQE no están regulados a partir del Reglamento Nacional de Agua Potable Primaria (NPDWR o estándares primarios) bajo la Ley de Agua Potable Segura y pocas agencias han propuesto métodos para establecer un nivel seguro provisional en plantas de tratamiento de agua (PTW). La investigación ha identificado compuestos farmacéuticos, un (CQE), detectado en las superficies de

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cuerpos de agua. Sin embargo, los métodos de análisis convencionales en las plantas de filtrado de agua, no son precisos para detectar partículas en partes por billón (ppb). El objetivo de esta investigación es analizar las concentraciones de penicilina G y V que contengan riesgo de derivados de metabolitos activos de antibióticos en la superficie de cuerpos de agua que alimentan la planta de filtración de agua potable en la Planta de Tratamiento de Agua Quebradas en Hatillo, y del Embalse Carraízo en Trujillo Alto. Desarrollamos un método para determinar la presencia de estos CQE utilizando espectrometría de masas en tándem y cromatografía líquida de ultra rendimiento (UPLC, por sus siglas en inglés) con un coeficiente de correlación de curva de calibración de r = 0,999804 para 5 concentraciones diferentes. Estos compuestos de metabolitos de antibióticos se analizaron y compararon con la curva de calibración de estándares de antibióticos y dentro de los límites detectados se encontraron cantidades de 0.7 ppb. Estas cantidades no se han podido cuantificar con la curva de calibración ya que las concentraciones están por debajo del LOD establecido en el método. En conclusión, utilizando la tecnología verde de UPLC-MS-MS, identificamos la presencia de metabolitos de penicilina G y V en el río Camuy en Hatillo. Los antibióticos de penicilina G/V son muy inestables en el medio ambiente y se espera que las cantidades sean bajas (ng/L) en agua. Otros tipos de antibióticos con una molécula más estable y menos biodegradables deben ser monitoreados en los cuerpos de agua.
*Palabras clave: Contaminantes emergentes, penicilina, cromatografía líquida, espectrómetro de masa, química verde.*

## Introducción

Emerging contaminants (EC's) in drinking water treatment processes are an arousing concern, mostly because there is not enough information recovered or studies on its toxicity, possible health impact, established threshold values of prescribe drugs (endocrine disrupting drugs) and pharmaceutical and personal care products. In the quest for a better health, endocrine disrupting (Benotti et al., 2009) are in the raise with the discovery of new developed pharmaceutical compounds, which are a class of drugs contaminants that are affecting the environment and are been detected in water bodies, especially, in surface, groundwater, estuarine, and drinking water as well as in wastewater (Pulicharla et al., 2015).

Pharmaceutical drugs (e.g. antibiotics) are designed to treat microbial diseases, control and prevention cause by bacterial infections in humans and in animals (Animal Antibiotics 2016), and also increase lifespan probability (Berkner, Konradi, & Schonfeld, 2014). Antibiotics are a type of chemical drug (derived from microorganism), that kills or slows the growth of bacteria used in many ways that may affect our health. They also play a key role in our food supply, making it safer, people healthier and controlling some diseases (Vikesland et al., 2017).

These types of chemical drugs have been approved by agencies like the Federal and Drug Administration (FDA) and the United States Department of Agriculture (USDA) for livestock and the Centers for Disease Control and Prevention (Animal Health Institute, 2016). Over the past years, concerns have raised regarding the increase in the use and over-use of pharmaceutical products, especially those containing antibiotics, used in food animals (livestock) that are also used in humans (Pulicharla et al., 2015). Therefore, the contamination of rivers and streams with pollutants of chemical pharmaceutical origins is an emerging public health concern (WHO, 2015).

Puerto Rico, as an incorporated territory of the United States, is not the exception. Relatively little is known of possible pharmaceutical chemical contamination from various sources or "hot spots" in surface waters. Even tough, Puerto Rico Water Authority (PRWA) complies with federal regulations and treatments for drinking water, there are not requisites for the detection of emerging chemical pollutants (ECP), such as pharmaceutical chemical substances.

Pharmaceutical residues are ECP's that may have risk asses to the environment, rivers and streams ecosystems by an endless number of pollutants of pharmaceutical origin (e.g. antibiotics). Since ECP's are unregulated as of the National Primary Drinking Water Regulations under the Safe Drinking Water Act (USEPA, 2004), few agencies have proposed methods to establish a provisional safe level in water treatment plants (WTP). Concern in the United States and many other countries around the world about emerging contaminants that may be present in water bodies in trace quantities, many drugs, had gone unnoticed, and according to the Center for Disease Control this public health concern is one of the biggest threats to global health that may estimate over 2 million human illnesses in the USA (United States Government Accountability Office, 2017).

*Identifying areas of agricultural activity with the potential in having antibiotic residues*

Agricultural activity (mainly related to the production or processing crops and or dairy products like poultry or live stock for commercial purposes in sampling area and residential sanitary downloads adjacent to water bodies were observed in Camuy river, that feed the Quebrada Filtration Plant and in Carraízo reservoir (Sergio Cuevas WFP) in Trujillo Alto, that is used for drinking water. We visited the rivers that deliver the water source to the filtration plants and we observed mayor agricultural activities, industrial and residential areas for possible contamination source or discharges and anthropogenic activities.

In order to determine the fate and spread of these emerging contaminants reaching to drinking water and filtration plants is important to identify possible areas of great anthropogenic activities and the kind of drugs that are taken by consumers or given to food animals that may remain un-metabolized in the digestive system that eventually the active drug is excrete. These active drugs go through a path from sewage to treatment plants or to ground waters via landfills sites or to soil for nutrient recycling and water from digest sludge process since modern water treatment plants in general are not designed for the detection and/or removal of these types of contaminants to reach drinking water quality (Heidari et al., 2013).

The early detection and analysis of antibiotics in the water cycle, including surface waters, waste waters, ground waters, lakes, rivers and even drinking water is important in order to assess risk to humans and the environment. Since humans are using antibiotics for any infectious disease, they are becoming strongly dependent on these type of drugs (Pulicharla et al., 2015).

Farmland animals (cow, pigs, chickens) are overexpose to antibiotics for disease control but mostly as grow promoters. These farm animals are overcrowded in such a small space for the number of animals that they eat and also defecate in the same area that all of these bacteria spread rapidly from one to another creating a bacteria resistance between them. According to the Canadian Integrated Program for Antibiotic Resistance Surveillance (Public Health Agency of Canada, 2007) about 1.8 million Kg of antibiotic were sold in Canada for human (17%), animal (82%) or agricultural use (less than 1%). Research studies, indicate, that not even the three stages of water filtration process are 100% effective to remove all the antibiotic residues from water. At the end of the WTP, all antibiotic residues become part of the waters returning to the rivers of as part of the sludge going back as fertilizers to landfills (Joss et al., 2006).

This research aims to identify and quantify the potential contaminants derived from pharmaceutical antibiotics, as penicillin V (more acid stable for oral use), and penicillin G (intravenous use), as contaminants and possible point sources in the norther area of Municipality of Hatillo Puerto Rico and Carraízo Reservoir in Trujillo Alto that may affect the drinking water filtration plants and the communities that are served from it. Analyze the water samples with a possibility of having active antibiotic metabolites of penicillin G and penicillin V pollutants residues are not yet regulated under the Resource Conservation Recovery Act (RCRA) nor the Safe Drinking Water Act (SDWA) standard limits (USEPA,

2003). Also, to identify areas that most affect the drinking water filtration plant system and calculate the concentration of these EC's with antibiotic penicillin V and penicillin G chemical characteristics in bodies of water in Hatillo, up-stream the Quebrada drinking water filtration plant and up-stream the Carraízo Reservoir Dam.

## Materials and methods

This study was conducted in brook drinking water filtration plant in the municipality of Camuy Puerto Rico, where water samples were collected utilizing EPA method 538 (US EPA, 2018) upstream and downstream at a determine area inside the filtration plant and directly adjacent to the Carraízo Dam, under the IBC protocol #B01-050-17.

### *Sampling*

Water samples were collected from the surface (approx. 10-12 inches from surface) at three sampling sites in acid-washed, (2)-1-L amber glass bottles from the sampling site at:

a) Parque Río Camuy, Camuy Puerto Rico Lat. = 18.347400 and long = 66.819403,

b) Up-stream and down-stream (influent and effluent) of the Quebrada Water Filtration Plant (WFP) in the Municipality of Camuy Puerto Rico and Carraízo Reservoir Dam in Trujillo Alto at the Sergio Cuevas WFP at sampling source inside the WFP facilities.

Before any chemical or biological treatment process was done, samples were collected packed and placed in ice cooler to preserve the samples and prevent possible evaporation of water and transported to the laboratory for analysis. The parameters documented in the sampling were of place to collect sample, date, time, climate conditions, turbidity; the pH was analyzed at site with a combination glass electrode and volume. Upon receipt at the laboratory water samples were filtered, to eliminate the particles, through a Whatman #3, 9.0 cm filters with a vacuum filtration process stored at -80°C, limiting UV light exposure and extracted within 24 hours of sample collection.

*Lyophilization method*

After the first filtration, water samples were frozen at -80°C for 24-48 hrs., then samples were lyophilized (Gao et al., 2010). The removal of bound water molecules was done through the process of desorption using a Labconco FreeZone 4.5 to eliminate all frozen molecules and separate any minor organics solvent present within the samples. Precipitate remaining organics and salts compounds obtained after freeze dry were diluted with 3 mL of methanol (MS-HPLC grade) followed by solid separation using centrifuge at 3,300 rpm and filtration through 0.45-um nylon filter. A second filtration was performed with a 0.22-µm nylon filters, Millipore, Billerica, MA and transferred to a 1mL glass vials for MS-MS-HPLC analysis.

*Analytical analysis*

Design and apply an analytical analysis to calculate the concentrations of these pharmaceutical chemical contaminants before and after the filtration plant process in the Quebrada Water Treatment Plant in Hatillo, in the Carraízo Reservoir Dam (Sergio Cuevas) in Trujillo Alto and directly from the river in Camuy with a UPLC-MS/MS.

Each 2,000 mL of water sample was frozen and lyophilized to evaporate all the water, separate any organics within the sample and dilute any remaining organics / salts compounds with 3 to 5 mL water (MS grade). A second lyophilize procedure was done to evaporate and purify any organics in the samples. Dilute any residues with 1 mL MS grade methanol + 0.1% MS-Formic Acid organic solvent and analyzed.

For quantification of pollutants, we utilized a Waters Xevo TQD mass spectroscopy instrument. Water sample analysis was performed using a Waters Instruments Acquity UHPLC Class (MS-MS-UPLC) for liquid chromatography, Acquity UPLC c-18 columns, 1.7 µm, and 2.1 x 50mm column with a Pre-Column an Acquity UPLC BEH Shield RP18 1.7 VanGuard 2.1 x 5mm column and Mass lynx V4.1 software installed. The program methodology for analysis was performed using a column temperature of 40°C and sample temperature of 15°C (inside instrument) with a source temperature of 150°C - 200°C (as desolvation temp.). Capillary voltage at 3.10(kV) and cone voltage (35V) using a collision energy of 3V and flow at 10 µL/min. using a mix gradient of solvents of water with 0.1% formic acid and acetonitrile with 0.01% formic acid and Argon gas. Prior to sample preparation the instrument was calibrated to its correct programmed temperature:

column temperature of 40°C and sample temp of 15°C with a source temperature of 150°C - 200°C (as desolvation temp.) and stabilized to a delta of -30.

For this research analysis, the advantage of ultra-high-performance liquid chromatography (UPLC) resolution coupling to mass spectrometry (UPLC/MS-MS), is that it has significantly improved the detection and quantification of antibiotics and other pharmaceuticals compounds at very low concentrations. In environmental samples, mass spectrometric methods have improved reproducibility, accuracy and reliability result for detection of trace amounts of antibiotics, including surface and groundwater, with modern mass spectrometric methods. Some antimicrobial treated water and PAHs in urban area are been detected with technological advances in methods of chromatography and mass spectrometer (Dasgupta & Sengupta, 2015). Liquid chromatography with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) are excellent methods for target compounds determination to the ng/L level and are commonly applied for the detection of drug compounds (as in emerging compounds) in surface waters. This method selection is dependent on the compounds physical and chemical properties. UPLC-MS/MS analysis is more suitable for measuring more polar and highly soluble compounds in water (Fatta et al., 2007), and because of its high sensitivity and ability to confirm compounds, we can detect compounds with same molecular mass but with different ion products.

*Stock preparation and solutions storage*

Stock solutions will be prepared by dissolving (X) amount of reference material penicillin V (Phenoxymethylpenicillin) and penicillin G (Benzyl penicillin standards) in an organic solvent (organic alcohol as Methanol-MeOH LC/MS-grade from ACROS Organics). We weighed 10 to 20 mg of ampicillin or penicillin G (penicillin g potassium salt 99.7% purity Vetranal) and V (penicillin v potassium salt 98.7%), analytical standard, in a 10mL volumetric flask and fill with methanol-(MeOH ms-grade solvent). After the compound dissolves completely, transfer the solution to a clean 15-mL vial with fluoropolymer-lined cap. When not being used, store standard solutions in temperature -10 ºC in under a non-reactive (inert) gas (e.g., nitrogen) in a sealed glass vial. Replace solvent loss by evaporation detected.

All of the sample preparation was done inside a chemical fume hood for air particle precaution and weighing the samples in an analytical balance Mettler Toledo of 0.001 mg sensitivity; Class A volumetric flask (5 – 10 mL) for volumetric measurements; Adjustable pipettes: Eppendorf 100 – 1000 µL (Brinkman

Instruments Inc.) for precision small amounts for MeOH measurement.

*Analytical method for aqueous matrices*

The analytical method for liquid (aqueous) samples were performed as follow, (grab samples were collected approximately 12cm below surface the surface): First a 2,000mL aqueous grab samples in amber glass were collected and place in ice for transportation to the laboratory for process and filtration. Whatman #3, 9.0 cm filters were used to separate and large particles and sediment. A secondary filtering was done using 0.45 and 0.2 microns for the water sample do not clock the analytical separation column. All the samples were freeze in a -80°C freezer for 24 hours and water molecules were separated from any solid salt with our potential metabolite was performed using a Lyophilization LABCONCO Free Zone 4.5 until completely dry. The residues from the first separation were diluted with organic solvent (methanol) for (clean-up); solvent exchange and doing a secondary lyophilization process was performed. Dilution of dried organic sample in 1mL MeOH/ Formic Acid 0.1% for the remaining salts after secondary lyophilization.

Analysis was performed using a Waters Instruments Acquity-H UPLC Class UPLC-MS/MS spectroscopy. Validate each determination by tracing the instrument output (peak height or area,) to the result. These data include: a) Extraction dates, b) Analysis dates and times, c) Sample numbers and other identifiers, d) Sequence of analysis, e) Sample physical appearance, f) Sample or extract volume prior to each cleanup step, g) Final volume prior to injection, (h) Injection volume.

## Results and discussion

In this research, we identified waterbodies that we expected had mayor impact of these (ECP) in northern Puerto Rico. Near Carraízo Reservoir Dam in Trujillo Alto, we found high density of anthropogenic activities in residential area, adjacent to the river that have a direct implication of contaminants from several routes, discharges of residential communities, runoff waters and some agriculture activities, to the river that serve the Sergio Cuevas Filtration Plant. In comparison to Carraízo, at Parque Río Camuy and Quebrada Water Filtration Plant (WFP) the residential properties were minimum. At Parque Río Camuy, the cattle, dairy and pig industry were common and well established, about 2.5 miles upstream near the river, which may result in an elevated concentration of antibiotics, that we expected has a higher potential of contaminant on the river. We identified a potential point source of these ECP's on the river waters that comes from the

excretion of agricultural activities (cattle and pigs) near the river. We analyzed the water samples to detect antibiotic residues, that may have a potential risk to the microbial community.

From three sampling sites in northern Puerto Rico, Parque Río Camuy, Lat. = 18.347400 and long = 66.819403, Quebradas Water Filtration Plant (WFP) in the Municipality of Camuy Puerto Rico and Carraízo Reservoir Dam in Trujillo Alto at the Sergio Cuevas WFP facilities, we were able to identify the presence of antibiotic pollutants in the water samples. From the 22 samples taken in three sites, four samples were from Camuy river. At Camuy river, the samples were taken directly from the surface of the river every two weeks at 9:30 to 10:30 am of each day with similar climate parameters.

At each sampling site, we analyzed the pH of water using a Mettler Toledo pH meter, (6.36 -7.23). All samples from the river had very high turbidity due to high content of solids and minerals that were visible to the human eye. The determination of turbidity was not determined in this study. For Quebrada Filtration Plant the pH range from 6.50 to 7.06 and in Sergio Cuevas the pH ranges from 6.55 to 7.94.

All 22 water samples were filtered using a Whatman #3, 9.0 cm filters; then were frozen at -80°C for 24 hours and lyophilization using a Labconco FreeZone 4.5 until dried completely. The solid organic salts were dissolved with methanol (MS grade), centrifuged at 3,000 rpm for 30 seconds to separate solids from solution and filtered using 0.45 and 0.2 micron filters. The remaining organic solvent (MeOH) with the organic compound were placed in (1 mL) glass vial for chemical analysis.

The analytical software method for analysis used in the UPLC-MS-MS was performed with a calibration curve range from 100 to 1 ppb. A correlation coefficient of $r$ = 0.999804 with linear calibration for limits of quantization for 6 different concentrations (n = 6) determined that the concentration in the Quebrada Water Filtration Plant (WFP) in the Municipality of Camuy Puerto Rico and Carraízo Reservoir Dam in Trujillo Alto at the Sergio Cuevas WFP facilities were at 0.2 ppb. In Camuy river was 0.2 ppb but a broader base line in the graph that indicates a stronger signal. The relative standard deviation ranges from 0.87 – 1.07 for penicillin G and from -0.3 to -1.8 for penicillin V standards concentration from 1 to 50ppb and above 50ppb the % dev. range up to 29.8.

To verify these results and justify the method developed for analysis, we made three different spike solutions with the 10 ppb standard solution. First spike sample was done with a 10 ppb concentration and the second with a 1 ppb, both in a 2,000 mL water sample volume and performed the same method of analysis as the real samples. The results from the analysis were repeated three times and the concentration found was (0.7 ppb), having a consideration that in the lyophilization process 0.5 ppb was lost, but since they all were under the LOD, we could not integrate the concentration results and compare them with the antibiotic standards used for calibration. After analyzing spike samples, we found that during the analytical process (filtering, freeze, lyophilization and centrifugation), was lost 0.5 ppb of sample concentration from the spike standard, so we determined that the concentration ranged at about 0.7 ppb without numerical integrations comparison.

However, from the UPLC-MS-MS concentration graphic of the penicillin standard for the Camuy river and Quebradas Water Filtration Plant and Carraízo Reservoir Dam facilities, we can compare concentrations and determine that the area (as concentration) shown from the Camuy river are wider, associating a higher particle detection. Despite the methodology for analysis, we found that penicillin G (β-lactam ring) is bio reactive in the presence of UV light, hydrolytic opening and many environmental conditions including a neutral pH, temperature and the possible presence of enzymes in aquatic live that it biodegrades easily and rapidly (Canzani & Aldeek, 2017). The most abundant metabolites may be penicilloic acids once the (β-lactam ring) rearranges (Canzani & Aldeek, 2017). For penicillin stability, it has to be kept between 0 o C and 52o C to prevent degradation and a citrate buffer at pH 7 (Canzani & Aldeek, 2017).

The presence of antibiotics particles at very low concentration in river and filtration water plants threats the ecosystem life and public health. According to the WHO (2017), in 2050, over 10,000,000 deaths are attributed to antimicrobial resistance and/or antibiotics. A wide spectrum of bacteria can mutate (ej. *E.Coli* strain) to a stronger resistance that eventually give rise to a bacterial strains that may be very difficult to treat and having the possibility to become a fatal infection. The European Medicines Agencies Guideline (2006) suggested a prediction of the possible drug (EC's) concentration or metabolites in surface waters of below 10 ng -1 for quantitate risk assessment but still in phase 1 for further laboratory tests, knowing that resistance can occur at even lower concentrations (of <1ng -1).

This study had some limitations. The analysis of antibiotic metabolites in the environment is challenging due to the variability of matrix presented at the sampling sites in Puerto Rico, as well as their weather conditions. Additionally, it is important to improve the lyophilization process by having a lower vacuum pressure for solids stabilization and higher amount of freeze samples to increasing the recovery of dry organics.

## Conclusions and further recommendations

In conclusion, using the green technology of UPLC-MS-MS, we detected the presence of penicillin compounds in surface water of Camuy river, Hatillo. Penicillin G/V antibiotics are unstable in the environment and their concentrations are expected to be low (ng/L) in water. Other types of antibiotics more stable and less biodegradable need to be monitor.

Our results showed that conventional water treatment process (technology & methodology) at the water filtration plants in Trujillo Alto and Quebrada, Camuy, has low efficiency towards the removal of residual antibiotics. Therefore, the combination of biological and chemical treatment strategy or advance oxidation treatment process (AOP) may improve drinking water quality (Reungoat et al., 2012).

We recommend to increase organic compounds concentration recovery for antibiotics metabolites and different type of antibiotic compound with lower fate in water. This might by possible by increasing the amount of water samples collected for analysis, sampling sites, extending time period and different climate conditions and sing a method with specific antibiotics column separation with a lower LOD capable of reaching low concentrations of 1ppb.

To protect drinking water sources is important to reduce the overuse of antibiotics in farm animals and to focus on monitoring residual waters and its pathways. Scientists must study the mechanism of bacteria resistance. The environmental agencies must establish regulations for these types of emerging contaminants and been able to educate to reduce the drug overuse.

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