NM WRRI Student Water Research Grant Program Final Report

1. **Student Researcher:** Yun Ma
2. **Faculty Sponsor: Dr.** Runwei Li
3. **Project Title:** Using Zebrafish as an animal model to evaluate PFAS-induced liver damage under the co-exposure of PFOA and HFPO-DA (GenX).
4. **Research Problem and Research Objectives**

Perfluoroalkyl substances (PFASs) are a class of synthetic organic compounds that have garnered significant attention due to their widespread use in industrial and consumer products[1-3]. PFASs are utilized in various applications such as water- and oil-resistant coatings, food packaging, firefighting foams, and chemical processing because of their unique chemical stability, heat resistance, and water repellency[4, 5]. However, these same properties make PFASs extremely persistent in the environment, leading to their designation as forever chemicals[6]. This environmental persistence has resulted in the widespread distribution of PFASs globally, with their presence being detected in aquatic organisms and human populations[7, 8]. Among PFASs, perfluorooctanoic acid (PFOA) is one of the most extensively studied. PFOA has been restricted in several countries due to its potential carcinogenicity, reproductive toxicity, and developmental toxicity[9]. However, because of its industrial significance, alternative compounds such as GenX have been developed to replace PFOA. GenX, chemically known as hexafluoropropylene oxide dimer acid (HFPO-DA), was developed by DuPont as a substitute for PFOA and is widely used in the production of non-stick coatings and other products[10]. Although GenX is considered a replacement for PFOA, preliminary research indicates that its accumulation and toxicity in the environment and biological systems are like, or even greater than, those of PFOA in certain contexts[11, 12].

Given the widespread presence of PFOA and GenX in the environment and their potential health risks, especially to aquatic organisms, there is an increasing focus on the ecotoxicological effects of these compounds[13]. Zebrafish (*Danio rerio*) is a commonly used model organism in toxicology studies, particularly for investigating the impact of environmental pollutants on aquatic life[14]. The zebrafish genome shares a high degree of similarity with the human genome, making it a valuable model for understanding the potential implications of these compounds on human health[15]. In this study, the selection of female zebrafish as the experimental subject is of particular

significance. Female zebrafish are often more sensitive to environmental toxins due to the metabolic and detoxification roles of estrogen, which can be impacted by PFASs[16]. Moreover, the expression patterns of genes related to lipid metabolism, hormone regulation, and inflammatory responses in female zebrafish liver may differ from those in males, providing unique insights into the effects of PFOA and GenX on endocrine systems[17]. Additionally, the physiological state of female zebrafish during reproductive periods may amplify the potential toxic effects of these compounds, offering more sensitive toxicity indicators[18].

The liver, as the primary organ for detoxification metabolism, is a common target for environmental toxicants[19, 20]. Previous studies have demonstrated that PFOA can induce liver toxicity by activating nuclear receptor pathways, such as PPARα, leading to disruptions in lipid metabolism and oxidative stress[9, 21]. Although GenX is used as a PFOA substitute, its potential hepatotoxic effects remain unclear, and studies on the synergistic toxic effects of co-exposure to PFOA and GenX are limited[22]. By studying female zebrafish, this research aims to gain deeper insights into the multifaceted impacts of these compounds on liver function, particularly concerning detoxification and endocrine regulation.

This study aims to evaluate the hepatotoxic effects of chronic exposure to PFOA, GenX, and their combined exposure in female zebrafish through long-term exposure experiments. The focus is on analyzing the expression of genes associated with toxicity in the liver, particularly those involved in oxidative stress, lipid metabolism, and inflammatory responses. By utilizing quantitative real-time PCR (qPCR), we seek to elucidate the changes in gene expression under different exposure conditions, providing new insights into the toxic mechanisms of PFOA and GenX in aquatic organisms. Through this research, we aim to reveal the specific hepatotoxic effects of PFOA, GenX, and their combined exposure in female zebrafish, and to investigate whether these effects exhibit synergistic interactions. These findings will contribute to the assessment of the long-term impact of these compounds on aquatic ecosystems and provide scientific evidence for future environmental policymaking. The use of female zebrafish as the study subject not only allows for the evaluation of potential impacts on reproductive health but also provides critical data for understanding the broader effects of these compounds in aquatic environments.

5. Methods

Fish Maintenance

Experimental Fish

Adult female zebrafish (*Danio rerio*) were selected as the experimental subjects due to their sensitivity to environmental pollutants and their significant relevance in toxicological research[23]. The fish were obtained from a certified supplier and acclimated under laboratory conditions for two weeks prior to the initiation of the experiment. During the acclimation period, the zebrafish were maintained in a recirculating water system with a temperature of 27 ± 1°C and a photoperiod of 14 hours light/10 hours dark. The pH was controlled at 7.0 ± 0.2, and dissolved oxygen levels were kept above 6.5 mg/L. The fish were fed twice daily with commercially available zebrafish feed to ensure their optimal health and well-being.

Exposure Setup

Female zebrafish were randomly divided into four groups: a control group, a 0.2 μg/L PFOA+GenX group, a 20 μg/L PFOA+GenX group, and a 200 μg/L PFOA+GenX group. Each group consisted of 15 fish, with 5 fish placed in each of three 2L glass beakers to avoid tank effects. These three concentrations were selected to cover a range from environmentally relevant levels to high exposure scenarios, allowing for a comprehensive assessment of the dose-dependent toxic effects of PFOA and GenX on the liver of zebrafish. This concentration range helps to thoroughly evaluate the dose-dependent toxicity of PFOA and GenX co-exposure, providing insights into the potential impacts of these compounds on zebrafish liver at both environmental and high exposure levels. Throughout the 90-day exposure period, the fish were continuously exposed to the respective PFOA and GenX mixtures. Every 24 hours, half of the solution in each beaker was replaced to maintain stable exposure concentrations and minimize chemical degradation. Water quality parameters, including pH, temperature, and dissolved oxygen, were monitored daily to ensure optimal living conditions for the fish during the experiment. These measures ensured consistency in experimental conditions, enabling reliable evaluation of the combined toxic effects of PFOA and GenX at different concentrations.

Dissection and Sample Collection

At the end of the 90-day exposure period, fish were anesthetized using 125 mg/L MS-222 (tricaine methane sulfonate) until unresponsive to stimuli, ensuring humane treatment during dissection. Under a dissection microscope, fish were dissected with sterile instruments, focusing on the liver as the primary organ of interest. The liver was quickly removed and placed in RNAlater solution to preserve RNA integrity, and all samples were stored at -80°C until RNA extraction. This study strictly adhered to ethical guidelines and animal welfare principles to ensure humane treatment of the fish. All procedures involving live animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines and were approved by the New Mexico State University Animal Ethics Committee. The research team followed the principles of the "3Rs" (Replacement, Reduction, and Refinement) to minimize the number of animals used while ensuring the scientific validity and ethical compliance of the experiment.

RNA Extraction and cDNA Synthesis

RNA Extraction

To extract RNA from female zebrafish liver tissue using the Pure Link® RNA Mini Kit, the frozen tissue is first quickly thawed and handled on ice to prevent RNA degradation. An appropriate volume of lysis buffer, typically 1 mL per 50 mg of tissue, is used to ensure efficient lysis. The liver tissue and lysis buffer, which includes 2-mercaptoethanol, are placed into an RNase-free tube and homogenized thoroughly using a homogenizer or by manual grinding to release RNA. After homogenization, the mixture is centrifuged to remove unlysed tissue, and then the clear supernatant is carefully transferred to a new RNase-free tube. This supernatant is applied to an RNA binding column and centrifuged to bind the RNA to the column. Subsequently, the column is washed with a wash buffer to eliminate non-RNA components. Finally, RNA is eluted from the column using a small volume of elution buffer or RNase-free water. The RNA's concentration and purity are assessed using a spectrophotometer to ensure suitability for further experimental analyses. This protocol ensures the efficient extraction of high-quality RNA from zebrafish liver tissue, facilitating accurate downstream molecular biology applications.

Before initiating cDNA preparation, DNase I treatment was applied to all RNA samples to eliminate any contaminating DNA. Specifically, 8 µL of each RNA sample was combined with 1 µL of 10X DNase I Reaction Buffer and 1 µL of DNase I, Amplification Grade (1 U/µL) in an RNase-free, 0.5 ml microcentrifuge tube. The samples were gently mixed and then incubated at

room temperature for 15 minutes. Subsequently, 1 μL of 25 mM EDTA solution was added to inactivate the DNase I, followed by heating the RNA sample at 65°C for 10 minutes, rendering it ready for reverse transcription.

First-strand cDNA synthesis involves preparing the RNA template and adding specific reagents in a sterile, nuclease-free environment. After thawing and briefly centrifuging the components, 1 μg of RNA is combined with 1 μL of primer (Oligo(dT)18 primer, random hexamer primer, or gene-specific primer) and nuclease-free water to a total volume of 12 μL. If the RNA is rich in GC content, the mixture can be heated to 65°C for 5 minutes and then cooled. Next, 4 μL of 5X Reaction Buffer, 1 μL of Ribo Lock RNase Inhibitor (20 U/μL), 2 μL of 10 mM dNTP Mix, and 1 μL of Revert Aid M-MuLV RT (200 U/μL) are added, bringing the total volume to 20 μL. The reaction is then incubated at 42°C for 60 minutes (or 25°C for 5 minutes followed by 42°C for 60 minutes if using random hexamer primers), and the reaction is terminated by heating at 70°C for 5 minutes. This protocol ensures the efficient synthesis of cDNA for subsequent analyses.

Quantitative Real-Time PCR (qPCR)

Primer Design and Validation

Specific primers for the target genes (*cyp1a*, *il6*, *vtg1*, and *fabp10a*) and the reference gene (*β-actin*) were designed using Primer-BLAST based on the zebrafish genome sequence. The primers were synthesized by a commercial provider, and their specificity was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. The primer sequences are shown in Table 1.

Table 1 Specific primer sequences of mosquitofish used in the qPCR experiments.

Gene	Forward primer sequence F（5'--3'）	Reverse primer sequence R（5'--3'）	Reference
cyp1a	TTCACGCCATCACTGCCACA	TCAGGGATGACCTTGCCAACAG	[24]
il6	CCATCTTCTTCATCAGGGACGC	GGGTTTGAGGGTTTCGCTTCT	[25]
vtg1	TGCTCGCCATCAATCCCAGG	AAGCACCGTAGGACTCGTTCAG	[26]
fabp10a	CCTCGCTGAAGATTTTGTCC	TGTTGAAGCGGTTGTTGAGG	[27]
β-actin	CTTGCCGACTCAGGAAGTGTTAC	TGACGCCAGTCTGTCGTTTGT	[28]

qPCR Procedure

Following cDNA synthesis, stock solutions of 100 mM for each gene were prepared by dissolving the appropriate solids in water and vortexing gently. The primers were then diluted 10-fold from the stock solution. A master mix was prepared for the qPCR using the SsoAdvanced™ Universal SYBR® Green Super mix; specifically, 10 µL of 2x SsoAdvanced™ Universal SYBR® Green Super mix was mixed with 1 µL each of forward and reverse primers (10 µM) and 6 µL of Nuclease-free water for each gene, ensuring gentle mixing to avoid foaming3. Subsequently, 18 µL of the master mix was added to each well of a qPCR plate, followed by 2 µL of cDNA.

The qPCR plate was then sealed with a microseal B adhesive sealer and placed in the Real-Time System-CFX Connect. The qPCR protocol included an initial DNA denaturation at 95°C for 30 seconds, followed by 15 seconds of denaturation at 95°C, annealing/extension at 60°C for 15 seconds, and fluorescence reading at the same temperature, repeated across 35-40 cycles. Gene expression was quantified using the ddCT (Delta-Delta-CT) method, with fold changes calculated and normalized relative to control values from zebrafish tissues in the absence of endocrine disruptors.

Data Processing and Statistical Analysis

The relative expression levels of the target genes were calculated using the $2^{\wedge\text{-}\Delta\Delta Ct}$ method, with *β-actin* as the reference gene[29]. Data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using origin 2024 Software. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. The results of the qPCR analysis provided insight into the expression patterns of key genes involved in detoxification, inflammation, and endocrine function in response to chronic exposure to PFOA and GenX. This detailed methodological approach ensured the reliability and accuracy of the data, contributing to a comprehensive understanding of the hepatotoxic effects of these persistent environmental contaminants.

6. Result

This study evaluated the effects of co-exposure to different concentrations of PFOA and GenX on the expression of key liver genes (*cyp1a*, *il6*, *vtg1*, and *fabp10a*) in female zebrafish. The results are summarized as follows:

Expression of *cyp1a* and *il6*

The expression levels of both *cyp1a* and *il6* increased significantly with higher concentrations of PFOA and GenX. In the control group, the expression levels of *cyp1a* and *il6* were 1.031 and 1.050, respectively. In the 0.2 μg/L, 20 μg/L, and 200 μg/L exposure groups, *cyp1a* expression levels were elevated to 1.402, 2.654, and 7.354, while *il6* expression levels rose to 2.819, 3.971, and 5.053, respectively. Compared to the control group, these changes were statistically significant in the 20 μg/L and 200 μg/L exposure groups ($p < 0.01$) (Fig.1). These findings suggest that higher concentrations of PFOA and GenX co-exposure strongly induce liver detoxification processes and inflammatory responses in zebrafish.

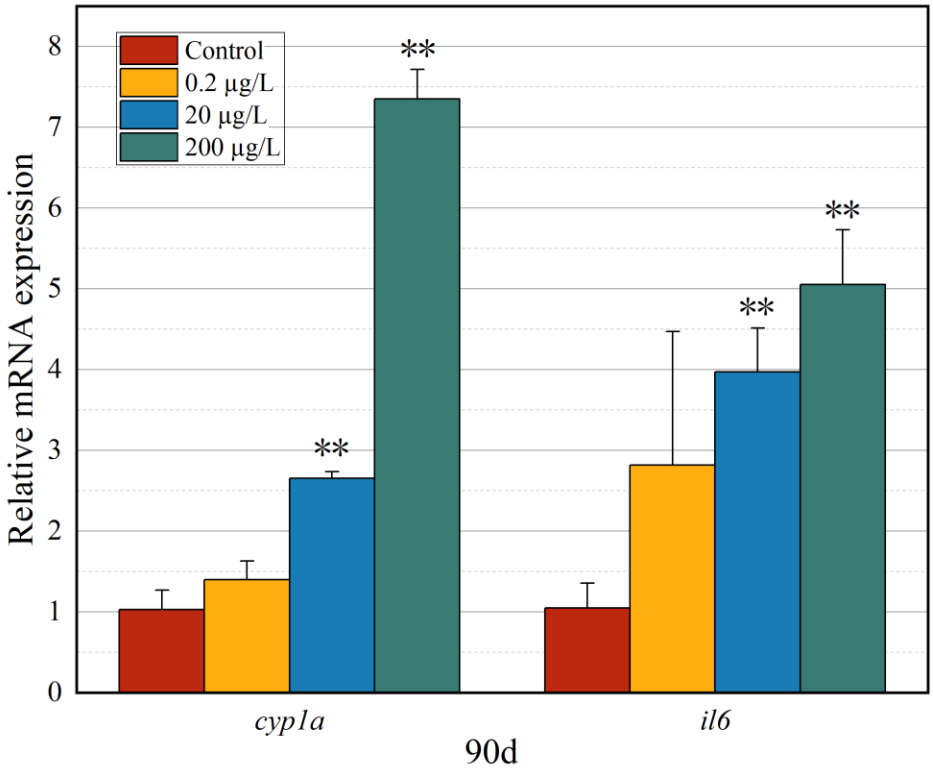

Fig.1 Effects of combined exposure to PFOA and GenX at different concentrations on the expression of *cyp1a* and *il6* Genes in the liver of female zebrafish for 90 days

Expression of *vtg1* and *fabp10a*

The expression of *vtg1* and *fabp10a* also showed significant upregulation with increasing concentrations of PFOA and GenX. In the control group, the expression levels of *vtg1* and *fabp10a* were 1.063 and 1.369, respectively. In the 0.2 μg/L, 20 μg/L, and 200 μg/L exposure groups, *vtg1* expression levels increased to 1.408, 3.815, and 6.339, while fabp10a expression levels rose to 4.415, 6.092, and 9.284, respectively. The differences in gene expression between the control group and the 20 μg/L and 200 μg/L exposure groups were statistically significant ($p < 0.05$) (Fig.2). The upregulation of these genes suggests that co-exposure to PFOA and GenX may adversely affect the reproductive system and induce strong stress responses in the liver, particularly at higher concentrations.

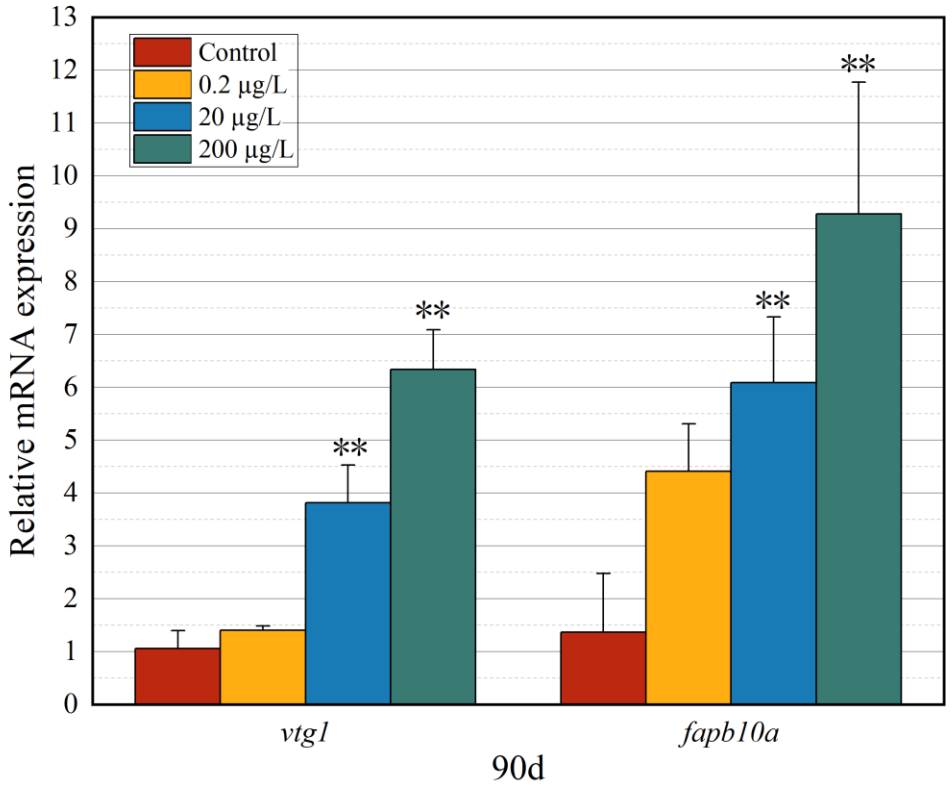

Fig.2 Effects of combined exposure to PFOA and GenX at different concentrations on the expression of *vtg1 and fabp10a* Genes in the liver of female zebrafish for 90 days

The results of this study demonstrate that co-exposure to PFOA and GenX leads to a significant upregulation of *cyp1a*, *il6*, *vtg1*, and *fabp10a* gene expression in the liver of female zebrafish, with the magnitude of these changes being dose dependent. Compared to the control group, these changes in gene expression were statistically significant ($p < 0.05$) in the medium and high concentration exposure groups. These findings suggest that co-exposure to PFOA and GenX may have significant effects on liver detoxification functions, inflammatory responses, and reproductive health in zebrafish, and that these effects are clearly dose dependent.

7. Discussion

The Effects of long-term exposure to PFOA and GenX on female zebrafish liver gene expression

This study investigated the effects of long-term exposure to varying concentrations of PFOA and GenX on gene expression in the liver of female zebrafish. Significant upregulation of *cyp1a*, *il6*, *vtg1*, and *fabp10a* genes was observed, especially at higher concentrations, indicating dose-dependent toxicity. *cyp1a*, closely associated with detoxification, was markedly increased, suggesting an attempt by the liver to counteract the toxicity of these perfluorinated compounds through enhanced expression of detoxification enzymes[30]. However, this could lead to an overburdened metabolic system and further cellular damage. The *il6* gene, a marker for inflammation, also showed increased expression particularly at the highest concentration, highlighting a potential inflammatory response which could lead to tissue damage or liver disease[31]. The upregulation of *vtg1* indicates potential endocrine disruption affecting reproductive functions, which could adversely affect population sustainability and ecological balance[32]. Similarly, the increase in *fabp10a* expression suggests a significant stress response, potentially leading to apoptosis or other cellular damage, particularly under high exposure conditions[27]. Collectively, the findings indicate a synergistic toxic effect when exposed to both compounds together, with possible pathways including increased detoxification load, inflammatory response, and endocrine disruption, ultimately leading to severe liver damage. While this study highlights significant effects of combined PFOA and GenX exposure, limitations include its focus solely on gene expression changes without assessing how these translate to protein levels and specific physiological impacts. Future research should consider responses in male zebrafish and assess long-term population dynamics and ecosystem impacts.

8. Conclusion

The results of this study demonstrate that co-exposure to PFOA and GenX leads to a significant upregulation of *cyp1a*, *il6*, *vtg1*, and *fabp10a* gene expression in the liver of female zebrafish, with the magnitude of these changes being dose dependent. Compared to the control group, these changes in gene expression were statistically significant ($p < 0.05$) in the medium and high concentration exposure groups. These findings suggest that co-exposure to PFOA and GenX may have significant effects on liver detoxification functions, inflammatory responses, and reproductive health in zebrafish, and that these effects are clearly dose dependent.

9. A paragraph on who will benefit from your research results. Include any water agency that could use your results.

My research will provide valuable insights and support to the Department of Civil Engineering at New Mexico State University and the New Mexico Water Resources Research Institute. These institutions can leverage my findings to optimize water management strategies and enhance the sustainable use of water resources. Additionally, both my mentor and I will benefit from this work by gaining a deeper understanding of and addressing the water pollution challenges faced by New Mexico and surrounding regions, thereby contributing to the advancement of knowledge and innovation in this field. These outcomes may also be utilized by other water agencies, government bodies, and industry decision-makers to help them develop more scientific and effective water resource management policies.

10. Describe how you have spent your grant funds. Also provide your budget balance and how you will use any remaining funds.

Table 2. Budget and justification.

Item	Amount	Justification
Salary		
Student PI	$3,282	1.5 summer month salary for the student PI at 0.5 FTE (Level 1)
Fringe Benefit		
Student PI	$16	0.50% fringe rate for student PI
Lab Supplies		

Zebrafish	$280	A purchase was made for 40 zebrafish, with 20 males and 20 females, each priced at $7. The supplier, American Aquatics, provided research-grade zebrafish, ensuring a balanced population for various experimental setups
Zebrafish food	$84	4 packs of zebrafish food at a unit price of $21 will be purchased from Amazon and used for zebrafish maintenance
Zebrafish circulatory system	$563	This $563 expenditure is primarily for constructing and maintaining the zebrafish circulatory system. It includes purchasing six fish tanks for $200, two iron racks for $80, six temperature sticks for $60, four filters for $120, several PVC pipes for $50, and two water pumps for $53. These items will ensure a stable living environment for the zebrafish, suitable for research related to their circulatory system.
Chemicals	$325	1 PFOA stock solution at a unit price of $150 and 1 GenX stock solution at a unit price of $175 will be purchased from Wellington LLC and used in zebrafish experiments and PFAS analysis
qPCR Reagents	$1320.20	The total expenditure of $1,320.20 was spent on essential reagents and supplies purchased from the Thermo Fisher Scientific website. These included a Pure Link® RNA Mini Kit $318.65, DNase I, Amplification Grade Kit $165.65, two First-strand cDNA Synthesis Kits $201.30, four sets of Primers $80, a pack of SsoAdvanced™ Universal SYBR® Green Supermix $205.30, a Microseal B Adhesive Sealer $187.65, and four 96-Well Plates $161.65. These items were crucial for conducting RNA extraction, cDNA synthesis, and qPCR analysis as part of the research project

Homogenizer	$1305.8	The Homogenizer $1305.8 was purchased from Thermo Fisher Scientific. This equipment is essential for homogenizing samples to ensure uniformity in RNA extraction and other preparatory processes within the research project.
Other		
Other Cost	$224	A budget of $224 will be used for other potential costs such as printing posters and purchase of office supplies.

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

Currently, I have not made any presentations related to the project. However, if I could present in the future, I will ensure that these presentations are properly documented and shared.

12. List publications or reports, if any, that you are preparing. For all publications/reports and posters resulting from this award, please attribute the funding to NM WRRI and the New Mexico State Legislature by including the account number: NMWRRI-SG-FALL2023.

Currently, there are no publications or reports in preparation. However, I plan to attribute any future publications, reports, or posters resulting from this award to NM WRRI and the New Mexico State Legislature, including the account number: NMWRRI-SG-FALL2023, as required.

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

Several individuals have played a crucial role in assisting me with my research project. I have been fortunate to have the support of student members Lin Wang and Dulith, who have contributed significantly to various aspects of the project. Additionally, faculty member Runwei Li has provided valuable guidance and expertise, helping to shape the direction of the research. Their collective efforts have been instrumental in advancing the project.

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

Currently, there have been no special recognition awards, notable achievements, or publicity, such as newspaper articles, related to my research. However, I am continuing to work diligently, and I hope to achieve significant milestones as my research progresses.

15. 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 on track to complete my degree as planned, with an expected graduation date of May 31, 2027. My research and academic journey have been focused on water environment management, particularly addressing the critical issues facing New Mexico and its surrounding regions. Looking ahead, I am committed to pursuing a career in water environment regulation, with a strong desire to remain in New Mexico. The state's unique challenges and opportunities in water resource management are areas where I believe I can make the most significant impact. Whether through continued research, collaboration with state or local water agencies, or contributing to policy development, I aim to apply the knowledge and skills I have acquired through my studies to benefit the future of New Mexico's water resources. The support from the New Mexico State Legislature has been instrumental in my academic and professional development, and I am dedicated to giving back to the state by working in a field that directly contributes to the sustainable management of its vital water resources.

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