NM WRRI Student Water Research Grant Progress Report Form

Progress Report due October 1, 2018
Draft Final Report due April 15, 2019
Final Report due May 15, 2019

1. Student Researcher: Xiaoxiao Cheng
   Faculty Advisor: Yanyan Zhang

2. Project title:

Developing new strategies to mitigate antimicrobial resistance for safe water reuse

3. Description of research problem and research objectives.

1) Research problem:

Massive use of antibiotics for human and veterinary purposes have accelerated the evolution of antimicrobial resistance in bacteria which promotes wastewater as an environmental reservoir of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs).

Water resources management in arid and semiarid southwestern United States has been a significant challenge due to limited fresh water supplies and chronic droughts. Water scarcity has a huge impact on food production since agriculture accounts for 70 percent of global freshwater withdrawals. Wastewater has been identified as potential water resource for agriculture food production such as irrigation, livestock watering, and aquaculture. However, the massive use of antibiotics for human and veterinary purposes have accelerated the evolution of antimicrobial resistance in bacteria which promotes wastewater as an environmental reservoir of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs). As treatment objectives focus on enabling higher value of treated water from wastewater, greater attention should be turned toward processes that have even greater capability in the removal of ARB and ARGs. Conventional wastewater treatment processes include a combination of physical, chemical and biological approaches to eliminate or reduce suspended solids, organic matter, nutrients and microorganisms. However, they are not effective in term of inactivating ARBs and destroy ARGs. Some processes might positively affect ARB strains' spread and selection as well as ARG transfer. Making matters worse, conventional wastewater treatment does not include a process to eliminate phages which may contain ARGs. Considering conventional wastewater treatment processes are not effective in term of inactivating ARBs and destroy ARGs, developing new treatment trains to minimize the ARB and ARG in the treated water is urgently needed to mitigate the dissemination of ARB/ARG to the environment and ensure safe use of treated water in agriculture food production. The overall objective of this proposed study is to develop new strategies to mitigate the ARB and ARGs in the effluent of WWTPs for minimizing the dissemination of ARGs into the environment and agricultural ecosystem.

2) Research objectives:

1) Investigate the efficiency of ARB and ARG removal through the existing treatment processes
2) Develop the new treatment trains for mitigating antibiotic resistance by targeting ARB and ARGs.

4. Description of methodology employed.

1) Sampling

Duplicated samples of primary treatment effluent (P), trickling filter effluent (TF), and activated sludge system effluent (S) were collected from the Las Cruces Wastewater Treatment Plant (WWTP) on October 2017 during normal operation.

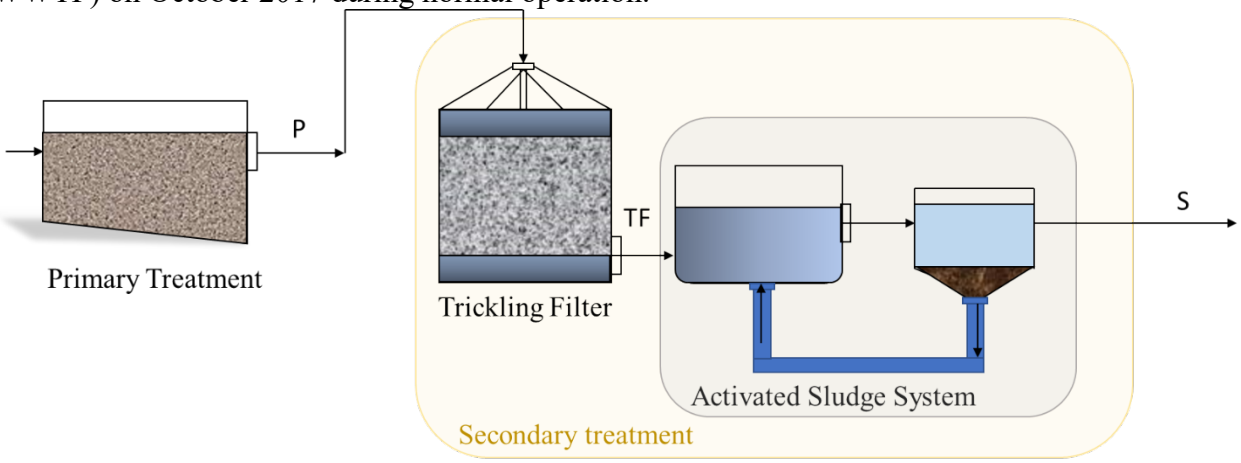

Fig 1. The diagram of sampling points (P, TF, and S)

2) Quantification for antibiotic resistance genes (ARGs)

Bacteria in 25 mL, 50 mL, and 215 mL of water samples respectively in primary treatment effluent (P), trickling filter effluent (TF), and secondary treatment effluent (S) were concentrated by membrane filtration with 0.22 µm pore-size membrane (Millipore, Billerica, MA) before DNA extraction using DNeasy PowerWater Kit (Qiagen, Germany).
12 antibiotic resistance genes were detected in this program including 4 quinolone resistance genes (*qnr*A, *qnr*B, *qnr*C, *qnr*S), 3 tetracycline resistance genes (*tet*W, *tet*M, *tet*O), 2 sulfonamide resistance genes (*sul1*, *sul2*), 1 erythromycin resistance gene (*erm*B), 1 multi-resistance to β-lactam antibiotics gene (bla_{TEM}) and 1 mobile element class 1 integron gene (*intI1*). Polymerase chain reaction (PCR) was used for screening the existence of the chosen ARGs. Quantification (qPCR) was used for quantifying the abundance of the detectable ARGs.
qPCR reactions for 8 ARGs were performed in 96-well plates with a system volume of 20 µl, which containing 10 µl SsoAdanved Universal SYBR Green Supermix (Bio-Rad), 1 µl of each primers (10uM), 2 µl of diluted DNA sample and 6 µl of DNA/RNA free water. qPCR reaction for 16s rRNA was performed in 20-µl system consisting 10 µl of SsoAdvanced Universal Probes Supermix (Bio-Rad), 1 µl of each primer (10 µM), 2 µl of diluted DNA sample, 1 µl of probe (2.5 µM) and 5 µl of DNA/RNA free water. Each reaction was performed in triplicate. Standard plasmids containing target genes were constructed using a TOPO TA cloning® kit (Invitrogen, by Thermo Fisher Scientific) for quantification. Reactions without the DNA template served as negative controls.

3) Plate count

Heterotrophic plate count (HPC) method was used to quantify the population of ARB in each sample. 5 types of antibiotics (ampicillin: 32 mg/L; tetracycline: 16 mg/L; ciprofloxacin: 4 mg/L; erythromycin: 8 mg/L; sulfamethoxazole: 50.4mg/L) were chosen for enumerating ARB. Each antibiotic represents a class of antibiotic: ampicillin (Penicillin class, broad spectrum); ciprofloxacin (Quinolone class, broad spectrum), and tetracycline (Tetracycline class, broad spectrum); erythromycin (Macrolide class, broad spectrum); sulfamethoxazole (Gantanol class, broad spectrum). R2A agar (BD) was used as culture medium with the above antibiotics. Plates were incubated for 48 h at 37 °C and then incubated 72 h at room temperature.

4) ARB removal by the combination of granular activated carbon (GAC) and chlorine

Granular activated carbon (GAC) filter combined with different concentration of NaClO is proposed to remove ARB from treated water before discharge. First, the optimal chlorine dose in ARB removal was investigated by adding the chlorine into the secondary effluent with a final concentration of 2, 8, 16 and 32 mg/L. Next, we attempt to use GAC column combined with 16 mg/L of NaClO and 32 mg/L NaClO to maximize the reduction of ARB.

Table 1. Operation condition of the GAC column

Volume of column (mL)	160
Volume of water sample (mL)	1000
Flow rate (mL/min)	20
Retention time (min)	8
Concentration of NaClO (mg/L)	16; 32

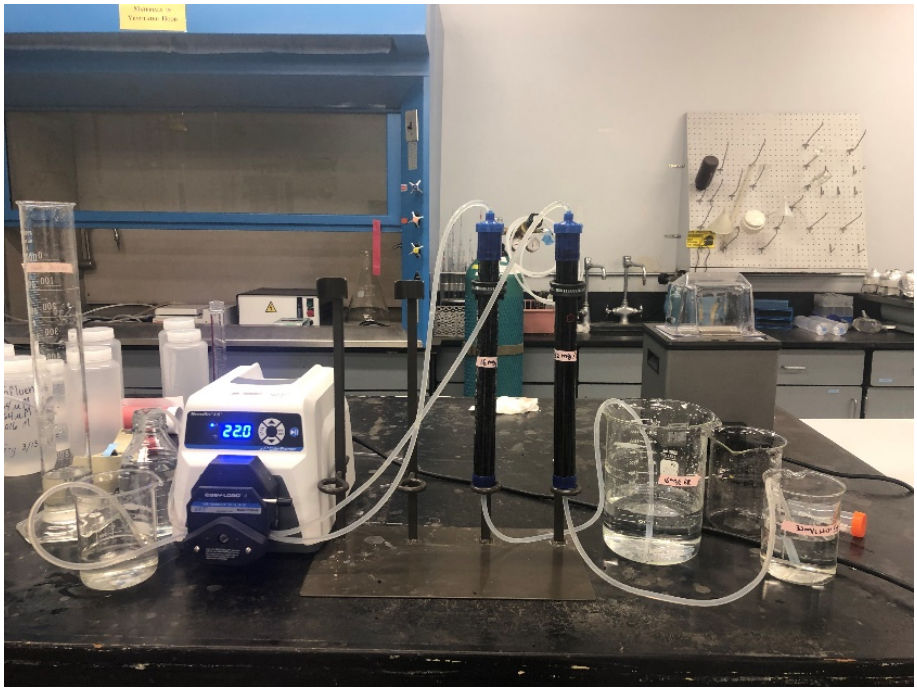

Fig 1. The GAC column setting for ARB removal

5) The retention of ARB in GAC filter

In order to investigate the retention of ARB in GAC filter, we took 15 mL of GAC sample from the upper part, middle part and bottom part of the GAC column separately. Then, 35 ml of sterile phosphate buffer saline (PBS) was used to release bacteria from each GAC sample by shaking overnight. 5 types of antibiotic resistance bacteria were detected by plate count.

5. Description of results; include findings, conclusions, and recommendations for further research.

1) Relative abundance of ARGs in primary treatment effluent, trickling filter effluent and secondary treatmnet effluent

Eight of twelve analyzed genes (*bla*$_{TEM}$, *intl1*, *tet*W, *qnr*A, *qnr*B, *qnr*S, *sul1*, *erm*B) encoding 5 classes of antibiotic resistance were detected in primary effluent by PCR. And the eight gene's ralative abundance (copy number of ARG / copy number of 16s rRNA) were showed in figure 2. The relative abundance of tetW and ermB gene decreased in secondary effluent while *qnr*S, *bla*$_{TEM}$ and *sul1* gene had a higher relative abundance in the effluent compared to influent. the lowest relative abundance of *qnr*A, *qnr*B, *qnr*S, *sul1*, and *bla*$_{TEM}$ were observed in primary treatment effluent whereas trickling filter increased their relative abundance slightly. The highest relative abundance of those ARGs were observed in activated sludge effluent where individual ratio ranged from 6.67×10^{-4} (*bla*$_{TEM}$) to 1.47 (*qnr*S). For *tet*W and *erm*B, contrarily, the lowest relative abundance of the genes occurred in activated sludge effluent which was 3.04×10^{-3} and 3.13×10^{-4}. Although considerable bacterial reduction occurred along the conventional treatment processes, the relative ratio of some ARGs in the surviving bacteria increased. There are some studies indicating that wastewater treatment systems especially biological treatment processes may promote the spread of ARGs through horizontal gene (Davies 2012, Kruse and Sørum 1994, Mach and Grimes 1982, Poté et al. 2003).

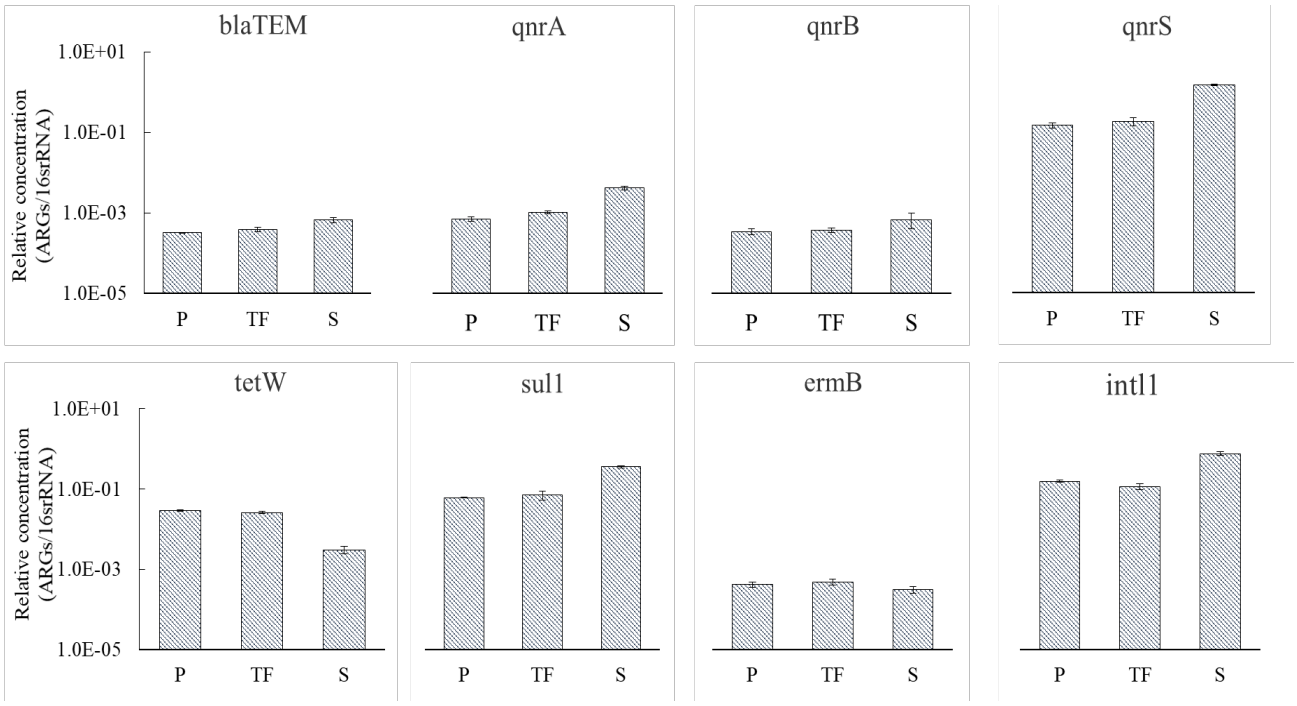

Fig 2. The relative abundance of ARGs (copy number of ARGs / copy number of 16s rRNA) along the treatment train

2) ARB in primary treatment effluent, trickling filter effluent and secondary treatmnet effluent

Unlike the total bacteria in the conventional system, the abundance of ARB peaked in the effluent of trickling filter, and then dropped notably in the effluent of activated sludge system (Figure 1). The high ARB concentration in the effluent of the trickling filter might be attributed to the horizontal gene transfer of ARGs in trickling filter. High numbers of bacteria in the biofilm and mixed liquor likely provided a favorable condition for the propagation of antibiotic resistance. Activated sludge system showed effectiveness in ARB removal. For ampicillin resistance, 3 log removal of ampicillin resistant bacteria in activated sludge effluent has been found compared to primary treatment effluent. In the case of ciprofloxacin, erythromycin and tetracycline resistant bacteria, 1 log removal was observed between primary effluent and activated sludge effluent. For sulfamethoxazole resistant bacteria, less than 1 log removal between primary and activated sludge treatment occurred. However, compared with 1.5 log removal of total bacteria by activated sludge system, the removal of ciprofloxacin, erythromycin, tetracycline resistance and sulfamethoxazole resistance were less, suggesting that the antibiotic resistance of surviving bacteria increased after activated sludge treatment.

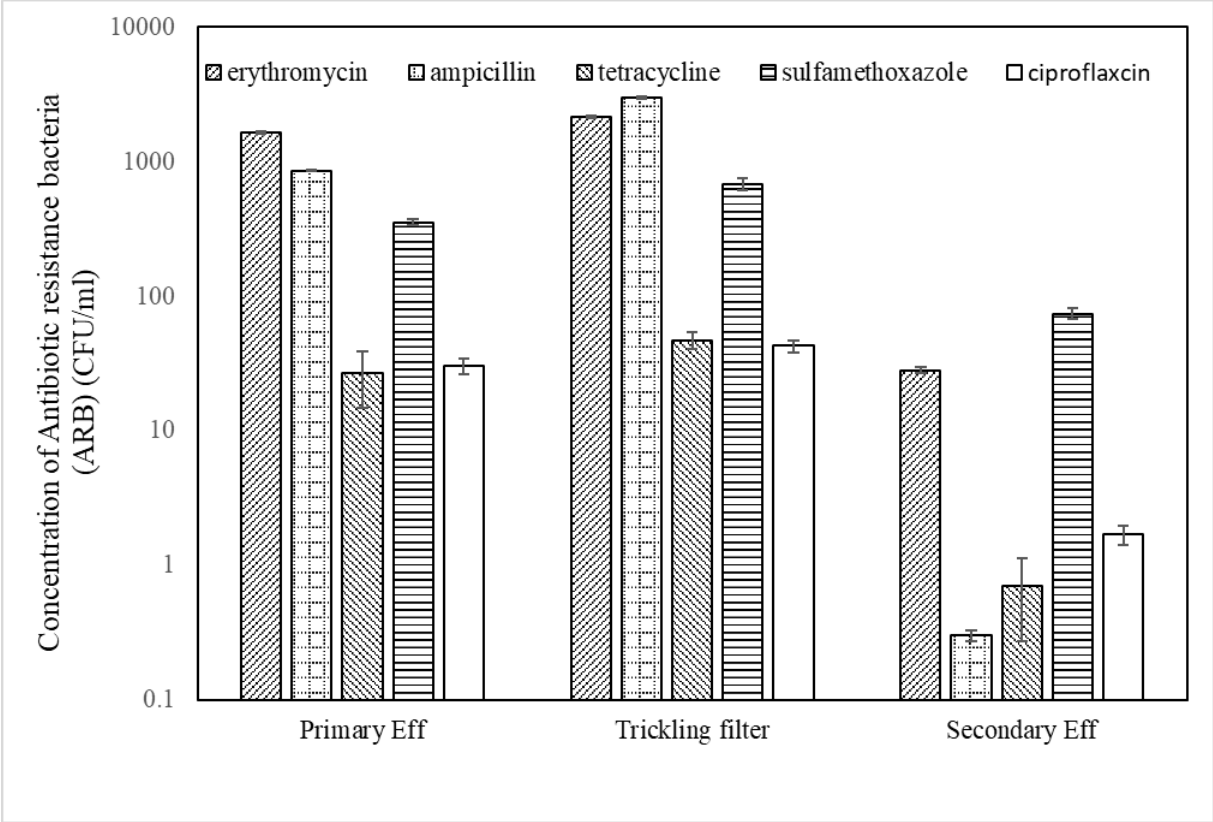

Fig 3. The concentration of antibiotic resistance bacteria (ARB) in existing wastewater treatment processes

3) ARB removal by the combination of granular activated carbon (GAC) and chlorination

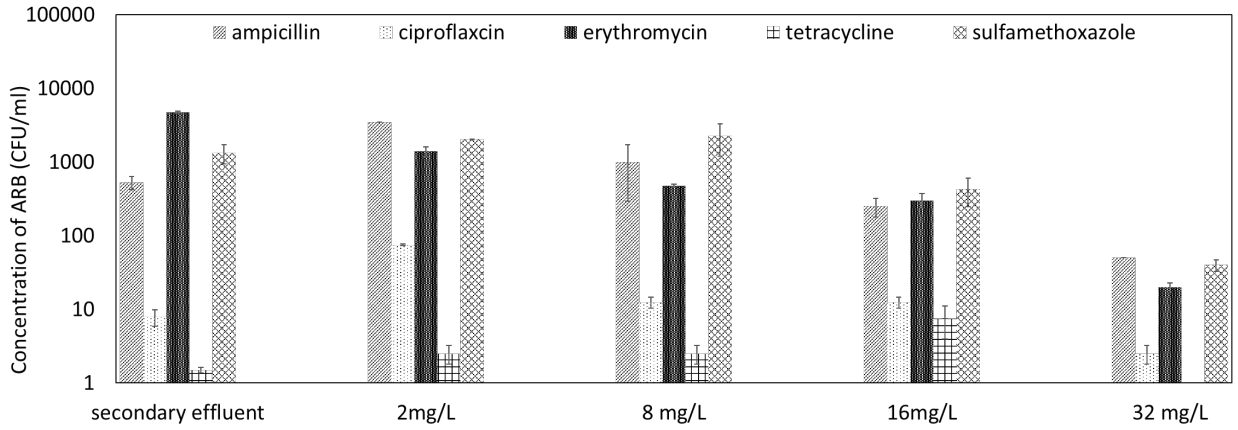

Fig 4. The concentration of ARB after different concentration of NaClO treated

Different concentration of NaClO were used to treat secondary effluent for ARB reduction. Although decreasing trend of ARB was observed with increasing chlorine dosage, sub-inhibitory concentrations of NaClO (2 mg/L and 8 mg/L) led to higher concentration of ARB when compared to the non-chlorinated secondary effluent sample. High concentration (16 mg/L and 32 mg/L) of NaClO performed better in inhibiting ARB (figure 4). Therefore, high concentration of

NaClO was chosen to combine with granular activated carbon for ARB removal in secondary effluent.

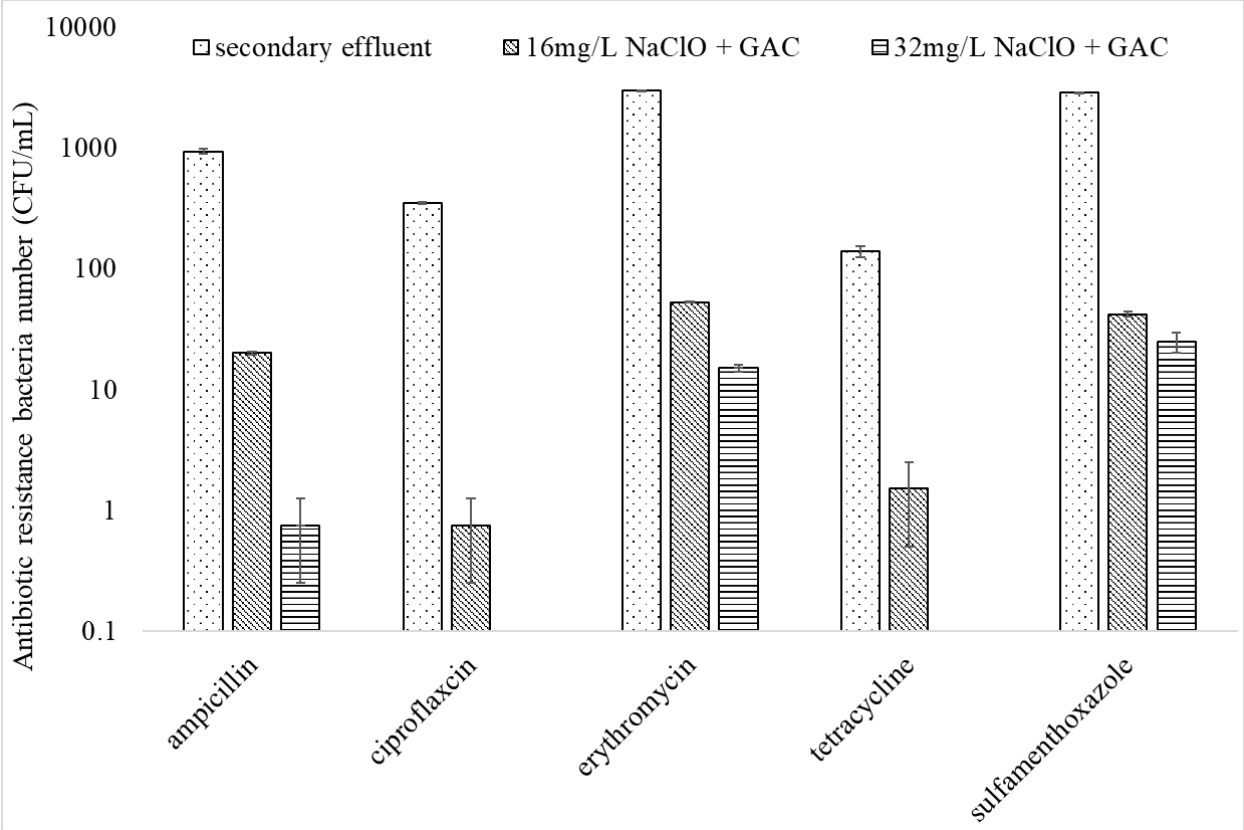

Fig 5. The antibiotic resistance bacteria concentration by using 16mg/L NaClO and GAC and 32mg/L NaClO and GAC

As the figure 5 shows, with the increase of NaClO concentration, a decreasing trend of ARB number was observed for all types of antibiotic resistance. 2-2.5 log removal was observed with the combination of 16 mg/L NaClO and GAC column while 2-4 log removal was observed when the concentration of NaClO increased to 32 mg/L. Specifically, concentration of the bacteria resistant to ciprofloxacin and tetracycline were blow detection limit in the effluent of GAC column when 32 mg/L of NaClO was used.

This result indicates that chlorination treatment with GAC adsorption is an effective method to reduce resistance bacteria. However, the retention of ARB in GAC is a concern to public health. Therefore, the spatial distribution of retained ARB in GAC was evaluated in our study as well.

5) The retention of ARB in GAC

As the figure 6 shows, for 16 mg/L NaClO/GAC treated water, the highest concentration of ampicillin, erythromycin and tetracycline resistant bacteria are observed in upper section of the GAC column and the lowest concentration of resistant bacteria are located in the bottom area. For 32 mg/L NaClO/GAC treated sample, all types of antibiotic resistance bacteria concentrated in upper area and lowest concentration are in bottom of the column. This can be explained by the

classic colloid filtration theory. This theory states that the number of deposited particles per mass of filter media decreases with the depth in the filter.
The high ARB concentration in GAC indicates the adsorption played major role in ARB removal. All of the 5 types of resistant bacteria, 32 mg/L NaClO can result in the lower ARB concentration in GAC compared with16 mg/L NaClO, suggesting the necessity of using chlorine to reduce the risk of ARB retention in GAC.

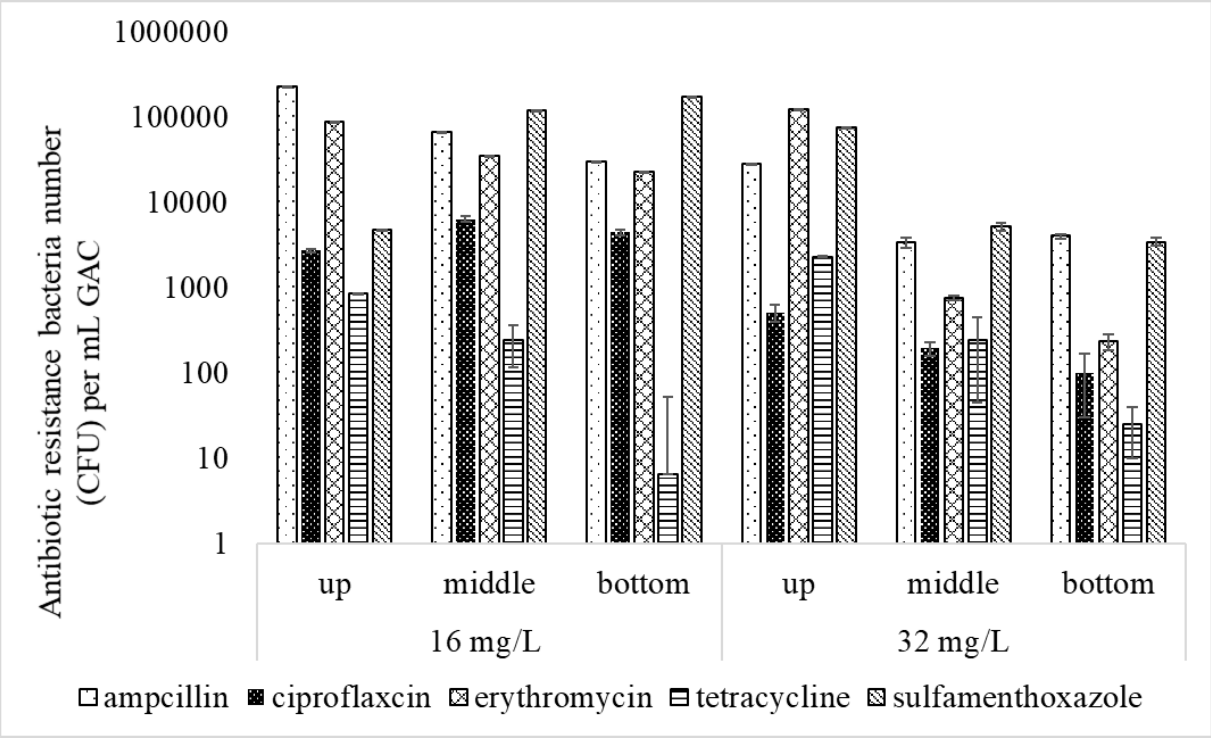

Fig 6. The bacteria number (CFU) in per mL GAC

6. Provide a paragraph on who will benefit from your research results. Include any water agency that could use your results.

The results from this study could advance the current understanding of the impact of wastewater treatment processes on the spread of antibiotic resistance. The new knowledge could be used to provide valuable information to stakeholders and policy makers for re-assessing the current wastewater treatment processes and reducing the environmental risks during wastewater management, reuse and disposal. The developed new processes in this study could be easily scaled up and applied by the management agencies and industries for the treatment on site to minimize the dissemination of antibiotic resistance into the environment and agricultural ecosystem.

7. List presentations you have made related to the project.

- Cheng, X., and Zhang, Y. (2018). Effects of Wastewater Treatment on the Prevalence of Antimicrobial Resistance 63rd Annual New Mexico Water Conference, Las Cruces, NM.

- Cheng, X., and Zhang, Y. (2018). Removal of Antibiotic Resistance in an Algal-based Wastewater Treatment System. RMSAWWA/RMWEA Joint Annual Conference, Denver, CO
- Cheng, X., and Zhang, Y. (2019) Metagenomic analysis reveals Algal system as novel method to reduce antibiotic resistance in wastewater treatment plants. Two Nations One Water Summit, Las Cruces, NM.
- Cheng, X., Zhang Y. (2019) Effect of Wastewater Treatment Processes on the Prevalence of Antibiotic Resistance. The 2019 AEESP Research and Education Conference, Phoenix, AZ

8. List publications or reports, if any, that you are preparing. Remember to acknowledge the NM WRRI funding in any presentation or report that you prepare.

Cheng, X., Delanka-Pedige, M. K., Srimali P. Munasinghe-Arachchige, Abeysiriwardhana-Arachchige, I.S.A., Smith, G. B., Nirmalakhandan, N., Zhang, Y., "Removal of Antibiotic Resistance Genes in an Algal-based Wastewater Treatment System: A Comparative Study." *International Journal of Medical Microbiology* (in prep)

9. List any other students or faculty members who have assisted you with your project.

Nagamany Nirmalakhandan (Civil Engineering faculty member);
Himali M. K. Delanka-Pedige1(Civil Engineering graduate student);
Srimali P. Munasinghe-Arachchige (Civil Engineering graduate student).

10. Provide special recognition awards or notable achievements as a result of the research including any publicity such as newspaper articles, or similar.

Not applicable

11. Provide information on degree completion and future career plans. Funding for student grants comes from the New Mexico Legislature and legislators are interested in whether recipients of these grants go on to complete academic degrees and work in a water-related field in New Mexico or elsewhere.

I am in my second year of my PhD study. I am planning to become a faculty member or research scientist in the field of wastewater treatment and reuse.