Program of Research Experiences for Undergraduates & Graduates at

Alabama A&M University

and

Nanjing Forestry University

Proceedings of the Student Research & Trip Reports May-July 2014

Center for Forest Ecosystem Assessment
Department of Biological and Environmental Sciences
College of Agricultural, Life, and Natural Sciences

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Research Experiences for Undergraduates and Graduates in China

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Students (from left to right) Andrew Lawhorn, Michael Kennedy, Linzi Thompson, Nicole Mihelich, Hollis Dahn, Morgan Dean, Rosie Long, and Angelica Durrah (missing is Mercedes Bartkovich, who arrived at a later date), along the Huangpu River in Shanghai, China, on our first day in the country after a long flight from the U.S., May 2014.

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Preface

Background

Citing the strategic importance of the U.S.-China relationship, in November 2009 President Barack Obama announced the "100,000 Strong" initiative, a national effort designed to dramatically increase the number and diversity of the composition of American students studying in China. In May 2010, former Secretary of State Hillary Clinton officially launched the initiative in Beijing (Clinton 2010). The "100,000 Strong" initiative seeks to prepare the next generation of American experts on China who will be charged with managing the growing political, economic and cultural ties between the U.S. and China. The initiative also seeks to develop specific opportunities and funding sources for under-represented students to study in China. The need for Americans to gain greater exposure to and understanding of China is clear: there is perhaps no more important or complex relationship in the world than that between the U.S. and China in terms of securing global peace and security. Virtually no major international issue, whether global economic recovery or climate change or nuclear non-proliferation, can be solved effectively without the active engagement of both the U.S. and China. However, American knowledge of Chinese society and customs is inadequate. Ten times more Chinese students come to the U.S. for educational programs than Americans who study in China, and 600 times more Chinese study the English language than Americans study Mandarin (Clinton 2013). This imbalance in knowledge can undermine trust, and thus the relationship, between the two countries. Redressing this imbalance is essential in ensuring that Americans have the cultural understanding and language skills that underpin effective diplomacy and foreign policy, which will also enhance our students' ability to succeed academically and professionally in the global environment.

Through the efforts of the Center of Forest Ecosystem Assessment, funded by National Science Foundation's CREST program (Grant number HRD-1036600) , Alabama A&M University (AAMU) was awarded a three-year grant by the USDA-National Institute of Food and Agriculture (NIFA) International Science and Education Program (ISE) in 2009 (Grant number: 2009-51160-05462) to develop an international exchange program with China. The program was designed to strengthen AAMU's ability to develop globally competent students and faculty through collaborative partnerships with higher education institutions and research organizations in China. The program focuses on the fields of agricultural and environmental sciences. The exchange program also aims to enhance courses with international contexts to prepare and mentor students for international opportunities in agricultural and environmental sciences, as well as to add new dimensions to scientific research and teaching capabilities of AAMU faculty via exposure to international resources and technologies. Our primary Chinese partner for this

program is Nanjing Forestry University (NFU), a comprehensive university with a tradition of forestry programs. The development and the opportunities created by this program led to a new three-year program: Research Experiences for Undergraduates (REU) in China, funded by the National Science Foundation (NSF) in 2011 (Grant number: DBI-106310). The primary goal of the NSF REU program is to expose undergraduate students with an interest in pursuing a graduate research degree in science to hands-on research experiences. In 2013, we were funded through the National Institute of Food and Agriculture's Capacity Grant (No. 2013-38821-21250) to further enhance our program by establishing the collaboration and involvement of students and faculty from another minority institution in Alabama: Tuskegee University (TU). This multi-institutional and multi-agency joint project have created great opportunities for many faculty and students, particularly those from minority serving or small institutions with limited research and international programs, to gain valuable research experience under a challenging, but enjoyable, international setting. In the summer of 2010 and 2011, a total of twenty-five students and faculty from AAMU participated in this program and travelled to China. In the summer of 2012, a total of twelve students from six different institutions across USA and five faculty/staff members joined the program; and in the summer of 2013, a total of thirteen students and six faculty/staff participated in the program.

This Year's Program

In the summer of 2014, eight undergraduate students from seven different institutions across the U.S. (including AAMU), three AAMU graduate students, and eight faculty/staff participated in the REUG program. These numbers also included one undergraduate student and two faculty members from Tuskegee University. The program started with a three day orientation at AAMU to prepare for international travel. Once we arrived in China, students and faculty engaged in a variety of courses including Chinese language, culture, and history classes taught by NFU faculty, as well as scientific writing, statistics, and how to create PowerPoint presentations and posters by AAMU faculty and graduate students. All students were paired with a primary mentor in the U.S. prior to travel. Mentors included graduate students and faculty from both AAMU and TU. Upon arrival at NFU, students were then paired with a second mentor from NFU, with similar research interests. Students then met with mentors to devise and conduct a research project. Research topics included a diversity of subjects such as "*Songbird Species in China: Evaluation of DNA Extraction Kits, Primers, and Feather Age*", "*Occurrence of Typical Antibiotics in Huai River and Hongze Lake, Eastern China*," "*Bat Forage and Insect Communities in Three Habitat Types in Nanjing, China*," and "*The Adsorption Behavior of Black Carbon in Urban Forest and Traffic District Soils Toward Heavy Metal Ions (Cu, Zn),*" "*Establishment of a Riparian Buffer Strip for Alleviating Lake Eutrophication*," among others.

Students learned how to design a research project, collect data, operate research equipment, analyze data, and communicate their research results to their peers.

The team took two extended educational/cultural trips in Jiangsu Province during the program. The first trip (2 days) brought them to Wuxi, Yixing, and the Tai Lake (*Tài Hú*) area. This trip was designed to help students and faculty understand bamboo ecology, the applications of bamboo in daily life, and bamboo as a biomass potential. During this trip, they visited a bamboo experimental forest and a bamboo processing plant; while at these sites, they were able to interact with researchers and workers. They also had opportunities to explore local culture and history: they watched a Chinese ballet ("*The Red Detachment of Women*," 红色娘子军), performed by the National Ballet of China at the Wuxi Grand Theater, visited the Huishan Clay Figurines Museum (惠山泥人博物馆) and enjoyed tea along the streets of Zhongshan Road. The second trip (3 days) took the team to Sheyang in northern Jiangsu Province. This trip was designed to help students and faculty to understand forest ecology, applications of forest research, the value of popular forests and their impact on local economies and ecosystems, and other conservation programs in the region. During this trip, they visited several poplar tree plantations and industry based in Sheyang County. In the early 1980s, faculty from NFU helped to introduce a hybrid poplar (genus *Populus*, hybridized in part from eastern cottonwood originating in Mississippi), to Sheyang. Once one of the poorest counties in China because of its high human population density, shortage of natural resources, and frequent flooding, Sheyang's economy has been transformed, and the poplar tree hybrid and the related agroforestry industry have played a major role in its economic growth. During this trip we also visited the Yancheng Dafeng National Milu Reserve (*Yánchéng Dàfēng mílù guójiā jízìránbǎohùqū*, 盐城大丰国家级自然保护区), which was established in 1983 to start a breeding program for the Père David's deer (*Elaphurus davidianus*), known simply as *mílù* in China, which is extinct in the wild in China. In addition, the team got to view the endangered red-crowned crane (*Grus japonensis*), also in a breeding program at the reserve.

While completing their research and collaborative lab or field work at Nanjing, the AAMU team experienced the culture, people, and a whole host of foods that they never in their lives dreamed they would eat! They explored the city by foot, bus, taxi, and subway and became intimately familiar with the city in a way that tourists almost never experience when visiting a foreign land. Students climbed Purple Mountain; visited Dr. Sun Yat-sen's Mausoleum; paid tribute to the fallen victims of the massacre by the Japanese during World War II, as commemorated in the Nanjing Massacre Memorial Hall; posed with enormous hand-carved mythical figures at the Xiaoling Mausoleum of the Ming Dynasty; celebrated the Chinese traditional Dragon Boat Festival with a big crowd of locals and foreign visitors; and visited a wide variety of stunningly beautiful gardens. Additionally, several students (Michael Kennedy, Andrew Lawhorn, Linzi

Thompson, and Mercedes Bartkovich) were invited to participate in the official Youth Olympics game advertisement, resulting in their appearance in an internationally viewed ad!

The team had the opportunity to visit other major cities such as Shanghai, Beijing, and Yangzhou and witnessed the effects of dramatic economic development during last 30 years. In Shanghai, we took a boat tour of the Bund on the Huangpu River and viewed the City from the top floor of the Oriental Pearl Radio and TV Tower (东方明珠塔). From Nanjing, we travelled to Beijing on a high-speed train at over 125 miles per hour. There, we climbed the Great Wall at the Mutianyu (**慕田峪**长城), walked through the halls of the (once) Forbidden City (**紫禁城**), maneuvered through a crowded Tiananmen Square (**天安**门广场) and observed the mile-long line of people waiting to see Chairman Mao's body, ate a wonderful homemade meal in the Lingdang Hutong district, and finished off our stay with a final Peking Duck meal that was phenomenally delicious.

This year, we did things a bit different from the previous two programs, based on our experiences and the suggestions of participants. Apart from having courses taught at NFU throughout the first few weeks, we increased the stay in China to almost eight weeks (May 22-July 14), and omitted the return to AAMU for a week-long stay. Thus, students had to complete in full all of their work in China, including a research report, a cultural report, webpages, a PowerPoint presentation, and a scientific poster. We feel this was a very successful strategy and we will likely implement it in the future. Students presented their research to a full audience at NFU prior to leaving for Beijing.

Overall, faculty and students in the program are still reeling from the exchange trip to China that has significantly broadened their research, educational, cultural and language experiences. This year's program was the best yet, and we look forward to many more.

The documents in this proceeding consist of the students' cultural reports and their scientific research papers. Students and mentors worked together to complete these documents. Each student also created a website with additional information, which can be accessed at: http://myspace.aamu.edu/users/sha.li/reu/reu2014/reu2014.htm

Acknowledgements

On behalf of all of the Research Experiences for Undergraduates and Graduates (REUG) program coordinators at Alabama A&M University (AAMU), we would like to thank the Alabama A&M University administration, the Nanjing Forestry University (NFU) administration, and most importantly, the National Science Foundation and National Institute of Food and Agriculturefor their support in the implementation of this program. This program was a great success, and it absolutely would not have transpired without the support and understanding of the aforementioned. Thank you to NFU for allowing the REUG program participants into your labs, your office spaces, and being so forgiving of our social faux pas. The students and faculty had a wonderful, once-in-a-lifetime educational and cultural experience in China, and you all contributed significantly to this occurring.

In addition to PI and Co-PI, the AAMU and TU professors, Drs. Khairy Soliman, Kozma Naka, William Stone, Guohao He (Tuskegee University), Ramble Ankumah (Tuskegee University), and Malinda Gilmore chose these eight undergraduate students and three graduate students from a highly competitive group of individuals from many universities across the nation. These students were chosen because of their academic strengths, their interest in the areas to be researched, and their compatibility with the project. We are proud of each of these students and all that they accomplished at Nanjing Forestry University and Alabama A&M University during this time.

Thank you!

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Department of Biological and Environmental Sciences
College of Agricultural, Life, and Natural Sciences

November 10, 2014

REUG Participating Students

Undergraduate Students

Rosie Shenice Long: Ms. Long is junior majoring in Animal Science at Alabama A&M University in Normal, Alabama. She was mentored by Dr. William Stone and Dr. Kozma Naka (AAMU) and Dr. Gao Cuiqing (NFU). Her research focused on bat foraging preferences along an urban gradient. The title of her research paper is "*Bat Forage and Insect Communities in Three Habitat Types in Nanjing, China.*"

Nicole Teresa Mihelich: Ms. Mihelich is a junior majoring in Biochemistry at the University of Wisconsin in Madison, Wisconsin. She was mentored by Dr. Khairy Soliman (AAMU) and Dr. Xu Lian and Wang Jianwen (MS candidate) (NFU). The title of her research paper is "*Isolation and Identification of a Peroxidase (TcPOD1) of* Tamarix chinensis*, a Crucial Gene in the Phenlanalanine Metabolic Pathway and its Potential Role in Salt Tolerance.*"

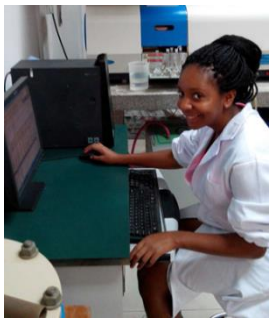
Hollis Anne Dahn: Ms. Dahn is a junior majoring in Biology at the University of Central Florida in Orlando, Florida. She was mentored by Dr. Yong Wang and Kevin Messenger (PhD candidate) (AAMU) and Dr. Ding Yulong (NFU). Her research compared the morphological and bioacoustics characteristics of two closely related frog populations to determine whether or not they are evolutionarily distinct. The title of her research paper is "*Two Potential Undescribed Species of the Genus* Xenophrys *(Anura, Megophryidae) from China.*"

Michael L. Kennedy: Mr. Kennedy is a junior majoring in Forestry Science at Humboldt State University in Arcata, California. He was mentored by Dr. Kozma Naka (AAMU) and Dr. Wu Yongbo (NFU). His research examined the effectiveness of varying buffer strip widths in alleviating lake eutrophication. The title of his research paper is "*Establishment of a Riparian Buffer Strip for Alleviating Lake Eutrophication.*"

Andrew Lawhorn: Mr. Lawhorn is a junior majoring in Forestry Sciences at Alabama A&M University in Normal, Alabama. He was mentored by Dr. Kozma Naka (AAMU) and Dr. Ding Yulong (NFU). His research focused on the effects of saline on bamboo rhizomes. The title of his research paper is "*Development of Bamboo Rhizome System in Jiangsu Province, China.*"

Linzi Renee Thompson: Ms. Thompson is a junior majoring in Environmental Health Science and Chemistry at East Central University in Ada, Oklahoma. She was mentored by Dr. Elica Moss (AAMU) and Dr. Li Wei (NFU). She examined the presence and sources of antibiotics in a lake and a river in the Jiangsu province of China. The title of her research paper is "*Occurrence of Typical Antibiotics in Huai River and Hongze Lake, Eastern China*."

Junqiao (Cicely) Wang: Ms. Wang is a sophomore majoring in Statistics at Oberlin College in Oberlin, Ohio. She was mentored by Dr. Yong Wang and Lisa Gardner (AAMU), and Dr. Lu Changhu (NFU). Her research focused on the effects of urbanization on bird nest predation. Due to illness, Ms. Wang was unable to complete her data analysis and science paper, and withdrew from the program. She helped us immensely during the program and wish her the best.



Morgan Dean: Ms. Dean is a sophomore majoring in Environmental Science at Tuskegee University (TU) in Tuskegee, Alabama. She was mentored by Drs. He Guohao and Ramble Ankumah (TU) and Dr. Yu Yuanchun and Yang Jingyu (NFU). She compared the adsorption behavior of black carbon in two soil types with two heavy metals. The title of her research paper is "*The Adsorption Behavior of Black Carbon in Urban Forest and Traffic District Soils Toward Heavy Metal Ions (Cu, Zn).*"

AAMU Graduate Students

Angelica Durrah: Ms. Durrah is an MS candidate in the field of genetics at Alabama A&M University in Normal, Alabama, under the guidance of Dr. Khairy Soliman. Ms. Durrah traveled to China as a research participant in the REUG-China program at Nanjing Forestry University. She was mentored by Dr. Soliman (AAMU), and Drs. Qiang Zhuge and Sun Weibo (NFU). Her research focused on …. The title of her research paper is "*Verification of the Expression of the Chloride Channel Gene GmCLC1, Incorporated into the Genome of Select* Populus deltoides *x* P. euramerucana *'Nanlin895'.*"

Mercedes Bartkovich: Ms. Bartkovich is an MS candidate in the field of Plant and Soil Science at Alabama A&M University in Normal, Alabama, under the guidance of Dr. Yong Wang. Ms. Bartkovich traveled to China as a mentor and research participant in the REUG-China program at Nanjing Forestry University. She assisted and was mentored by Dr. Yong Wang (AAMU) and Zhang Zhengwang (Beijing Normal University). Her research focus was to compare the effectiveness of several DNA extraction methods on bird feathers of a variety of ages for the purpose of determining sex. The title of Ms. Bartkovich's research paper is "*Songbird Species in China: Evaluation of DNA Extraction Kits, Primers, and Feather Age.*"

Kevin Messenger: Mr. Messenger is a PhD candidate under the guidance of Dr. Yong Wang. His research focuses on the conservation and natural history of herpetofauna in the heavily populated areas of southern China. He spent more than half of the year in China surveying areas for herpetofauna. Mr. Messenger mentored one of the REU students, Hollis Dahn, who focused his research attention on the potential split of a single toad species into two species in China. Both traveled from the home base of Nanjing Forestry University to southern and western China to survey selected areas, then back to Nanjing to compile and analyze the data collected, and to present it to the NFU community. Mr. Messenger's research is ongoing and does not have a research paper in these proceedings.

Participating REU Mentors

Alabama A&M University Mentors

Yong Wang, PhD: Dr. Wang is professor of biometry and wildlife ecology at AAMU. His research interests are behavioral ecology of avian migration; wildlife and habitat relationships and conservation; and natural resource modeling with statistics, Geographic Information System (GIS), and remotely acquired data. His recent research efforts include (1) stopover ecology of migratory birds at the southern Cumberland Plateau of northern Alabama; (2) wildlife (amphibians, reptiles and birds) response to anthropogenic habitat and landscape alternations such as forest management practices and urbanization; (3) breeding distribution and biology of Cerulean Warblers; (4) classification of land types for forest management based on GIS, remotely sensed data and statistical models; and (5) avian biology and conservation in China. Dr. Wang's research has been supported by organizations or agencies such as NSF, USDA, Forest Service, EPA, State of Alabama, TNC, and private landowners. Thus far, Dr. Wang has mentored seven undergraduate students across all three years of the program, with the assistance of his PhD candidate Kevin Messenger (2012-2014) and Postdoctoral fellow Dr. Jianqiang Li (2012). In 2012, Dr. Wang mentored David Farris and Iwo Gross; in 2013, he mentored Christopher Griffith, Jacob Drucker, and Justin Waraniak; and in 2014 he mentored Hollis Dahn and Junqiao Wang. **(Dr. Changhu Lu, Dr. Zhen Wang, and Dr. Ding Yulong)**

Elica Moss, PhD: Dr. Moss is assistant professor of environmental microbiology. Her research interests are in identifying microbes that contribute to the environment and subsequently effect human health. Her recent research efforts include: Arsenite oxidizing/resistance genes in soils; presence and abundance of pathogenic fecal bacteria in water systems; and biogeochemical nutrient cycling in a disturbed forest ecosystem. She was instrumental in the accreditation of the Environmental Health Science program at Alabama A&M University after only one year in existence; rendering it the only accredited Environmental Health Science Program in the state of Alabama. She is heavily involved in undergraduate research, which is evident in her involvement in the REU and URM programs. Additionally she oversees a program that takes undergraduates to scientific meetings to present their research. Dr. Moss's research has been supported by organizations or agencies such as NSF, USDA, and the State of Alabama. Thus far, Dr. Moss has mentored four undergraduate students and one graduate student across all three years of the program. The graduate student, Jonjala Jackson also assisted with mentorship of the undergraduate students (MS candidate, 2012-2013). In 2012, Dr. Moss mentored Rakeyta Scales and Nara McCray; in 2013, she mentored Antionette Fowlkes; and in 2014, she mentored Linzi Thompson. **(Dr. Fang Shengzuo and Dr. Ye Tian)**

Khairy Soliman, PhD: Dr. Soliman is professor of plant molecular genetics. His research interests are focused on plant molecular genetics and evolutionary genetics, gene resource conservation and utilization, cytogenetics, and plant breeding. His forest related research is focused on studying the genetic diversity and dynamics of oaks and pool breeding amphibians. Thus far, Dr. Soliman has mentored four undergraduate students and three graduate students across all three years of the program. Graduate students Rashidah Farid (MS candidate, 2012),

Abreeotta Williams (PhD candidate, 2013), and Angelica Durrah (MS candidate, 2014), also assisted with mentorship of the undergraduate students. In 2012 Dr. Soliman mentored Joanna Kukla and Calvin Means; in 2013 He mentored Melissa DellaTorre; and in 2014 he mentored Nicole Mihelich. **(Dr. Yin Tongming, Dr. Xu Lian, Dr. Zhuge Qiang, Dr. Sun Weibo)**

Kozma Naka, PhD: Dr. Naka is associate professor of forest mensuration and forest operations at AAMU. He also teaches biometric statistics. His research interests are focused on biomass harvesting, forest products, and forest management. Dr. Naka is new to the program as of 2014, and mentored two undergraduate students, Andrew Lawhorn and Michael Kennedy. Additionally, he taught statistics to the students and co-mentored Rosie Long with Dr. Stone. **(Dr. Ding Yulong and Dr. Wu Yongbo)**

William Stone, PhD: Dr. Stone is an Associate Professor of Forest Wildlife at Alabama A&M University. He is a certified wildlife biologist and belongs to several natural resources professional societies. His teaching and research focus on habitat relationships of forest-dwelling wildlife, especially bats and other small mammals. Thus far, Dr. Stone has mentored two undergraduate students between 2013 and 2014. In 2013, he mentored Sarah Katherine Springthorp; in 2014 he co-mentored Rosie Long with Dr. Naka. **(Dr. Hao Dejun and Dr. Gao Cuiqing)**

Malinda Gilmore, PhD: Dr. Gilmore is an assistant professor of chemistry. Her research interests are primarily in the effects of atmospheric pollutants on environmental systems (i.e. humans, animals), and determining the effects of agricultural emissions on ambient air quality. Dr. Gilmore mentored students Tangelia Hatch and Maya Rudolph in 2013, and co- mentored Linzi Thompson in 2014 with Dr. Moss (she did not travel to China in 2014). **(Dr. Zhang Yinlang and Dr. Ding Yulong)**

Guohao He, PhD: Dr. He is research professor of plant genetics and genomics at Tuskegee University. His research interests focus on peanut genomics and improving peanut production through genetic and genomic tools, with an emphasis on peanut resistance genes and gene transfer. Dr. He collaborated with Dr. Ramble Ankumah (also of TU), Dr. Yu Yuanchun and Yang Jingyu (NFU) to mentor Morgan Dean (of TU), whose research compared the adsorption behavior of black carbon in two soil types with two heavy metals. **(Dr. Yu Yuanchun and Dr. Yang Jingyu)**.

Ramble Ankumah, PhD: Dr. Ankumah is professor and assistant dean in the College of Agriculture, Environment and Nutrition Sciences at Tuskegee University. His research interests focus on impacts of anthropogenic process on soil and environment quality. Dr. Ankumah collaborated with Dr. He (also of TU), Dr. Yu Yuanchun and Yang Jingyu (NFU) to mentor Morgan Dean (of TU), whose research compared the adsorption behavior of black carbon in two soil types with two heavy metals. **(Dr. Yu Yuanchun and Dr. Yang Jingyu).**

China Mentors

This year, most students had a faculty mentor from the College of Forest Resources and Environment at NFU. One student was mentored by a faculty from Beijing Normal University. More information on NFU faculty can be found at http://eng.njfu.edu.cn/info.php?id=125

Lin Cao, PhD: Mr.Cao is faculty in GIS Nanjing Forestry University. He helped to mentor one REU student and lead field trips.

Zhengwang Zhang, PhD: Dr. Zhang is a professor of ornithology at Beijing Normal University. He mentored Mercedes Bartkovich with her project which examined the feather age and DNA extraction kits for successfully sexing songbirds. Ms. Bartkovich worked in his lab and with his graduate students.

Yulong Ding, PhD: Dr. Ding is former dean of the College of International Education and professor of forestry ecology of Nanjing Forestry University, and is currently the Director and professor of Bamboo Institure at NFU. Dr. Ding is well-known bamboo ecologist. He has been instrumental in getting this Program established at NFU. Dr. Ding has mentored students across all years. This year, he mentored Kevin Messenger and Hollis Dahn, and Andrew Longhorn. Additionally, he facilitated each of the educational and cultural tours and accompanied the team on each trip, including the bamboo research farm near Wuxi and the poplar plantations of Yangzhou.

Yongbo Wu, PhD: Dr. Wu is an associate professor of forestry ecology of the College of Forestry and Environmental Sciences of Nanjing Forestry University. He collaborated with Dr. Kozma Naka from AAMU on research projects. Together, they mentored Michael Kennedy on his research that focused on riparian buffer strips for alleviating lake eutrophication.

Qiang Zhuge, PhD: Dr. Zhuge is a professor of molecular biology and biotechnology in the College of Forestry and Environmental Sciences of Nanjing Forestry University. Dr. Zhuge worked with Dr. Soliman to mentor Ms. Angelica Durrah on her research examining the chloride channel gene GmCLC1 in a poplar hybrid.

Weibo Sun, PhD: Dr. Sun is a post-doctoral fellow in Dr. Zhuge's lab. He helped to mentor Ms. Angelica Durrah on her research examining the chloride channel gene GmCLC1 in a poplar hybrid.

Lian Xu, PhD: Dr. Xu is a professor of forest genetics and breeding in the College of Forestry and Environmental Sciences, Nanjing Forestry University. He worked with Dr. Soliman to mentor Nicole Mihelich on her research in isolating and identifying a peroxidase of *Tamarix chinensis*, to determine its role in salt tolerance. His MS student, Wang Jianwen, also helped Ms. Mihelich with her research.

Wei Li, PhD: Dr. Li is a professor of environmental science of the College of Biology and Environment of Nanjing Forestry University Her research is currently focused on antibiotic pollution. She worked with Dr. Elica Moss to mentor Linzi Thompson, whose research focused on antibiotic presence in the Huai River and the Hongze Lake in eastern China.

Cuiqing Gao, PhD: Dr. Gao is an entomologist and professor of the College of Forestry and Environmental Sciences at of Nanjing Forestry University. She worked with Dr. William Stone to mentor Rosie Long, in her research project exploring bat foraging and insect communities in three habitat types in Nanjing, China.

Yuanchun Yu, PhD: Dr. Yu is a professor of soil biology and chemistry of the College of Biology and Environment of Nanjing Forestry Universitywith an interest in soil carbon sequestration. He assisted Dr. He Guohao with mentoring Ms. Morgan Dean on her research comparing the adsorption behavior of black carbon in urban forest soils versus traffic district soils. His student, Yang Jingyu, also helped with the process.

Changhu Lu, PhD: Dr. Lu is a professor of wildlife ecology of the College of Forestry and Environmental Sciences at of Nanjing Forestry University. His research interests are on plant-animal interactions, and bird biodiversity and behavior. He worked with Dr. Wang and his student Wang Junqiao, who investigated the effect of urbanization bird nest predation.

Other Participants and Institutions

From Alabama A&M University

Sha Li, PhD: Dr. Li is a professor from the School of Education of AAMU. He provided training for REU students on webpage development; he is also serving as an evaluator of the REU program and conducted the exit survey.

Dawn Lemke, PhD: Dr. Lemke is a GIS specialist and assistant professor at AAMU. She traveled to China in 2010, and participated in the pre-travel discussion panel on what to expect in China as well as presenting a workshop on how to design posters for oral presentations.

Lisa Gardner: Ms. Gardner was program coordinator and assisted Drs. Wang and Moss in coordinating the REUG program on the AAMU side. She helped set up the website, created documents, organized the three-day pre-travel orientation and the two-week post-China program. She traveled to China to assist students and faculty with their needs and to act as liaison between NFU staff and faculty and AAMU staff and faculty when Dr. Wang was not available. She created the REU Proceedings, and assisted in creating and submitting the REU Annual Report.

From Nanjing Forestry University

Shengzuo Fang, PhD: Director, Office of International Cooperation and Exchange, and Professor of Silviculture, Department of Forestry and Environmental Science. Dr. Fang's research is focused on the effects of silvicultural regimes on the biomass production, carbon sequestration, and wood quality. He is also interested in the physiological and ecological performance of trees.

Chaonian Feng, PhD: Dr. Feng is the Chairman of Nanjing Forestry University. He is in charge of the operation of the university, and provided leadership role for helping AAMU REU-China program. He visited AAMU in August 2012 to promote the strengthening and expansion of collaborative efforts.

Guofen Li, PhD: Dr. Li is professor of civil engineering of Nanjing Forestry University. In August 2012, she visited AAMU to explore opportunities for further collaboration.

Pingping Li, PhD: Dr. Li is the Vice President of Nanjing Forestry University and a professor of Agronomy. She provided guidance and support for the operation of AAMU REU program at NFU.

Xueqin Liu, PhD: Dr. Liu is an associate professor of the Department of Chinese Language and Literature of Nanjing Forestry University. She trained REU students for Chinese language.

Honghua Ruan, PhD: Associate Dean of the College of Forest Resources and professor of soil ecology, Department of Forest Resources and Environmental Science. He coordinated collaboration between AAMU REU faculty and students and the NFU faculty and students.

Dongrong Shi, PhD: Dr. Shi is the new Director of the Office of International Cooperation at Nanjing Forestry University. He is in charge of the operation of the international program and assisted the development and implement of the AAMU REU-China program. He visited AAMU in August 2012 to promote the strengthening and expansion of collaborative efforts.

Ms. Qingyu Wang: Ms.Wang is former director of the Office of International Cooperation at Nanjing Forestry University. She helped to develop AAMU REU-China program, and provided logistic support.

Ms. Zhiyun Wang: Ms. Wang is Director of the Office of College of International Education at Nanjing Forestry University. She provided logistic support for AAMU REU China program.

Jianren Ye, PhD: Dr. Ye is the Dean of the College of Forest Resources and Environment and a professor of forestry pathology. As the dean, he supervised his staff and faculty to collaborate with AAMU REU-China team. In August 2012, he visited AAMU to explore opportunities for further collaboration.

Zhu Jiangang, PhD: Dr. Zhu is the new dean of the College of International Education of the Nanjing Forestry University. He helped to arrange REU program and provided logistic support.

Other Participating Institutions in China

Nanjing Forestry University

Beijing Normal University

Dafeng National Wildlife Reserve

Wuyishan Nature Reserve

Hainan Normal University

Beijing Forestry University

Shenyang Forestry Bureau

Shennongjia Nature Reserve

Research

BAT FORAGING AND INSECT COMMUNITIES IN THREE HABITAT TYPES IN NANJING, CHINA

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Abstract

Bats are economically, ecologically, and culturally important to countries around the world. Yet, they are understudied, and very little is known about their response to urbanization. Studying bats in China is an ideal place to see the response that rapid urbanization has had on bat foraging activity and dietary preference. China's rapid growth over the past 30 years may have drastic effects on insect communities and therefore food availability for bats. To determine the effects of urbanization, three habitats types were chosen: developed, forested, and riparian. Three sites were distributed uniformly throughout each of the three habitat types. To determine foraging activity, echolocation calls were recorded for one minute in five minute intervals from 7:30 p.m.to 8:30 pm at a total of nine sampling sites. There was significantly more ($p<0.05$) foraging activity in the riparian habitats than developed habitats and forested habitat, which displayed the least foraging activity. Insects were collected once from 7:30 p.m. to 8:30 p.m.at each habitat using a white tarp and a bright light. These insects were identified to taxonomic order and then compared to guano samples from a previous study conducted in Nanjing, Jiangsu, China (June 4th, 2013-June 21st, 2013) to determine dietary preference. Seven orders of insects were collected in the habitats. A significant difference ($p<0.05$) was observed in the abundance of insects collected in different habitats types. Approximately 57% of the insects were collected from forested habitats, 23% from riparian, and 20% from developed. Hemipteran insects comprised 47% of the specimens collected in the developed habitat and 70% of the specimens captured in forested habitat. Diptera (flies) comprised 78% of the insects collected in the riparian habitat. The dietary preference for each species mirrored previous studies with *E. serotinus* displaying distinct preference for Coleopterans and *P. abramus* having more versatile diet with a overall preference for Coleopterans. There was no significant difference ($p>0.05$) found in dietary preference of either species when compared to the guano samples collected from the previous study.

Keywords: *Bat, Insects, Forage, China*

Introduction

Bats comprise almost one-fourth of all mammal species in the world. They are a major insect controller and plant pollinator to ecosystems worldwide. They have long been a contributing factor ecologically and economically to the world, but most people view them as only being a carrier of rabies. Therefore bats are viewed as a threat by some. Bats economically benefit the Earth through research, biological pest control, plant pollination and guano fertilizer. A study conducted in 2011, determined that the loss of bats could cost the US agricultural industry $22.9 billion a year (Boyles et al 2011).

Bats are not only economically important, but culturally important as well. In China, where this study was conducted, bats contribute significantly to the Chinese culture. Chinese artists have long used bats to represent five blessings: health, long life, prosperity, love of virtue, and a tranquil, natural death (Kern 1988). Not only do bats signify cultural blessings in China, but they can be seen in history around the world. Ancient Egyptians believed bats could cure poor eyesight and fevers. Bat gods were important to pre-Colombian civilizations in the New World, and bats have been used in voodoo worships in parts of Africa and the Caribbean (Kunz 1984).

Despite the cultural significance, there is very little information known about bat foraging and their echolocation calls, especially in China. Bats use echolocation calls to hunt for prey. They "see" with their ears by emitting vocal calls in ultrasonic range, inaudible to humans, and listening for a return echo. Since these vertebrates are highly understudied, many have questioned their dietary preference. Bats are known for their gluttonous appetite: a single bat can eat up to a thousand insects in one night (Tuttle 2006). Previous studies indicate that different species of bats prefer to consume different types of insects (Griffin et al 1960; Corrigan 2010). Their dietary preference consists mainly of beetles, wasps, ants, flies, stoneflies, mayflies, moths, mosquitoes, and grasshoppers. However, many species of bats prefer mosquitoes over larger prey because they are the easiest to capture (Tuttle 2006). Yet, researchers have argued that mosquitoes generally make up only a small percentage of a bat's diet (Gonsalves et al 2013) and other studies indicate lepidoptera are the most common ubiquitous nocturnal insects and are preferred most by certain species of bats (Summerville and Crist 2003, Dodda et al 2012, Springthrope and Stone 2013).

Not only are their foraging habits understudied, but their habitat preference is uncertain as well. However, it has previously been determined that bats respond poorly to urbanization. A previous study showed that the bat activities in low human density areas displayed higher foraging activity than those located in high human density areas (Gaisler et al 1998). Another study conducted in Nanjing, China contradicts this conclusion. The study in Nanjing showed that more bats were captured on Nanjing Forestry University, a developed habitat, versus Purple Mountain, a forested habitat (Springthrope and Stone 2013). This contradiction suggests that urbanization may have only caused detrimental effects to certain bat species, while others have adapted and continue to thrive. Consequently, the goals of this study are to determine bat forage preference for insect prey from available insects in the environment and to document the preferred foraging areas in riparian, forest and developed habitats. Since Nanjing, China is where the contradiction occurred, it would be an ideal place to continue the study. Given that insects prefer light, we hypothesize that there will be more foraging activity on Nanjing Forestry University Campus, (urban developed habitat) than Purple Mountain (forest habitat), and Xuanwu Lake (riparian habitat).

Methods

The study was conducted in Nanjing, China between May 30, 2014 and June 28, 2014. Study sites were located in three different habitat types on Nanjing Forestry University, Xuanwu Lake, and Purple Mountain. There were a total of nine sites. Insects were captured one time per habitat type. The sites were distributed uniformly throughout each habitat type. The three sites located on Nanjing Forestry University were considered developed. In order for a site to be considered developed, the site had to be located within 100 m of human activity including roads, streetlights and buildings. The three sites located on Xuanwu Lake were considered riparian habitat. Sites were classified as riparian if they were at least 100 m of Xuanwu Lake. The sites located on Purple Mountain were identified as a forested habitat. To be classified into this habitat type the site was to be located more than 100 m away from human activity. This included roads, streetlights and buildings.

Bat Foraging Activity

Bat foraging activity was measured as the mean number of the echolocation call per minute using a Anabat SD2 CF Bat Detector. The numbers of calls heard were counted for one minute every five minutes from 7:30 to 8:30 pm. Foraging activity was then compared between habitat sites using a one-way ANOVA test in Microsoft Excel Spreadsheet.

Insect Capture

A white tarp with a bright light was set up one time per habitat type to catch insects. The insects were collected using jars filled with an inch of ethyl alcohol. The insects were then brought back to the lab, identified to taxonomic order, and then compared to guano analysis results from a previous study (conducted Nanjing, China from June 4th, 2013-June 21st, 2013) using a Chi-square test in Microsoft Excel Spreadsheet. Since there were orders of insects not captured that were found in the previous experiment, the numbers were omitted to avoid a division by zero when using the Chi-Square test. In order to do this, the percentages observed were arranged accordingly and then multiplied them by the total of the expected frequencies. The number obtained was then use to calculate the chi-square statistics.

Results

Bat Foraging Activity

The results from the single factor ANOVA showed that there was a significant difference ($p<0.05$) in foraging activity by the habitat type. The riparian habitat mean (n=3) of 9.39 calls per minute (n=36), was considerably greater than both the means of developed habitat (n=3) with 5.34 call per minute (n=36), and the forest habitat (n=3) with 0.14 calls per minute (n=36). A Tukey test determined that the there was a significant difference ($p<0.05$) between the riparian habitat and the developed habitat.

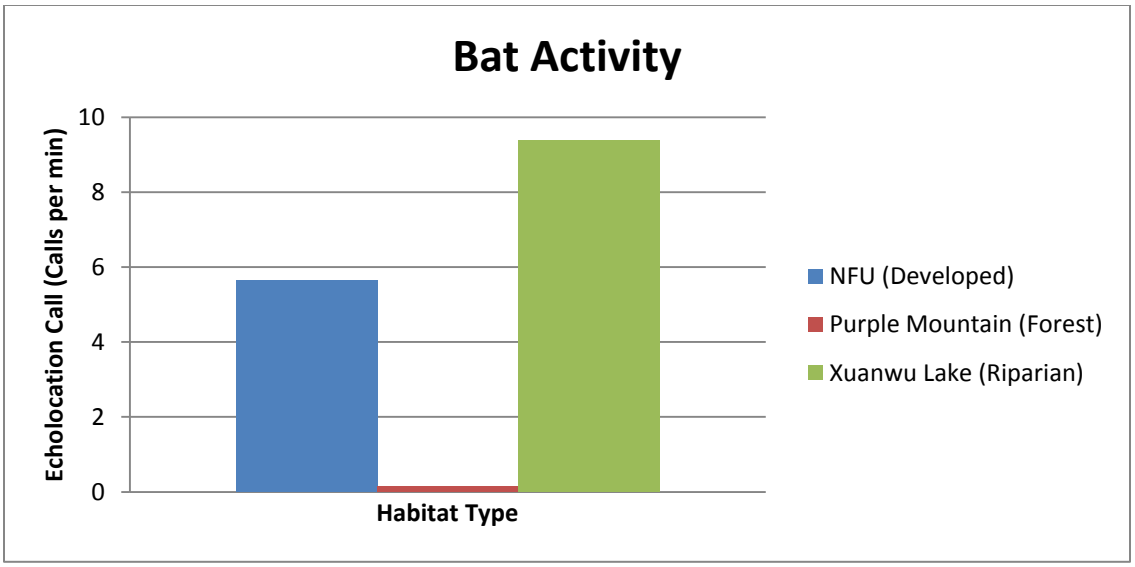


Figure 1. Comparison of forage activity measured by echolocation calls per minute in five minute intervals. 9.3 calls per minute was observed at Xuanwu lake, riparian habitat (n=3), which was significantly more ($p<0.05$) than calls observed at Nanjing Forestry University, developed habitat (n=3), with 5.3 call per minute and calls observed Purple Mountain, forested habitat (n=3), with 0.13 calls per minute.

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	36	203	5.638889	29.09444
Column 2	36	5	0.138889	0.123016
Column 3	36	338	9.388889	65.67302

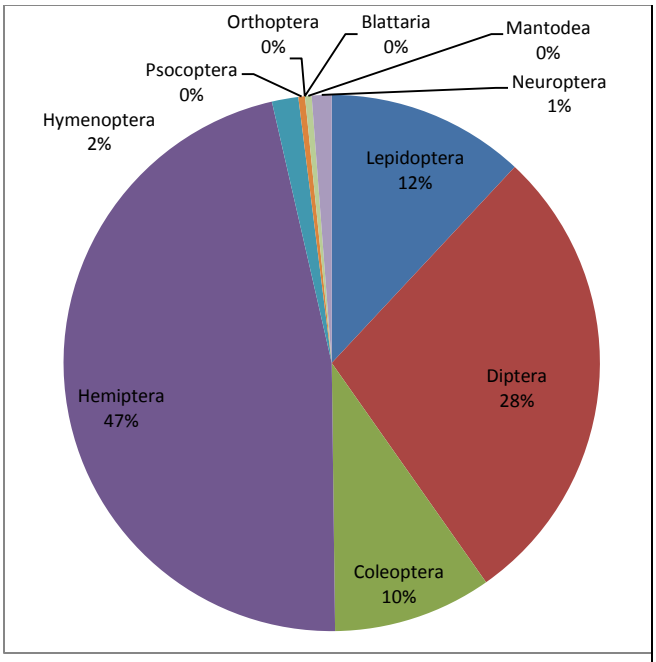
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-val*	*F crit*
Between Groups	1558.5	2	779.25	24.6363	1.69E-09	3.082852
Within Groups	3321.167	105	31.63016			
Total	4879.667	107				

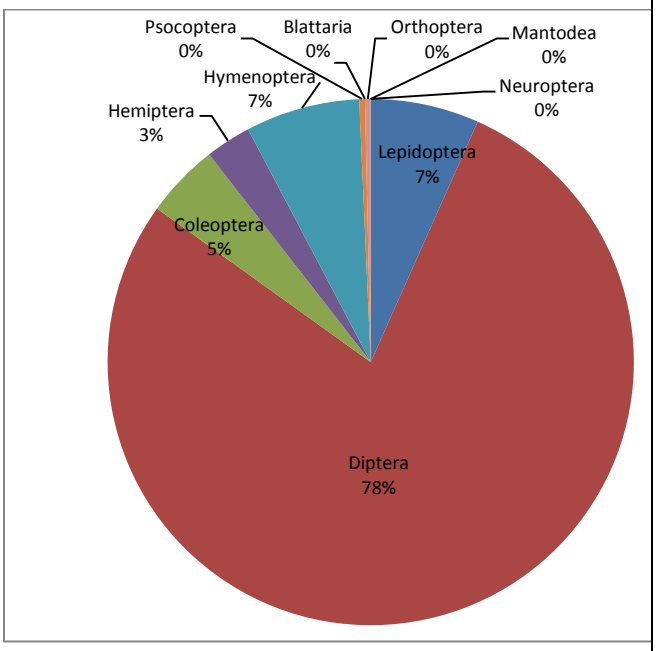
Table 1. ANOVA single factor test between riparian, developed, and forest habitats.

Insects Captured per Habitat

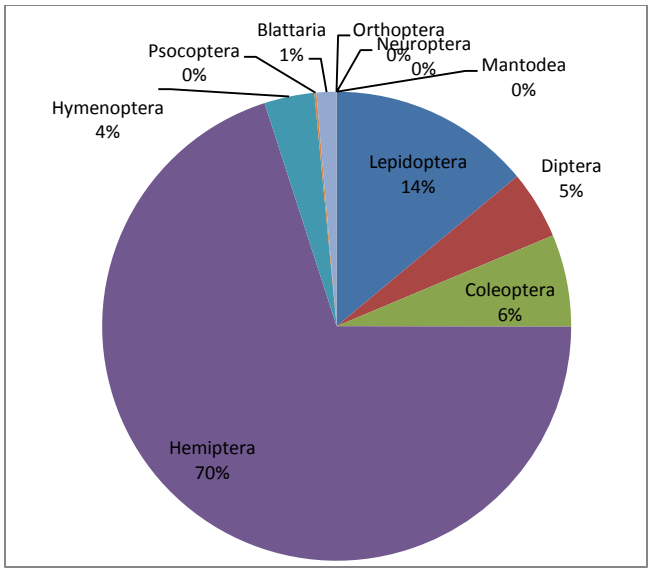
There were seven different Orders of insects captured at each site. Hemiptera was a common order in each habitat type: 47% of the specimens collected in the developed habitat and 70% of the specimens captured in forested habitat were Hemipteran. However in the riparian habitats, Diptera made up 78% of the insects collected. A Chi-square test determined that there was a significant difference ($p<0.05$) of insects populations composition in each habitat type.



Developed Habitat



Riparian Habitat

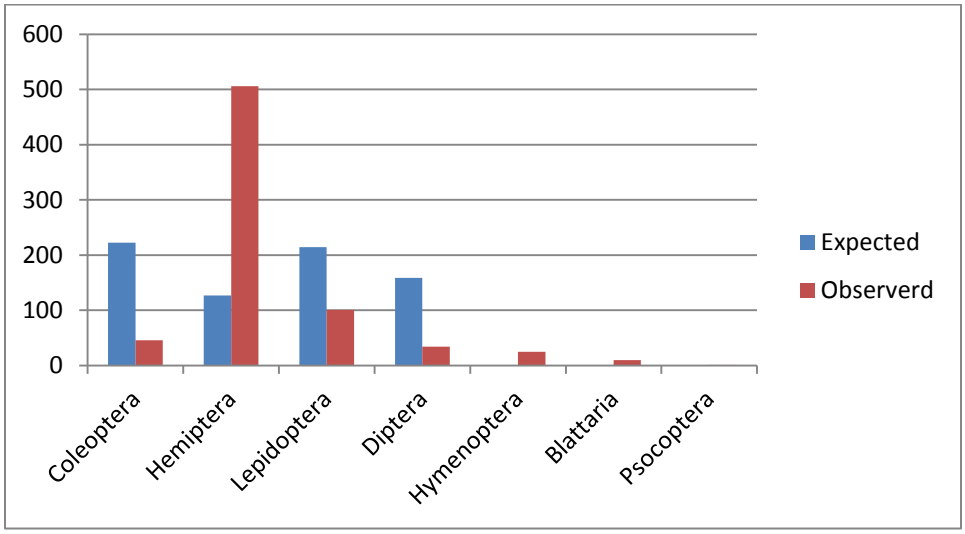


Forested Habitat

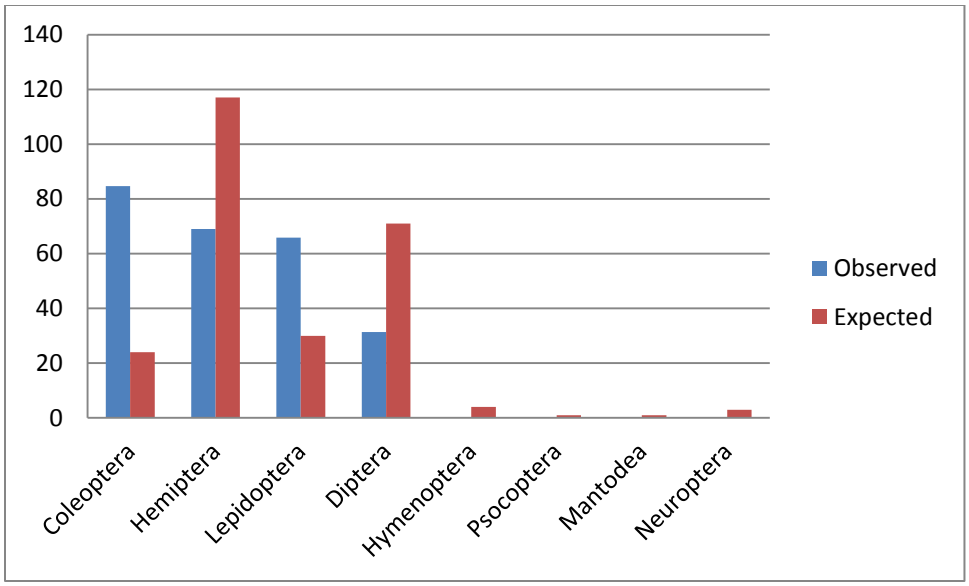
Figure 2. A) shows the percentage of insects collected in developed habitat. (B) shows the percentage of insects collected in riparian habitat (n=285). (C) shows the percentage of insects collected in forested habitat (n=723). A significant difference ($p<0.05$) was found in insects per habitat.

Bat Dietary Preference

To determine if bats have a dietary preference, insects captured in the habitats were compared to the taxonomic composition of insects identified in guano samples from the forest and developed habitats from a previous experiment (Springthorpe and Stone 2013).The insects found in the guano samples were measured as the observed value and the insects collected in the habitat determined the expected value. Since the *Pipistrellus abramus* was the only species found in both developed and forested habitats in the previous study, it was the only one that could be used for diet preference per habitat. The diet preference for the *P. abramus* varied in both habitats types. However, the Order Coleoptera, was preferred over all other Orders in each habitat. (In the forested habitat 31% of Coleopterans were found in the diet but only 10% were available and in developed habitats 34% of Coleopterans were found in the *P. abramus* diet, but only 5% was available.). The *P. abramus* did not have a preference for Hymenoptera, Blattaria, Psocoptera, Neuroptera in either habitat type. Because no bats were captured in the riparian habitat and thus no guano samples were obtained, this habitat type was not included.



Developed (a)



Forested (b)

Figure 3. Dietary preference for *P. abramus* in developed habitat (a) and forest habitat (b). The overall preference for insects varied. However the order Coleoptera was mostly preferred.

Eptesicus serotinus (Common Serotine) was only captured in developed habitats and therefore dietary preference was only compared to insects found in developed habitats. The *E. serotinus* displayed a distinctive preference for Coleopterans which 72% of its diets consisted of with only 9% being available in developed habitats. Other Orders of insects found in the *E. serotinus* diets included, Hemiptera (9%), Lepidoptera (7%), Diptera (9%), and Neuroptera (3%). The E. *serotinus* did not have a preference for Hymenoptera.

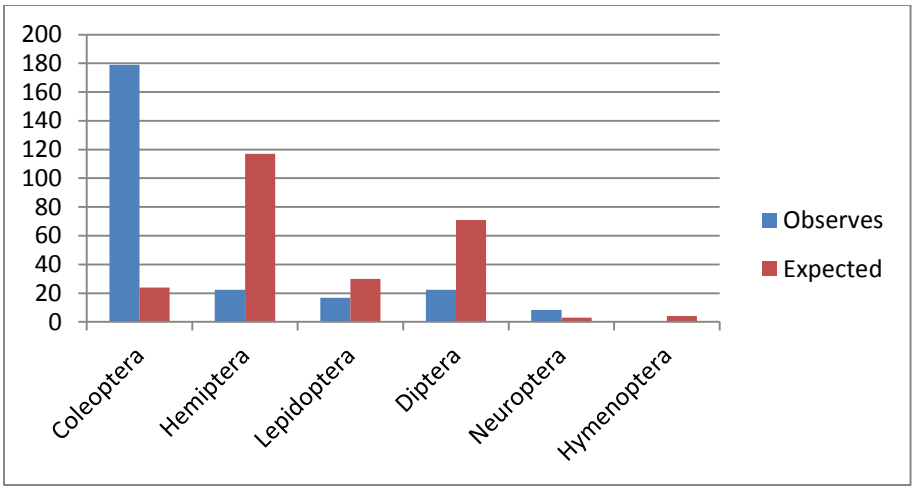


Figure 4. Dietary preference for E. *serotinus* in developed habitat. The E. serotinus displayed a high preference for Coleopterans which (72%) of its diet consisted of.

Discussion

Previous studies indicated that urbanization could have detrimental effects on bat foraging activity. However a study in conducted in Nanjing China proved that 82 % more foraging activity occurred in developed habitats versus forested habitats (Springthope and Stone 2013). Therefore more foraging activity in the developed habitat was expected. However results demonstrated that there was significantly more ($p<0.05$) forging activity in the riparian habitats. A plausible explanation for this is riparian habitat having both street lights and water, which attract insects. A previous study also specifies that streetlights were vital to foraging activity (Tomassin et al 2013). Our study also mirrored studies who expressed more forage activity was recorded in riparian habitats (Myslajek et al 2007 and Salsamendi et al 2012)

There was a diverse population of insects at each habitat type. However, each species had a significant preference ($p<0.05$) for insects that their diet consisted of. *E. serotinus* had distinct preference for Coleopterans and *P. abramus* diet was more versatile. Past studies also indicate that coleopterans accounted for the majority of identified prey for *E. serotinus* (Kervyn and Libois 2008) and *P. abramus* having a very broad diet with Coleopterans being most preferred (Lee and Lee 2005). Insect's Orders such as Blattaria, Hymnoptera, Psocoptera, and Mantodea were not preferred by either E. serotinus or P. abramus.

Finally, a significant difference ($p<0.05$) was observed in the insects collected in different habitats types. 57% of the insects were collected from forested habitats, 23% from riparian, and 20% from developed. Hemiptera was a common Order in each habitat type: 47% of the specimens were collected in the developed habitat and 70% specimens were captured in forested habitat were Hemiptera. However in the riparian habitats, Diptera made up 78% of the insects collected. Coleopterans comprised 6% of insects collected in forested habitats and 9% in developed habitat but was the ideal prey for both *E. serotinus* and *P. abramus*.

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TWO POTENTIAL UNDESCRIBED SPECIES OF THE GENUS *XENOPHRYS* (ANURA, MEGOPHRYIDAE) FROM CHINA

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Abstract

It has been hypothesized that the species richness of the genus *Xenophrys* has been underestimated within the mountainous regions of China, due in part to frequent ecological divergences within China's highly heterogeneous montane stream habitats. We surveyed two areas in China: the subtropical Wuyishan National Nature Reserve and transitional north-central subtropical Shennongjia National Nature Reserve for *Xenophrys* in the summer of 2014. We collected morphological and bioacoustic data to identify each individual *Xenophrys* encountered to species. In Shennongjia National Nature Reserve, we encountered a single species of *Xenophrys* of morphology inconsistent with diagnoses of known *Xenophrys* species in the region. In Wuyishan National Nature Reserve we encountered *Xenophrys boettgeri*, *X. kuatunensis*, and a third sympatric species inconsistent with diagnoses of known species in the region.

Keywords: Xenophrys, *Megophryidae*, *morphology*, *bioacoustics*, *Shennongjia National Nature Reserve*, *Wuyishan National Nature Reserve*

Introduction

The genus *Megophrys* was first established by Kuhl and van Hasselt (1822) with *Megophrys montana* as the type species. In 1864, Günther then named *Xenophrys* as a subgenus within *Megophrys* with the type species *Xenophrys monticola* (now *Xenophrys parva*). Rao and Yang (1997) then proposed the genus *Panophrys* be founded based on the type species *Megophrys omeimontic*. This genus would include many species formerly within *Megophrys*. Dubois and Ohler (1998) then proposed that *Panophrys* be considered synonymous with *Xenophrys*. Ohler (2003) and Dubois (2007) subsequently chose to elevate *Xenophrys* from subgenus to genus status. Discordance in the diagnosing of *Megophrys* versus *Xenophrys* has been observed in some species of Megophryid (Xie and Wang 2000; Zheng et al., 2004; Wei et al., 2010), suggesting possible paraphyly. Until more thorough analyses are conducted to address this, we follow the treatment of Li and Wang (2008) as well as Pyron and Wiens (2011) wherein *Xenophrys* is considered to be a genus apart from *Megophrys* with all previous *Megophrys* species present in China being transferred to *Xenophrys*.

Xenophrys is housed within the subfamily Megophryinae and family Megophryidae under Anura. These are frogs of typically small body size with the type species, *Xenophrys parva*, measuring 7.0-44.0mm snout-to-vent in males and 45.0-54.0mm in females (Li et al., 2014). Frogs of this group range throughout southern China and parts of India (Zhao and Adler, 1993; Fei et al., 2012). *Xenophrys* utilize mountain streams for breeding, with tadpoles occurring near the surface of the water where they filter feed (Huang et al., 1991). The tadpoles possess umbelliform oral disks covered with fingerlike papillae. The morphology of these disks and other associated mouthparts vary within Megophryinae with correlation to microhabitat, diet, and feeding behavior (Huang et al., 1991; Li et al., 2011). Because of this, it is hypothesized that larval evolution in this group is driven by ecological divergence (Liu and Hu, 1961; Li et al., 2011). A species of Megophryid's larval morphology may potentially be adapted for specific larval microhabitat conditions such as fast versus slow moving water, substrate type, and benthic versus surface feeding behavior (Liu and Hu, 1961; Altig and Johnston, 1989; Li et al., 2011). This implies a potential mechanism for the support of a high level of species richness within Megophryidae spread over a diversity of habitat types.

It has been posited that the species richness of *Xenophrys* has been underestimated within China (Wang et al., 2012; Li et al., 2014). The rate of description of this diversity has increased in recent years with the additions of several new species (Fei and Ye, 1992; Mo et al., 2010; Wang et al., 2012; Li et al., 2014; Wang et al., 2014). Many known species within the genus occur in relatively small geographic ranges (Zhao and Adler, 1993; Fei et al., 2012). To elucidate the full extent of this group, further exploration of China's diverse mountainous habitat is required.

In this study, we survey two regions for *Xenophrys* and identify those populations encountered via morphological and bioacoustics comparison. If the population encountered cannot be confidently placed under a current species designation, its validity as a new species will be considered. Because of the theorized high species richness of *Xenophrys* in mountainous areas with appropriate stream habitat and underrepresentation of this richness in existing sampling, we predict the occurrence of potentially novel taxa during our sampling effort that do not align with diagnostic characters for existing species.

Materials and Methods

Study sites - In 2014, we surveyed two regions within China for *Xenophrys*. These regions were Wuyishan National Nature Reserve (NNR; N27°33′ –54′, E117°27′–51′) in Fujian province and Shennongjia National Nature Reserve (N31°15′–31°57′, E109°56′–110°58′) in Hubei province.

Wuyishan NNR is located in northern Fujian province and southern Jiangxi province in China. The reserve is comprised predominantly of mountainous, subtropical evergreen forest (Lan, 2003). Our primary survey site in Wuyishan was near the village of Guadun (=Kuatun). The stream adjacent to this village is the type locality of both *Xenophrys boettgeri* and *Xenophrys kuatunensis*.

Shennongjia NNR is located in east-central China near the Three Gorges area of Hubei province. Habitat within the reserve consists primarily of steep slopes hosting deciduous and conifer-deciduous forest intermingled with evergreen broadleaf forest, meadows, and long-established agricultural areas (Wang et al., 2004; Chen et al., 2005). The Shennongjia Mountains are part of a transitional zone between the central sub-tropics and northern sub-tropics of China and contain highly varied habitats with many vegetational zones (Zhao et al., 2005). Numerous small streams

can be found on the slopes within the reserve, with *Xenophrys* observed during this study to congregate in and near them for courtship displays.

Survey and collection – Surveying was conducted and individual animals collected in June and July of 2014 from Wuyishan NNR and Shennongjia NNR. In Wuyishan NNR , suitable locations were surveyed at night for a period of four days (suitable being defined as areas within approximately 2km of a stream with particular emphasis being placed on the banks of the stream). In Shennongjia NNR, suitable locations were surveyed at night for eight days. All *Xenophrys* encountered were captured by hand and recorded via GPS (accurate to ± 3 m; Garmin 60CSX). Time and environmental data were gathered at each location of capture including temperature, humidity, elevation, and weather condition. Vocalizations were recorded in situ using a Samson C01u cardioid microphone then compared and analyzed with the program Audacity, version 1.3 (The Audacity Team, www.audacity.sourceforge.net) for note duration, duration of time between notes, and repetition rate (notes per second).

Morphological measurement - Collected specimens of ambiguous specific identity were measured using calipers to the nearest 0.1 mm for the following characters with definition and abbreviations according to Li et al. (2014): Snout-to-vent length (SVL), head length (HDL) from the articulation of the jaw to the tip of the snout, head width (HDW) between left and right articulations of the quadratojugal and maxilla, snout length (SNT) from tip of snout to the anterior corner of the eye, eye diameter (EYE) from the anterior corner of the eye to posterior corner of the eye, internasal distance (IND), tympanum diameter (TMP), tympanum-eye distance (TEY) from anterior edge of tympanum to posterior corner of the eye, hand length (HND) from distal end of radioulna to tip of distal phalanx of III, radioulna length (RAD), foot length (FTL) from distal end of tibia to tip of distal phalanx of III, tibia length (TIB). Adult morphological data were compared to reference values (Li et al. 2014) using standard 95% confidence interval method. Further statistical measures of significance could not be obtained in comparison of our data to reference values as the reference data presents only ranges. For tadpoles we measured total length from tip of snout to posterior end of tail (TOT), head-body length from tip of snout to base of tail (HBL), and tail length from base of tail to tip of tail (TL). Tadpole measurement definitions are based on Liu and Hu (1961). Tadpole TOT measurements were compared to reference values given in (Li et al., 2011) using a standard t-test. In addition to these

measurements, other morphological characters were recorded including presence/absence of a horn-like tubercle above the eye, vomerine teeth, vocal sac, and toe webbing.

Specimens were sacrificed, measured, stored in 75% ethanol with liver tissue samples in 95% ethanol, and deposited in the respective museum collections of Wuyishan NNR and Shennongjia NNR. Collections were permitted by the senior management at each respective reserve.

Results

Survey - In Shennongjia NNR, a single species of *Xenophrys* was encountered. The *Xenophrys* with a geographic range most nearly placing them in Shennongjia NNR is *Xenophrys wushanensis* (Fei et al. 2012). However, the presence of a horn-like tubercle above the eye observed on those individuals encountered was immediately inconsistent with descriptions of *X. wushanensis* by Fei et al. 2012. Eleven adult individuals were collected exhibiting courting behavior on stream banks (n = 10 males and n = 1 female). Five *Xenophrys* tadpoles were also collected from the same location. Because only one population of a single species of adult *Xenophrys* was encountered during the survey, we will attribute these tadpoles to the same species as the adults collected. They were encountered at elevations ranging from 1320 to 1630 m above sea level. This elevational range is inconsistent with that of *X. wushanensis*: 945 to 1200 m (Fei et al., 2012).

In Wuyishan NNR, we encountered and identified *X. boettgeri* and *X. kuatunensis* in abundance, the presence of which is predicted in the known ranges of those species (Fei et al., 2012). However, a third population of *Xenophrys* was also encountered that was inconsistent with descriptions of and visually distinct from *X. boettgeri* and *X. kuatunensis*. Four adults of the unidentified population were collected (n = 4 males) from agricultural areas adjacent to a mountain stream. These individuals were found existing sympatrically with both *X. boettgeri* and *X. kuatunensis* at elevations ranging from 1242 to 1302m above sea level. Representative individuals from all Xenophrys populations encountered are shown in Figure 1.

Figure 1. All *Xenophrys* species encountered in this study. A, unidentified *Xenophrys sp.* in Shennongjia NNR; B, unidentified *Xenophrys sp.* in Wuyishan NNR; C, *Xenophrys kuatunensis* in Wuyishan NNR; D, *Xenophrys boettgeri* in Wuyishan NNR.

Morphology – In comparison of the unidentified *Xenophrys* found in Shennongjia NNR to recorded measurements of *X. wushanensis* (Li et al. 2014), the unidentified *Xenophrys* diverged from *X. wushanensis* in tympanum to eye ratio (95% Confidence Interval (CI) [0.63, 0.71]mm, reference *X. wushanensis* = 0.5mm), and the presence of a horn-like tubercle above the eye (observed population = present, reference *X. wushanensis* = absent). The tadpole total length given by Fei et al. (2009) is 39.7mm. This value falls outside our calculated confidence interval, (95% CI [27.4, 31.66]mm) from our observed measurements of five tadpoles. The tadpoles collected in Shennongjia differed in TOT from reference values given by Li et al. (2011) for *X. kuatunensis* ($p < 0.0001$, $df = 13$) and *X. boettgeri* ($p < 0.0001$, $df = 13$) (Table 1).

Table 1. Larval morphological data gathered for unidentified *Xenophrys* population in Shennongjia NNR (n = 5).

Character	*Xenophrys sp.* Shennongjia
TOT	29.53 ± 2.43
HBL	16.60 ± 2.64
TL	12.92 ± 0.66

In a comparison of the unidentified *Xenophrys* from Wuyishan NNR to *X. kuatunensis* reference data from Fei et al. (2014), the unidentified *Xenophrys* were found to diverge from *X. kuatunensis* in measures of tympanum to eye ratio (95% CI [0.69, 0.77]mm, *X. kuatunensis* = 0.44mm). In a comparison of the unidentified *Xenophrys* from Wuyishan and *X. boettgeri* reference data from Fei et al. (2014), the unidentified *Xenophrys* diverged in the measures of SVL (95% CI [27.54, 32.22]mm, *X. boettgeri* = 34.5 – 37.8mm), tympanum to eye ratio (95% CI [0.69, 0.77]mm, *X. boettgeri* = 0.4 – 0.67mm), and tibia to SVL ratio (95% CI [0.39, 0.43]mm, *X. boettgeri* = 0.45 – 0.49mm) (Table 2).

Bioacoustic call structure - Because no recording of the mating call of *X. wushanensis* was available, vocalization could not be compared to the unidentified population in Shennongjia NNR.

Table 2. Adult morphological data gathered for both unidentified populations of this study. *Xenophrys sp.* Shennongjia: n = 11, *Xenophrys sp.* Wuyishan: n = 4. With "-" indicating absence of a trait, "+" indicating presence, and "+ +" indicating strong presence.

Character	*Xenophrys sp.* Shennongjia	*Xenophrys sp.* Wuyishan
SVL	33.16 ± 1.79	29.88 ± 2.39
HDL	10.93 ± 0.69	10.85 ± 1.76
HDW	11.46 ± 0.47	11.63 ± 0.95
SNT	4.55 ± 0.46	3.75 ± 0.29
EYE	3.32 ± 0.16	3.00 ± 0.00
IND	4.09 ± 0.32	3.84 ± 0.09
TMP	2.22 ± 0.15	2.18 ± 0.09
TEY	1.91 ± 0.28	2.31 ± 0.40
HND	8.38 ± 0.41	7.20 ± 0.47
RAD	8.33 ± 0.58	7.69 ± 0.90
FTL	22.46 ± 1.48	18.25 ± 0.50
TIB	15.65 ± 0.77	12.19 ± 0.94
TMP/EYE	0.67 ± 0.06	0.73 ± 0.04
TIB/SVL	0.47 ± 0.02	0.41 ± 0.02
Horn-like tubercle	+	++
Vomerine teeth	-	-
Vocal sac	+	+
Toe webbing	+	+

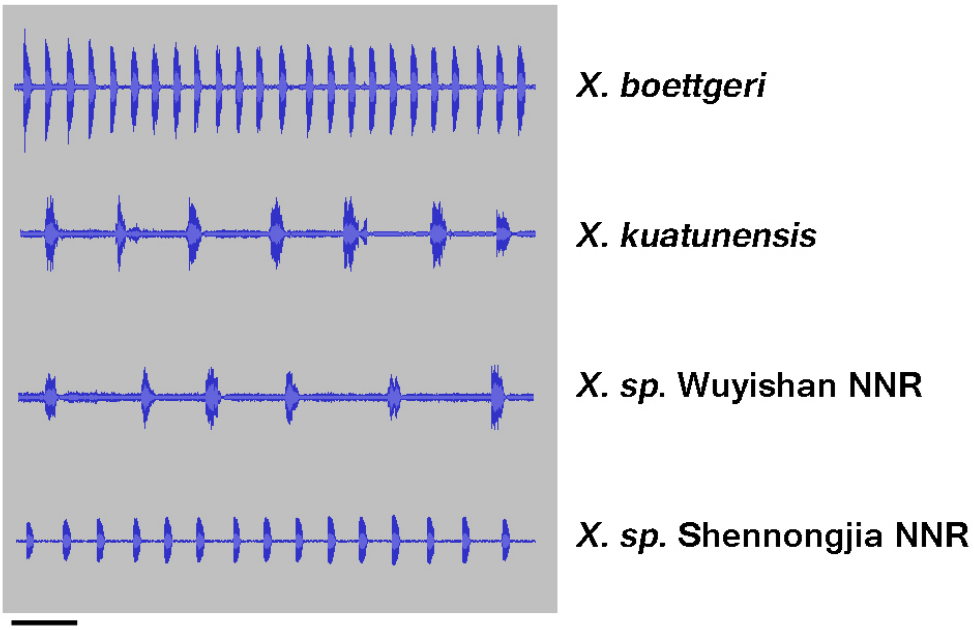

Figure 2. Wave forms of Xenophrys vocalizations. Scale bar = 1 second.

Table 3. Acoustic data gathered from one call strophe of each species.

	Xenophrys boettgeri	*Xenophrys kuatunensis*	*Xenophrys sp.* Wuyishan	*Xenophrys sp.* Shennongjia
Note duration (s)	0.127 ± 0.00	0.247 ± 0.06	0.247 ± 0.01	0.137 ± 0.03
Duration between notes (s)	0.267 ± 0.08	0.917 ± 0.10	1.145 ± 0.26	0.390 ± 0.07
Repetition rate (note/s)	3.00 ± 0.00	0.875 ± 0.00	0.755 ± 0.01	1.875 ± 0.09

In comparing unidentified *Xenophrys* from Wuyishan with its two sympatric congeners (Figure 2), the call was found to be distinct from *X. kuatunensis* in the duration between notes ($p = 0.0045$, $df = 4$) and repetition rate ($p = 0.0259$, $df = 4$), distinct from *X. boettgeri* in note duration

($p < 0.0001$, $df = 4$), duration between notes ($p < 0.0001$, $df = 4$), and repetition rate ($p < 0.0001$, $df = 4$) (Table 3).

Conclusions

Based on these data, we conclude that there are possibly three different species of *Xenophrys* existing sympatrically in Wuyishan NNR: *X. kuatunensis*, *X. boettgeri*, and a third possible species yet undescribed. The third species is distinct from *X. kuatunensis* and *X. boettgeri* in its morphology and vocalization and can be readily distinguished in the field from its congeners. Description of this potentially novel taxon will require more thorough sampling in addition to high-resolution morphometric and molecular analysis.

The unidentified *Xenophrys* encountered in Shennongjia can not conclusively be distinguished from *X. wushanensis* with these data. We estimate that, based on some incongruence of morphology and habitat preference, that there is notable disparity between *X. wushanensis* and the unidentified individuals measured in Shennongjia NNR. However more thorough analysis is needed to elucidate the degree of this disparity, whether it is more consistent with species or sub-species distinctions.

The existence of these two cases of undescribed diversity supports the hypothesis that the species richness of *Xenophrys* within China is currently underestimated. It is widely known that species with relatively small geographic ranges are particularly vulnerable to severe decline (McKinney, 1997). As deforestation and ever-increasing agricultural land use in China progresses, exploration of the remote montane habitat of the genus *Xenophrys* must be concurrently accelerated to document the species of China in these regions under threat.

As molecular techniques and technologies are simplified and made more widely available to researchers, we anticipate more species of *Xenophrys* to be described phylogenetically as well as morphologically. This will allow for more accuracy and consistency in description of the genus. While the means to gather data become more workable with time, access to field sites remains a challenge. However, these two instances of undocumented diversity in the genus were discovered after less than two weeks of surveying in the study areas. While the steep montane

habitat in the regions may not be immediately conducive to exploratory investigation, this exploration is needed to more accurately understand the diversity of China's amphibian taxa.

Acknowledgements

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DEVELOPMENT OF BAMBOO RHIZOME SYSTEM IN JIANGSU PROVINCE, CHINA

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Abstract

Bamboos are one of the largest members of the grass family, Poaceae, and one of the most economically important crops in Asia. China's bamboo forest is dominated by one monopodial species, *Phyllostachys heterocycla* var. *pubescens*, also known as moso bamboo. This species accounts for approximately 72% of the total bamboo area in China, and over 90% of the country's bamboo production (Buckingham et. al. 2011). This study examined the age structure and morphological features of several bamboo species to compare biomass growth. We also measured diameter of rhizome, length of internode, and bud development to a rhizome, to a shoot, to a root, or nothing and compared growth of containerized grown and open grown rhizomes of *Pleioblastus argenteostrialus*.

Introduction

Bamboos are among the fastest growing, most productive, most versatile, multipurpose plants in the world. These characteristics make them one of the most important sources of non-timber forest products in the world (Peng et. al 2013). Bamboos represent a major lineage of grasses that are native primarily to subtropical forests. Taxonomically, they belong to the Bambusoideae subfamily of Gramineae (Poaceae, or true grass) family. Plants belonging to this family produce a continuous growth axis that extends at its apex and produces successive lateral shoots (i.e. monopodial).

There are two distinct groups of bamboos: woody bamboos (Bambuseae) and herbaceous bamboos (Olyreae). The woody bamboos are characterized by a lignified culm (i.e. stem) with complex branching patterns and bisexual flowers. The herbaceous ones, distributed mainly in the tropical rainforest of the New World, have an unlignified culm with unbranched or simple branching pattern and unisexual flowers. The bamboo's rhizome can be classified into three distinct forms: pachymorph (clumping), leptomorph (diffuse) and metamorph (mixed), based on the characteristic of the branching pattern of the rhizome. When a rhizome is cut, it does not die, as a root would, but it generates several new plants. Studying rhizomes is important to to understand certain bamboo species growth pattern.

China's bamboo forest is dominated by one species, *Phyllostachys heterocycla* var. *pubescens* or moso bamboo. This species accounts for approximately 72% of the total bamboo area in China and over 90% of the bamboo production (Buckingham et. al. 2011). The plant's incredible growth rate and large size contributes to its use as food, paper, plywood, furniture and flooring, making it the most utilized bamboo in China.

The rapid growth a rate of bamboo (75–1,000 mm per day in the peak of the growing season) means that it can be harvested more frequently than comparable short-rotation silvicultural species such as eucalyptus (Buckingham et. al 2011). A primary advantage of bamboo harvesting is that it takes a relatively short time to establish a mature commercial bamboo plantation after planting; for instance, only three years for sympodial (clumping) bamboo and six years for monopodial (running) bamboo.

While bamboos are beneficial to the majority of South, Southeast, and East Asia's forests, the species can also be an economic and laboring nightmare if poorly managed. Most bamboos species have an infrequent flowering process. In fact, lots of bamboos have flowering intervals that are as long as 65 to 120 years. When flowering occurs, it can prove to be both a blessing and or devastation depending on circumstances. The flowering process can cause a rise in rodent populations, which can lead to an increase in disease and famine in human populations. In either case, flowering delivers masses of seeds that pass on a new generation of species with a genetic code that it is not identical to the parents.

Due to their biological characteristics and growth habits, bamboo forests have ecological and environmental functions in soil erosion control, water conservation, land rehabilitation, and carbon sequestration. With numerous rhizomes and evergreen leaves, bamboo is a valuable ally in the fight against soil erosion and water loss (Zhao-hua 2004). There are a number of universities analyzing the use of bamboo for aesthetic appeal, afforestation/reforestation and as environmentally friendly building materials. The Nanjing Forestry University Xiashu Research Farm (NFUXRF) has been conducting bamboo research for the past thirteen years, however government ownership of the farm goes far back for several decades. In spite of more than one hundred species of bamboo at NFUXRF and over 1400 hundred species in China, data on bamboo rhizome system are limited.

Due to their clonal life history, bamboo rhizomes may optimize the efficiency of light use in their growth either by spreading or by morphological plasticity (Wang et. al.2006). This study examined species age structure and morphological features for biomass growth. We also examined and compared the rhizome development of open grown (OG) and containerized grown (CG) bamboos.

Materials and Methods

Our experiments were established in the Nanjing Forestry University Xiashu Forest Farm (NFUXRF), a forestry research site for the school in Jurong, Jiangsu (31°56′N 119°09′E). Bamboo plantations were established for experimental purposes thirteen years ago. Twelve plots of various bamboo species were measured during June 4-7, 2014. Measurements included height,

weight, and diameter of culm at ground level. The tools we used include GPS equipment, scales, measuring tapes, calipers, handsaws, hatchets, spades, and soil extraction plunges. A theodolite was used to determine angles for the plots.

The decision on which culm to cut was based on age structure and environment conditions. Based on age structure, we removed culms that were two to three years old from a plot. The bamboo's age were characterized by a shade variation of lightness or darkness on the outside of culms. Although there are not any annual rings on bamboo, age of the plots chosen was provided by a research faculty. The plots ranged from a few months old to twelve years of age. The environmental conditions focused on drought, weathering and soil conditions of the plots. For each plot, we took samples of soil from the center point and a few random spots within the plot. We mixed soil well to make a composite sample and send to the laboratory.

We examined two types of growing methods for Pleioblastus argenteostrialus: containerized grown (CG or type 1) and open grown (OG or type 2). Pleioblastus is a genus of monopodial bamboos native to China and Japan. Plants of this genus spread by vigorous underground rhizomes that run along just beneath the soil surface, producing plantlets at the nodes. These can be used to propagate new plants, but if not removed they can become invasive.

A standard procedure was used to grow both bamboo samples at Nanjing Forestry University (NFU) campus until they were three years old. At that time the OG bamboo was transplanted in the ground at a bamboo plantation site in Yixing, while CG bamboo continued to grow in a container at NFU. In May 2014, the CG bamboo was removed from its container while the OG bamboo was dug out of the ground on June 14, 2014. These specimens were randomly chosen from their population to compare rhizome characteristics of one another. Variables of CG and OG that were measured included diameter of rhizome, length of internode, and bud development to a rhizome, to a shoot, to a root, or nothing. The reason we compared these characteristics was to gain an understanding in growth patterns and to recognize how containerizing bamboo plants affects the development arrangement of their rhizome system.

Discussion

The findings of twelve various plots collected at Xiashu were not analyzed statistically. Due to factors of time constraint and publication rights, the use of specific data will not be shared at this time. However, Table 2 indicates overall averages of a specific plot.

There was over one hundred species of bamboo in NFUXRF and the most important are as follows:

Phyllostachys aureosulcata was thriving in several different areas. This bamboo is a leptomorph and has the capability of tolerating extreme environment conditions (i.e. drought and air pollution). It grew in a dense area on plots 1 and 3, in which we only took a few samples.

Another species, moso bamboo (*P. heterocycla)*, which is characterized by its culm's strength and edible shoot. This species was planted on a large plot due to its average height of 10.7m and average diameter at ground level of 9cm.

The species *Phyllostachys bambusoides* grew on a plot that was surrounded by 1m deep and 0.3m wide trenches to keep it separated from other bamboo species. We sampled 202 bamboo specimens because many of the plants from this plot were not maintained well and we needed to clear the way for new plants to sprout. This species needs regular harvesting; otherwise damage to their offspring can increase substantially.

Table 1. Plot location, size, altitude, and age of bamboo species at Nanjing Forestry University Xiashu Forest Farm (NFUXRF).

Plot	Latitude & Longitude	Plot size	Species	Samples	Altitude	Age
1	N 32’07.13.19” E 119’13’07.29”	5m*5m	*Phyllostachys aureosuleata*	30	104.9m	N/A
2	N 32’07.46.9” E 119’12’09.9”	30m*30m	*Phyllostachys heterocycla*	30	124.6m	N/A
3	N 32’07.14.04” E 119’13’09.09”	6m*6m	*Phyllostachys aureosulcata*	34	100.0m	N/A
4	N 32’07.14.72” E 119’13’17.48”	5m*5m	*Phyllostachys aurea* Mask Bamboo	35	102.8m	1,3 & 5
5	N 32’07.11.4” E 119’18’04.8”	3m*3m	*Pseudosasa japonica* Arrow Bamboo	62	108.7m	N/A
6	N 32’07.12.5” E 119’13’06.7”	4m*4m	*Hibanobambus tranguillans*	74	107.0m	N/A
7	N 32’07.10.02” E 119’13’11.92”	10m*10m	*Phyllostachys iridescens*	30	101.0m	3
8	N 32’07.08.2” E 119’13’10.3”	2m*2m	*Pseudosasa japonica*	31	104.0m	10
9	N 32’07.10.38” E 119’13’07.85”	5m*5m	*Phyllostachys makinoi*	36	117.0m	9
10	N 32’07.11.7” E 119’13’13.8”	5m*9.7m	*Phyllostachys vivax*	33	89.6m	11
11	N 32’07.14.35” E 119’13’17.98”	3m*3m	*Indosasa shibataeoides*	76	107.0m	11
12	N 32’07.14.41” E 119’13’19.14”	5m*5m	*Phyllostachys bambusoidesvar*	202	101.0m	11

Table 2. Plot species, soil type and average height, diameter and volume.

Plot	Species	Height (m)	Diameter at Ground Level (cm)	Volume (kg)	Soil Type
1	*Phyllostachys aureosuleata*	6.24	1.88	1.284	Yellow loam
2	*Phyllostachys heterocycla*	10.73	9.1	15.96	Yellow loam
3	*Phyllostachys aureosulcata*	5.98	1.81	1.11	Yellow loam
4	*Phyllostachys aurea*	3.15	1.7	0.361	Yellow loam
5	*Pseudosasa japonica*	3.49	1.15	0.289	Yellow loam
6	*Hibanobambus tranguillans*	1.66	0.908	0.279	Yellow loam
7	*Phyllostachys iridescens*	3.52	2.4	1.108	Yellow loam
8	*Pseudosasa japonica*	0.75	1.188	0.066	Yellow loam
9	*Phyllostachys makinoi*	4.68	1.53	0.674	Yellow loam
10	*Phyllostachys vivax*	5.94	2.882	1.79	Yellow loam
11	*Indosasa shibataeoides*	3.59	1.3	0.452	Yellow loam
12	*Phyllostachys bambusoidesvar*	2.45	1.374	0.417	Yellow loam

Most of the data collected and compared from CG and OG bamboos showed significant differences while for other data was not as major. In figures 1 and 2, when examining CG, it was found that diameter and length of internodes were significantly different from those of OG. The diameter of CG on average measured at 0.31 cm while OG diameter 0.37cm. Then, CG detection with length of internodes on average was 0.86 cm while in OG was 1.39 cm. This finding indicates CG lack of an ability to receive adequate growth ability in comparison with OG. Figures 3, 4 and 5 show that in variables such as percentage of buds forming into rhizomes, of

buds forming into shoots (culms) and buds forming into roots there is no statistical significant difference between the two types.

Based on NFUXRF previous studies, CG bamboo had an average of 100% percent survival rate, while OG had just 70%. Furthermore, OG bamboo soil content normally weighs 5 kg while CG weighs 1.75 kg. In particular, during transporting these plants, variables such as survival rate and weight are intensely taken into consideration. In other words, bamboo is shipped all over the world; if a species weighs too much and will not survive to its destination then it will be an economic loss.

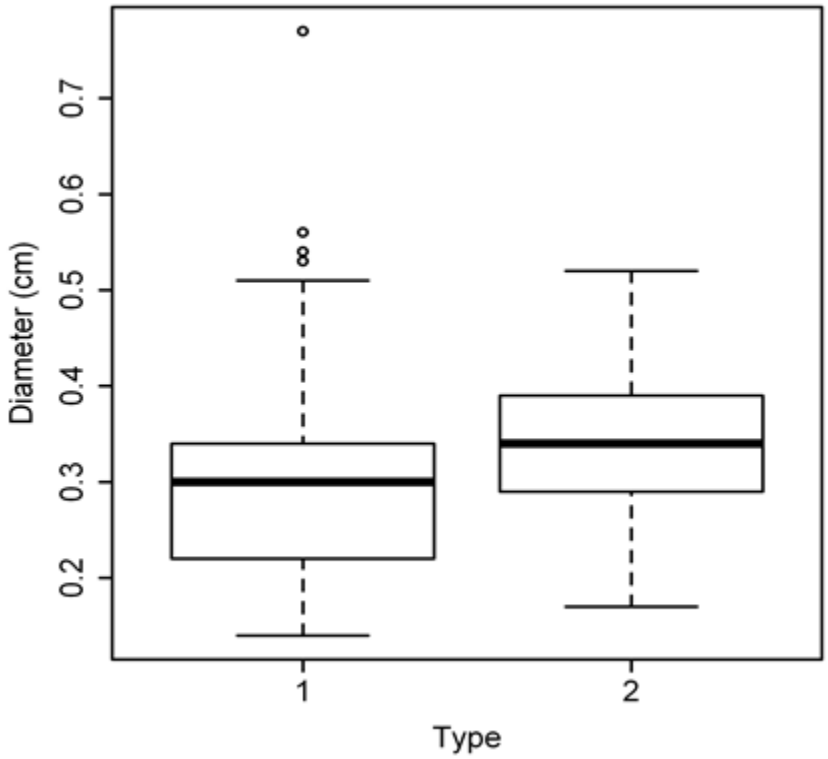

Figure 1. Comparison of diameter of the rhizome node between two types.

	DF	SS	Mean Sq	F-value	Pr(>F)
Type	1	0.311	0.31096	45.18	4.19e-11***
Residuals	599	4.122	0.00688		

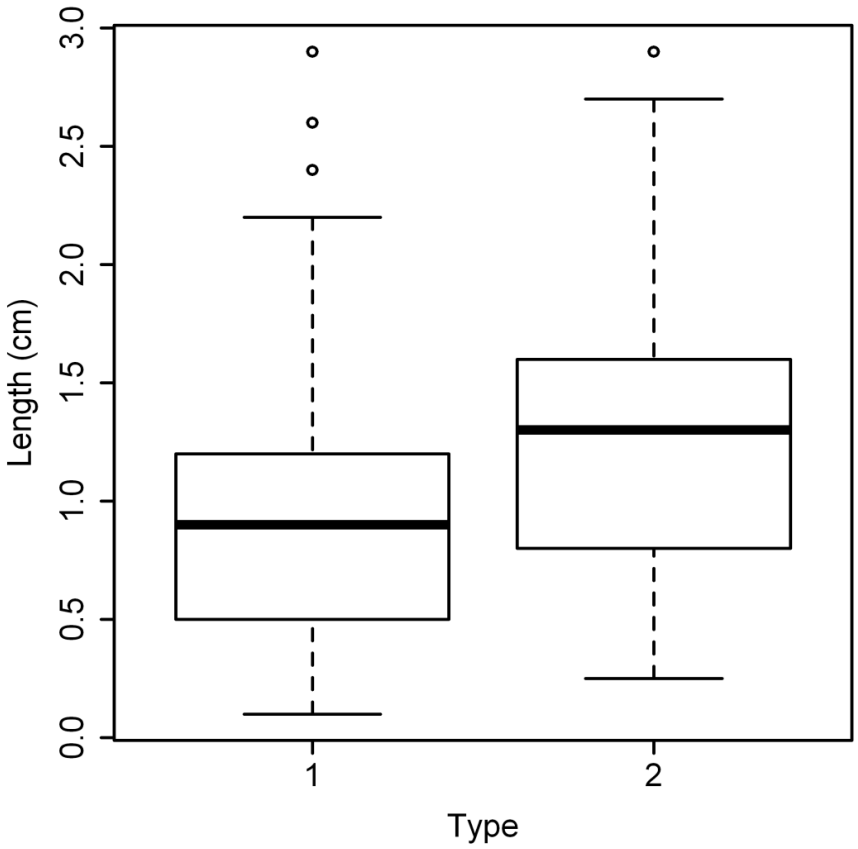

Figure 2. Comparison of the distance between two rhizome nodes between two types.

	DF	SS	Mean Sq	F-value	Pr(>F)
Type	1	13.55	13.547	42.25	1.69e-10***
Residuals	599	192.07	0.321		

Note: The length of Type 2 is highly significant than that of Type 1.

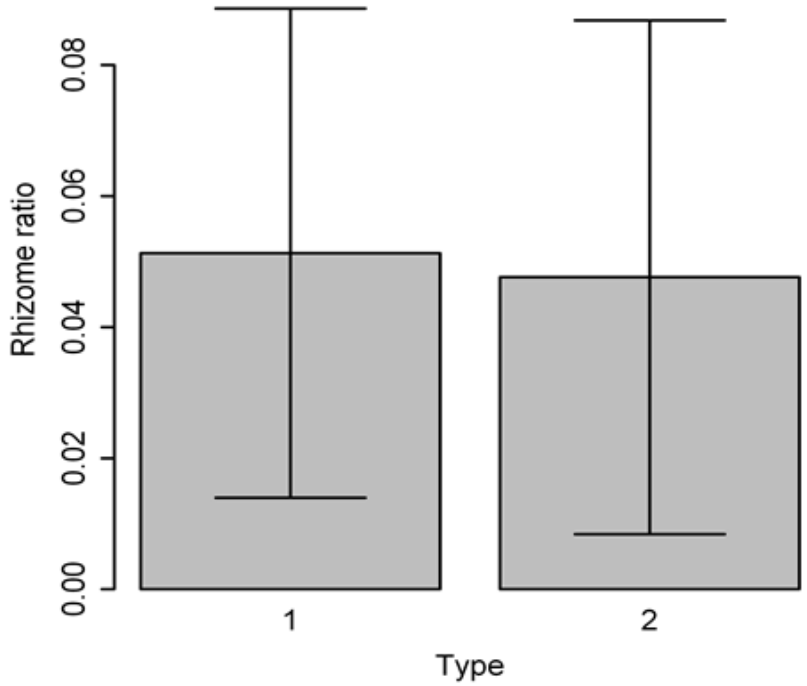

Figure 3. Comparison of rhizome ratios between two types.

	DF	SS	Mean Sq	F-value	Pr(>F)
Type	1	0.00006	0.0000611	0.019	0.891
Residuals	599	0.05661	0.0031448		

Note: There is no statistically significant difference in the rhizome ratio between two types.

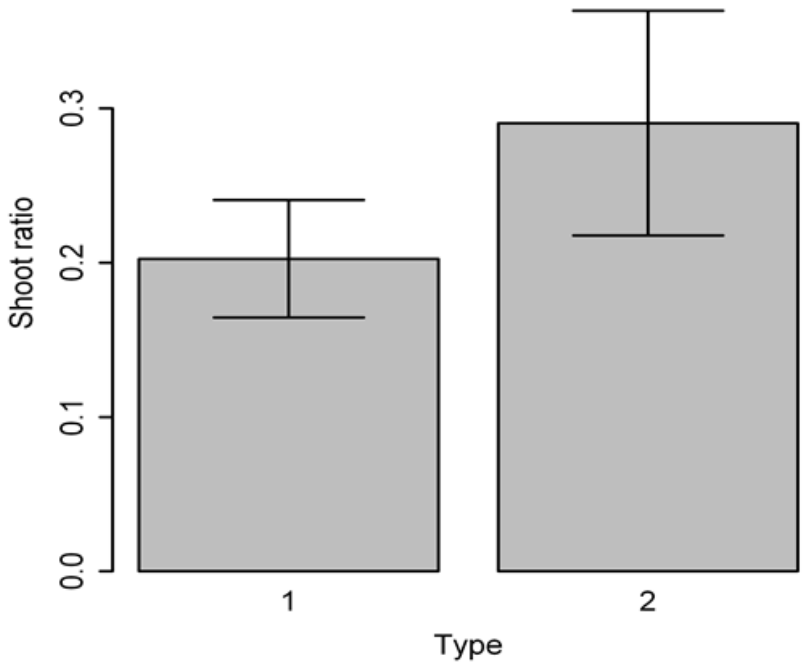

Figure 4. Comparison of shoots ratios between two types.

	DF	SS	Mean Sq	F-value	Pr(>F)
Type	1	0.03516	0.03516	73461	0.0137*
Residuals	18	0.08484	0.00471		

Note: The shoot ratio of Type 2 is highly significant than that of Type 1.

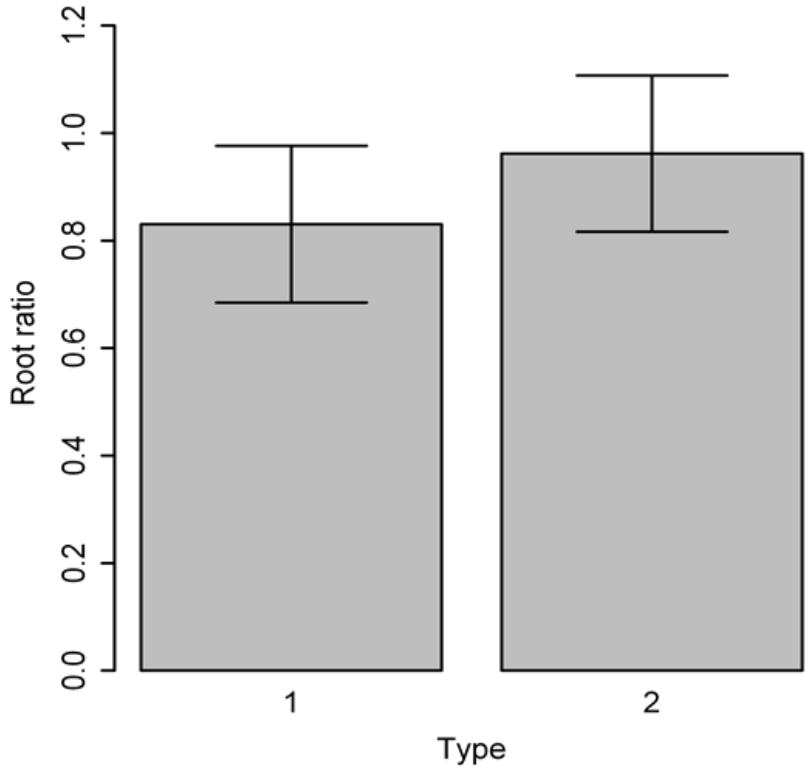

Figure 5. Comparison of root ratios between two types.

	DF	SS	Mean Sq	F-value	Pr(>F)
Type	1	0.0782	0.07824	1.664	0.213
Residuals	18	0.8464	0.04702		

There is no statistically significant difference in the root ratio between two types.

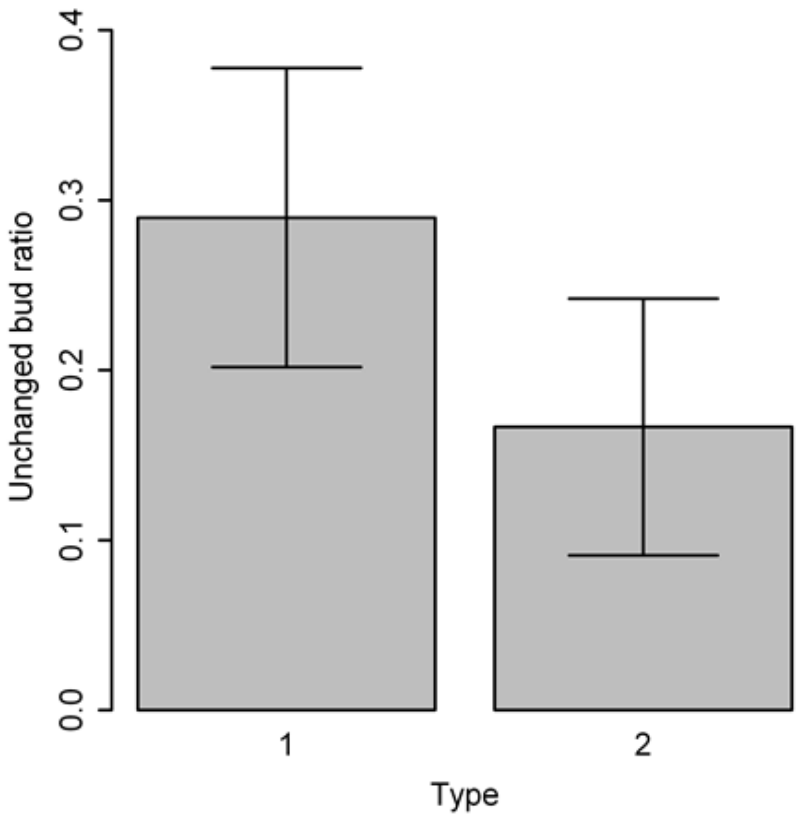

Figure 6. Comparison of unchanged bud ratios between two types.

	DF	SS	Mean Sq	F-value	Pr(>F)
Type	1	0.06892	0.06892	4.217	0.0548
Residuals	18	0.29419	0.01634		

Note: There is no statistically significant difference in the unchanged bud ratio between two types. However, the unchanged bud ratio of Type 2 is slightly lower than Type 1.

Conclusion

This study compared containerized and open grown bamboo to one another. The benefits of containerized are less amount of soil attached to its rhizomes. Also their soil contents on rhizome, weight less than OG bamboo. Therefore, it is better for management cost to stay within a given weight range. From previous studies, the open grown had a survival rate of 75% while CG had a 100%. The nutrients in CG were bettered monitored and less contaminated from

external factors. Further studies will need to be done on more species with similar environmental conditions and age structures.

Acknowledgements

We would like to thank the Nanjing Forest University faculty, students and staff for their help in this project and their hospitality. This research was supported by the National Science Foundation (REU II-China Grant No. 1063101) and the National Institute of Food and Agriculture, through a joint program of Alabama A&M University (USA) and Nanjing Forestry University (China).

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ESTABLISHMENT OF A RIPARIAN BUFFER STRIP FOR ALLEVIATING LAKE EUTROPHICATION

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Abstract

Riparian buffer strips are a growing conservation practice to control and mitigate non-point source pollution in Asia. China has seen rapid population growth and economic development in the last fifty years, coupled with a rapid increase in environmental pollution. Freshwater ecosystems have been particularly affected. Lake Tai, China's third largest freshwater lake by volume has seen a severe reduction in water quality since economic reforms began in the 1970s. Thus, significant interest for establishing riparian buffer strips in agricultural watersheds and freshwater systems within China is warranted. Eight 50 m x 20 m plots adjacent to a rice-phragmites farm were cleared within the Lake Tai basin region in Jiangsu Province, China. Seven plots were planted with either a poplar hybrid, cypress hybrid or a combination of both at varying densities, while the control and final plot allowed only for local vegetation to grow naturally. Soil, tree and groundwater samples were collected from all plots and analyzed for nitrogen and phosphorus concentrations. At this time in the study, results have been analyzed only for nitrogen concentrations using the ANOVA procedure. Results for both nitrogen and phosphorus concentrations are currently being analyzed.

Introduction

Non-point source pollution has exponentially grown as a cause of concern over the past century amid rapid global industrialization. There has been an increased availability and heavy use of nitrogen and phosphorus beginning in the early 20^{th} century, specifically with fertilizers in agricultural settings. Over-enrichment with phosphorus and nitrogen of aquatic ecosystems causes a wide range of problems, including toxic algal blooms, loss of oxygen, fish kills, loss of aquatic vegetation, resulting in loss of biodiversity - including species important to commercial and sport fisheries .

By 1999, The People's Republic of China had become the world's largest producer of nitrogen fertilizer and consumer of mineral fertilizers (Yan et. al). This is not surprising considering that China must feed 22% of the world's human population while only possessing 9% of total worldwide arable land. A systematic over-application of fertilizers has become a standard in order to meet the food demand of such a large population; on average 180 kg/ha of nitrogen fertilizer is applied to rice production, which is roughly 75% higher than the global average (R. Wang, 2009).

The process when a body of water rapidly accumulates available nutrients, such as nitrogen and phosphorus, and encourages the rapid growth of aquatic plant life is called eutrophication. This stimulated plant growth also accelerates the rate of decay, which requires large amounts of dissolved oxygen, and quickly diminishes the quality of water, possibly leading to large fish die-offs and even preventing safe human consumption (Le et al, 2010).

The use of fertilizers and their impact on food production and population growth in developing countries cannot be overlooked. Food production in China has more than doubled in less than 40 years, from 240 mt/year in 1970 to 530 mt/year in 2009 (Yang et. al, 2012). However, this expansion has often been coupled with poor management strategies leading to increased nutrient depositions in watersheds, which in turn, increases the likelihood of eutrophication and hypoxia in freshwater systems.

China has 2759 lakes with a surface area of 1 km^2 or greater and about one-third of these are freshwater lakes. These aquatic bodies constitute nearly 70% of all freshwater lakes in China and

are primarily located on the eastern coast of the country and in the middle and lower reaches of the Yangtze River (Le et al, 2010). Prior to the 1980's, the vast majority of these lakes were rated as grade I (suitable for human consumption) or grade II (suitable for fishing or bathing) (T.B.A., 2008). Surveys carried out from 1984 until 1985 at Lake Dianshan, within the densely populated Shanghai municipality, found nitrogen and phosphorus concentrations within the water up to 58 times greater than the recommended limit for preventing large algal blooms (Kung and Ying, 1991).

China's third largest freshwater lake by volume and the object of our study, Lake Tai, has also seen a severe reduction in water quality since economic reforms began in the 1970s. The Lake Tai Basin region is administered predominantly by Jiangsu Province (51.8%), Zhejiang Province (33.7%), the Shanghai Municipality (14.1%), and Anhui Province (0.4%), and is considered one of the most important regions for human development in China (Wang 2009). Located in the Yangtze River Delta, it is China's third largest freshwater lake by volume and is considered one of the five most famous lakes in China. Historically, the region surrounding Lake Tai was primarily an agrarian center, and up until the mid-1960's, lake water quality was graded as level I or level II with virtually no eutrophication (Le et al, 2010). As early as 1977, investigations into the increasingly eutrophication of Lake Tai as a consequence of rapid urbanization had already begun (Carlson, 1977). Since then, the frequency of eutrophication events and overall quality of the Lake Tai basin has only continued to deteriorate as economic development has increased. From 1979 to 1989, the amount of wastewater discharged into the lake nearly doubled (Le et al, 2010).

While citizens of the Lake Tai region have prospered due to economic development and increased per capita income, the effect of eutrophication on economic output cannot be flouted. There were nine eutrophication events recorded just in 1998. This negatively impacted investment and tourism in the region and resulted in an economic loss of USD $6.5 billion, thus accounting for 5.9% of the GDP that year for the region (Le et al, 2010).

June 2007 marked the worst eutrophication event in Lake Tai to date and forced the Wuxi municipal government to cut off the water supply for its four million inhabitants. It also prompted the national government to shut down all industrial activities immediately surrounding

the lake until a solution could be found to address the problem (Dr. Wongbo-personal communication 12 June 2014). At that time, there were an estimated 49.17 million people living in the Lake Tai region, accounting for 3.7% of the total population in China and 11.6% of the nation's GDP (T.B.A. 2008)

Progress for better agricultural management has been slow since the blanket closure of industrial activity in the Lake Tai basin in 2007. In the years prior to 2008, general fertilizer use in agricultural land within the Lake Tai Basin had reached 578 kg/km^2, 41 percent higher than the national average (Wang, 2009) and already well above the global average as mentioned above. In 2011, a direct survey was carried out in 15 farming villages within 5 km west of Lake Tai within Yixing and Changxing municipal districts to discern current fertilizing practices and to spread awareness of recommended best management practices (BMPs). Among the 139 farmers interviewed face-to-face, mean nitrogen fertilizer application rates were reported at 353 kg/ha and 281 kg/ha for rice and wheat respectively. It is important to note that only 11 farmers, out of 118 responders, were even aware of BMPs, and none of whom responded as practicing BMPs (Yang et. al, 2012).

Strong indications of over-fertilization and inappropriate application methods has begun to direct research focus on mitigating the growing non-point source pollution in agricultural watersheds as pressure to use more fertilizer for producing higher yielding crops mounts as China's population continues to swell. Little information exists on current research being conducted on capturing and immobilizing nutrient runoff and non-point source pollution within China, but promising results have been shown elsewhere in the world.

A joint Chinese-American study conducted in Taiwan in 2009 examined the width and slope of both natural and anthropogenic riparian buffers in various agricultural settings and found average removal rates of phosphorus and nitrogen (10-30%) to be significantly correlated with riparian widths less than 30m as the local government recommends (Chang et. al, 2010). While only examining the width and length of riparian buffers in this study, the authors recommended larger widths for said buffers to be implemented only within heavy agriculture watersheds due to the cost-effectiveness.

Research carried out in 2013 at the Dongting Lake basin, China's second largest freshwater lake, indirectly indicated forest riparian buffer strips are the most efficient method for removing non-point source pollution. Of the ten watersheds sampled, only one sample, largely comprised of natural forest, was measured to have phosphorus concentrations lower than the eutrophication criterion of 0.05 mg P/L (Li et. al, 2013), suggesting for further study into forest riparian buffers as a valid management strategy for non-point source pollution.

Most of the research in this field has been carried out in the U.S. due to the large percentage of the country's land dedicated to agriculture (Nickerson et. al, 2011). A recent comparison of mixed hardwood and native bamboo riparian buffers in a southern Illinois agricultural watershed showed a slight preference for managing the uptake of nitrogen and phosphorus between vegetation type, and most importantly demonstrated the robust efficiency of woody riparian buffers to mitigate non-point source pollution. In every plot measured, concentrations of nitrogen and phosphorus concentrations decreased up to 80% over distances as short as twelve meters (Blattel et. al, 2009), suggesting further research into woody riparian buffers is justified.

The objectives of this study were to compare concentrations of nitrogen and phosphorus in soil, groundwater and trees of varying densities in 50 m wide poplar and cypress riparian buffers on an agricultural watershed in the Lake Tai basin, and to examine if one particular species or a combination of both at a given density would be more or less effective at the uptake of nitrogen and phosphorus. Specifically, the hypotheses surmised that: (1) both poplar and cypress would reduce N and P concentrations; (2) higher tree densities would be more efficient at reducing N and P concentrations; (3) a combination of both poplar and cypress would be the most effective in reducing N and P concentrations relative to tree density.

Materials & Methods

Site Description & Field Methods

The study site was situated between the shores of Lake Tai and a rice-phragmites rotation farm at coordinates 31.435371N, 120.026328E, in the town of Zhoutie, Yixing municipal district in Jiangsu Province, China. During the study, the bulrush phragmites crop was in the current rotation. The land was provided by Jianwei Chen, a local plantation owner who primarily grows and manages hybrid poplar on 400 hectares. The site belongs to the subtropical monsoon climate region with an annual precipitation of 1200 mm and an average annual temperature of 16°C.

All eight plots were adjacent but spatially separated by an irrigated, one meter wide shallow ditch with natural grass acting as a buffer strip. Slope was negligible, accounting only for a 0.3% gradient. Hybrids of Poplar (*Populus deltoides* X *euramericana*) and Cypress (*Taxodium ascendens* X *mucronatum*) were chosen as study species due to their growing economic importance for industrial use in China (Zhang, 1999) and their tolerance for soils with high water content. Trees were planted at the depth of 60 cm, and each tree was roughly 1 m in height when purchased and planted in January of 2014.

Treatments consisted of: (1) poplar, 2 m x 3 m; (2) cypress, 2 m x 3 m; (3) poplar and cypress, 2 m x 5m; (4) poplar, 2 m x 5m; (5) cypress, 2 m x 5 m; (6) poplar, 5 m x 5 m; (7) cypress, 5m x 5m. The last plot had no planted trees and acted as the control, allowing only natural vegetation to grow on it. Samples were collected on June 10th, 2014 along a transect of 0 (field edge), 5, 15, 30, and 50 m from the crop field edge perpendicular to the farm, near both boundaries and in the middle of each plot. The trees had reached an average height of 2.7 m at the time of data collection.

Estimates for average annual fertilizer application rates in the adjacent farm were 240 kg/ha, administered by spraying during the spring. Average pH level at the study site was measured to be 7.0. Soil samples were collected using a 1 m soil auger at depths of 0-20 cm and 20-40 cm along both edges and along the middle transect. Water samples were collected in 100 mL bottles only along the middle transect of each plot using a hand-action vacuum pump from three, 10 cm-diameter PVC pipes placed directly on the ground and adjacent to one another. Each PVC pipe

was placed below the soil at a depth of 20, 40, and 60 cm, respectively. All water left in the PVC pipes was pumped out and removed after the initial sample was taken, allowing them to be filled again for future collections. Tree leaves were collected by hand along both edges and in the middle transect of all seven plots containing trees. One plot, acting as the control, had no trees planted thus allowing the natural vegetation to grow.

Laboratory Methods

Due to time constraints, only analyses to determine the concentration of Total Nitrogen (TN), the sum of nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), ammonia-nitrogen (NH_3-N), and organically bonded nitrogen within water and soil were completed.

Water samples of 1 mL were extracted from the collected samples and placed into 25 mL test tubes. Then 12.5 mL of a 2.25% solution of potassium peroxydisulfate (20 mL) and sodium hydroxide (3 mL) were added to each sample before being wrapped and sealed by a cotton cloth and placed into an autoclave for sterilization. After 2.5 hours, the samples were removed from the autoclave, and 11.5 mL of distilled water was added to each sample and thoroughly mixed by hand. Each sample was then individually analyzed for TN concentrations using a Shimadzu brand Ultraviolet-Visible Spectrophotometer, model #UV-2550.

Nitrate concentrations within the soil were also determined using a Shimadzu brand Ultraviolet-Visible Spectrophotometer, model #UV-2550. Collected samples were kept in a refrigerator to maintain initial water content at time of collection before analysis of concentration. About 5 g was extracted from each collected soil sample, cleaned of visible organic material, weighed within an accuracy of 0.01 g, and then placed into 200 mL plastic tubes. A solution of 50 mL of 15% potassium chloride (KCl) was added to each 5 g sample, and then placed into an electric shaker to thoroughly mix each sample for 30 minutes or until attaining a homogeneous state. Afterwards each sample was poured into a 150 mL Erlenmeyer flask through standard filter paper to remove any particulate matter. Each sample was then run through the UV spectrophotometer.

Determination of ammonium concentrations within the soil was conducted using the same UV spectrophotometer. After each sample was poured into a 150 mL Erlenmeyer flask through

standard filter paper to remove any particulate matter for nitrate concentrations for nitrate analysis, 5 mL was extracted and placed into 50 mL flasks. A series of three chemical solutions was then added to each flask before being placed into the machine:

1) 5mL [10g (C_6H_5OH) + 100mg ($Na_2Fe(CN)_5NO \cdot 2H_2O$) + 1L H_2O]

2) 5mL [10g (NaOH) + 7.06g($Na_2HPO_4 \cdot 7H_2O$) + 31.8g ($Na_3PO_4 \cdot 12H_2O$) + 52.5g$\cdot 1L^{-1}$(NaOCl) + 1L H_2O]

3) 1mL [400g$\cdot L^{-1}$ ($KNaC_4H_4O_6 \cdot 4H_2O$) + 100g$\cdot L^{-1}$ ($C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$)]

For every 100 mL of resulting solution produced, 0.5 mL of a 10% sodium hydroxide solution was added. Then each sample was allowed to sit without disturbance for one hour. After one hour, 34 mL of water was added to each sample and mixed thoroughly by hand. Then we filled each flask to the 50 mL limit before being placed into the spectrophotometer for analysis.

Statistical Analysis

Initial results were determined using an ANOVA and Tukey test comparisons for both NO3 concentrations within soil, and TN concentrations within water.

Groundwater and soil nutrient data were analyzed according to a randomized block design with measurements through time and space (distance from the field edge) using a mixed model procedure. The eight plots were the blocks in the model and were considered a random effect. Fixed effects included tree species, density of trees, and distance from field edge. Log transformation is done to meet the normality assumption. A constant of one was added to the raw data to avoid negative and zero values prior to log transformation.

The least squares means procedure was used to compare nutrient concentrations between tree densities at each distance (theta = 0.05). Changes in nutrient concentrations over distance was also analyzed separately for each site using a simple linear regression procedure.

Analysis of variance with a Tukey's mean separation procedure was used to test for differences in TN in groundwater and soil among the eight plots.

Results

Nitrogen

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0.03348	7	0.004783	0.553586	0.788487	2.249024
Columns	0.243397	4	0.060849	7.042966	0.000217	2.605975
Interaction	0.22587	28	0.008067	0.933688	0.56924	1.758583
Within	0.345589	40	0.00864			
Total	0.848335	79				

Table 1. Initial results of nitrate (NO3) in ppm concentrations within soil, where columns (distances from field edge) had a significant effect on reducing NO3 at 95% confidence interval, with a P-value of 0.000217.

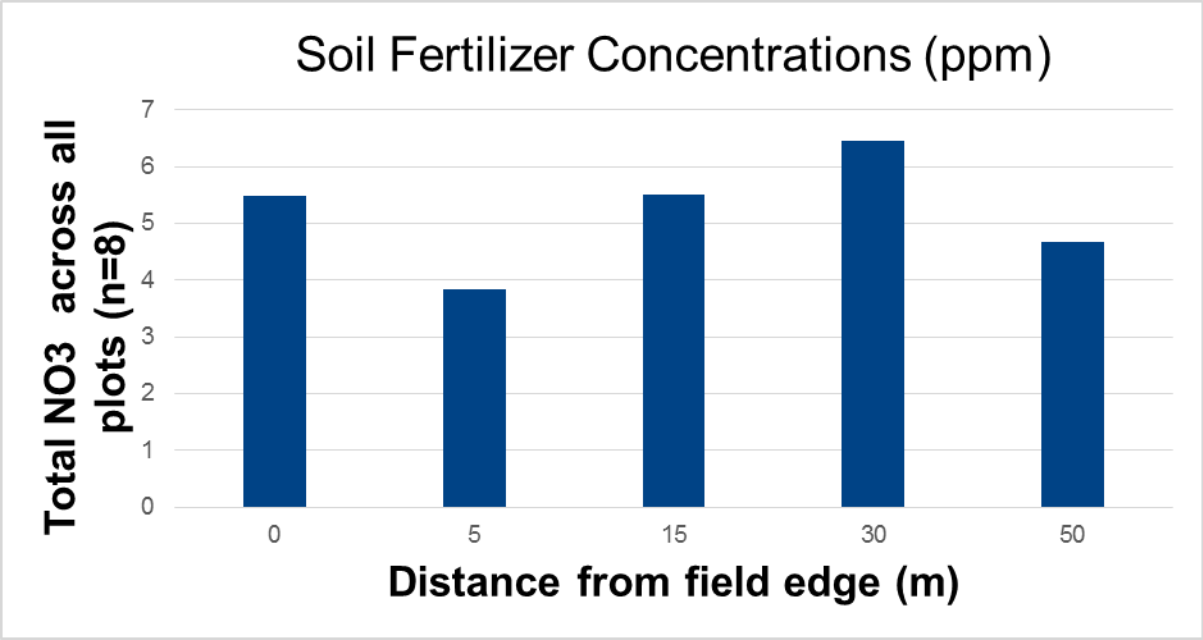

Figure 1. Average totals of nitrate (NO3) concentrations in ppm at the five distances from the samples collected from across all plots.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	11.54191	7	1.648844	1.789488	0.100778	2.126324
Columns	31.99	4	7.9975	8.679676	7.17E-06**	2.485885
Interaction	39.47392	28	1.409783	1.530035	0.072696	1.617112
Within	73.71243	80	0.921405			
Total	156.7183	119				

Table 2. Initial results of Total Nitrogen (TN) in ppm concentrations within water, where columns (distances from field edge) had a significant effect on reducing TN at a 95% confidence interval, with a P-value of 7.17E-06.

Conclusions

Initial examination of nitrogen concentrations in groundwater and soil within the eight plots did not reveal site specific effects of plantation species preference or tree density on nutrient attenuation.

Variation between the plots, specifically with respect to the calendar date when samples were collected, and the relatively young age of the planted trees were likely responsible for inconsistencies in nitrogen and phosphorus concentrations through the riparian buffer strips. In this northwestern region of Lake Tai basin within lacustrine plains, riparian buffer strips may be critical for the uptake and immobilization of nutrients derived from non-point source pollution in agricultural watersheds, where there may be a greater potential of nutrient runoff relative to non-agricultural watersheds.

Initial results strongly suggest each plot was able to significantly attenuate nitrogen across a gradient of 50 m. However, there was no significant difference among all eight plots, thus suggesting that there was no preference of species or tree density for the uptake and immobilization of nutrients. There are several possible reasons for a lack of plot preference. Since the trees were planted quite recently (in January 2014), their ability to uptake nutrients is limited by their size and young age. This, in turn, limited the effect that tree density has on each plot. As this is just an analysis of one data collection apart of a long-term study, it is suggested that species preference and tree density will display a significant effect on nutrient attenuation in the years to come as the trees age and their ability to uptake nutrients increases.

Future research in this subject should consider securing a vehicle to and from the study site, as some samples were lost due to shipping and human errors. Removal of excess water from the PVC pipes should be reconsidered, as similar research in this field has rarely mentioned the removal of excess water, and it could be affecting the results. The method for removing water should be re-evaluated if this study continues to do so; excess water was initially being removed and tossed onto the adjacent soil and trees, which has the potential to affect the results of this study. It was then suggested to toss the excess water into the adjacent irrigation ditch where it would have less potential to affect the results. Distances in which samples were collected from

the field edge should also be reassessed, as an unexplained increase in nutrient concentrations was found in samples collected at the 30 m mark. It is this author's suggestion that equally spaced distances from the field edge, and possibly more than five distances, should be taken into consideration to help explain this unaccounted variation.

Additional research should consider tree species locally native, especially when the use of commercial plantation species would not be economically feasible or ideal due to geographic location, topography, and water content relative to the study site in question. Investigations into other species of plants tolerant of high water contents and possessing high economic value within China, such as bamboo, should also be taken into account when feasible.

In addition, greater scrutiny of soils comprising the study site including texture, bulk density, and classification is highly recommended. Especially in watersheds with very high water depths, soils greatly influence the type of vegetation capable of growing within a given site. Long-term experiments should also be included as there are very few publications attempting such investigations within China; the only current long-term research in this field known to the author at the time of this paper's writing was Dr. Yongbo Wu of Nanjing Forestry University, who provided the field site and made this study possible.

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ISOLATION AND IDENTIFICATION OF A PEROXIDASE (TCPOD1) OF *TAMARIX CHINENSIS*, A GENE IN PHENYLALANINE METABOLIC PATHWAY, AND ITS POTENTIAL ROLE IN SALT TOLERANCE

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Abstract

Salt tolerance in plants is becoming an increasingly sought after trait as the salinity of soils are increasing around the globe and threatening the availability of agricultural land. *Tamarix chinensis* is a halophytic plant native to China that is prolific in many different soil environments, including soils of high salinity. However, the salt tolerance mechanisms within this species and its relatives are still poorly understood. In previous studies, it has been shown that enzymes involved in phenylalanine metabolic pathway are up regulated under salt stress conditions. In this present study, the sequence of a secretory peroxidase gene (TcPOD1) from the phenylalanine metabolic pathway was cloned from *T. chinensis* using RNA Ligase Mediated Rapid amplification of cDNA ends polymerase chain reaction. The sequence was then analyzed using bioinformatical tools to predict its protein structure. Furthermore, the predicted protein was compared with other peroxidases to elucidate its role in salt tolerance mechanisms. The results showed that TcPOD1 has very strong predicted structural similarities to other salt tolerance-associated plant peroxidases. The results also support that TcPOD1 from *T. chinensis* is homologous to the salt tolerance gene ThPOD5 in *T. hispida.* The evidence presented here suggests that TcPOD1 is a valuable gene for the genetic engineering of plants for salt tolerance. This may ultimately lead to the expansion of useable land for more abundant and efficient production of plants for food and other resources.

Keywords: Tamarix chinensis, *Salt tolerance, PCR, Peroxidase, Bioinformatics, Phylogenetic tree*

Introduction

The *Tamarix* genus, belonging to the Tamaricaceae family, is comprised of about 54 species, and is a native to more arid regions across North Africa and Eurasia (Cui et al., 2010). Florae of the *Tamarix* genus are woody plants that can grow as a small tree or shrub. Although they are native to drier areas, plants of the *Tamarix* genus have been successful at survival in other environments such as semi-arid areas, mountains, wetlands, and beaches (Jiang et al., 2012).

China alone has 16 species of *Tamarix* distributed nationwide. An important species among those in China is *Tamarix chinensis*, which is native to the Anuhui, Hebei, Jiangsu, Liaoning, and Shandong provinces (Yang and Gaskin, 2007). Although *Tamarix* is considered a noxious invasive species in the American Southwest, *T. chinensis* is harmless to its native environment. In China, it has many useful applications such as erosion control, windbreak, and honey production, and ornamental purposes (Jiang et al., 2012). The wood can also be cultivated as used for firewood and lumber (Everitt, 1980). The dried twig of *T. chinensis* is also used in Chinese herbal medicine to treat measles without adequate eruption, rheumatoid arthritis, and neuralgic pain in joints (Liu et al., 2010).

In addition to the many different uses for *T. chinensis*, the cultivation and investigation of the species is important because it is a halophyte, a type of plant that can survive and reproduce in environments of high soil salt concentration (Busch and Smith, 1995). About 1% of plants in the world are considered halophytes and can successfully complete their life cycles in concentrations of about 200mM NaCl (Flowers and Colmer, 2008). The high salt tolerance of halophytes are becoming even more important to study as soil salinity has been found to be increasing in many parts of the world, causing a decrease in conventional crop yields due to low quality irrigation water, high evaporation-to-rainfall ratios, and rising coastal lowland sea levels related to global warming (Munns, 2005). Therefore, studying the salt tolerance characteristics and mechanisms in halophytes like *T. chinensis* are crucial in discovering ways to protect crop yields and allow for the utilization of arable land.

When exposed to high salinity, the salt stress induces several interconnected types of resulting stress: osmotic, ionic, and reactive oxygen species (ROS). Osmotic stress occurs early in high

salinity exposure, which decreases water potential and leads to inhibitions in water uptake, water transport, cell elongation, and leaf development (Munns and Tester, 2008). The high concentration of ions in the soil environment draws water out of the roots, causing plant dehydration (Horie et al., 2012). To cope with ionic and osmotic stress, plants accumulate different ions, solutes, and organic compounds to reestablish osmotic pressure. This strategy also helps in reestablishing ion homeostasis by sequestering ions into vacuoles and compartmentalizing others into older cells for eventual sacrifice (Parida and Das, 2005). Lateral root formation to increase total root surface area has also been observed (Zolla et al., 2010). Although ion buildup is a method for dealing with osmotic stress, accumulating ions and ROS creates a toxic environment, causing leaf chlorosis and necrosis, and decreases in activity of essential cellular metabolisms like photosynthesis, protein synthesis, and enzyme activity (Hasegawa et al., 2000; Parida and Das, 2005; Horie et al., 2012). However, at high enough salt concentrations, even these common strategies are not sufficient for the survival of 99% of all plant species that are non-halophytic, the most sensitive of which are main agricultural crops like rice (Parida and Das 2005). Salt stress inhibits dry matter accumulation of the plant, as growth of plant tissues and organs are negatively affected due to the deterioration of metabolic processes and diminishing photosynthetic efficiency (Flowers and Yeo, 1995; Parida and Das, 2005; Munns and Tester, 2008). Although growth is inhibited, the overall life cycle of the plant is more rapid as developmental stages such as the vegetative and flowering phases are induced sooner and last for shorter periods of time, ultimately stunting the life of the plant (Xia et al., 2002; Li et al., 2008).

Besides the common strategies of regulating osmotic balance, ion homeostasis, and ROS scavenging, halophytes like *T. chinensis* have evolved more special and effective salt tolerance strategies that non-halophyte plants do not have. The root systems of *Tamarix* species have mechanisms to resist salinity in the environment more effectively with increasing salt concentration. Li (2002) showed that when in 200mM NaCl, 96.8% of the salt was not absorbed by the root. *T. chinensis* is also able to actively excrete excess salt through glands in its leaves that would otherwise continue to accumulate to toxic levels in non-halophytes (Wilkinson, 1966; Storey and Thomson, 1994). About half of the total salt in the stems and leaves are secreted to the external of the salt glands, then are blown off by wind or washed off by rain (Zhang et al.,

2003). Members of the Tamaricaceae family also have succulent leaves which house extra secretory cells and vacuoles compared to regular leaves (Dang et al., 2013). About half of the salinity absorbed by the roots is kept in the plant cell. Besides the need for physiological activity, a high salt concentration of a vacuole creates a higher osmotic potential for plant (Ding et al., 2007). Other *Tamarix* species have also responded to salt stress by synthesizing solutes to protect enzymatic activity and cellular osmotic potential such as amino acids and modified amino acids, as well as antioxidants to help scavenge and neutralize ROS (Cui et al., 2010; Carter and Nippert, 2011).

However, the full extent of halophyte mechanisms for salt tolerance is still widely unknown, as there is no one mechanism and set of genes that is solely responsible. There is also a great deal of salt tolerance mechanism variability between species even within a genus such as *Tamarix*. Other species such as *T. hispida* and *T. ramosissima* have been the most commonly studied of the *Tamarix* genus, mostly with respects to its prevalence as an invasive species in the New World (Natale et al., 2010; Carter and Nippert, 2012). Since not much is known about *T. chinensis*, this research is valuable to expand the study of this plant to investigate its salt tolerance characteristics to apply to the development of superior transgenic salt-tolerant plants.

Many pathways have been proposed to be involved in salinity stress response in halophytes, including but not limited to the mechanisms associated with salicylic acid, broassinosteroids, abscisic acid, gibberellic acid, jasmonic acid, auxin, ethylene, sucrose non-fermenting related protein kinase, mitogen activated protein kinase (MAPK), and phenylalanine (Geng et al., 2013; Deinlein et al., 2014; Golldack et al., 2014). According to the result of Illumina transcritome sequencing of *T. chinensis*, a potential genes was chosen from the phenylalanine metabolic pathway. This pathway was selected for this study because it has been reported to be highly active under high salt concentration in compared with the control.

The gene in this study is referred to as TcPOD1, a potential secretory (class III) plant peroxidase associated with the phenylalanine metabolic pathway (Mei et al., 2009). In previous studies, salt stress has been associated with an increase in phenylalanine and an up regulation of genes in its synthesis pathway among broad transcriptomic profiles (Sanchez et al., 2004; Sahi 2006; Kim 2007; El-Samad 2010; Dang 2013). Dang et al. (2013) recently conducted transcriptomic

profiling on *Reaumuria trigyna* and concluded that phenylalanine metabolic pathway has a large ratio of differently expressed genes when put under salt stress. *R. trigyna* is also a member of the Tamaricaceae family and is therefore closely related to *T. chinensis*. The study on *R. trigyna* provides evidence that the phenylalanine metabolic pathway may be associated with salt tolerance in *T. chinensis*. The phenylalanine metabolic pathway contains plant peroxidases (PODs), including secretory class III PODs, which have been shown to play key roles in plant cellular ROS scavenging and detoxification under salt stress conditions, and are up regulated by salt stress in plants (Sreenivasulu et al. 2000; Menezes-Benavente et al. 2004; Gao 2010). Gao et al. (2010) also observed up regulation in the secretory peroxidase ThPOD5 gene in *T. hispida* roots with high salinity treatment compared to the control. This *T. hispida* study provides evidence that ThPOD5 may be homologous to TcPOD1 in *T. chinensis*. The objective of this current study is to clone the TcPOD1 gene and analyze its sequence to determine its potential role in salt tolerance. Delineating the response of *T. chinensis* to salt stress will be vital to subsequent engineering of more salt tolerant crops that will prevent crop loss while increasing the potential for high-salinity land to become arable.

Materials and Methods

NaCl Treatment for Salt Response Gene Detection

Three *T. chinensis* trees were selected from a clonal population to ensure they are genetically identical. The roots were harvested from perennial plants and all roots were cut into smaller sections. These root sections were submerged in a salt solution of 2% NaCL for 0.5, 1.5 hours. As a control, some roots were submerged in water for 1.5 hours. Following treatment, these roots were flash-frozen in liquid nitrogen and stored at -80°C for future use. A sample from each of the three groups of roots were sent to Novogene for Illumina transcriptome RNA sequencing to detect the level of gene expression in response to increased time submerged in the 2% NaCl solution. Putative salt tolerance genes were detected based on their differential expression. This Illumina sequence was the basis for primer design and subsequent gene sequence alignment.

RNA Extraction & Purification

Frozen *T. chinensis* leaf tissue (0.1g) was ground to a fine powder with a mortar and pestle and regular application of liquid nitrogen. Total RNA was extracted from the ground leaf powder using the Qiagen RNeasy Plant Mini Kit (Qiagen Co., Ltd., China). It was possible to use RNA from untreated leaves because the Novogene analysis showed that the expression of TcPOD1 was present in the control, but to a much lesser extent. RNA was purified by the addition of DNase I to digest any residual DNA, followed by several steps of phenol chloroform extraction and isopropanol precipitation. The pellets were dissolved in 20µl DEPC-treated water and RNA concentration was determined with the NanoDrop 2000c Spectrophotometer (NanoDrop Technologies, New Zealand). Quality and size of the RNA was checked by electrophoresis in a 1% agarose TAE gel.

3'RLM-RACE by nested PCR cDNA Synthesis

Complimentary DNA synthesis of the gene of interest was carried out using Ambion FirstChoice® RNA Ligase Mediated Rapid amplification of cDNA ends (RLM-RACE) kit (Thermo Fisher Sientific, U.S.A.). This step required one PCR run with an outer primer and a second with an inner primer, functioning as a pair of nested primers. In addition to the supplied adapter primers, outer and inner gene-specific primers (GSPs) were designed specific to each gene to be amplified from the full length sequence for the 3' RLM-RACE cDNA as shown in Table 1. Oglio 6 software was used to design and analyze the primers to ensure an appropriate annealing temperature and to minimize hairpins, mismatches, and primer dimers. A 3' RLM-RACE PCR was conducted for the gene with Takara polymerase (Takara Bio Inc., Japan). The size and quality of the PCR product were observed using a 1% agarose TAE electrophoresis gel. The bands of the successfully amplified gene cDNA sequence were cut out of the gel and recovered using a Generay Biotech DNA Gel Cleanup Kit (Generay Biotech Co., Ltd., China).

Table 1. Designed RACE GSPs.

Primer Name	**Sequence 5'-3'**
TcPOD1 3' Outer Primer 239-259	TGGTATGCCTCGTCTGGTTGT
TcPOD1 3' Inner Primer 532-561	AAGTCTAAAGTGGAGAGCGTCTGTCCTGGT
TcPOD1-CORF-Forward	AACCCAAACTTTCTCCTTCCCTATTC
TcPOD1-CORF-Reverse	CACCTTGCCTGTCCGATTGT

Gene Cloning

The purified 3' RACE PCR product was ligated into a pMD19-T plasmid (Takara Bio Inc., Japan). The plasmid was then transformed into Top Ten E. coli (Tiagen, China) cells by heat shock. The cells were then grown on an LB plate with ampicillin to enable the growth of only cells that contain the antibiotic resistant plasmid that was constructed. Multiple colonies from each plate were selected and cultured in liquid LB with ampicillin. A PCR of the liquid bacterial cultures were run and the sizes of the bands were determined on a TAE 1% agarose gel. Liquid cultures possessing the correct bands were sent for plasmid extraction sequencing (Bejing Genomics Institute, China) to confirm that the correct gene was amplified.

Bioinformatics Analysis

Bioinformatical analysis was carried out to identify the gene of interest and determine its similarity with genes from other plant species. The primer sequence was removed prior to analysis. The gene sequence was entered into NCBI BLAST and the degree of similarities in the sequence with other plant species peroxidases was determined. This information was used to construct a phylogenetic tree to diagram species similarities with the gene sequences using Mega 6. Further analysis was employed to study the protein structure in detail and the results were compared with other plant species. A list of Bioinformatics tools are given in Table 2.

Table 2. Bioinformatics Programs Used for TcPOD1 Gene Sequence Analysis.

Title	Abbreviation	Analysis Type	Source
Basic Local Alignment Search Tool	BLAST (nucleotide) BLASTP (protein)	Sequence alignment	Altschul, et al., 1990
ProtParam	-	Physical and chemical protein sequence parameters	Gasteiger, et al., 2005
ProtScale	-	Protein Hydrophobicity/ hydrophilicity	Kyte and Doolittle, 1982; Gasteiger, et al., 2005
Self-optimized Prediction Method	SOPMA	Protein secondary structure analysis	Geourjon and Deleage, 1995
Transmembrane Prediction	TMpred	Protein transmembrane region and orientation prediction	Hofmann, 1993
Transmembrane hidden Markov model	TMHMM	Protein transmembrane	Sonnhammer, et al., 1998
SignalP-4.1	-	Signal peptide cleavage sites prediction	Peterson, et al., 2011
SWISS MODEL	-	Homology modelling tertiary structure prediction	Arnold, et al., 2006; Guex, et al., 2009; Kiefer, et al., 2009; Biasini, et al., 2014
Mega6 (Unweighted Pair Group Method with Arithmetic Mean)	UPGMA	Phylogenetic tree	Tamura, et al., 2013

Results

Primary Structure and Alignment

A comparison between the sequence obtained with 3'RACE to that obtained by the initial Illumina sequence were nearly identical. Consequently, bioinformatics tools were employed. ProtParam predicted that TcPOD1 exhibited one open reading frame (ORF) that had the potential to be the complete coding DNA sequence (CDS). The CDS encodes a polypeptide that is 324 amino acids in length, with a predicted molecular mass of 34.1kDa and pI of 8.34. The instability index was computed to be 35.02, indicating that he protein is stable. Furthermore, BLASTP characterized the TcPOD1 gene as a member of the plant peroxidase-like superfamily, and specifically matched with secretory (class III) peroxidases. Other sequences producing significant alignments to TcPOD1 from the BLASTP were also used for subsequent sequence and structure analysis alongside TcPOD1. The top match was the class III plant peroxidase

ThPOD5, which showed 95% nucleotide sequence identity and 85% amino acid sequence identity (Fig. 1) to TcPOD1. ProtScale predicted five troughs below a score of -0.5, predicting that the protein is hydrophilic, though two peaks above 1.5 were also present, which predicted distinct hydrophobic regions (Fig. 2).

Score	Expect	Method	Identities	Positives	Gaps
516 bits(1329)	0.0	Compositional matrix adjust.	272/320(85%)	282/320(88%)	5/320(1%)

```
Query  10   FEFPTVVVCLVWLFSGILASAQLTTSFYSTTC--PNALSTIQTE-VKKAVAKEKR-MGAS  65
            EFPTVVVCLVW FSGILASAQLTTSFY TTC   +A+   +TE  +KAVA E+   G
Sbjct  1    MEFPTVVVCLVWFFSGILASAQLTTSFYKTTCSGKSAVDHFKTEGEEKAVANEENAWGLP  60

Query  66   LLRLHFHDCFVNGCDASVLLDDTANF-TGEKTALPNNGSLRGFDVVDTIKSKVESVCPGV  124
                 FHDCFVNGCDAS  +          KTA PNNGSLRGFDVVDTIKSKVESVCPGV
Sbjct  61   CFGFIFHDCFVNGCDASGSIRRHCQLHRKRKTAQPNNGSLRGFDVVDTIKSKVESVCPGV  120

Query  125  VSCADILAVAARDSVVALGGKSWGVLLGRRDSTTASLSAANTGIPAPTLNLSGLITSFSN  184
            V CADILAVAARDSVVALGGKSWGVLLGRRDSTTASLSAANTGIPAPTLNLSGLITSFSN
Sbjct  121  VPCADILAVAARDSVVALGGKSWGVLLGRRDSTTASLSAANTGIPAPTLNLSGLITSFSN  180

Query  185  VGLSTKDLVVLSGAHTIGQARCTSFRARIYNETNINSSFAKSLQSNCPSTGGDNNLSPLD  244
            VGLSTKDLVVLSGAHTIGQARCTSFRARIYNETNINSSFAKSLQ+NCPSTGGDNNLSPLD
Sbjct  181  VGLSTKDLVVLSGAHTIGQARCTSFRARIYNETNINSSFAKSLQANCPSTGGDNNLSPLD  240

Query  245  TSSPTTFDIGYYTDLVGQKGLLHSDQQLYNGGSTDSQVKSYSSSSSTFLTDFGTSMINMG  304
            TSSPTTFD+GYYTDL+GQKGLLHSDQQLYNGGSTDSQV SYSSSSSTFLTDFGTSMINMG
Sbjct  241  TSSPTTFDVGYYTDLIGQKGLLHSDQQLYNGGSTDSQVTSYSSSSSTFLTDFGTSMINMG  300

Query  305  NISPLTGSSGQIRTNCRKTN  324
            NISPLTGS GQ+RTNCRKTN
Sbjct  301  NISPLTGSRGQVRTNCRKTN  320
```

Figure 1. BLASTP alignment with TcPOD1 (Query) and ThPOD5 (Subject) showing high degree of identity.

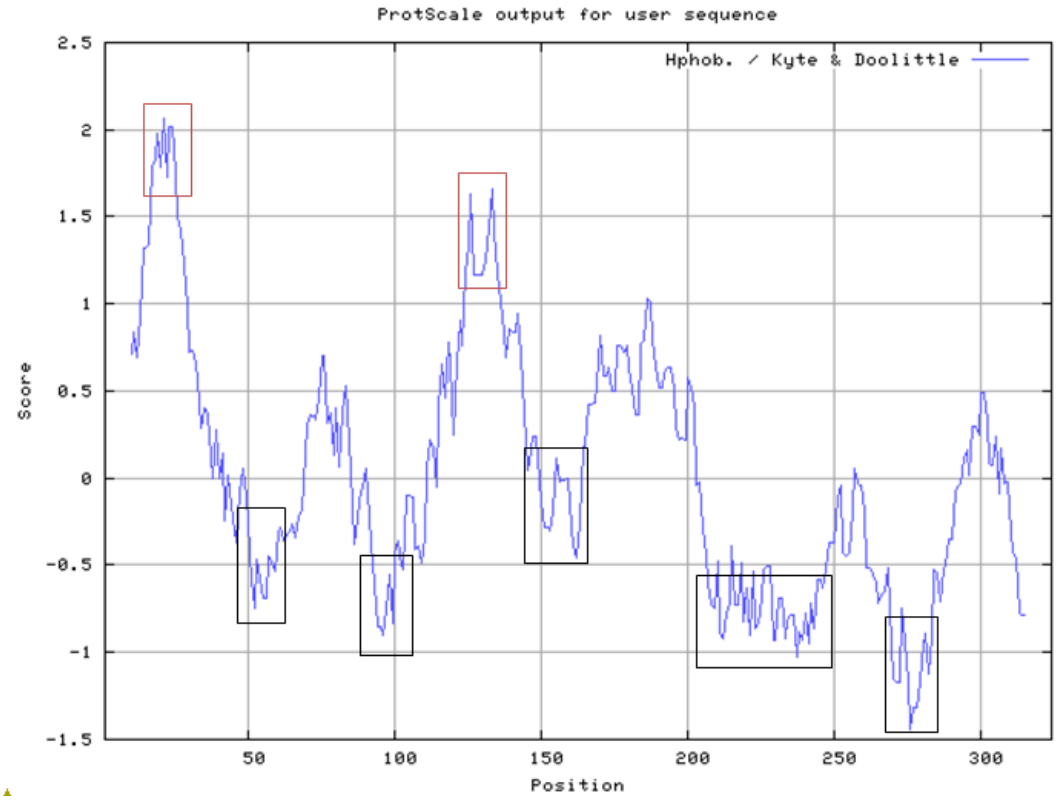
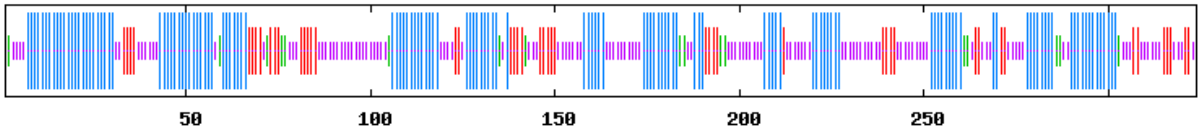


Figure 2. ProtScale hydrophobicity test of TcPOD1. The graph shows five troughs below a score of -0.5 (black boxes), predicting that the protein is hydrophilic, though two peaks above 1.5 were also present (red boxes), which predicted distinct hydrophobic regions.

Secondary and Tertiary Structure

SOPMA predicted the TcPOD1 protein would contain 41.67% alpha helices, 38.58% random coils, 14.51% extended strands, and 5.25% beta turns (Fig. 3). Transmembrane prediction was conducted with TMpred and TMHMM, which suggested that there was a high probability of a hydrophobic transmembrane helix near the N-terminus spanning amino acid 12-34 (Fig. 4 and 5, respectively). The SignalP-4.1 (3.0 Parameters) predicted a signal peptide in the N-terminus and a cleavage site between amino acid position 28 and 29 (Fig. 6a). 14 of the 17 significant sequence alignments from the BLASTP also had predicted signal peptide in the N-terminus, with cleavage sites located between the 19-34 amino acid positions (Fig. 6b). Upon using the more stringent 4.0 Parameters to a higher threshold, TcPOD1 was the only gene that did not pass threshold level that confirmed signal peptide was present. A 3D model of the predicted TcPOD1

tertiary structure from amino acid position 31-324 was constructed on SWISS-MODEL (Fig. 7). Peanut peroxidase (model number 1schB) was used as a template for the modeling of TcPOD1 because it showed 75% identity and an Evalue of 4.58e-117. Typically template sequence with identity of at least 40% is considered a good match. The coloration ranged from blue (highly probable structure) to red (highly uncertain structure).



BLUE: Alpha helix 41.67%
RED: Extended strand 14.51%
GREEN: Beta turn 5.25%
PURPLE: Random coil 38.58%

Figure 3. SOPMA secondary structure analysis of TcPOD1 exhibiting a main composition of alpha helices and random coils.

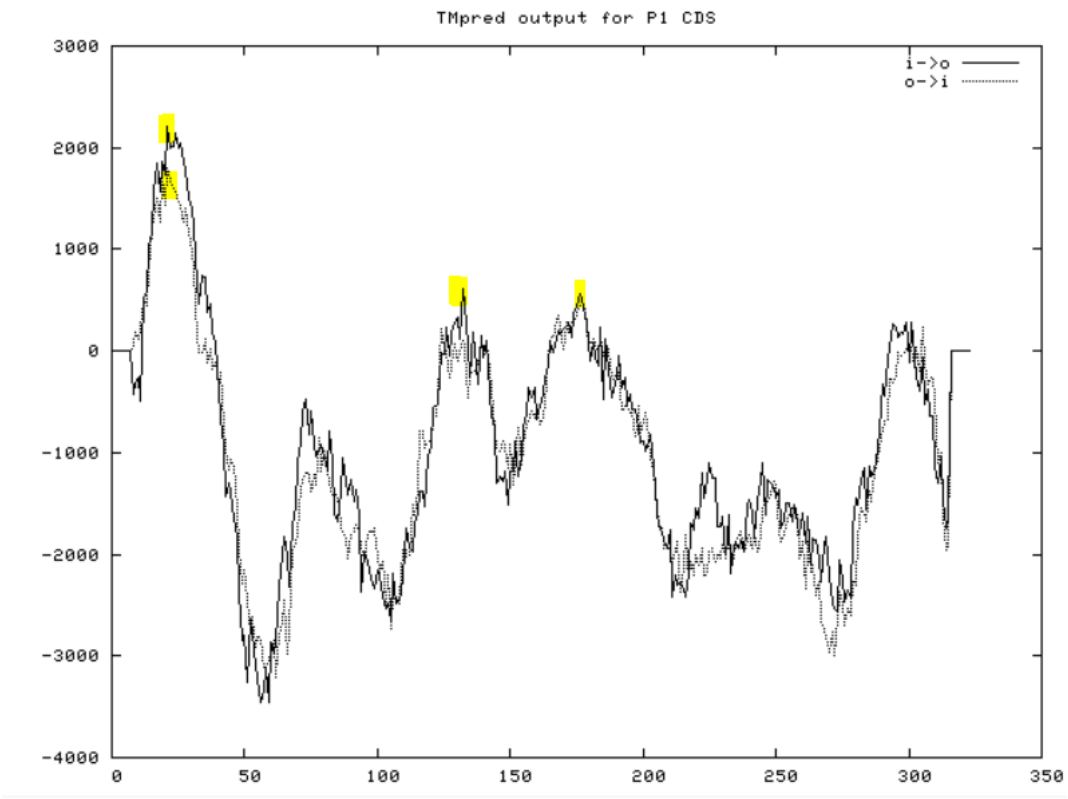


Figure 4. TMpred transmembrane prediction and orientation analysis of TcPOD1. Scores above 500 are considered significant potential transmembrane regions (in yellow).

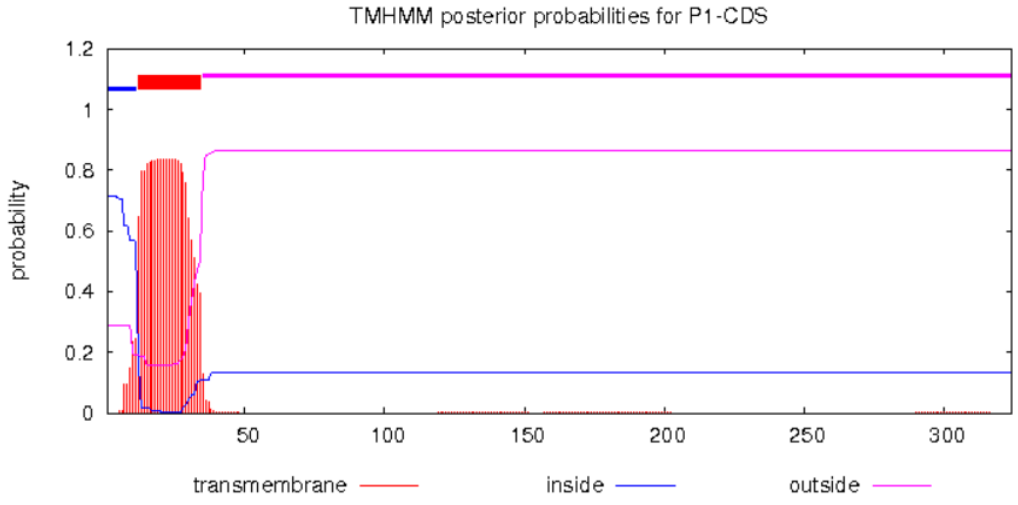
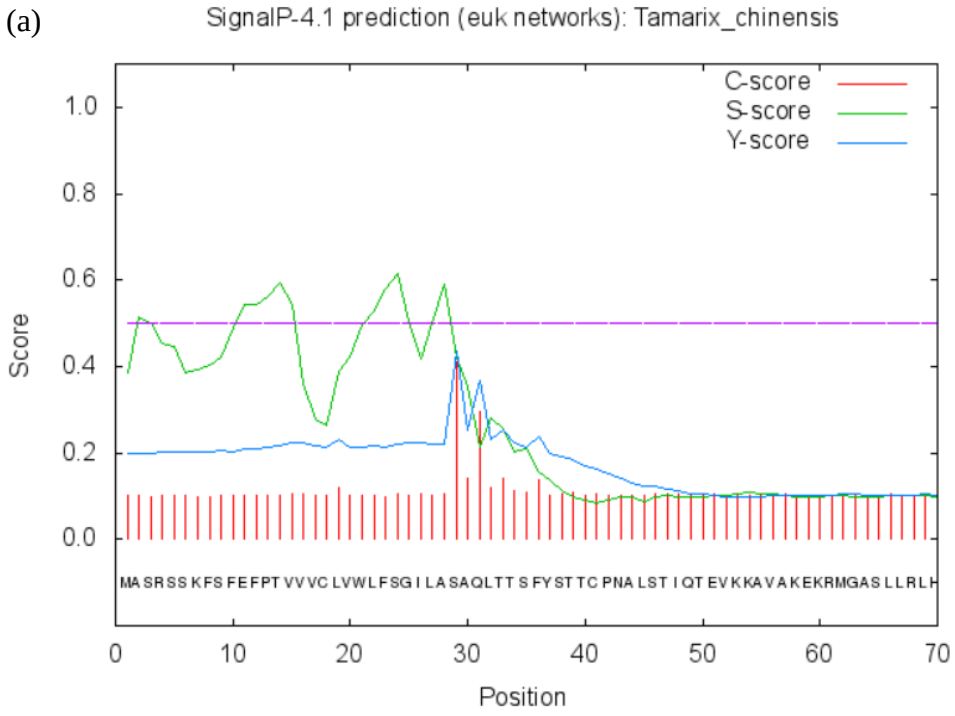


Figure 5. TMHMM transmembrane analysis of TcPOD1 showing a high probability of a transmembrane region near the N-terminus.

(a)



(b)

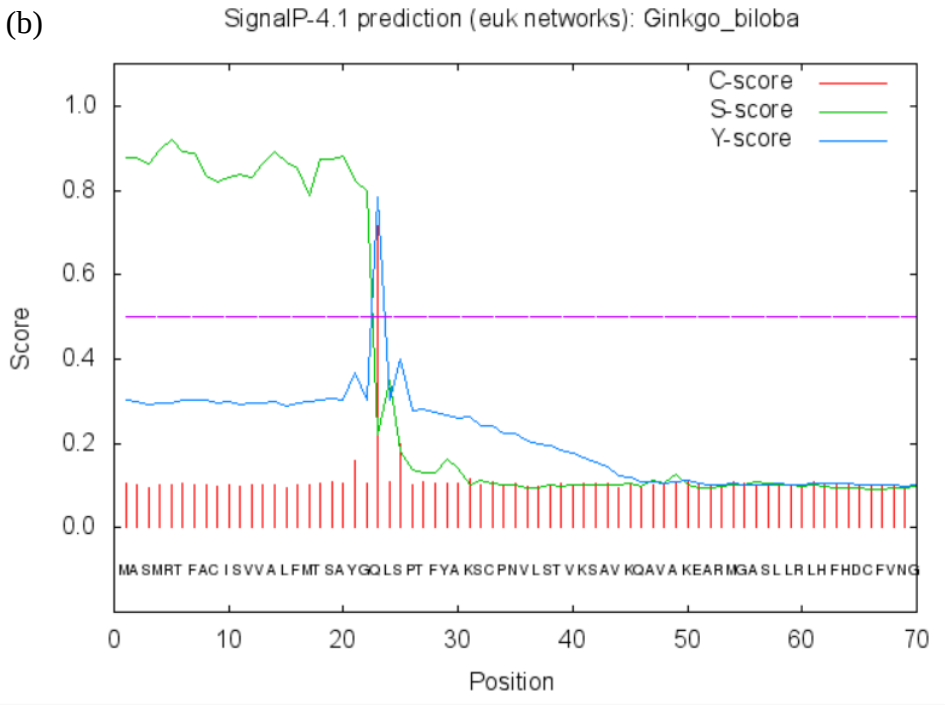


Figure 6. The SignalP-4.1 (4.0 Parameters) prediction for a signal peptide in (a) TcPOD1 and (b) Ginkgo biloba as an example of the stronger signal peptide predictions of high identity match peroxidases compared to TcPOD1.

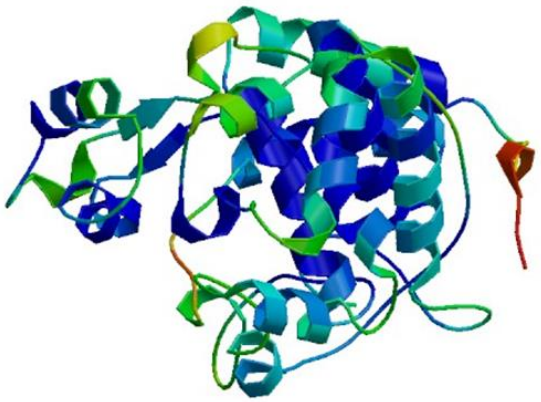


Figure 7. SWISS-MODEL 3D prediction of TcPOD1. Peanut peroxidase (model number 1schB) was used as a template for the modeling of TcPOD1 because it showed 75% identity and an Evalue of 4.58e-117. Typically template sequence with identity of at least 40% is considered a good match. The coloration ranged from blue (highly probable structure) to red (highly uncertain structure).

Phylogenetic Analysis

Mega 6 was used to construct a phylogenetic tree for TcPOD1 and 17 other peroxidase-like genes of different plant species that exhibited high identity from the BLASTP (Fig. 8). The tree grouped the peroxidases T. chinensis and T. hispida together. It also grouped peroxidases closely with similar plant species, such as branches of grouped legumes, gymnosperms, and monocots.

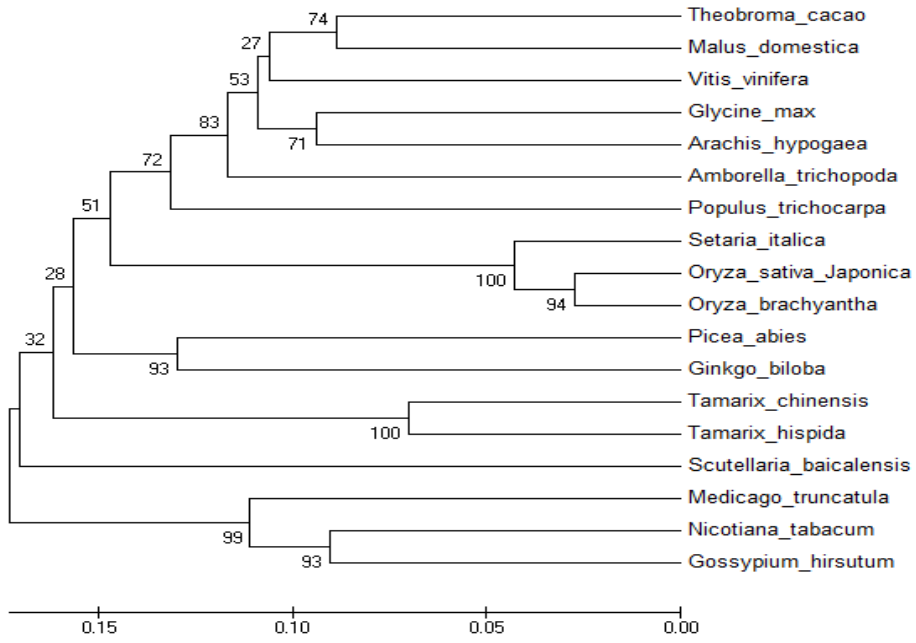


Figure 8. UPGMA phylogenetic tree of secretory plant peroxidase genes of *T. chinensis* and 17 other peroxidase-like genes of different plant species that exhibited high identity from the BLASTP.

Discussion

The primary protein sequence was consistent with the typical characteristics of a class III plant peroxidase (Hiraga et al., 2001; Guo 2009; Meng et al., 2011), and the BLASTP characterized it as such, showing high identity to many other secretory plant peroxidases. The very high identity and sequence predictions between TcPOD1 and ThPOD5 from Gao et al. (2010) further supports homology between *T. chinensis* and *T. hispida*; both exhibit up regulation in roots during salt treatment compared to the control. The prediction of an N-terminal sequence in TcPOD1 further implies that it is a secretory protein. This suggests that after being fully translated, the protein will be transported to the endoplasmic reticulum for further modification before being secreted. Conversely, the analysis also showed that this same N-terminus region in TcPOD1 could be a membrane bound alpha helix instead of a signal peptide. These predicted structures can often be difficult to distinguish (Peterson et al., 2011), however, it was concluded that this structure was a signal peptide and not a transmembrane helix because signal peptides are common in the other

secretory peroxidase genes selected from the BLASTP. However, it is worth nothing that the TcPOD1 signal peptide prediction had the least confidence out of all of the genes confirmed to have such a structure, and that the signal peptide on TcPOD1 is unique within its close gene matches. This uniqueness could be a focus of further analysis. The 3D model of TcPOD1 with the peanut peroxidase template was mostly dark blue and light blue, showing that the predicted model has a high degree of certainty, and is therefore a good prediction of the tertiary structure. The phylogenetic tree of the 18 plant peroxidases grouped many of the species according to their overall species similarity, including legumes, gymnosperms, and monocots, showing a general connection between the evolution of similar species and their peroxidases. *T. chinensis* and *T. hispida* showed the shortest pair-wise distance between each other, further supporting the homology between TcPOD1 and ThPOD5. The results of the sequence analysis strongly support that TcPOD1 is a gene that encodes a class III plant peroxidase that is associated with salt tolerance response.

In this study, the 3'UTR and the CDS of TcPOD1 were obtained and analyzed. To further validate this sequence and subsequent analysis, cloning will continue to be done to obtain the complete CDS and 5'UTR so that the entire gene can be cloned and analyzed. Following the successful cloning, the total DNA will be extracted and analyzed alongside the cDNA sequence to detect the presence or absence of introns. More genes that showed up regulation under salt stress compared to the control in the Illumina sequence can also be cloned and characterized just as TcPOD1 has been. From there, transgenic functional analysis in *T. chinensis* root and leaf can be conducted and the different transgenes can be compared to test for increased salt tolerance. The same kind of transgenic functional analysis could also be conducted in other organisms, such as glycophtye species.

This study of TcPOD1 provided evidence that this enzyme can be characterized as a secretory plant peroxidase that is up regulated in the phenylalanine metabolism pathway in the presence of salt stress. This may lead to the development of salt tolerant transgenic cultivars and further expansion of arable land for more abundant and efficient production of plants for food and other resources.

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THE ADSORPTION BEHAVIOR OF BLACK CARBON IN URBAN FOREST AND TRAFFIC DISTRICT SOILS TOWARD HEAVY METAL IONS (CU, ZN)

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Abstract

In China, many pollutants due to urbanization, traffic, and mining activities contaminate soil in urban areas. These pollutants include black carbon and persistent organic pollutants (POPs). They can remain in urban soils for a long time, which may play an important role in further pollution in urban environments, and pose a potential threat to human health and ecological systems. Adsorption of Cu and Zn in single metal solutions by traffic district and urban forest soils was investigated in batch sorption experiments. The black carbon (BC) of soils was also investigated. The soil samples were taken over 0-10 cm depths from traffic district and urban forest in Nanjing, China main urban areas (118°22′–119°14′E, 31°14′–32°37′N). The results showed complete differences in adsorption capacities of the studied soils. Traffic district soil had higher adsorption capacities than urban forest soil. The adsorption sequence Cu > Zn was established for the traffic district and urban forest soil. The Freundlich and Langmuir models described the experimental isotherms for the metal sorption. Traffic district soil containing a high amount of BC is characterized by the higher adsorption capacity towards the metals, compared to urban forest soil with a lower BC content. Properties of the soils, such as pH and the content of clay and organic matter, showed a significant influence on adsorption capacities of the studied soils.

Keywords: *heavy metals, traffic district soil, urban forest soil, adsorption, black carbon*

Introduction

Urbanization in China

Soil is a vital component to our ecosystem, thus it is important to maintain good quality of soil. However, in China, maintaining soil quality has been a challenge because it is rapidly becoming more urbanized and industrialized. As the largest developing country in the world, China has achieved rapid economic development, averaging an annual growth rate of 10% in gross domestic product (GDP) over the past two decades. Urbanization in China has taken place at an unprecedented pace in the last three decades, and will continue at a striking rate over the next couple of decades (United Nations, 2010; Fig. 1) [8]. As a result of urbanization, overloaded traffic and industrialization have a negative effect on the soil quality.

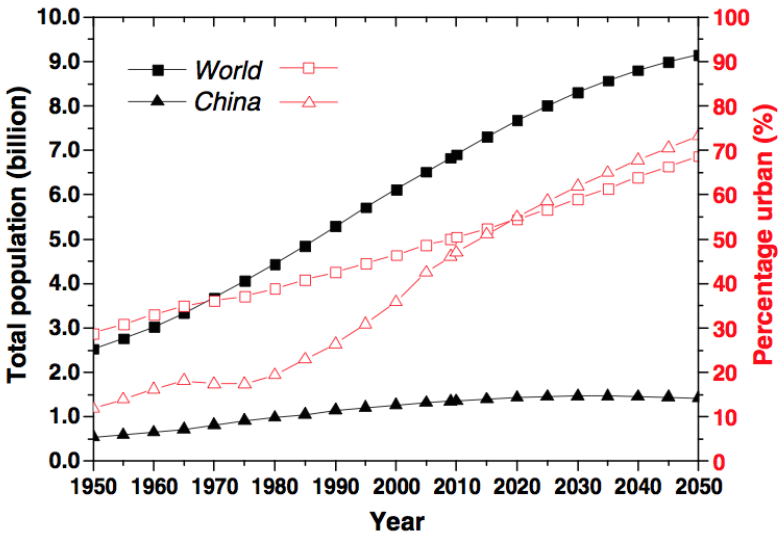


Figure 1. World urbanization prospects and rapid urbanization in China. United Nations, 2010.

The Effects of Urbanization

Heavy Metals (HM) in Soil. Urbanization also has an effect on the environment, one being heavy metal pollution in soil. Heavy metals (HM), such as Cd, Cr, Cu, Ni, Pb and Zn, in urban soils, urban road dust and agricultural soils has become significant with the rapid industrialization and

urbanization of China over the last two decades. Urban soils are contaminated by heavy metals due to modern industries, traffic and mining activities in urban areas. The pollutants can be released in many ways such as vehicle emission, chemical industry, coal combustion, municipal solid waste, the sedimentation of dust and suspended substances in the atmosphere [14]. Urban environmental quality is of vital importance because the majority of people now live in cities. Urban soils are therefore an important indicator of human exposure to heavy metals in the urban terrestrial environment [15]. As the urban area has high population density and intensive anthropogenic activities, there are a great number of sources of heavy metals in cities, placing a considerable influence on human health [14].

Environmental and Health Effects. Urbanization and its environmental impacts has caused environmental issues to become more prominent, specifically the effect on the health of the citizens that are being impacted. Urban environments have thus become supremely important with regard to human health and wellbeing [9]. Industrial and economic activities are more concentrated in urban areas, and cities have become the geographic focus of resource consumption and chemical emissions, which may cause many environmental problems [12]. Soil is a crucial component of urban ecosystems, contributing directly or indirectly to the general quality of life for city residents [10]. In urban areas, soil directly affects structural support for buildings, the filtration of water, and also roads. Because of the rapid industrialization and urbanization, environmental pollution, including urban soil pollution, has become a very important issue in China.

Urban Forest. Urban forests, which include woodlands, parks, gardens, street and square vegetation, and other green areas within urban agglomerations [16], have become a paramount and overarching scholarly focus in the 21st century as a result of rapid urbanization and the contribution of urban forests to the sustainability of urban ecosystems and urban life quality [17-19]. Urban landscapes in China are undergoing unprecedented transformation because of rapid urbanization accompanied by extensive institutional reconstruction.

Sources of Urban Soil Pollution

Traffic District. Urban traffic is one of the major sources for urban soil pollution. The main sources of these pollutants are industrial discharges, traffic emissions, and wastes from municipal

activities [13]. Many pollutants can remain in urban soils for a long time, which may act as a source of further pollution in urban environments, and pose a potential threat to human health and ecological systems [12].

Black Carbon. Black carbon (BC) is a generic term associated with a group of carbon containing materials including graphitized soot to partially combusted residues of plants that are formed through incomplete combustion of fossil fuels, biofuels and biomass [21,22]. It is typically formed in industrial wastes, vehicle emissions, coal burning waste and other sources [21, 23]. Black carbon typically has an asymmetric spherical structure. The particles can range in size from nanometers (ultrafine) to 10's of microns (coarse). The smaller particles can remain airborne for much longer periods of time and as a result, they can be transported over long distances. The larger particles on the other hand, typically settle to the ground and can be found in soils, runoff water, rivers and oceans. Black carbon is relatively stable in the soil.

Environmental and Health Effects of Black Carbon. Smaller particles can be more detrimental to human health than larger ones. Figure 2 shows the size distribution of particles from different sources. Studies have shown that soot BC that has PAHs adsorbed to its surface can get trapped in the lungs. The PAHs can subsequently be absorbed into the bloodstream. The soot BCs may take years to be cleared from the lungs. The size of particles and their loading also has an effect. Particles less than 0.1 mm can also enter organs such as the liver.

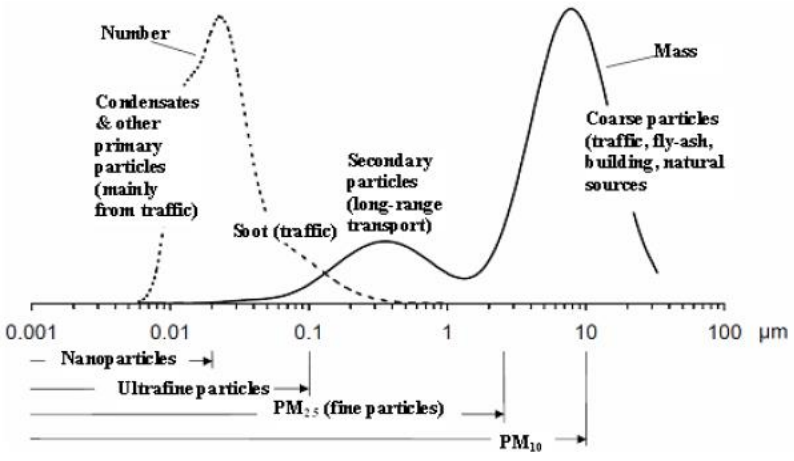


Figure 2. Size distribution of particles from different sources.

BCs have been shown to affect the climate, primarily through warming. This effect occurs via radioactive scattering and absorption in the atmosphere and to changes to system albedo (fraction of solar energy (shortwave radiation) reflected from the Earth back into space) at the land surface.

Recent Studies and Objective of This Study

Recent studies have tested the various HM in diverse soil locations, however for this study, Zinc (Zn) and Copper (Cu) in urban forest and traffic district soil samples were tested for their adsorption ability and compared to black carbon concentration. [20] Reviewed studies of heavy metal contamination in several Chinese cities over the past 10 years and have found that contamination with Cr, Ni, Cu, Pb, Zn and Cd is widespread in urban soils and in urban road dust. Once in the soils, HM can desorb into soil water and migrate to the lower soil horizons and groundwater. The risk of HM uptake by plants and the subsequent accumulation in the food chain is also possible. Therefore, investigation of processes of HM movement and accumulation in soils has received much attention [1-7].

The aim of this study was to examine the adsorption ability of Cu and Zn onto traffic district and urban forest types of soil using classical adsorption models based on the Freundlich and Langmuir equations and comparing it to the concentrations of black carbon. Relationships between soil properties and the adsorption behavior of heavy metal in cases of single metal, effects of soil organic matter content on heavy metal sorption, relations between heavy metal sorption, pH, mechanical composition of soils, and relations between heavy metal sorption and black carbon content in soil were investigated. The hypothesis for this study is traffic district soil samples will have higher black carbon concentration and higher adsorption ability in Zinc and Copper than urban forest soil samples.

Materials and Methods

The adsorption of urban traffic district soil and forest soil to metal ions was conducted in this study. Urban traffic district soil contains a high level of black carbon due to the pollution by exhaust emissions while forest soil is usually enriched with organic matter.

Description of study site and soil sampling

Nanjing (118°22′–119°14′E, 31°14′–32°37′N), the capital of Jiangsu Province, is located in the lower reaches of the Yangtze River. Nanjing is characterized by the subtropical monsoon climate, where it has four distinctive seasons, hot and wet in the summer, cold and dry in the winter. Its annual average temperature is 16.2°C, the annual rainfall is 1298.4 mm, and the annual sunshine is 1899.3 h. The city has been undergoing rapid urbanization and economic development in the last 30 years. Nevertheless, industries with high-energy consumption and high pollution, such as petrochemical, steel smelting and thermal power plant, are thriving in Nanjing. The consumption of coal was 2.8×10^7 t in 2011, accounting for 80.5% of the total energy consumption in Nanjing, and emissions of industrial dust were about 5.6×10^4 t in that year (SBN, 2012). Moreover, the local vehicular fleet has increased rapidly in recent years with an annual rate of over 20% and reached 1.4 × 106 vehicles in 2011.

Soil samples were taken over 0-10 cm depths from traffic district and urban forest in Nanjing main urban area. Soil samples were air-dried, crushed, and passed through a 2-mm sieve prior to analysis.

Soil Analysis

The pH values of the soil samples were measured in a water suspension using a soil: ultra pure water ratio of 1:2.5 (w/w). The PHS-3C pH Meter (Shanghai, China), a laboratory pH meter, was used for pH determination in aqueous phase. Soil porosity (n) was estimated as the volume of ultra pure water for complete dampening of a give soil volume. The mechanical analysis of the soil samples was carried out using the hydrometric method with isolation of clay, silt, and sand fractions.

Table 1. Characteristics of the soils sampled from different areas

Different areas	pH	Soil organic carbon(%)	Soil black carbon(%)	Available potassium (mg/kg)	Available phosphorous (mg/kg)	Clay (%)	Silt (%)	Sand (%)
Traffic district soil	4.84 (±0.09) *	1.65 (±0.47)	0.80 (±0.14)	21.7 (±0.33)	21.14 (±6.00)	29.50	53.66	16.84
Urban forest soil	4.72 (±0.24)	1.41 (±0.18)	0.44 (±0.04)	21.22 (±0.88)	18.16 (±2.29)	20.64	76.82	2.54

*. ±Standard deviation

Sorption Experiments

To carry out the adsorption experiments, solutions of Cu and Zn were prepared using their nitrate salts (supplied by Nanjing Chemical Reagent Co,, Ltd., China). Stock solutions of metal ions and all other solutions used in the study were prepared using ultra pure water. A 0.01 M $CaCl_2$ solution was used as a background electrolyte. For this sorption experiment, 10.0g portions of each soil sample were added in 100 mL plastic centrifuge tubes, mixed with 50 mL solutions of either Zn (0, 150, 300, 450, 600, and 750 mg/L) or Cu (0, 150, 300, 450, 600, and 750 mg/L). The tubes were shaken with a rotary shaker at 130 rpm for 24 h at 30°C. After shaking for 24 h, the equilibrium mixture samples were filtered using a 0.45μm cellulose membrane filter and the filtrate was assayed for the remaining Zn or Cu metal concentration by using the TAS-990 Atomic Absorption Spectrophotometer (Beijing, China). All measurements were made in triplicate.

The equilibrium amount of metal adsorbed in the soil during the experiments was calculated as the difference between the concentration of the initial solution and the concentration after reaching equilibrium using the mass-balance equations:

$$q_e = (V (C_0 - C_e)) / m$$

where q_e is the amount of metal ions sorbed at equilibrium (expressed in *mmol/g* sorbent); m is the mass of the soil (*g*); C_0 is the initial concentration of metal ions (expressed in *mmol/*L); C_e is the equilibrium concentrations of metal ions (mmol/L); V is the volume (in liters) of solution from which adsorption occurs. The sorption effectiveness (E, %) was calculated by the following formula:

$$E = 100\ m_s / m$$

where m_s is the mass of cation sorbed onto soil at equilibrium (*mmol*); *m* is the initial mass of cation in solution (*mmol*).

Determination of soil Black Carbon

In this study, the Black Carbon (BC) contents in soils were quantified using the chemical oxidation (CO) method applied by Lim & Cachier [12]. In brief, the method consists of six steps: (1) weigh out 3.0g of dried soil sample, sieved by 100 mesh sieve, (2) removal of carbonates (acid treatment with 3mol/L HCl for 24 h), (3) removal of silicates (acid treatment with15mL 10mol/L HF: 1mol/L HCl for 24 h) (4) removal of CaF_2 (acid treatment with 15 mL 10mol/L for 24 h) (5) removal of organic carbon (CO with 15 mL 0.1 mol/L K2Cr2O7: 2 mol/L H2SO4, 55°C for 60 h) and (6) quantification of residual carbon as BC by (Elementar IV, Germany). All data were determined in triplicate.

Statistical Analysis

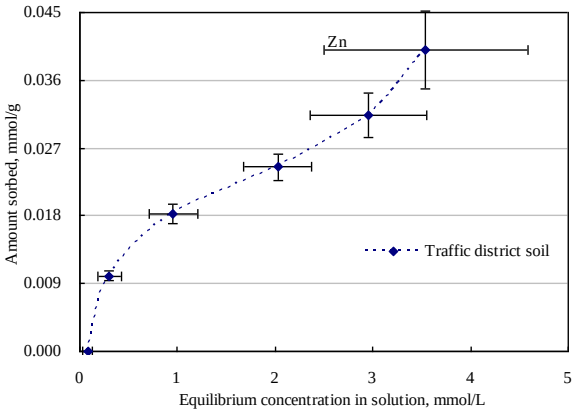
All experiments were conducted with three replicates and the mean were used to plot isotherms. The data descriptive and statistical analyses were carried out with Microsoft® Excel 2003 and IBM® SPSS® Statistics 19 software. T-test was used to compare the means of different soil characteristics, variability in the data was expressed as the standard deviation, and a $p < 0.05$ was

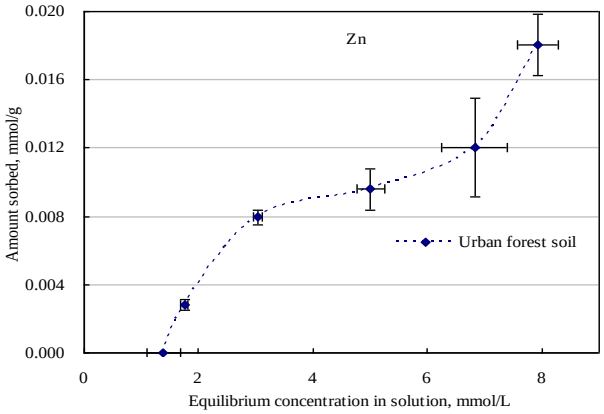
considered to be statistically significant. Pearson correlation analysis was used to describe correlation between soil adsorption capacity and soil pH, SOC, BC, respectively.

Results and Discussion

Metal Adsorption Isotherms and Fitting of Adsorption Models

The adsorption isotherm is a function describing the dependence between the amounts of adsorbed matter and its equilibrium concentration and may provide useful information about the





strength of soil interaction with the adsorbate at different concentrations. It can be also be the basis for prediction of screening properties of soils towards the pollutants. Adsorption isotherm equations have therefore been widely used to describe adsorption of different organic or inorganic compounds by soils [27,28]. That was the reason why the Freundlich and the Langmuir equations for equilibrium adsorption of heavy metals onto traffic district and urban forest soil types were applied in this study. The single metal sorption isotherms are shown in Figure 1.

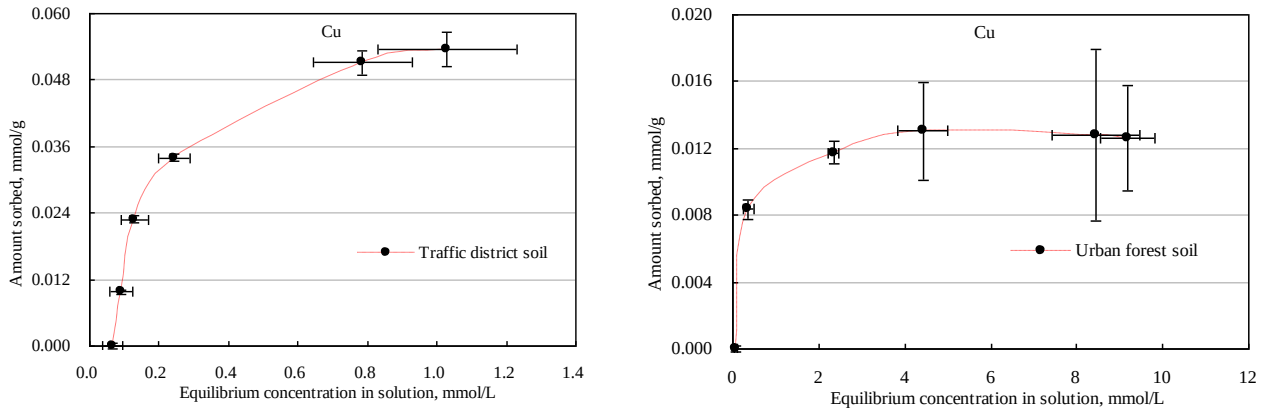


Figure 1. Isotherms of HM sorption from metal solutions.

It can be observed in Figure 1 that adsorption isotherm of Zn shows a refraction point at an equilibrium concentration of about 450 mg/L with a quick increase of the isotherms after that point in traffic district soil, and the refraction point of adsorption isotherm is at an equilibrium concentration of about 600 mg/L, with a rapid increase of the isotherm after that point in urban forest soil. However, the adsorption isotherms of Cu were different than Zn, the refraction points appeared at an equilibrium concentration of 450 mg/L and 150 mg/L in traffic district and urban forest soils, respectively.

A considerable difference in metal adsorption capacities for the two soil types can be observed from Figure 1. The adsorption capacities of the Traffic District soil towards both Zn and Cu are higher than corresponding capacities of the Urban Forest soil. The ranges of adsorption capacities of the soils are presented in (Table 2).

Table 2. The adsorption capacity ranges of the soils towards initial concentrations of the metals.

CONCENTRATION RANGES	SOIL TYPES	
	Traffic district soil	Urban forest soil
Ranges C_0, mmol/L		
Zn^{2+}	0-11.54	0-11.54
Cu^{2+}	0-11.72	0-11.72
Ranges Q_{max}, mmol/g		
Zn^{2+}	0-0.0400	0-0.0181
Cu^{2+}	0-0.0534	0-0.0126

The data for metal adsorption by the soils have been fitted to both Freundlich and Langmuir adsorption equations. The parameters predicted from these equations are shown in Table 3. It can be observed that Freundlich and Langmuir isotherms may describe the HM sorption by the studied soils acceptably. This is confirmed by the high values of the determination coefficient ($R^2 > 0.90$). Generally, the obtained values of determination coefficient suggest that experimental results fit better to the Freundlich isotherm (Table 3). Zn sorption onto traffic district and urban forest soils, however, fitted the Freundlich equation better than Cu. The maximum sorption capacities values predicted by the Langmuir equation are considerably higher than the experimental values obtained for Cu and Zn sorption by both the traffic district soil and urban forest soil.

Table 3. Model parameters for the adsorption of heavy metals by different soils.

Soil	Heavy metal	Freundlich model			Langmuir model		
		K_f	n	R^2	q_{max} (mmol/g)	b	R^2
UF	Zn^{2+}	0.0015	1.1809	0.9200	0.0500	0.0536	0.8680
	Cu^{2+}	0.0079	0.2556	0.7690	0.0135	2.9084	0.9370
TD	Zn^{2+}	0.0168	0.6397	0.9680	0.0642	0.3738	0.9570
	Cu^{2+}	0.0563	0.5735	0.8790	0.0778	2.3655	0.9240

Good agreement of the experimental data with Langmuir and Freundlich formulas allows for the creation of models of the studied HM sorption onto the soils and to predict the HM distribution in the soil—solution interface**.** It should be noted that the different levels of fitting in the mathematical model describe the same ranges of applied initial concentrations of different metals.

Effect of Soil Organic Carbon Content on the Heavy Metal Adsorption

The relationship between the content of soil organic matter (SOC) and adsorption capacity of the soils towards HM is shown in Figure 2. The content of organic carbon in soils is presented in (Table 1).

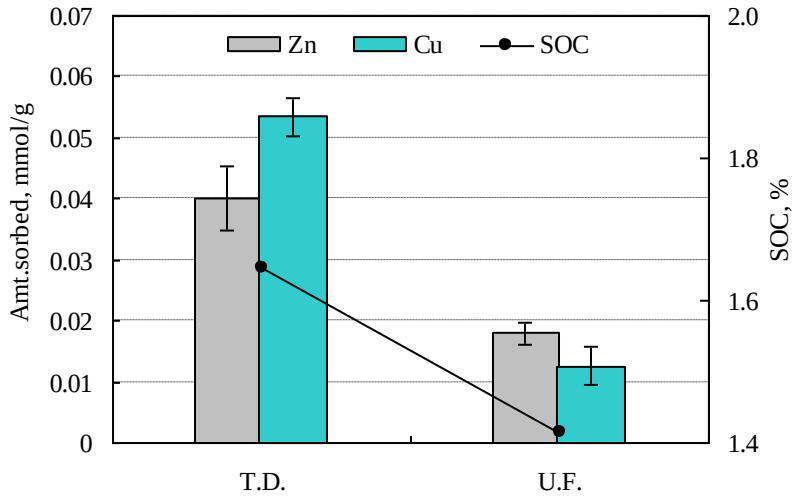


Figure 2. The relationship between SOC in soils and HM sorption capacity; (C_0—750 mg/L of Cu, Zn)

It can be observed in Figure 2 that the traffic district soil has the higher sorption capacity towards Zn and Cu compared to urban forest soil with a lower SOC content. Traffic district soil containing a high amount of SOC is characterized by the higher adsorption capacity towards the metals. It seems to be related with different contents of SOC in the traffic district and the forest soils.

Relations between HM adsorption and pH, and HM adsorption and Mechanical Composition of Soils

The relationships between the soils adsorption capacity towards HM, the pH value, and the Mechanical Composition of soils have also been investigated (Table 1).

As shown in Figure 3, the high adsorption capacity of the traffic district soils corresponds to higher pH values whereas in urban forest soils, the low adsorption capacity of both Zn and Cu corresponds to the lower pH values. According to the data reported by Zhang and Zheng [29] the pH value of the soil plays a crucial role in the control on solubility and mobility of metals in soils.

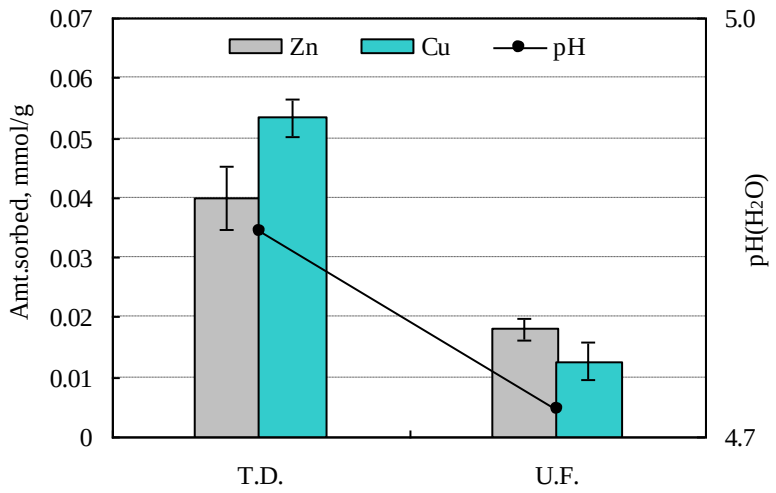


Figure 3. The relationship between pH in soils and HM sorption capacity; (C_0—750 mg/L of Cu, Zn).

The dependence of the metal adsorption capacity of the soils on their mechanical composition is shown in Figure 4. The traffic district soil contains about 29.5% of clay particles (Table 1) and is characterized by the higher adsorption capacity. The same fraction content in the urban forest soil is 20.64%. Silt particles in Urban Forest soil contain 76.82% whereas traffic district soil only contains 53.66%. Traffic district soil tends to have higher sand fractions (16.84%), however urban forest soil only has 2.54% of sand particles. The metal selectivity orders for both soil types are different at high initial concentrations of the metals, where the order is Zn<Cu in traffic soil and Zn>Cu in urban forest soil.

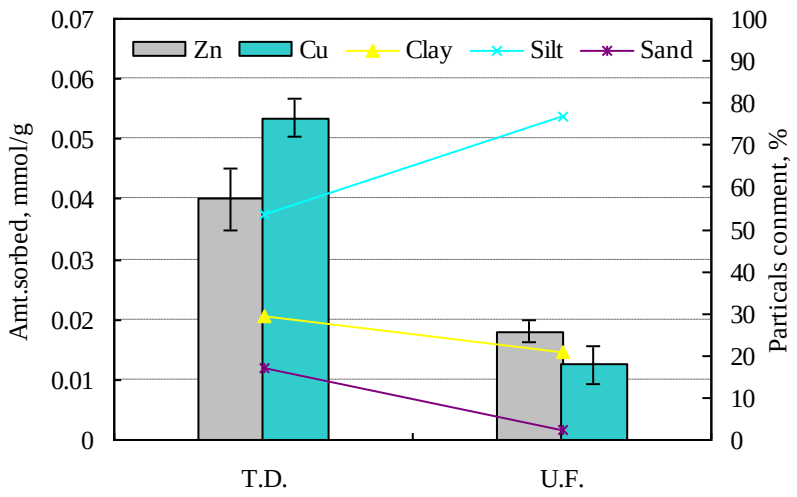


Figure 4. The relationship between Mechanical Composition in soils and HM sorption capacity; (C_0—750 mg/L of Cu, Zn).

Relation between Heavy Metal Sorption and Black Carbon Content in Soil

The relation between the soils adsorption capacity and black carbon content has also been investigated (Table 1).

It can be observed in Figure 5 that the traffic district soil with a higher BC content has the higher sorption capacity towards Zn and Cu compared to urban forest soil with a lower BC content. Black carbon with very high sorption capacity towards HM plays a dominant role in the total sorption capacity in traffic district soil. Traffic district soil containing a high amount of BC is characterized by the higher adsorption capacity towards the metals. It seems to be related with different contents of BC in the traffic district and the forest soils.

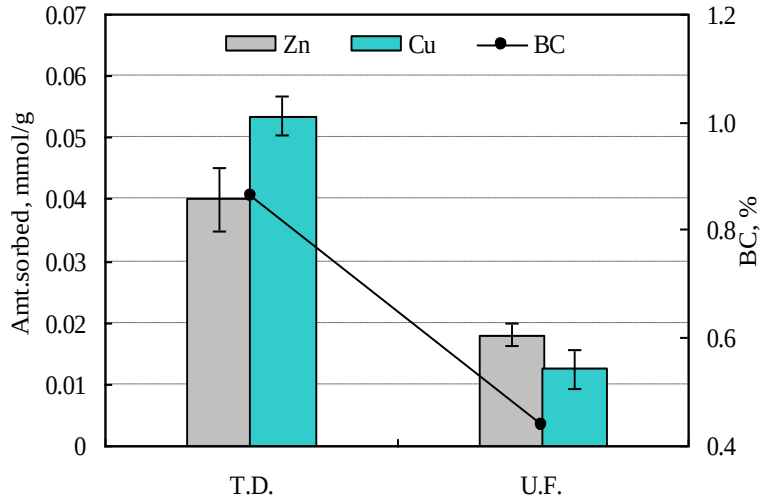


Figure 5. The relationship between BC in soils and HM sorption capacity; (C_0—750 mg/L of Cu, Zn).

Conclusion and Summary

The Freundlich and Langmuir models can describe the metal sorption onto the soils acceptably, while the Freundlich isotherm is fitted better to the experimental data of Zn in traffic district soil. The hypothesis of this study was supported; traffic district soil samples had a higher adsorption capacity and higher BC content than urban forest soil samples. The adsorption isotherms of the metals consist of two segments with different slopes: the initial segment with the steep slope and the second segment with the gentle slope that reflects different adsorption properties in different HM concentrations.

Heavy metals adsorption capacity of the different soil types depends absolutely on the soil properties. Unlike the urban forest soil, the higher adsorption capacities of the traffic district soil towards the metals are associated with higher pH and clay-sand fraction content. The higher sorption capacity of the metals is not connected directly with the higher content of soil organic matters, but depends on the different contents of BC.

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OCCURRENCE OF TYPICAL ANTIBIOTICS IN HUAI RIVER AND HONGZE LAKE, EASTERN CHINA

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Abstract

The presence of antibiotics in the aquatic environment, particularly in lakes and rivers, has increased with the rapid development of the world economy (Cheng et al., 2014). Today, between 100,000 to 200,000 tons of antibiotics are consumed globally per year (Wise, 2002) with China alone consuming 12-25% of this value (Gao et al., 2012a; Xu et al., 2007). Research on antibiotic contamination in many water bodies of China has been conducted. The economically important Hongze Lake, the Huai River, and the wastewater treatment plant (WWTP) of Laozishan Town in eastern China were examined for background antibiotic concentrations in this study. Water and sediment samples were collected from six locations in this region, including near fish farms and the WWTP outlet waters, influent, and effluent. These samples were analyzed for five common antibiotics: norfloxacin (NOR), oxytetracycline (OTC), enrofloxacin (ENF), ofloxacin (OFL), and ciprofloxacin (CIP). OTC was not detected in any samples, and OFL was only detected in the WWTP influent and effluent, indicating this location as a potential source of OFL contamination into the environment. NOR and ENR were found in all samples, with NOR occurring in the greatest concentrations. The WWTP was over 75% efficient at OFL and ENR removal, but only 4% efficient at NOR removal. Based on these results, NOR appears to be of greatest concern to environmental contamination in this region. These background levels and results should be useful to future study in this region and on this subject.

Literature Review

Water quality, whether for drinking, recreation, or natural areas, is highly dependent on factors such as pH, oxidation-reduction potential (ORP), conductivity, temperature, and nutrient levels (primarily nitrates and phosphates). Water quality is also dependent on surrounding anthropogenic activity, as heavy metal, nutrient, and other chemical contamination may occur. Because of growing environmental concern throughout the 20th century, much research on these contaminants and their remediation has been conducted. However, in just the last few decades, concern of a new contaminant has been on the rise. Today, antibiotic contamination in our planet's aquatic environments is a developing issue. While much research on the topic has yet to be conducted, antibiotic concentrations have been found at levels that may harm the local ecosystem and affect public health, particularly by promoting the growth of antibiotic-resistant bacteria (Gao et al., 2012b; Bouki et al., 2013).

Antibiotic Use and Consumption

The presence of antibiotics in the aquatic environment, particularly in lakes and rivers, has increased with the rapid development of the world economy (Cheng et al., 2014). As the planet's population has pushed to over 7 billion, studies have shown that between 100,000 to 200,000 tons of antibiotics are consumed globally per year (Wise, 2002). Consequently, an increasing number of studies on the input, occurrence, fate, and effects of antibiotics in the environment have been published in the past decade (Kümmerer, 2009b).

Antibiotics are an important group of pharmaceuticals that are further divided into sub-groups based on their structure or mechanism of action. First used in agriculture in the 1950s, there are over 250 types of these chemicals used in human and veterinary medicine (Kümmerer and Henninger, 2003). While antibiotics were originally developed from natural sources, today they are often produced synthetically with most being small molecules weighing less than 1000 daltons (1.66×10^{-15} ug). To be used for disease control, antibiotics must be able to tolerate oxidation, UV irradiation, rainfall, and high temperatures, thus making them very resistant to degradation (Kümmerer, 2009a). Additionally, antibiotics are non-toxic to the host and are intended to inhibit and stop microorganism growth (i.e., bacteria, fungi, protozoa, and viruses)

within humans, plants, and animals; however, once in the environment their therapeutic properties may pose potential environmental risks.

Once consumed, both by humans and animals, antibiotics are processed in the liver and converted into metabolites. Metabolites tend to be more water soluble than the original compound, thus allowing them to be excreted with urine more easily. Some metabolites are also more toxic than the original antibiotic (Kümmerer, 2009a). However, in a study conducted by Kümmerer and Henninger (2003), it was found that the average human liver can only metabolize approximately 30% of the antibiotic being consumed. This means around 70% of antibiotics consumed are excreted as the original compound with no biochemical changes. A similar study in Germany also found that approximately 70% of antibiotics consumed in the country are excreted unchanged and unmetabolized. However, this percentage is an average and varies by individual and by compound: some compounds may be 90% metabolized while others may be metabolized by as little as 10% (Kümmerer, 2009a).

Entry of Antibiotics into the Environment

Once consumed and excreted, antibiotics enter the environment through various routes including sewage wastewater, hospital and medical waste, industrial wastewater, agricultural waste and runoff, and aquaculture farms (Kümmerer, 2009a). However, the amount that each of these sources contributes to the environment varies. In Europe, hospitals accounted for only 5-20% of total antibiotic use (de Wirth et al., 2004), with community use tripling that number at 70% (in the UK) (House of Lords, 1998). In fact, hospitals are typically only a major source of cephalosporins in wastewater (Kümmerer, 2009a), an antibiotic class that targets cell walls ("Cephalosporins"). Generally, as suggested by research done in the United Kingdom, wastewater treatment plants (WWTP) tend to release the greatest amount of antibiotics into the environment, mainly due to most WWTP's inability to remove antibiotics from effluent (Chang et al., 2010; Jia et al., 2012). If not eliminated during wastewater treatment, then the antibiotics tend to enter the environment. However, concentrations of antibiotics are normally some orders of magnitude lower in the free water phase than in therapeutic use (Lorian, 2005).

Although humans use many of these agents, it was found that a large portion of antibiotics are used by livestock producers (Mellon et al, 2001), particularly for disease prevention, for therapy,

or to promote fattening. While antibiotics used for fattening are fed in low doses (Cromwell 2002), it has been theorized that even using small amounts of antibiotics may encourage the selection of antibiotic-resistant bacteria. This is because sub-therapeutic doses change the microbial populations within the animal's gut, thus allowing it to eat more. Low doses of an antibiotic can have this magnitude of an ecological affect within an animal, thus it is likely to cause a similar effect once in the environment. Because of this, the European Union and several other countries have recently banned the use of antibiotics as growth promoters (Kümmerer, 2009a). Another study found that by taking the manure of livestock treated with antibiotics and applying it to agricultural fields, the runoff and overflow can pollute water bodies with antibiotics (Jacobsen et al., 2004; Wei et al., 2011). Kim and Carlson (2007) also found various human and veterinary antibiotics such as tetracyclines, sulphonamides, and macrolides in river sediments that were influenced by agriculture. This indicated that runoff from nearby fields was a main source for antibiotics entering the environment.

Another important source of antibiotics in the environment is their use in aquaculture to prevent and treat microbial infections (Lalumera et al., 2004). These antibiotics (primarily oxytetracycline, florfenicol, premix, sarafloxacin, and erythromycin sulphonamides potentiated with trimethoprim or ormethoprim (Serrano, 2005)) are often fed to fish by throwing the agents directly into the water. Due to this, these substances easily enter the ecosystem without undergoing any kind of metabolic changes. This was first noticed over twenty years ago when several studies found large concentrations of antibiotics directly below fish farms (Jacobsen and Berglind, 1988; Björklund et al., 1991; Coyne et al., 1994; Migliore et al., 1995). Little information has been published on this specific issue since this study.

While some soil bacteria such as *Streptomyces* can produce antibiotics naturally, bacterial density is much lower in the free water phase when compared to sediments and sludge. Therefore it is unlikely to expect measurable concentrations of antibiotics being produced naturally. In fact, no studies have been published reporting the natural production of antibiotics in the aquatic environment (Kümmerer, 2009a). Therefore it can be assumed that any antibiotics found in aquatic environments have come strictly from anthropogenic sources.

Fate of Antibiotics in the Environment

Once in the environment, the fate of antibiotics may vary depending on the surrounding soil characteristics. For example, it was found that antibiotics may lose antimicrobial activity by binding to sediment particles. However, contradictory results have been found, although these differences may have occurred due to variations in sediment composition (Kümmerer, 2009a). Additionally, little is known about these compounds effectiveness after sorption to sediment or whether they are released back into the environment at a later point. Despite this, it was shown that the concentration of antibiotics may be much higher if the active compounds are persistent and sorb to solid surfaces (Kümmerer, 2009b) because sediment and water interactions are highly important in determining the persistence of contaminants in the aquatic environment (Zhou et al., 1999; Maskaoui and Zhou, 2010). This is largely because compounds that are strongly bound to sediments are more likely to be preserved from potential degradation (Chen and Zhou, 2014). Additionally, the natural degradation process of antibiotics in the aquatic environment depends highly on the water's pH and water hardness (Werner et al., 2006).

Another important concern is the ability of antibiotics to be rereleased into the environment through sediment disruption. As stated previously, many antibiotics sorb to aquatic sediment; therefore, sediment could act as a significant secondary source of antibiotics if it is disturbed, particularly through changes in water flow, pH, temperature, or physical disruption (Gong et al., 2012; Kümmerer, 2009a; Rosen et al., 2010; Westerhoff et al., 2005). The vertical distribution of antibiotics in sediments (with higher concentrations near sediment's surface) discovered in Cheng et al's (2014) research also suggests that sediment may be acting as a secondary source of some antibiotics. However, further study is needed on this subject.

Of primary importance with these studies is understanding how to eliminate these antibiotics from aquatic environments. Antibiotics are removed naturally from the environment through sorption (particularly to Ca or Mg), photolysis (Werner et al., 2006; Turiel et al., 2005b), hydrolysis, thermolysis (Halling- Sørensen, 2000; Kümmerer, 2009a), oxidation, and ozonation (Li et al., 2008c; Dantas et al., 2007). Aerobic biodegradation, on the other hand, has not been found to be a means of eliminating antibiotics from the environment (Richardson and Bowron, 1985; Al-Ahmad et al., 1999; Wiethan et al., 2000; Kümmerer et al., 2000; Ingerslev et al.,

2001a; Ingerslev and Halling-Sorensen, 2001b; Thiele-Bruhn, 2003; Alexy et al., 2003, 2004; Gartiser et al., 2007; Li et al., 2008c). In fact, one study found that out of 16 antibiotics studied only one (benzyl penicillin) was capable of complete oxidation through biodegradation (Gartiser et al., 2007). Other antibiotics such as ampicillin, doxycycline, oxytetracycline, and thiamphenicol were significantly degraded, though not completely (Maki et al., 2006). Additionally, many antibiotics used in aquaculture were found to have long half-lives in sediment (Jacobsen and Berglind, 1988; Hansen et al., 1992; Samuelsen et al., 1992, 1994; Hektoen et al., 1995; Capone et al., 1996; Marengo et al., 1997; Lai et al., 2008), were very persistent, and many were only partially degradable (Donoho, 1984; Gilbertson et al., 1990; Samuelsen et al,. 1991, 1994; Capone et al,. 1996; Thiele-Bruhn, 2003).

Effects on the Ecosystem

Upon entry into the ecosystem, antibiotics can affect the soil's ability to decompose, may have hazardous effects on aquatic organisms, and may enhance bacterial resistance (Gao et al., 2012b; Bouki et al., 2013). Additionally, upon exposure to unneeded antibiotics through drinking water, humans may experience allergic reactions and negative impacts on the digestive system (Kümmerer, 2009a).

Toxic effects of antimicrobial on higher-order fish are rarely documented, with effects only occurring at unrealistic environmental concentrations (Kümmerer, 2009a). However, antibiotic concentrations below 1 mg/L of furazolidone were shown to have toxic effects on smaller organisms including the larvae of mosquito species. (*Culex pipiens* and *C. molestus)*, the planktonic crustacean *Daphnia magna*, and the bring shrimp *Artemia salina*. While these organisms are small and low on the food chain, their disappearance could negatively affect higher organisms that depend on them as a food source (MacrÌ et al., 1988). Additionally, antibiotic exposure may also affect the reproduction and early life stages of lower-order aquatic organisms (Kümmerer, 2009a) such as *D. magna*. Antibiotics may also significantly depress the hatching rate for *Artemia sp.* cysts, may cause a high mortality rate for *Artemia sp.* nauplii (MacrÌ et al., 1988; Migliore et al., 1993, 1997; Brambilla et al., 1994; Wollenberger et al., 2000), and may alter the pigmentation of *A. salina* nauplii (Brambilla et al., 1994).

Algae and cyanobacteria are also affected by antibiotics, though their sensitivity varies widely between species. For example, *Microcystis aeruginosa* (a cyanobacteria) was inhibited at antibiotic concentrations of less than 0.1 mg/L, a level that is 2-3 orders of magnitude more sensitive than *Selenastrum capricornutum* (an algae) (Halling- Sørensen, 2000); similar results were found in an experiment by Holten-Lützhøft et al. (1999). Additionally, a risk assessment in the Yongjiang River of southern China showed that sulfamethoxazole and erythromycin pose high ecological risks to *Synechococcus leopoliensis* (a cyanobacteria) and *Pseudokirchneriella subcapitata* (an algae), both of which are highly sensitive organisms. Other selected antibiotics in this study were shown to cause high ecological risks (risk quotients up to 95) in sediment samples (Xue et al., 2013). However, it was found that antibiotic toxicity susceptibility varied across multiple algal and plant species, especially between cyanobacteria, green algae, and higher plants; of these, cyanobacteria tend to be the most sensitive to antibiotics (Brain et al, 2008).

In water surrounding aquaculture systems, concentrations of antibiotics have been found at high enough levels to inhibit bacterial growth (Kümmerer, 2009a). For example, a study found that antibiotics applied directly to water significantly affected nitrifying bacteria populations in sediments below the fish farms. In fact, this disruption to the nitrification process occurred at concentrations that may be found in common fish tanks (Klaver and Matthews, 1994). In a study conducted by Hansen et al. (1992), it was shown that adding antibiotics to sediment also causes a temporary effect on sulfate reduction by sulfate reducing bacteria. In addition, antibiotics may negatively affect the microbial community in wastewater and sewage systems. Several have found that when commonly used antibiotics were added to a model sewage system in concentrations similar to that found in hospital wastewater, bacterial levels were reduced (Stanislawska, 1979; Al-Ahmad et al., 1999; Kümmerer et al., 2000). While this study was conducted in a model before the water had entered the environment, these results indicate that a similar result could occur upon introduction to an aquatic ecosystem.

There are many limitations on the study of antibiotic effects on bacteria. Of greatest hindrance to the study is the determination of using acute or chronic effects as endpoints. Thomulka and McGee (1993) for example, found that the toxicity of several antibiotics (novobiocin, tetracycline, chloramphenicol, ampicillin, streptomycin) on *Vibrio harveyi* showed almost no

toxic affects over a short incubation period when luminescence was used as an endpoint. However, when a long-term study was conducted that used reproduction as an endpoint, a toxic affect in environmentally-relevant concentrations could be detected for almost all substances. This finding was similar to the results found by Froehner et al. (2000). Additionally, similar to Kümmerer's findings (2004), Backhaus and Grimme (1999) found that toxicity varied between short- and long-term studies with long-term results showing greater toxic effects on the bacteria. Because of this, short-term tests with various endpoints may underestimate the effects and risks of antibiotics on bacteria in the environment; this may also cause difficulty in comparing studies. However, limited study has been conducted on this subject and should be an important focus for future research.

In addition to bacterial and ecosystem damage, the widespread use of antibiotics has also been associated with the development of antibiotic resistance in pathogenic bacteria, particularly in *Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Edwardsiella ictaluri, Vibrio anguillarum, Vibrio salmonicida, Pasteurella piscicida,* and *Yersinia ruckeri* (Serrano, 2005). Because of the limitations for antibiotics to degrade naturally, the development of an unfamiliar, worldwide challenge of antibiotic-resistant bacteria is currently underway (Wellington et al., 2013). In fact, antibiotic resistant bacteria were detected in drinking water as early as the 1980s and later in the 1990s (Kümmerer, 2009b). Since then continuous exposure to small doses of antibiotics in the environment may lead to the emergence of this resistance (Schwartz et al., 2003). Resistant bacteria can transfer to humans if contaminated water or food—especially meats—are consumed (Perretin et al., 1997; Khachatourians, 1998; Salyers, 2002; Dolliver and Gupta, 2008).

Others, such as Al-Ahmad et al. (1999) and Withan et al. (2002), believe that the input of antibiotics into the environment is of minor importance regarding the development of bacterial resistance. Instead, they suggest that the continuous input of already-resistant bacteria into the environment (particularly from the localized application of antibiotics in medical treatment (Hingst et al., 1995; Russel, 2000)) is of greater concern than resistance caused by continuous bacterial exposure to low concentrations of antibiotics. Supporting this, previous research had found that bacterial resistance is very high in hospital effluents and in WWTPs (Wiethan et al., 2001; Kümmerer, 2004) before even reaching the environment. Because of these contradictory

beliefs, the debate on whether bacteria become resistance before or after they enter the environment is a growing topic, and further research on the subject is expected to continue.

Related Studies on Aquatic Environments in China

Of the large amount of chemicals produced today, it is estimated that China alone uses 25,000 tons of antibiotics per year (Gao et al., 2012a; Xu et al., 2007), or about 12 to 25% of the planet's total consumption. However, reliable data providing information on the usage patterns of antibiotics only exist for a few countries, of which China is not included. Additionally, some antibiotics do not require a prescription and thus total consumption is not well-documented (Kümmerer, 2009a). Because of this, it is essential to monitor and evaluate water bodies individually in order to determine baseline contamination levels, as well as to identify potential sources of antibiotic entry into that aquatic ecosystem.

Several studies have been conducted in China to determine the current antibiotic concentrations in many of the country's water resources. A study by Chen and Zhou (2014) determined the concentrations of antibiotics in the water and sediments of the Huangpu River, a major drinking water supply for Shanghai. Of the 20 antibiotics tested in this water, the authors found that sulfonamides had the highest concentrations in water samples (34-859 mg/L) while tetracyclines (18 ug/kg) and macrolides (12 ug/kg) had the highest concentrations in sediment samples. These concentrations were mainly linked to animal farming sites and to the river's tributaries. Overall, of the 20 antibiotics tested in the water, two had low detection rates while 11 had high detection rates at over 80%. In sediment, *all* antibiotics showed high detection rates (12.4 to 101 µg/kg on a dry weight basis). A relationship between the concentrations of antibiotics in water and soil was then created to determine the partition coefficients of the samples, thus indicating the importance of the contaminant properties in inter-phase conditions. This study also highlighted the spatial variability of antibiotic concentrations between samples along the river, as supported by significant changes in concentrations near discharges from animal farming sites and tributaries downstream of the Yuanxie River. In another study on the Huangpu River, 11 antibiotic resistant genes (ARGs) in bacteria were found, with the highest concentration of these ARGs found in suburban areas. (Jiang et al., 2013). This supports the hypothesis that resistant bacteria are present in Chinese waters; however, while the study does indicate that these ARGs

may be a concern to public health, it does not indicate if these ARGs developed before or after the bacteria entered the environment.

In another study, the seasonal variation of antibiotics in Lake Baiyangdian in northern China was researched (Cheng et al., 2014). This study involved lateral sampling by collecting surface water, overlying water, pore water, and sediment samples at each location. All of the antibiotics that were sampled for were detected in 100% of the samples. The high residuals of norfloxacin (NOR) in aquatic commercial animals (23.8 ng/g) confirmed that a large amount of NOR was consumed by aquaculture in Baiyangian Lake (Li et al., 2012). The concentrations of NOR were also much greater than what the natural degradation and dilution rates are in the aquatic environment (Wiwattanapatapee et al., 2002). In another study, significantly high concentrations of NOR were found to occur mainly in the summer near aquaculture farms, thus suggesting that this practice is a major source of this particular antibiotic (Zou et al., 2011). Additionally, it was found that antibiotic concentrations in surface water were significantly different between seasons. However, all antibiotic concentrations found in this study were of moderate levels compared to other sites globally (Cheng et al., 2014).

Another study in China aimed at monitoring pharmaceuticals in the tap water of over one dozen Chinese cities; the results showed 17 of the monitored chemicals in 89% of samples. While the majority of these pharmaceuticals showed little risk to humans, four were found at levels that may pose risk to infants and children. Because of this study, cities within the Yangtze River region and Guangzhou are now regarded as pharmaceutical contamination "hot spots," largely due to the high concentrations and frequent detection of these chemicals in the water. Thus Chinese tap, especially that coming from lakes and rivers, may be viewed as an additional route of exposure to pharmaceuticals, although the risk to human health is low based on current toxicity data (Leung et al., 2013). Another study on the same river found 15 of 20 selected pharmaceutical and personal care products (PPCPs) in the central and lower parts of the Yangtze River. These PPCPs were also detected in four large lakes within the Yangtze River basin, including Dongting Lake, Poyang Lake, Tai Lake, and Chao Lake. In this region, high environmental risks of erythromycin and clarithromycin in the Yangtze River, clarithromycin in Chao Lake, and clindamycin in Tai Lake were found (Wu et al., 2014). The source of high pharmaceutical concentrations in the Yangtze River region may be supported by a study

conducted by Qi et al. (2014). During their 14-month project, it was determined that the Yangtze River unloads 152 tons of pharmaceuticals into the East China Sea annually; this quantity is likely due to the large watershed of the Yangtze River basin, which collects over 20% of China's total run-off. While 152 tons may seem moderate when compared to the river's discharge rate of 30,000 m^3/s into the sea, these concentrations are still suspected to pose a health threat to the marine coastal ecosystem (Qi et al., 2014).

Further north, in a study at a wastewater reclamation plant (WRP) in Beijing, the dominant antibiotics in all samples were quinolones, sulfonamides, and macrolides (Li et al., 2013). Additionally, through analysis of the receiving waters of a nearby WWTP, it was found that the removal process for 12 of the tested antibiotics was very ineffective. However, the concentration of the pharmaceuticals in the receiving water was still higher than the WWTP effluent, thus suggesting that other sources may also be linked to the river's pharmaceutical contamination (Dai et al., 2014). Similarly, in southern China, Zhou et al. (2003) found that lagoon and anaerobic digesters for waste treatment were ineffective in the elimination of antibiotics.

Limited studies on pharmaceutical contamination in other water bodies in China besides those listed above have been conducted. However, all studies conducted have found some sort of contamination. For example, sulfonamides were found to have the highest concentration of any antibiotic in both the Yangtze River (Yan et al., 2013) and the Pearl River (Yang et al., 2011) reaching 56.8 and 616 ng/L respectively (Chen and Zhou, 2014). Ofloxacin and norfloxacin have also reached high levels in Pearl River (Yang et al., 2011) while Lu et al. (2013) found multiple pharmaceuticals contaminating Zhushan Bay and Meilang Bay of Tai Lake in eastern China (Lu et al., 2013).

Other studies in China have been focused on locating the potential sources of this contamination. A study in Yongjiang River, which flows through the developing city of Nanning, found that concentrations of most antibiotics increased with proximity to urban areas, supporting the correlation between human activities and environmental antibiotic contamination. Additionally, the concentrations in tributaries were higher than those within the main river, indicating that tributary discharge is also a source of pollution (Xue et al., 2013). In Jiangsu Province, however, veterinary antibiotic residues from nearby large-scale livestock and poultry farms are one of the

prime sources of antibiotic contamination in that region. In fact, rivers nearby to the farms were contaminated with 9 different monitored antibiotics. The most frequently detected of these antibiotics were sulfamethazine (75%), oxytetracycline (64%), tetracycline (60%), sulfadiazine (55%) and sulfamethoxazole (51%) (Wei et al., 2011). While this may be true for Jiangsu Province, a country-wide study found that much of China's pharmaceutical pollution was related to megacities with a high density population. However, PPCP levels China's surface waters are lower than or comparable to the levels found in other countries (Bu et al., 2013). Regardless, in order for this finding to remain true and to ensure that pharmaceutical levels in untested waters are not high or rising, monitoring should continue particularly in water bodies of high importance to society (i.e. drinking sources). Additionally, because a large portion of China's surface waters remains untested and because the subject is still relatively new, it is suggested that pharmaceutical contamination continue to be a focus for future research in China and worldwide.

Introduction

Antibiotics have been found in the environment around the world, particularly in lakes, rivers, and other aquatic ecosystems. Some research on this subject has been conducted on the water bodies of China, such as on the Huangpu River near Shanghai and at Baiyangdian Lake in northern China. However, because many regions have yet to be examined, a study on Hongze Lake and Huai River in Eastern China was conducted (Figures 1 and 2). Hongze Lake, located on the western edge of Jiangsu Province, is the 4th largest freshwater lake in China with its primary inflow coming from Huai River. Because of its great importance to the local village culture and economy, particularly for its use in aquaculture, this lake and river was selected for study.

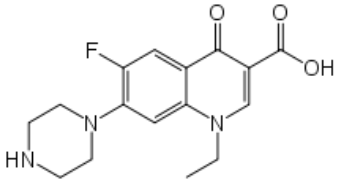
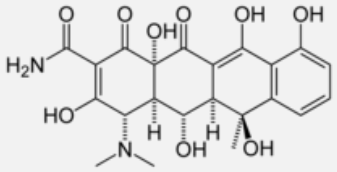
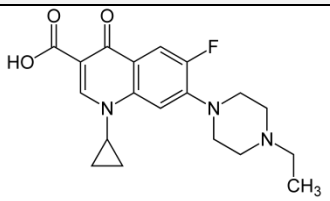
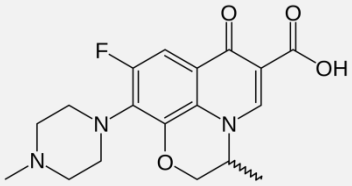
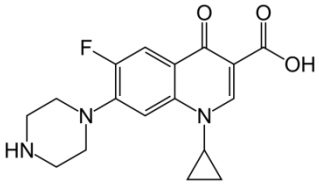
In this study, water and sediment samples from the lake's southern shore and from the Huai River discharge region were collected and analyzed for five common antibiotics: norfloxacin (NOR), oxytetracycline (OTC), enrofloxacin (ENF), ofloxacin (OFL), and ciprofloxacin (CIP). While NOR, OTC, OFL, and CIP are used to fight a variety of bacterial infections in humans, ENF is primarily for veterinary use (Table 1). Some of these antibiotics may also be used in aquaculture. The fluoroquinolone class, of which NOR, ENF, OFL, and CIP are included, is important as it kills bacteria by inhibiting bacterial DNA-gyrase, an enzyme necessary for DNA

synthesis and repair (Mehta). Tetracyclines, including OTC, inhibit the synthesis of essential proteins needed for bacterial growth and reproduction, thus causing the bacteria to die (Chopra and Roberts, 2001).

These five antibiotics are wide-spectrum antibiotics and are used to treat a variety of bacterial infections. NOR is primarily used to treat urinary tract and prostate infections, as well as some stomach and intestine infections ("Norfloxacin"). In order to treat the urinary tract, however, high levels of norfloxacin pass through the kidneys and into urine; this means that much of the medicine passes into the environment unused without any metabolic changes ("Utinor"). Not only is this antibiotic used for humans, but it is also commonly used to treat fish in Chinese aquaculture systems (Li et al., 2012; Zou et al., 2011). OTC is primarily used to treat acne and respiratory infections as well as rarer infections like Rocky Mountain spotted fever and brucellosis. However, some strains of bacteria have already developed resistance to this antibiotic ("Oxytetracycline"). ENF is a veterinary antibiotic that is effective against both gram-positive and gram-negative bacteria. Because of this, it is used to treat difficult bacterial infections in pets (primarily skin and ear infections). It is also effective against *Pseudomonas* and *Staphylococcus*, which were formerly very difficult to treat ("Baytril"). Its counterpart for human use is CIP. OFL is used to treat many infections including pneumonia, bronchitis, skin infections, gonorrhea, chlamydia, UTIs, and prostate infections. However, some strains of *Streptococcus*, *Enterococcus*, and anaerobic bacteria are resistant to ofloxacin (Ogbru). CIP works best against gram-negative bacteria including *Salmonella*, *Shigella*, *Campylobacter*, *Neisseria*, and *Pseudomonas* ("Ciproxin"). In fact, this antibiotic is used to prevent and treat anthrax (Ciprofloxacin") as well as to treat infections of the skin, sinuses, bone, lungs, ear, abdomen, kidney, prostate, bladder, as well as some STDs and typhoid; it may also be used as a single dose treatment for gonorrhea ("Ciproxin").

Of these five antibiotics, only OTC (Terramycin®) is legal for aquaculture use in the United States, with all antibiotics from the fluoroquinolone class being banned. This is partly due to a public health concern regarding the development of antibiotic resistant bacteria. In China, however, the use of fluoroquinolones such as NOR is permitted (Lumpkin). Additionally, the regulation of aquaculture antibiotic use in China has proven to be much more difficult to enforce than anticipated; several antibiotics that are banned by the Chinese government have still been

Table 1. Antibiotic Names, Common Brand Name, Antibiotic Class, Structure, and Primary Consumer Information.

Antibiotic Name	**Common Brand Name(s)**	**Antibiotic Class**	**Chemical Structure**	**Primary Consumer(s)**
Norfloxacin (NOR)	NOROXIN®	Fluoroquinolone		Humans; Aquaculture (China)
Oxytetracycline (OTC)	TERRAMYCIN®	Tetracycline		Humans; Aquaculture
Enrofloxacin (ENF)	BAYTRIL®	Fluoroquinolone		Animals (Pets)
Ofloxacin (OFL)	FLOXIN® TARIVID®	Fluoroquinolone		Humans
Ciprofloxacin (CIP)	CIPRO®	Fluoroquinolone		Humans

detected in aquaculture products ("An Overview"). This may be due to China's status as the largest producer of aquacultured seafood in the world (~70% of the world's production) (Lumpkin).

The goal of this study was to establish the baseline concentrations of five antibiotics (norfloxacin, oxytetracycline, enrofloxacin, ofloxacin, and ciprofloxacin) in Lake Hongze and Huai River. Objectives included (1) to determine the presence and differences in antibiotic concentrations in six locations on the southern shore of Lake Hongze near the discharge site of Huai River ((a) fish farm on the Huai River that is separated from the river by a dike (Site 1), (b) fish farm in the Huai River (Site 2), (c) fish farm in Hongze Lake (Site 3), (d) downstream from the Laozishan Town WWTP outlet into Huai River (≈500-1000m from outlet) (Site 4), (e) upstream from the Laozishan Town WWTP outlet into Huai River (≈500-1000m from outlet) (Site 5), and (f) the influent, effluent, and sludge from the Laozishan Town WWTP) (Site 6), (Figures 1 and 2)) and (2) to detect the level of transference of antibiotics from water to sediment. With this data, the regions with the highest concentrations of antibiotics will be determined, thus aiding in understanding the fate and source of these antibiotics in the lake's ecosystem.

Materials and Methods

Site Assessment

Sampling was conducted at Hongze Lake, Huai River, and the wastewater treatment plant (WWTP) of Laozishan Town, all located near the western border of Jiangsu Province in eastern China (Figures 1 and 2). Hongze Lake is the 4th largest freshwater lake in China with a size of approximately 2069 km^2 and a maximum depth of 5.5 m (Nielsen, 2009). Several rivers flow into the lake, but its primary inflow comes from the Huai River found on the lake's southern end. While the surrounding province has over 106 cities and 1242 towns, Hongze Lake is situated between two major cities: Sihong and Huaian ("Hongze"). Additionally, the recently approved 16.7 km^2 Hongze Lake Wetland National Reserve is found on the lake's northwestern shore. Due to the lake's location in an ecological transition zone between a warm temperate and subtropical

climate, this reserve was established for its importance to over 140 species of migrating and wintering birds, 9 of which are internationally protected (Nielsen, 2009).

Hongze Lake (translating to “flooding marshland”) comprises over half of Jiangsu Province’s area. While the county currently has a population over 500,000 and is one of the most economically developed areas in China, the region is still widely known as the "county of fish and rice" and the "land of rivers and lakes,” and is highly important for local irrigation and hydropower generation. In fact, the lake has traditionally been relied on for agriculture and aquaculture, just as many villages such as Laozishan Town still do today. It is also noted as one of China’s principal lakes for freshwater farming (including for fish, crab, shrimp, shellfish, oyster, and aquatic vegetables). This is largely because manufacturing and other industries are not yet abundant in this region (“Hongze”).

The lake also serves as one of the county’s greatest tourist attractions and is the main focus of many local customs and folklore (“Hongze”). For example, the city of Xuyi, locally known as the “Crawfish Capital of the World,” holds its international crawfish festival every summer with crawfish caught fresh from both the Huai River and Hongze Lake. This event is so important that many Chinese celebrities visit the area for the opening ceremony. Another important festival is the Golden Autumn Crab Gourmet Festival (Nielsen, 2009). For these reasons, Hongze Lake and the inflowing rivers are a highly important part of the surrounding ecosystem as well as of great importance to the daily life in the local villages and cities, both for economic and cultural reasons; thus, environmental monitoring on this lake and river may be seen as highly advantageous.

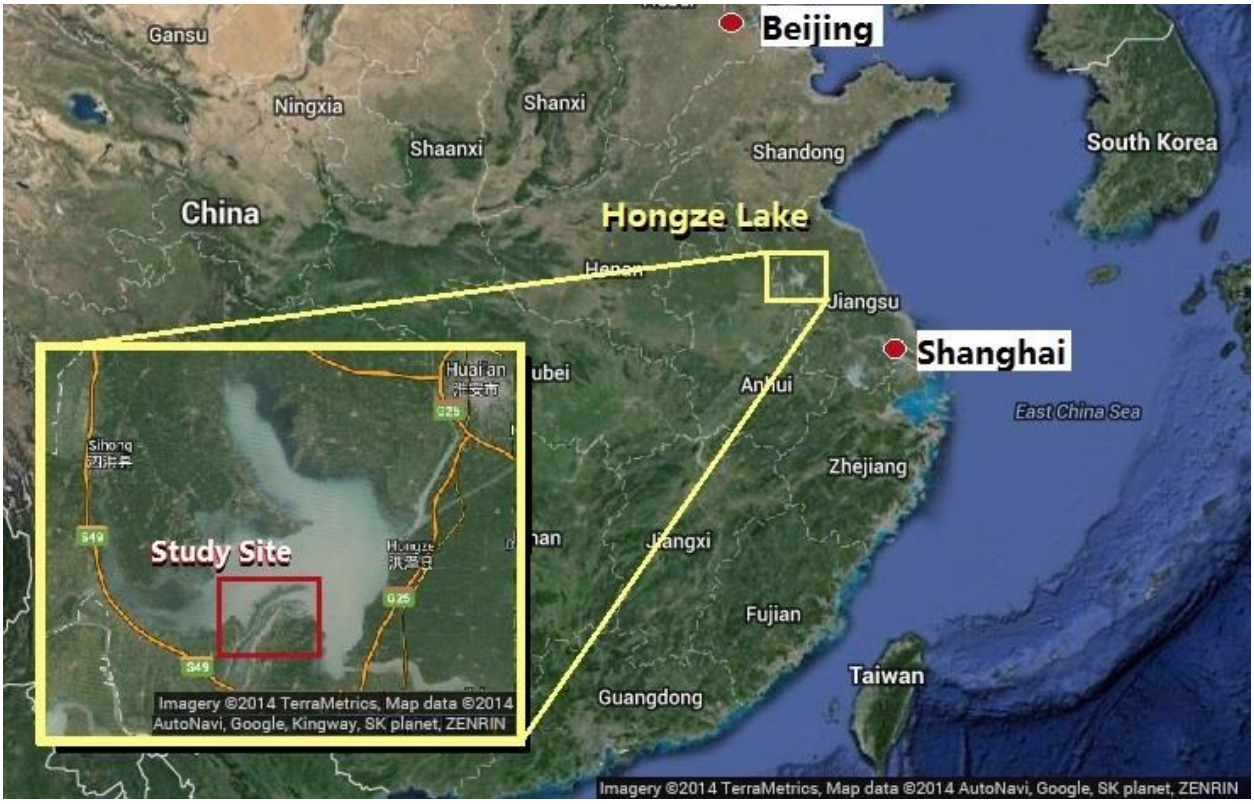


Figure 1. Location of Hongze Lake within China (inset: study site focus area on the lake and Huai River) (Map Data: TerraMetrics, AutoNavi, Google, SK planet, Zenrin and TerraMetrics, AutNavi, Google, Kingway, SK planet, Zenrin; edited by Linzi Thompson).

Water and Sediment Sampling

Samples were taken in June 2014 at six different locations on the southern shore of Hongze Lake: (1) fish farm on the Huai River that is separated from the river by a dike, (2) fish farm on the Huai River that is not separated from the river, (3) fish farm on Hongze Lake that is not separated from the lake, (4) downstream from the Laozishan Town WWTP outlet into Huai River (≈500-1000m from outlet), (5) upstream from the Laozishan Town WWTP outlet into Huai River (≈500-1000m from outlet), and (6) the influent, effluent, and sludge from the Laozishan Town WWTP (Table 2, Figure 2).

Table 2. Sample site number, matrices, locations, coordinates, and site characteristics (i.e. relation to fish farm or WWTP location).

Sample Site #	Matrix	Location	Coordinates	Site Characteristics
1	Water Sediment	Huai River	33.169723° N 118.575231° E	Fish Farm Separated from River
2	Water Sediment	Huai River	33.136259° N 118.526151° E	Fish Farm **NOT** Separated from River
3	Water Sediment	Hongze Lake	33.210464° N 118.54944° E	Fish Farm **NOT** Separated from Lake
4	Water Sediment	Huai River	33.181075° N 118.623063° E	Downstream from WWTP (≈500-1000m from outlet)
5	Water Sediment	Huai River	33.184657° N 118.613207° E	Upstream from WWTP (≈500-1000m from outlet)
6	Influent Effluent Sludge	Laozishan Town WWTP	33.188097° N 118.604091° E	

Figure 2. Location of sampling points on Hongze Lake and Huai River (Map Data: Google, Landsat; edited by Linzi Thompson).

At sites 1, 2, 3, 4, and 5, water and sediment samples were collected. Four 500 mL plastic containers of water (2 L total) were collected from approximately 10 cm below the lake's surface. Sediment samples were collected at two different depths (1-10 cm and 10-20 cm below the soil's surface) using a coring rod and were placed in plastic bags.

At these five sites, a Hach HQ30d multi-parameter meter was used to collect measurements of water conductivity (InteliCAL CDC401 probe), oxidation-reduction potential (ORP) (InteliCAL MTC101 probe), and dissolved oxygen (DO) (InteliCAL LDO101 probe) levels. At all six sites, a GPS (Trimble GeoXT, GeoExplorer 3000 series) was used to collect coordinates.

At site 6, four 500 mL plastic containers (2 L total) of influent (before anaerobic treatment) and four 500 mL containers (2 L total) of effluent (after aerobic treatment) were collected. Sludge from pre-treatment sedimentation was also collected in plastic bags.

Overall, 18 samples total were collected from six different locations: seven water samples (in quadruplicate) and eleven sediment samples. All samples were immediately refrigerated between 1°C and 4°C upon return to the laboratory.

Preparation of Samples

Sediment. Sediment was filled into four 50 mL plastic beakers to approximately the 40 mL mark before being placed in a 0°C freezer overnight. The sediment was then placed in a VirTis Benchtop SLC freeze dryer (between -40°C to -60°C) until dry. Once dry, all samples were ground and sifted through a 60 mesh/0.28 mm pore sieve.

Due to time constraints, preparation of sediment and sediment antibiotic analysis will be conducted at a later date.

Water. Water samples were filtered using 0.45um filter paper. After filtration, pH, total nitrogen (N), and total phosphorus (P) concentration data were collected.

To extract and concentrate antibiotics from each water sample in preparation for high performance liquid chromatography/mass spectrometry (HPLC/MS) analysis, samples were run through a Solid Phase Extraction (SPE) cartridge (Waters Oasis HLB 6cc (500 mg) LP Extraction Cartridge) (Figure 3). Procedures for SPE extraction were based on the methods of Cheng et al. (2014) with some modification. One cartridge was used per 500 mL sample. Before use, each cartridge was conditioned by running 5 mL of MeOH, 5 mL of 0.5 N HCl solution, and 5 mL of deionized (DI) H_2O through the cartridge, sequentially.

Each 500 mL sample was amended with 2 ml of 5% (w/w) Na_2EDTA for chelation. The pH was then adjusted to pH 3 using 30% (v/v) H_3PO_4 before the sample was run through one SPE cartridge under a pressurized rate of 5-10 mL/min. Once filtered, the eluents were collected and adjusted to pH 5 using 2.5 M NaOH. The extraction process was then repeated with this adjusted sample. Overall, the same 500 mL sample went through the SPE cartridge twice, once with a pH

of 3 and once with a pH of 5, to ensure that all antibiotics were collected within the hydrophobic-lipophilic cartridge.

Once complete, the cartridge was rinsed with 10 mL DI H_2O. The system was then run under pressure (with no sample) for 20 minutes to allow the cartridge to partially air dry. Each cartridge was then eluted with 12 mL MeOH (2 mL under pressure and 10 mL through gravity) to wash the collected antibiotics into solution. This resulting effluent was collected in a vial and then placed in a nitrogen evaporator until the MeOH had evaporated. Once only the solid antibiotics were left, 1 mL of MeOH was added to the solid before being transferred to a 2 mL vial for HPLC-MS/MS analysis.

This process was repeated for all seven water samples. Once complete, the process was repeated using duplicates of all seven samples.

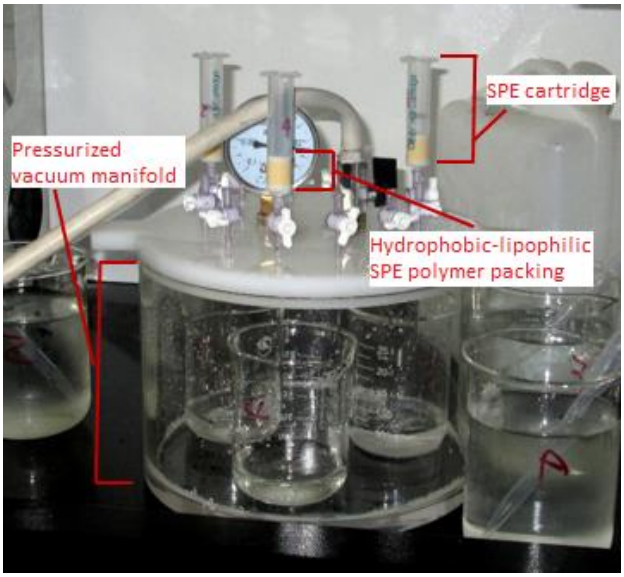


Figure 3. Pressurized vacuum manifold setup for antibiotic extraction, including the SPE cartridges and the hydrophobic-lipophilic SPE polymer packing.

Standards. Five antibiotics were used to create solution standards: norfloxacin, 98% (J&K Scientific, #309655), oxytetracycline, 95% (Acros Organics, #123841000), enrofloxacin, 98%

(Fluka, #17849), ofloxacin, 98% (J&K Scientific, #104510), and ciprofloxacin, 98% (J&K Scientific, #250693).

A 200 mg/L stock of each antibiotic was created in a 1:1 H_2O to MeOH solution (with 0.2% formic acid (CH_2O_2)). The stock was then diluted to 100 ug/L and sent for analysis as a standard.

Analysis of Antibiotics

The target antibiotic standards (norfloxacin (NOR), oxytetracycline (OTC), enrofloxacin (ENF), ofloxacin (OFL), and ciprofloxacin (CIP)) and samples were analyzed by a ThermoScientific LTQ Orbitrap XL and UltiMate 300 high performance liquid chromatography-mass spectrometry (HPLC-MS) system. This HPLC technique places analytes under high pressure in a chromatography column to allow for separation. The masses of the separated particles are then determined by the MS to determine the specific identity and quantity of each analyte. During analysis, Elution A (0.1% formic acid) and Elution B (acetonitrile) were used. A flow rate of 0.300 mL/minute was used with an elution gradient as follows: 0-4min, 90%A + 10%B; 4-7min, 30%A + 70%B; 7-7.1min, 70%B to 10%B; 7.1-10min, 90%A + 10%B. The total time for a sample was 10min with sampler temperature at 25°C.

Results

Preliminary Results

Both the water characteristics at each site and antibiotic concentrations in each sample were analyzed. During field sampling in June 2014, the average lake temperature was 28.7°C. Water characteristics for each site are shown in Table 3.

Due to time constraints, only the first set of water samples were analyzed. All water samples were analyzed for norfloxacin (NOR), oxytetracycline (OTC), enrofloxacin (ENF), ofloxacin (OFL), and ciprofloxacin (CIP). In all seven water samples, no OTC was detected. In the five river and lake samples (Sites 1-5), no OFL or CIP were detected; OFL, however, was detected in the WWTP influent and effluent and CIP was detected in the WWTP influent (Table 4). NOR

and ENR were detected in all seven samples; however, NOR occurred in the greatest concentrations overall (Chart 1 and 2). The Laozishan Town WWTP was 78.6-79.5% efficient at removing OFL and ENR, but less than 4% efficient at removing NOR (Table 4, Chart 3).

Table 3. *Water characteristics at each site, including conductivity, DO, ORP, pH, and Total Nitrogen (N) concentrations.*

	Conductivity *(µs/cm)*	***DO*** *(mg/L)*	***ORP*** *(mV)*	***pH***	***Total N*** *(mg/L)*
Site 1	*38.4*	*9.83*	*120*	*8.39*	*1.4*
Site 2	*36.2*	*14.25*	*200*	*8.69*	*1.0*
Site 3	*31.5*	*8.46*	*-858.4*	*8.36*	*0.5*
Site 4	*30.7*	*8.30*	*-460*	*8.24*	*2.0*
Site 5	*31.3*	*7.29*	*204.7*	*8.12*	*1.9*
Site 6 *(influent)*	*N/A*	*N/A*	*N/A*	*8.04*	*8.4*
Site 6 *(effluent)*	*N/A*	*N/A*	*N/A*	*8.02*	*17.5*

Table 4. Antibiotic concentrations (OTC, OFL, CIP, NOR, ENR) at each of the six sites, including the WWTP influent and effluent and the WWTP's antibiotic removal efficiency.

Antibiotics (ug/L)	**Site 1** River – SFF	**Site 2** River – NSFF	**Site 3** Lake – NSFF	**Site 4** DSO	**Site 5** USO	**Site 6**[(1)] influent	**Site 6**[(1)] effluent	**WWTP Antibiotic Removal Efficiency**[(2)]
OTC	ND	ND	ND	ND	ND	ND	ND	N/A
OFL	ND	ND	ND	ND	ND	**0.303**	**0.068**	**77.8%**
CIP	ND	ND	ND	ND	ND	**0.011**	ND	N/A
NOR	**0.579**	**0.161**	**0.036**	**0.047**	**0.014**	**0.280**	**0.270**	**3.57%**
ENR	**0.001**	**0.001**	**0.001**	**0.001**	**0.005**	**0.044**	**0.009**	**79.5%**

SFF - Fish Farm Separated From River/Lake; **NSFF** - Fish Farm Not Separated From River/Lake; **DSO** - Downstream from WWTP outlet; **USO** - Upstream from WWTP outlet; **ND** - Not Detectable

(1) Site 6 samples were diluted with 2mL MeOH to be within detectable range. Thus, the results were divided by 250 to account for this dilution.

(2) Percentage of antibiotics removed based on WWTP influent and effluent concentrations.

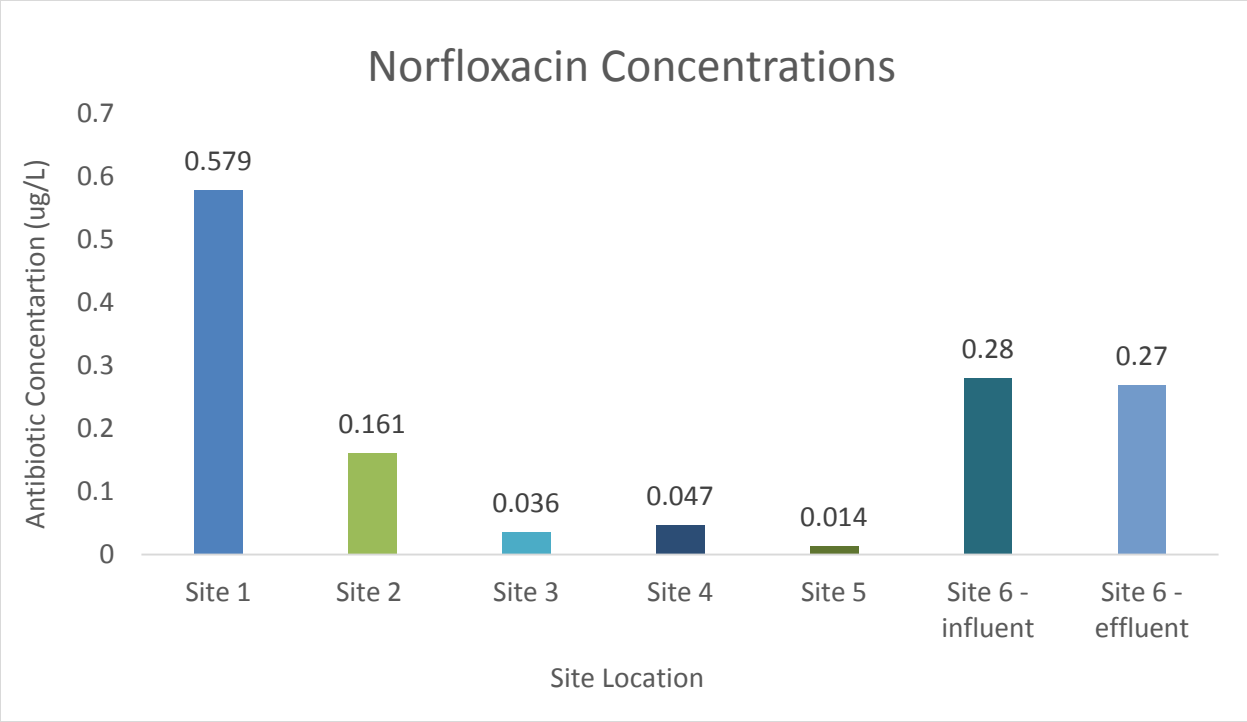


Chart 1. Norfloxacin concentrations at each site in the Hongze River and Huai River.

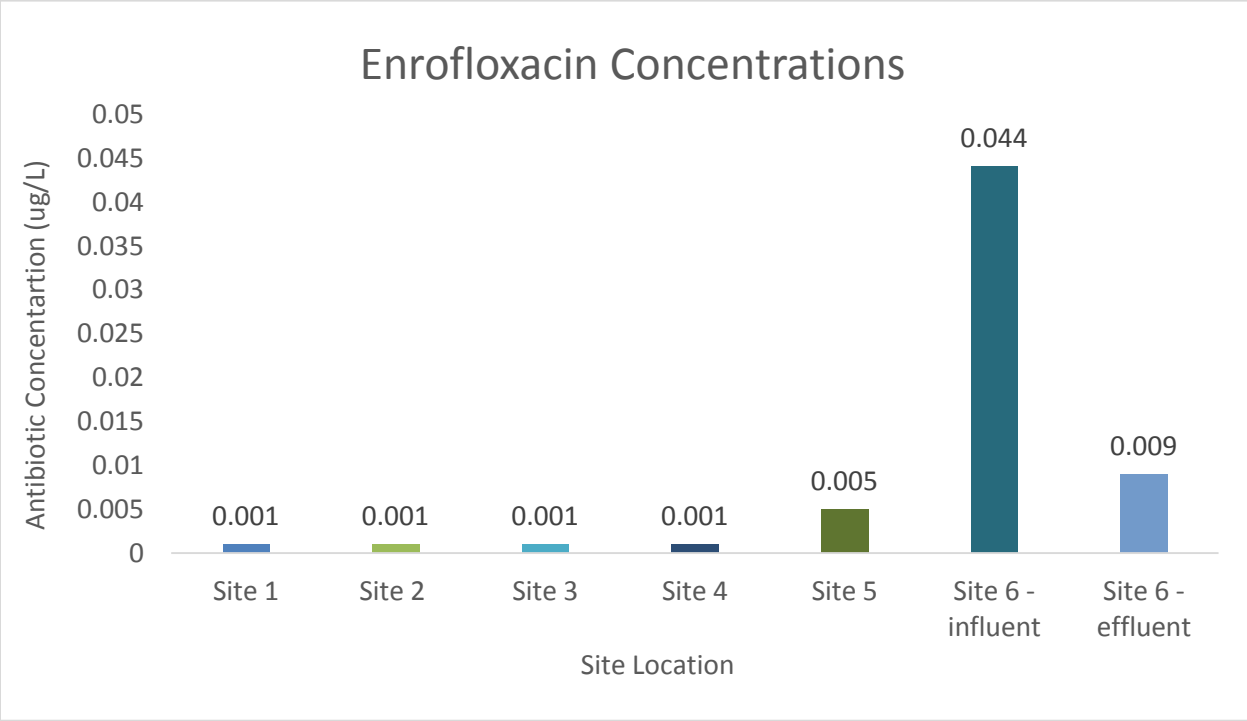


Chart 2. Enrofloxacin concentrations at each site in the Hongze River and Huai River.

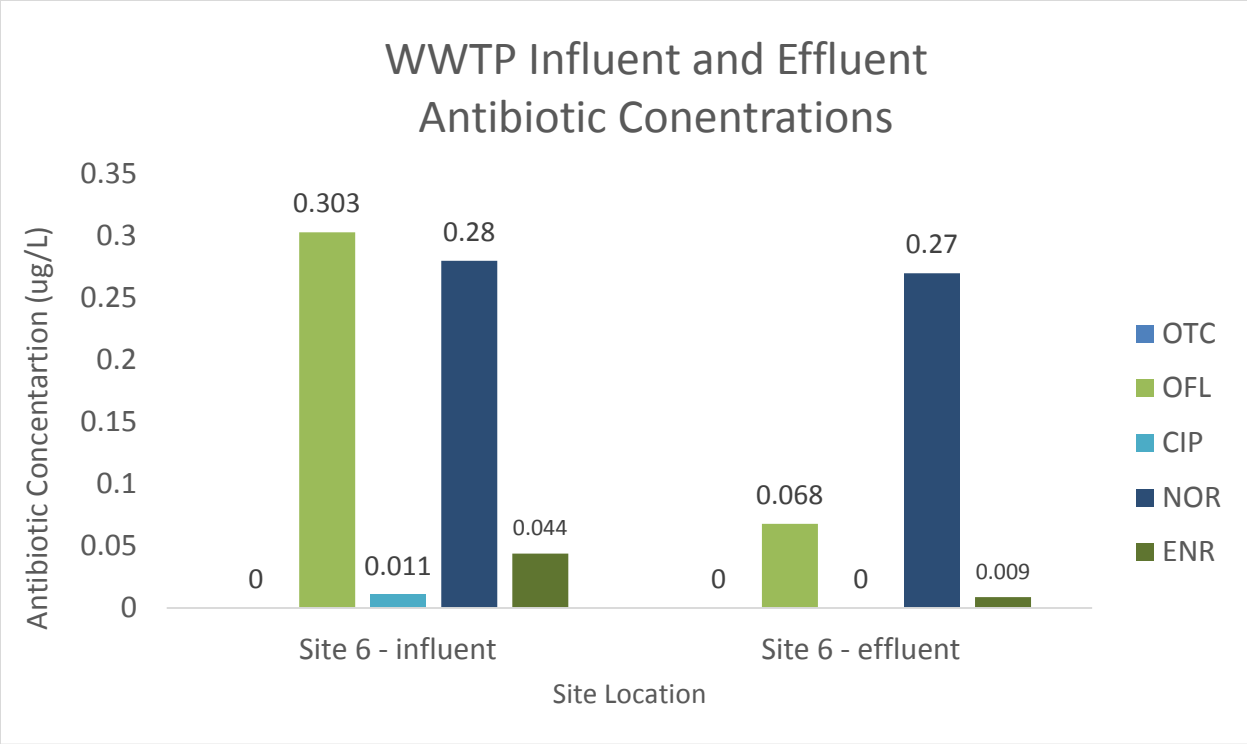


Chart 3. Comparison of the five antibiotic concentrations between the Laozishan Town WWTP influent and effluent.

Discussion

Because this is a time-constrained preliminary experiment leading to a multi-year study, only limited results are available from this data. However, some conclusions can be derived. As shown in Table 4, OTC does not occur at detectable levels in the study region. Thus, it can be suggested that future regional studies do not need to consider this antibiotic as a potential pollutant. Additionally, OFL and CIP are not present at detectable levels in this environment, but were found in WWTP influent and/or effluent. This indicates that these antibiotics may be more commonly used by households rather than fish farms in this region. However, because of the high removal efficiency of these chemicals by the WWTP (over 75%), these chemicals do not leave the site nor enter the environment at detectable levels. This is also suggested by the undetectable levels of OFL and CIP downstream from the WWTP outlet (Table 4, Chart 3).

Because NOR and ENR were present in all 7 samples, these antibiotics should be focused on in future studies of Hongze Lake and Huai River. However, ENR was found at near undetectable levels and may be of less concern than NOR (Table 4, Charts 1 and 2). The greatest concentration of NOR (0.579 ug/L) was found at Site 1, a fish farm that is separated from the Huai River by a dike. Site 2, a fish farm within the river, had lower concentrations (0.161 ug/L). This may indicate that antibiotics stay better concentrated within the confines of a dike-separated farm and become greatly diluted when not separated; however, samples outside the dike need to be collected and analyzed in order to support this conclusion. NOR's presence in these samples may be due to the permitting of some fluoroquinolone for aquaculture use in China, despite them being banned in the USA and other countries (Lumpkin). Additionally, high concentrations of NOR were found to occur in the summer near aquaculture farms in China (Zou et al., 2011).

The second and third greatest concentrations of NOR (0.280 and 0.270 ug/L) were found in the WWTP influent and effluent, respectively (Table 4, Chart 1). NOR's common usage as a treatment for urinary tract and prostate infections may account for these levels, largely because the antibiotic must pass through the body's system unmetabolized to treat these target areas (Kümmerer and Henninger, 2003; "Utinor"). Data on samples near the Laozishan Town WWTP outlet into Huai River were also analyzed. There was approximately 300% more NOR downstream from the outlet than upstream, suggesting that Laozishan WWTP may be a source for NOR contamination in the environment as suggested earlier. Additionally, only ≈3.5% of NOR was found to be removed from influent during the treatment process (Table 4, Chart 3). Oppositely, upstream from the WWTP outlet had a slightly higher concentration of ENR than downstream, indicating that the WWTP effluent is not a major source of this contamination in the environment. Additionally, it was found that ≈80% of ENR is removed from the influent during the WWTP treatment process (Table 4, Chart 3), thus supporting this conclusion. Because of this, it may be concluded that ENR is of less concern than NOR contamination in the aquatic environment.

Results from this study are not similar to findings in the Huangpu River by Chen and Zhou (2014) in which sulfonamides were the most prominent antibiotics in water samples. Additionally, these sulfonamides were found at mg/L levels, a much higher concentration than the ug/L levels found in this study. Also, previous results in Jiangsu Province showed that

oxytetracycline and tetracycline were some of the most frequently detected antibiotics at 64% and 60% detection, respectively (Wei et al., 2011). Because neither of these antibiotics were found in the five environmental samples of this study, Wei et al.'s results are not accurate for all of Jiangsu Province.

However, other studies in China also found NOR to be a common contaminant. For example, a study by Yang et al. (2011) found norfloxacin to be one of the highest concentrated antibiotics in Pearl River. Because a study by Li et al. (2012) found high residuals of NOR (23.8 ng/g) in aquatic commercial animals in Baiyangian Lake, similar results may be found in Hongze Lake and Huai River. Thus, it can be suggested that commercial animals in this region be analyzed for NOR and other antibiotic concentrations. Concentrations of NOR were also much greater than natural degradation and dilution rates in a study by Wiwattanapatapee et al. (2002); thus a similar fate may be occurring in Lake Hongze and Huai River and further study is suggested.

Taking samples in the other parts of the lake away from fish farms and other anthropogenic activity are recommended in order to determine the degree that human activity plays on antibiotic levels in the lake. Because a study in Yongjiang River found that concentrations of most antibiotics increased with proximity to urban areas, it is also recommended that samples be collected closer to cities on the lake and river (Xue et al., 2013). Overall, despite the current time limitations on soil analysis, the goal and some objectives of this study were met. The baseline concentrations of five antibiotics (norfloxacin, oxytetracycline, enrofloxacin, ofloxacin, and ciprofloxacin) in Lake Hongze and Huai River were established and the objective to determine the presence and differences in antibiotic concentrations in six locations on the southern shore of this region were determined. Because of this lake and river's great importance to the local village culture and economy, particularly for its use in aquaculture, antibiotic monitoring in this region will continue at a greater scale. Future study will include the collection of samples at different locations on the lake and river at various times of year. Additionally, SPE and HPLC-MS methods will be further optimized and more antibiotics will be targeted. With the preliminary results from this study, however, background levels of these five antibiotics have been established and will be used to provide further guidance in this multi-year study of Hongze Lake and Huai River.

Acknowledgements

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VERIFICATION OF THE GMCLC1 GENE, A CHLORIDE CHANNEL GENE, BEING INCORPORATED INTO THE GENOME OF SELECT TRANSGENIC *POPULUS DELTOIDES* X *P. EURAMERICANA* 'NANLIN895'

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Abstract

The purpose of this research was to verify that previous research to incorporate a chloride channel gene, GmCLC1 gene, into the genome of the *Populus deltoides x P. euramericana* was successful. In the ecological system of eastern China, not only is the *Populus deltoides x P. euramericana 'Nanlin895'* important for agricultural purposes but they are also used in the wood industry. One factor in particular that has hindered the growth of these poplars on plantations in south east China is that they are located near coastal, or salt, waters. Because these ions can be toxic to their growth, it is vital to find a way to make them more salt resistant. Two leaves were taken from two different transgenic plants for RNA extraction to take place using the E.Z.N.A. Plant RNA Extraction Kit by OMEGA. Following this step, results were processed by using BioDrop µLITE 0.5nm and gel electrophoresis. RNA to cDNA transformation followed using the Promega GoScript™ Reverse Transcription System A5001 protocol 4A: First-Strand cDNA Synthesis with modifications. Master mixes were made according to the primer sets for GmCLC1 gene detection as well as the reference gene and then qPCR was performed. The results from each test concluded that the gene was present. Therefore, it is safe to deduce that the gene was successfully incorporated into the genome of the Populus deltoides x P. euramericana.

Keywords: *GmClC1 gene*, Populus deltoides *x* P. euramericana

Introduction

The important role that vegetation plays within the eco system cannot be ignored, this can be said to be especially true when it comes to trees. Considering that trees provide almost 30% of the oxygen that living organisms need in order to survive, they play a vital role in the environment. Besides producing oxygen through the process of photosynthesis as a waste product, trees are also used as building materials and one of the many ways to control erosion of the soil.

In the ecological system of eastern China, not only does the *Populus deltoides x P. euramericana 'Nanlin895'* produce oxygen which every aerobic organism requires and help to prevent erosion, they are also used in the wood industry. Next to bamboo, the wood that is used from these trees can be found in almost anything. From chopsticks to desks and chairs the poplar plays a great role in this society. Because this group of populous plays such an important role to the ecosystem as well as the economy, it is understandable why improvements would be made in any way possible in order to insure that the tree is performing and growing to its full capacity.

One factor in particular that has hindered the growth of these poplars on plantations is south east China is that they are located near coastal, or salt, waters. The stress that is caused by the salt is thought to be because of the accumulation of the Na^+ and Cl^- ions within the plants, interfering with the processes of photosynthesis and growth because they are toxic (Weibo, 2013). Another reason to want to increase Cl^- ion tolerance specially would be because of its role in the movement of ions inside the cell that are waste so that they are not interrupting the regular processes. Because of this, it is vital to find a way to make this group of *Populus* resistant to the adverse effects of salt.

The gene GmCLC1 was originally discovered in soybeans and transgenic tobacco. The GmCLC1 gene used in the study done by Dr. Sun Weibo was provided in its plasmid form by Dr. Hon-Ming Lim of Chinese University of Hong Kong. This plasmid was inserted into the binary vector pGWB402Ω, provided by Dr. Tsuyoshi Nakagawa at Shimane University. Female poplar hybrid leaves were inserted with *Agrobacterium tumefaciens* strain EHA105 which had gone through bacterial transformation in order to incorporate the gene into its genome. The

objective of this study was to verify that the GmCLC1 gene was successfully incorporated into the genome of the Poplar deltoides hybrids in order to make their tolerance for salt, most specifically chloride ions, existent whereas it had none before.

Materials & Methods

RNA Extraction, BioDrop, and Gel Electrophoresis

In order to extract the RNA from the leaves of the transgenic poplar, the protocol from the OMEGA E.Z.N.A Plant RNA kit R6827-01's standard protocol. There were a total of 4 extractions that took place with two sub groups, two of the samples came from the same specimen therefore it was believed that the results would be similar. After using this kit, the quality of each sample was analyzed using the BioDrop µLITE 0.5µm followed by gel electrophoresis. For each reaction, 3.0 µL of the loading dye 6x loading buffer A5301A and 7 µL of the respective RNA sample were placed into the well of a 1% agarose mini gel with the exception of sample 2B based off of the BioDrop results. The gel was made using 0.2g agarose, 20mL of 1xTAE buffer, and 3µL EtBr. The mixture was poured into the mold, the appropriate combs were placed into it to form the wells, and allowed to cool for 20 minutes. After the gel was loaded it was allowed to run at 120 volts for 30 minutes. Once the run was completed, it sat for 10 minutes and then was observed using the Benchtop UV Transilluminator and the BioRad Molecular Imager ChemiDoc^{TM} XRS^{t} with Image Lab Software. The UV Transilluminator was used to check for the presence of 3 bands while the BioRad Molecular was used to verify their presence.

RNA to cDNA Transformation

Following the process of verification, each sample was then transformed from RNA to cDNA using the Promega GoScript^{TM} Reverse Transcription System A5001 protocol 4A: First-Strand cDNA Synthesis with modifications being made. The first modification made was to the desired concentration of the RNA that was used for each sample. These changes were based on the BioDrop results that were received. Normally, the desired concentration is 200µg/nL for each

sample; however, this was changed to be 30µg/mL per sample. In order to determine how much of the RNA was to be added for each sample's mixture the BioDrop reading was taken and divided into 30µg/mL. The equation that was used was:

$$\mu L = \frac{30}{\frac{\mu g}{nL}\ of\ RNA}$$

That amount was taken out and placed into a 1.5mL sterile tube that was labeled for each sample. This was followed up with 1µL of oligod T (Promega GoScript™ Reverse Transcription System A5001). The total volume for each reaction was to be 6µL, so the remaining volume was met by using RNA grade H_2O. The next modification was centrifuging each mixture using the Eppendorf mini centrifuge at 2000 x rpm for 3 minutes followed by incubation at 70°C for 10 minutes and an ice bath for 2 minutes. Next, a total volume of 4µL was added to each sample bringing the total volume for each sample to 10µL by adding the following chemicals in their respective amounts: 2µL 5x M-MLV, 1µL RNA dd H_2O (lab), 0.5µL dNTP, 0.25µL RNase Inhibitor, and 0.25µL MMLV. This step is also a part of the modifications made to the kit during the process of transforming the RNA to cDNA for each sample. The final modification to the Promega protocol was exclude the incubation at 25°C and move on to incubate each sample in the Eppendorf Thermomixer comfort for an hour at 42°C, followed by another incubation at 72°C for 15 minutes. The final product is then stored on ice if it was going to be used immediately or in a 20°C freezer for long term storage.

Preparation for Real Time qPCR

The final experiment that was performed on the samples was real time quantitative PCR. In order to do this the protocol and all of the necessary materials had to be assembled. Four of the most important ingredients were the primers that were used in order to detect whether or not the gene was present. These primers were NDPK2 Q1, 5'-3' sequence TGTGTATGGCATGGGAAGGT, and NDPK2 Q2, 5'-3' sequence CGCTTTCCATTTTCAGGGCT as well as, ACTIN Q1, 5'-3' sequence GCCATCTCTCATCGGAATGGAA and ACTIN Q2, 5'-3' sequence AGGGCAGTGATTTCCTTGCTCA (Invitrogen). NDPK2 was used in order to detect the GmClC2 gene while ACTIN was used as the reference.

Because there was a total of two sets of primers, the number of total reactions was doubled taking the number from 4 to 8. There were two sets, one with the primer ACTIN and the other containing the primer NDPK2. The protocol was performed as follows: in a sterile 1.5mL tube, one for each of the respective reactions, 28µL of DNA dd H2O was added, followed by 4µL of the appropriate forward and reverse primer. The tubes were then labeled according to which sample was to be placed inside of them followed by 4µL of that sample. The last material to be added to the sample mixture was 40µL of the SYBR Green Dye bringing the total volume to 80µL. SYBR Green is a dye is light sensitive and for this purpose this step was done in the dark.

All of the complete sample mixtures were then centrifuged in order to insure all components mixed together properly. 4 rows of 0.2mL 8 tube strips were placed on ice. In the first 3 tubes on each row, 20µL of a sample in the first sub group was added, the 4^{th} and 5^{th} tubes were skipped, and 20µL of the samples in the second sub group were placed in the last 3 tubes. Caps were then placed on each strip of tubes so that they could be spun in a vortex in order to insure that everything was at the bottom of the tube. After this the strips were placed inside of the Applied Biosystems StepOne Plus RealTime PCR System. The samples were run under advanced set up, as being a quantitative experiment containing SYBR Green. The qPCR protocol was preprogramed into the system and is as follows: step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds, step 3: 60°C for 1 minute, go back to step 3 40x, step 4: 95° for 15 seconds, step 5: 60° C for 1 minute, step 6: 95° for 15 seconds.

Results

RNA Extraction, BioDrop, and Gel Electrophoresis

The RNA extraction results were checked by using the BioDrop µLITE 0.5nm program and gel electrophoresis on a 1% agarose gel with Ethidium Bromide. The results for the BioDrop varied for each sample. Sample 2B had the highest reading at 502.2µg/nL followed by sample 1B at 34.24µg/nL, sample 2A at 29.61µg/nL, and sample 1A with a reading of 6.828µg/nL.

Sample Name	µg/mL	260/230	260/280
1A	**6.828**	**1.585**	**2.064**
1B	**34.24**	**1.634**	**2.103**
2A	**29.61**	**0.327**	**1.947**
2B	**502.2**	**2.130**	**2.094**

Figure 1: The table is a simple representation of the readings that were received for each sample from the BioDrop. The 260/230 category represents the measurement for pure nucleic acid with an ideal range being from 2.0-2.2. The 260/280 category represents the amount of RNA to DNA, a number in the 1.8-2.0 range is ideal with 2.0 meaning pure RNA.

It is perceived that the higher the concentration of RNA present, the better the qPCR results will be; however, because RNA was detected these samples were used in the transformation process with modifications to the protocol in order to yield the best possible results. The gel electrophoresis, despite the BioDrop results, did show that a considerable amount of RNA was present even in the samples that had low concentration results by the presence of bands.

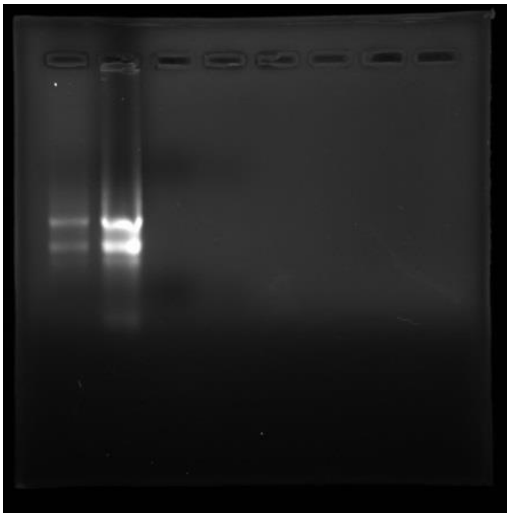

Figure 2: This figure shows a 1% agarose EtBr gel with RNA samples 1A and 1B in wells 1 and 2 respectively. The bands that are present verify that the RNA extraction from those leaves was successful.

Although three bands is the ideal result, two bands being produced was still enough to determine that RNA was in fact present therefore again verifying that the RNA extraction was successful.

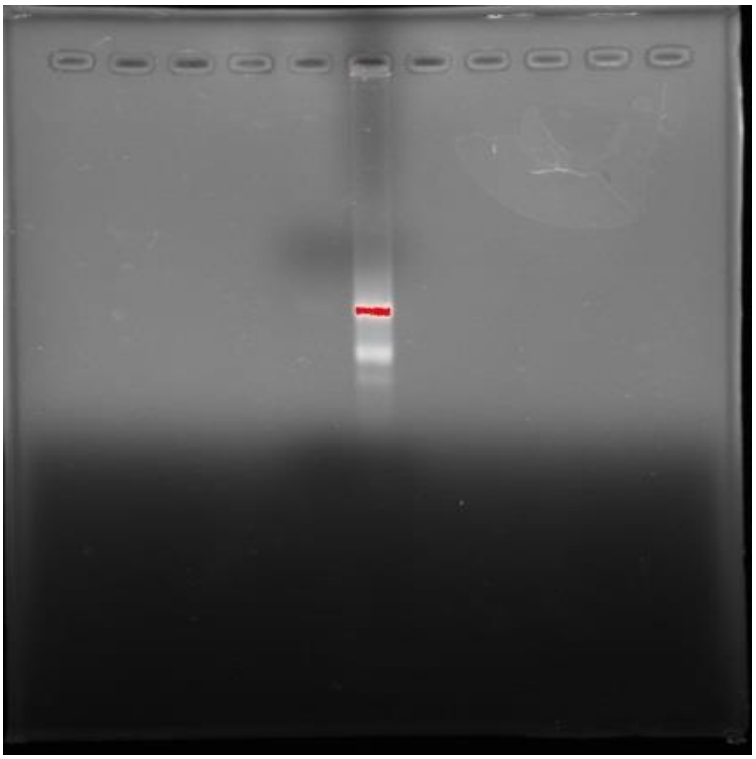

Figure 3. This gel depicts the electrophoresis that was done on samples 2A and 2B. Sample 2A did not show up at all during the process; however, in well 5 sample 2B can be seen. This sample displays the desired 3 bands for RNA.

RNA Transformation and qPCR

Out of the 24 samples that were run under the qPCR conditions, only 15 samples produced favorable results. The determining factors in whether or not a reaction is successful are first indicated by the Ct that is produced once a full run is completed. Ct simply stands for when the reaction started to take place or surpassed the threshold, background, by no longer being even in the amount of genetic material that was present. This point on a graph will first be a straight line and then a curve will form. Where the formation of this curve takes place is simply a representation of where in the annealing cycles the SYBR Green dye binds to the cDNA along

with the primer and the replication process begins. An example of this can be seen in Figure 3 below which was produced for one of the samples after the qPCR program was complete.

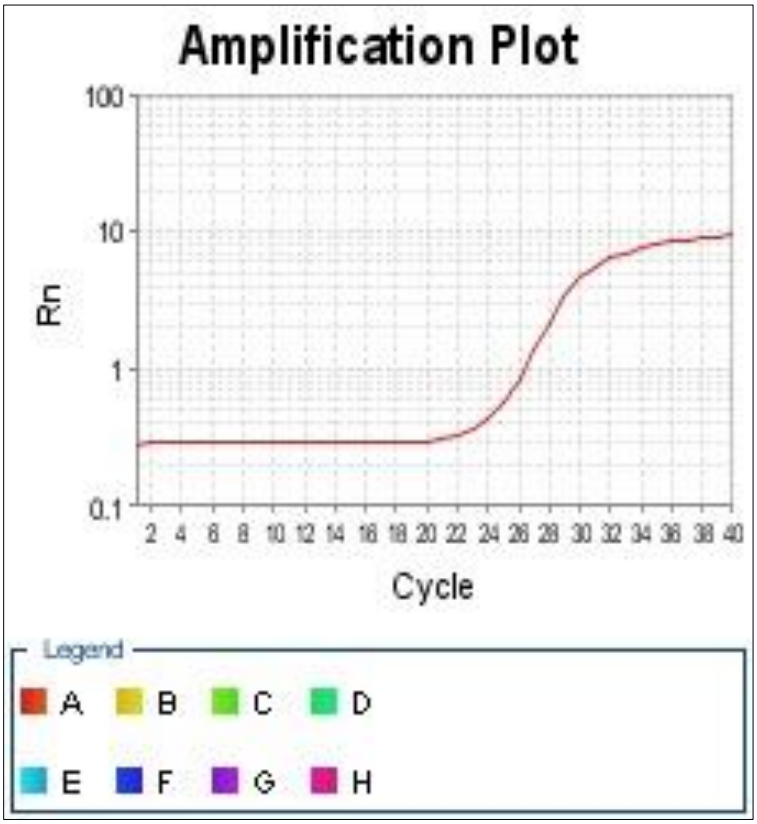

Figure 4. This figure is an illustration of sample $1A_1$, or simply sample A1, and the Amplification curve that was produced from the qPCR reaction. This curve shows that the sample's replication surpassed the background during cycle 26.

Rn on the graph represents the fluorescence of the reporter dye, in this case NDPK2, reaching the same level of fluorescence as the ROX, or background reference. In this case the background is what the samples were being compared to in order to see if the GmClC1 gene was present. This is an important value because it provides another way of verifying the results that a reaction did take place producing a product as well as if the Ct value is accurate.

Another factor in whether results are favorable is in what cycle number the sample covers over the threshold. An ideal Ct number will be no more than the total number of cycles in the run minus 10. This is an important factor because the Taq polymerase will either be used up at this

point depending on the quantity that was placed into each reaction, or it may no longer be active. Therefore it is believed that anything produced after 30 cycles, or 34 to 35 in order to be on the safer spectrum of things is not used. This is what caused 9 samples to be considered not favorable for the sake of the results. An example of this can be seen in Figure 4A in the multicomponent graph.

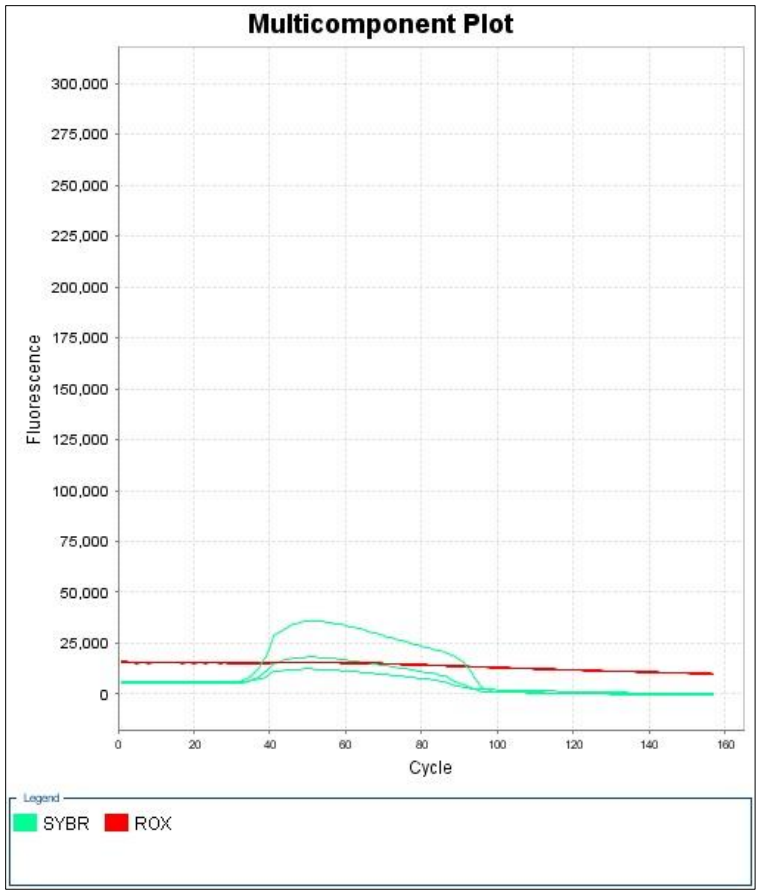

Figure 5A: The multicomponent graph shows samples 2A with the reference primer, wells 3F-3H on the 96 well plate.

As seen in the figure above, the amplification starts around the 35th cycle of the qPCR. Considering that the program was only set to run 40 cycles overall, it is safe to say that the Taq polymerase could have been used up at this time. A desirable multicomponent graph will show the reaction starting from cycle 15 to cycle 30, giving the reactions 15 cycles worth of annealing for the primers to bind and the SYBR Green to fluoresce. The fluorescence of the SYBR Green

dye is very important to the overall results because this is what allows the production of product to be seen while the reaction is still taking place. It allows for the amount of cDNA that is present to be measured through how strong the fluorescence itself is along with how much product is being produced within each reaction. This can also be seen in the multicomponent graph.

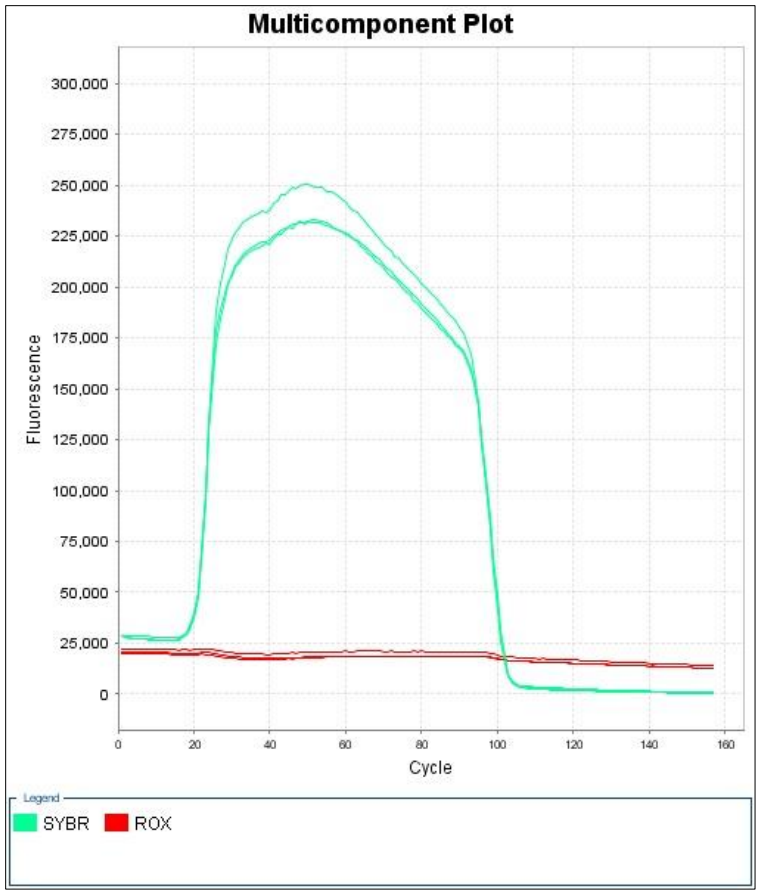

Figure 5B: This multicomponent graph shows samples 2F-2H. The start of the replication of the cDNA can be seen when the curve starts to form around cycles 18 and 19 of the program.

These results can be used to verify that the RNA to cDNA transformation was successful even though all of the qPCR results were found to be reasonable. This can be said based on the fact that in order for the qPCR to produce some type of reaction the cDNA had to present, meaning that the RNA was successfully transformed. Since there is no other way to check this process except through doing a PCR reaction the overall experiment is considered to be successful.

Sample name	Well number	Subgroup (primers)	Ct number
$1A_1$	**1A**	**NDPK2**	**26.36**
$1A_1$	**1B**	**NDPK2**	**26.13**
$1A_1$	**1C**	**NDPK2**	**26.11**
$1A_2$	**1F**	**ACTINQ**	**17.62**
$1A_2$	**1G**	**ACTINQ**	**17.8**
$1A_2$	**1H**	**ACTINQ**	**28.38**
$1B_1$	**2A**	**NDPK2**	**23.72**
$1B_1$	**2B**	**NDPK2**	**23.47**
$1B_1$	**2C**	**NDPK2**	**23.53**
$1B_2$	**2F**	**ACTINQ**	**15.84**
$1B_2$	**2G**	**ACTINQ**	**16.39**
$1B_2$	**2H**	**ACTINQ**	**16.47**
$2B_1$	**3A**	**NDPK2**	**33.56**
$2B_1$	**3B**	**NDPK2**	**27.93**
$2B_1$	**3C**	**NDPK2**	**28.32**
$2B_2$	**3F**	**ACTINQ**	**31.82**
$2B_2$	**3G**	**ACTINQ**	**32.98**
$2B_2$	**3H**	**ACTINQ**	**35.06**

$2A_1$	**4A**	**NDPK2**	**Undetected**
$2A_1$	**4B**	**NDPK2**	**Undetected**
$2A_1$	**4C**	**NDPK2**	**27.33**
$2A_2$	**4F**	**ACTINQ**	**Undetected**
$2A_2$	**4G**	**ACTINQ**	**35.96**
$2A_2$	**4H**	**ACTINQ**	**Undetected**

Figure 6. The contents of the table are the results of the qPCR that was performed. Each sample is first listed by their sample number and subgroup then by the well number that they occupied in the 96 well plate.

Conclusion

Based on the table provided in Figure 5, as well as the other results, it is safe to conclude that the experiment was successful. 62.5% of the reactions produced numbers and graphs that could confirm that the reaction did produce a product that could be measured through the Ct number and verified with the supplemental graphs. The Ct numbers obtained for each reaction, along with the graphs, BioDrop results, and gel electrophoresis results do prove that the null hypothesis is correct: the GmClC1 gene was successfully incorporated into the genome of the *Populus deltoides x P. euramericana* making it transgenic.

Discussion

Although the experiment was a success, with a 62.5% yield, I do believe that there were some things that could be improved upon in order to make this number higher. For example, when the BioDrop results were received and the majority of them were low, I would have suggested going back and collecting more samples in order to perform the RNA extraction again. This also would have been helpful because when looking at the gel electrophoresis results as well. Another improvement for the gel electrophoresis would have been allowing the mini gel to run longer

than the allotted 30 minutes because it's possible that 3 bands could have been present for samples 1A and 1B but didn't show because the molecules didn't move fast enough through the gel with the dye. When it comes to the transformation of RNA to cDNA, I don't believe that there was anything wrong with the process. However, when preparing all of the samples for the final protocol of qPCR there are a few recommendations. The first would be to be sure that the lab is almost completely dark and that the SYBR Green has no chance to come in contact with light at any point before being placed into the instrument where the reactions will take place. Because SBYR Green fluoresces in the present of light, this could have caused some the reactions to start before being placed into the Applied BioSystems StepOne Plus Real time PCR System. Therefore exposing the reactions to the least amount of light as possible could have caused the resulting products to be higher thus raising the percent yield and amount of product produced, better confirming the presence of the GmClC1 gene in the samples.

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EXAMINATION AND COMPARISON OF MOLECULAR SEXING PROTOCOLS FOR TWO SONGBIRD SPECIES IN CHINA – EVALUATION OF DNA EXTRACTION KITS, PRIMERS, AND FEATHER AGE

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Abstract

Accurate sex identification of birds is important for many wildlife studies ranging from behavioral studies to forensic studies. With many bird populations in the USA declining, it is imperative to gain a full understanding of a species' natural history, including any sex-related patterns. Studying these patterns can be difficult with over half of all Passeriformes species being sexually monomorphic; however, the development of molecular sexing techniques through polymerase chain reaction (PCR) has reduced the difficulty. This new development has given rise to a number of different DNA extraction protocols and primer sequences. We tested three DNA extraction kits and four primers commonly used for avian molecular sexing. We plucked feathers from a Black-throated Tit (*Aegithalos concinnus*) and two Long-tailed Tits (*Aegithalos caudatus)* and tested the Qiagen DNeasy Blood & Tissue Kit (Qiagen), Tiangen TIANamp Genomic DNA Kit (Tiangen), Omega MicroElute Genomic DNA Kit (Omega) for quantity and quality of DNA, and primers P2/P8, 2718R/2550F, sex1'/sex2, 2718R/2376F for sex identification success rate. Our primary objective was to determine which kit and primer produced the most accurate sex identification results. We also investigated the effect feather age has on the quantity and quality of DNA extracted. Results indicated that feathers taken from the oldest specimen produced a significantly larger amount of DNA than that of the young specimen. We found the Omega kit produced the largest average quantity (5.63ng/µL) and the best quality (1.8 260/280). Overall, primer 2718R/2550F had the most successful sex identification rate. When primer 2718R/2550F was paired with the Qiagen kit it had a 4/6 success rate, and when primer sex1'/sex2 was paired with the Omega kit it had a 5/6 success rate. We concluded the Omega kit was the best for DNA extraction and primers 2718R/2550F and sex1'/sex2 were the best primers for sex identification.

KEYWORDS: *genetic analysis, molecular sexing, avian, China, DNA extraction, primers*

Introduction

Monitoring data collected over the last three decades have shown population declines in many land-birds in USA (Gonzalez-Prieto et al. 2011). Effective conservation of migratory birds relies on understanding each aspect of a birds' annual cycle (Gonzalez-Prieto et al. 2011), including sex-related patterns. Sex-related patterns can be studied under a number of different research domains including behavioral studies (Lewis et al. 2002, Genovart et al. 2008), offspring sex ratio studies (Whittingham & Dunn 2000), management and reintroduction of species (Bose et al. 2007, Zhao et al.), evolutionary studies (Freed et al. 2009), and forensic studies (An et al. 2007). Unfortunately, studying these patterns can be difficult because the basic information of individual's sex is not always easy to obtain.

At least 60% of all Passeriformes species have males and females that have similar phenotypic traits (Griffiths et al. 1998), also referred to as sexual monomorphism . Previously, it has been difficult to investigate sex-based variations for these sexually monomorphic species. The development of molecular sexing techniques through polymerase chain reaction (PCR) amplification has reduced the difficulty (Griffiths et al. 1998).

Recent development of molecular techniques using molecular markers allows identifying a bird's gender inexpensive. PCR-based methods in avian molecular sexing use the sex-linked gene, chromodomain helicase DNA binding 1 (CHD1) gene (Griffiths et al. 1998), for sex identification. Through PCR amplification, Z and W alleles can be amplified and the products should appear as a single band in males (ZZ) and two bands (ZW) in females (Griffiths et al. 1998). For birds a common source of DNA is blood, but in recent studies, feathers have proven to be an equally reliable source of DNA (Harvey et al. 2006, Bayard de Vollo et al. 2008, Wang et al. 2009, Johansson et al. 2012). This provides the opportunity to obtain gender related information that could be used for the conservation and ecological studies of birds (Smith et al. 2003) with limited physical impact to the birds.

With this new development of non-invasive sexing techniques for birds, a number of different protocols for DNA extraction have been published over the years (Morin et al. 1994, Eguchi & Eguchi 2000, Horvath et al. 2005., Bayard de Volo et al. 2008). DNA has shown to be

successfully extracted from plucked feathers (Mundy et al. 1997, Harvey et al. 2006, Wang et al. 2009) and molted feathers (Horvath et al. 2005, Bayard de Vollo et al. 2008, Johansson et al. 2012). As feathers grow they are supplied with blood, and although the blood flow stops once the growth is complete, residual red blood cells remain inside the feather shaft (Horvath et al. 2005). Harvey et al. (2006) compared plucked feathers to blood as a concrete DNA source for sex identification, and provided evidence that feathers are a reliable source of DNA, matching the sex determinations of blood reactions 100%. Molted feathers also prove to be a good source of DNA (Segelbacher 2002, Bayard de Volo et al. 2008, Kerr and Voelker 2010, Johansson et al. 2012). Essentially the feather shaft provides a microenvironment that protects the DNA from any degradation caused by solar radiation, hydrolysis, and microorganisms (Bayard de Volo et al. 2008). On the contrary, with dried blood cells being the only source of DNA in the feather, the DNA that is extracted tends to be low quality and quantity (Horvath 2005). With that being said, having an impeccable DNA extraction method is essential in determining the quantity and quality of DNA obtained.

Scientists often prefer to perform the extraction methods using their own mixed buffers, oppose to kits with premade buffers (Bayard de Vollo 2008, Mino et al. 2009). Bayard de Vollo et al. (2008) created a new extraction protocol that involved 1xTNE, 1M Tris-HCl, Proteinase K, 25% SDS, and 1M DTT, followed by the use of a protein precipitation reagent (Ammonium acetate) and a hydration buffer (TE). All together, the DNA extraction process for this protocol could take anywhere from 6-10 days, 3-7 days for incubation and 3 days for extraction. Mino et al. (2009) followed a similar method and used a Phenol:Chloroform (PC) extraction procedure that had an incubation period of 7 days and then used a PC liquid-liquid procedure to extract the DNA. Each of these studies successfully extracted DNA from their feather samples, but a lot of time was lost preforming a single DNA extraction. DNA extraction kits may reduce the amount of time spent performing the protocols, usually only taking 1-2 days for a full extraction.

DNA extraction kits vary by country and even more so, vary by the specimen or sample being used – museum or live specimen: blood, muscle, egg, or feathers. Johansson (2012) tested the accuracy of molecular sexing using molted feathers versus plucked feathers and concluded that molted feathers are a good source of DNA as long as they are in good condition. Horvath et al. (2005) tested a new DNA source for genetic analysis in birds. Most previous studies extracted

DNA from the basal tip of the calamus (Mundy et al 1997), but Horvath et al. (2005) tested the blood clot of the superior umbilicus for DNA and found that clot samples yielded higher amounts of DNA than tip samples. Both studies used DNeasy Tissue Kit. Other kits have also been found in literature. Naim et al. (2011) used the Tissue DNA kit by Genispin to test the reliability of molecular sexing for the white-bellied sea eagle (*Haliaeetus leucogaster*), and similarly, Costantini et al. (2008) used the GenElute TM Mammalian Genomic DNA miniprep Kit to test the non-invasive techniques of molecular sexing on the endangered Humboldt Penguin (*Spheniscus humboldti*). Wang et al. (2010) used multiple kits to extract DNA. There are many considerations that go into choosing a DNA extraction kit: the size of the sample (e.g. large or small feather), the age of the sample, and where the sample comes from (Wang et al. 2010). Researchers may choose extraction method based on the sample they were extracting from – feather, egg shell membranes, muscles, or blood.

Although the age of a sample should be considered when choosing a DNA extraction kit, in some studies, there was little regard to the age of the feather samples. Bayard de Volo et al. (2008) successfully extracted viable DNA from feathers that ranged from 1-10 years old, and Kerr & Voelker (2010) from feathers that were approximately 120 years old. Bayard de Volo et al (2008) collected molted feathers from free-ranging Northern Goshawks (*Accipiter gentilis*) in Northern Arizona weekly from May to August of 1991-2000 and stored feather samples at room temperature in paper bags. Kerr & Voelker (2010) were able to identify the originating species of a Comanche artifact using molecular methods. The artifact was estimated to be over 120 years old and had been stored over a fireplace in a ranch house for over 60 years. Other studies, like Johanson et al. (2012) found that shed feathers, if in good condition, are a good source of DNA but if they have long-term exposure to the elements and are shed long before collection then the success rate of DNA extraction goes down. Johanson et al. (2012) suggests minimizing the time between shedding and collection to have good quality DNA. Many questions still surround the effect feather age has on DNA extraction and PCR success.

Along with numerous extraction methods, there are also a number of primers that have been proposed for avian sex identification. Commonly used primers are P2/P8 (Griffiths et al. 1998), sex1/sex2 (Wang & Zhang 2009), and 2550F/2718R (Fridolfsson & Ellegren 1999). Based on

the sequences of these primers, even more primer sets have been developed for molecular sexing (Lui et al. 2010, Wang et al. 2010).

In 1998 Griffiths et al. developed a single set of primers P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCCAAGGATGAGRAAYTG-3') that amplified homologous sections of both the CHD-W gene and CHD-Z gene. The primers P2 and P3 (Griffiths & Tiwari 1995) that are used to identify the CHD genes in domestic chickens, provided a basis for developing primers P2 and P8. They tested the primers accuracy on 28 species from 23 families, and included 11 of the 23 avian orders. They found that the P2/P8 sequences were able to correctly sex 27 of the 28 bird species by simply using PCR and agarose electrophoresis. This study provided the baseline for many other studies to use PCR methods to sex birds. Ramsey et al. (2003) and Harvey et al. (2006) both used primers P2/P8 to successfully sex Black-capped Chickadees (*Poecile atricapilla*). Harvey et al. (2008) used it on both blood and feathers. Other studies have used these primers for more than just genetic studies. Wilson et al. (2008) used primers P2/P8 to study the different migration patterns in males and females using a single feather collected.

Other primers like 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') (Fridolfsson & Ellegren 1999), sex1/sex2 (Wang & Zhang 2009) and their modified versions (Wang et al. 2010, Liu et al. 2010) have also been developed to increase the accuracy of sex identification. Fridolfsson & Ellegren (1999), similar to Griffiths et al. (1998), analyzed 50 avian species from 11 avian orders. A known male and known female were used for most species, but in three cases only a female was available. Fridolfsson & Ellegren used a different PCR (or thermal) profile than other molecular sexing methods use. They used what is termed a "touch-down" (Don et al. 1991) with the annealing temperature decreased by 1°C per cycle, starting from 60°C until 50°C. After 50°C, additional 25-35 cycles ran at that constant temperature. Of the 50 species that they analyzed, 47 of them could be accurately sexed with primers 2550F and 2718R. Lui et al. (2010) discovered that Fridolfsson's 2550F primer mismatched the CHD-Z sequences in the Chinese gamecock chickens. They used a new chicken CHD-Z primer 2376F (5'-GCTACTGATTCGTCTGCGAGA-3') paired with Fridolfsson's original 2718R primer.

Wang & Zhang (2007) designed primers sex1 (5'-CTCCCAAGGATGAGAAACTGTGCAAAACAGGTA-3') and sex2 (5'-CCTTCACTTCCATTAAAGCTGATCTGGAATTTC-3') specifically to improve the reliability of molecular sexing for the brown eared pheasant (*Crossptilon mantchuricum*), and then they applied the new primers across a variety of species. The successful identification rate was 100% using these primers for the brown-eared pheasant, and it was also successful when sexing five other species in the Passeriformes family. Wang et al. (2010) took primers sex1/sex2 even further and modified them to improve their sex identification abilities for Passeriform species. They tested 99 individuals that belonged to 17 bird species in ten families. Wang et al. (2010) modified sex1 by deleting three base pairs at the 3'-end (referred to as sex 1') and substituted four base pairs in sex2 that did not match with CHD-Z (referred to primer sex-mix). They then recombined primer sets as sex1'/sex2 and sex1'/sex-mix. After running the PCR products through electrophoresis, they found that the sex identification rate with P2/P8 was 85.6%, sex1/sex2 was 89.6%, sex1'/sex-mix was 92.7%, and sex1'/sex2 was 98.9%. They concluded that sex1'/sex2 for sexing species in Passeriformes. These improved PCR methods and proper DNA extracting methods provide a simple, rapid, and inexpensive procedure for the sexing of many avian species.

In this study, we tested the quantity and quality of DNA extracted from feathers of bird specimens of three different ages (8 years, 5 years, and 4 years) using three different DNA extraction kits – Qiagen DNeasy Blood & Tissue Kit, Tiangen TIANamp Genomic DNA Kit, Omega MicroElute Genomic DNA Kit. In addition, we tested the accuracy of PCR products based on the primers – P2/P8, 2718R/2550F, Sex1'/Sex2, 2718R/2376F. The study intended to improve our understanding of molecular sexing and help researchers who are unfamiliar with these methods. The results from this study could increase our ability to do more sex-based avian studies on sexually monomorphic species, which could help develop more effective conservation strategies for these bird populations. We hypothesized that the kit used for DNA extraction would affect the quantity and quality of the DNA; the primer pair used in PCR will not affect the accuracy of the sex identification, but it will affect the clarity of the bands; feather age will have an adverse effect on the quantity and quality of DNA, meaning as the feather age increases the quantity and quality decreases.

Materials and Methods

Feathers were analyzed in the Avian Molecular Ecology Lab of the College of Life Sciences at Beijing Normal University, Beijing China. Feathers were collected from two species: Long-tailed Tit (*Aegithalos caudatus)* and Black-throated Tit (*Aegithalos concinnus*), both were collected locally. The two Long-tailed Tit specimens were a male and female collected in 2009 and 2010, and the Black-throated Tit was a female collected in 2006. All specimens were stored whole in a freezer set at -20°C. Two retrices (Figure 1) were taken from each individual for analysis.

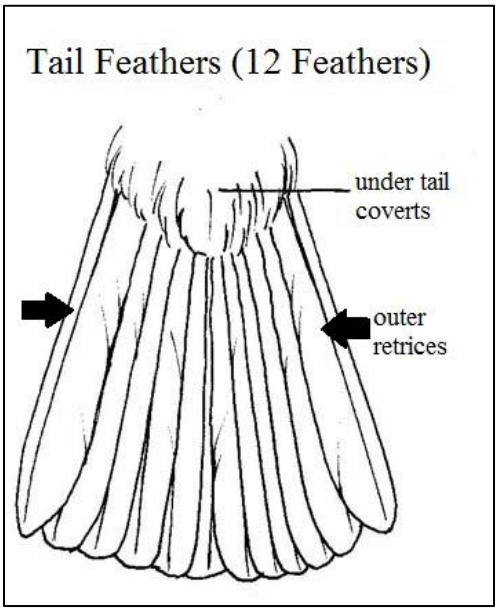

Figure 1. (modified from All About Birds 2014) A diagram of under tail of common songbird.

Genetic Analysis

The sex of the individuals was determined using molecular markers. DNA from the feather samples was extracted and used for PCR.

DNA extraction: Three kits were used for DNA extraction – Qiagen DNeasy Blood & Tissue Kit (Qiagen), Tiangen TIANamp Genomic DNA Kit (Tiagen), and Omega MicroElute Genomic

DNA Kit (Omega). The entire feather calamus was used for all three extractions (Figure 2) (Horváth et al. 2005). Two feathers were used for each extraction. In addition, minor modifications were made to the protocol that was given in each kit. These modifications were based on the experience of the experts in the lab (Appendix A).

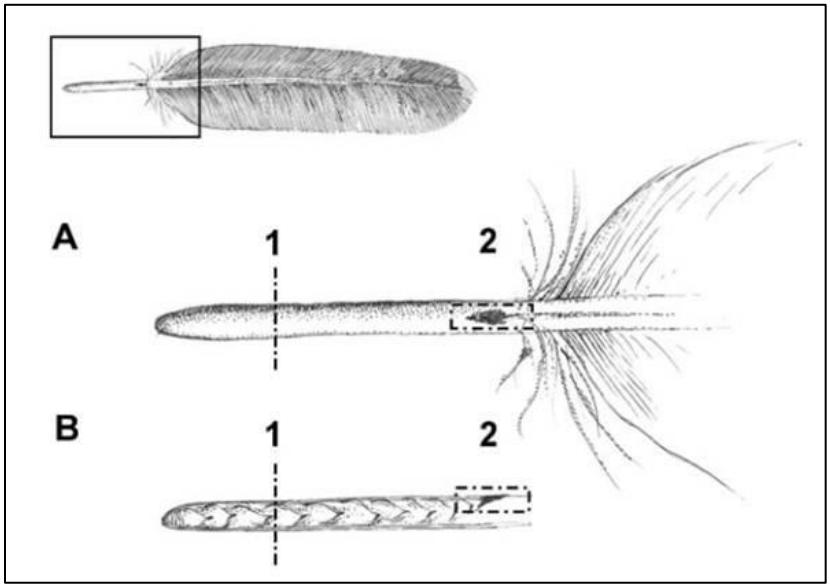

Figure 2. (Horváth et al. 2005) Diagram of a typical flight feather. Two sampling areas are shown: (1) basal tip of the calamus and (2) blood clot in the superior umbilicus.

DNA quantification: Following DNA extraction, both the quantity and quality of DNA were tested using two different machines: Thermo Fisher Scientific Nanodrop 2000 (Nanodrop) and BIOtek Synergy H1 Hybrid Reader (H1 Hybrid Reader). We measured the quantity of the DNA by looking at the concentration in ng/µl. The ratio of absorbance at 260 and 280nm was used to assess quality of DNA, also known as the amount of DNA relative to RNA. A ratio of 1.8 is generally accepted as "pure" DNA. If the ratio is appreciably higher (2.0) then it indicates the presence of RNA, and if the ratio is lower than it may indicate contamination by either protein, phenol or other contaminants that absorb at 280nm. DNA concentration and quality for each sample was measured three times using the Nanodrop and twice using the H1 Hybrid Reader. To test whether there was a difference between quantity and quality values given by the two machines, we ran an ANOVA test. To test whether the DNA extraction kit, feather age, or an

interaction of the two had an effect on the quantity and quality values we used two-way factorial ANOVA, followed with Tukey Test for comparison. All statistical analysis was done in Microsoft Excel.

PCR: The primers used for sex-identification include: P2/P8, 2718R/2550F, Sex1'/Sex2, 2718R/2376F (Table 1) (Griffiths 1998).

Table 1. Primer sequences used for molecular sexing

P2/P8		Sex1'/Sex2	
P2	5' -TCTGCATCGCTAAATCCTTT-3'	Sex1'	5'-CTCCCAAGGATGAGAAACTGTGCAAAACAG-3'
P8	5' -CTCCCAAGGATGAGRAAYTG-3'	Sex2	5'-CCTTCACTTCCATTAAAGCTGATCTGGAATTTC-3'
2718R/2550F		2718R/2376F	
2718R	5'-ATTGAAATGATCCAGTGCTTG-3'	2718R	5'-ATTGAAATGATCCAGTGCTTG-3'
2550F	5'-GTTACTGATTCGTCTACGAGA-3'	2376F	5'-GCTACTGATTCGTCTGCGAGA-3'

These primers were the most frequent used in avian molecular sexing methods. We used TaKaRa Bio Inc. Premix Taq that contained 25mM TAPS (pH 9.3 at 25C), 50mM KCl, 2 mM $MgCl_2$, 0.1mM DDT, 200µM of each dATP dGTP dCTP, 100µM dTTP, and 0.25mg/ml activated salmon sperm DNA. The PCR mixture used had a total volume of 10µl containing 2µl DNA, 0.5µl per primer, 5µl Taq mixture, and 2µl ddH_2O. Two PCR profiles were used for this analysis to ensure that our results were not based solely on the PCR profile. The first was labelled as "PCR-1," and consisted of 5 min denaturation step at 94°C, 34 cycles of 30-s denaturation at 94°C, 45-s annealing at 50°C, and 45-s extension at 72°C, and a final extension for 5 min at 72°C. The second PCR profile was labelled as "PCR-2." This PCR profile was similar to PCR-1, but had more steps and is referred to as a "touchdown PCR" (Don et al. 1991). It consisted of a 5 min denaturation step at 94°C, 22 cycles of 30-s denaturation at 94°C, 45-s annealing at 63°C with auto delay of -0.5°C for each cycle, and 45-s extension at 72°C, 13 cycles of 30-s denaturation at 94°C, 45-s annealing at 52°C, and 45-s extension at 72°C and a final extension cycle for 7 min at 72°C. Following PCR, the PCR fragments were made visible on a 2% agarose gel containing ethidium bromide using DL1000 and DL 2000 markers. Females were

identified by two bands in the gel electrophoresis (CHD1Z allele and CHD1W allele), and one band for males (two CHD1Z alleles) (Figure 3) (Dubiec & Zagalska-Neubauer 2005).

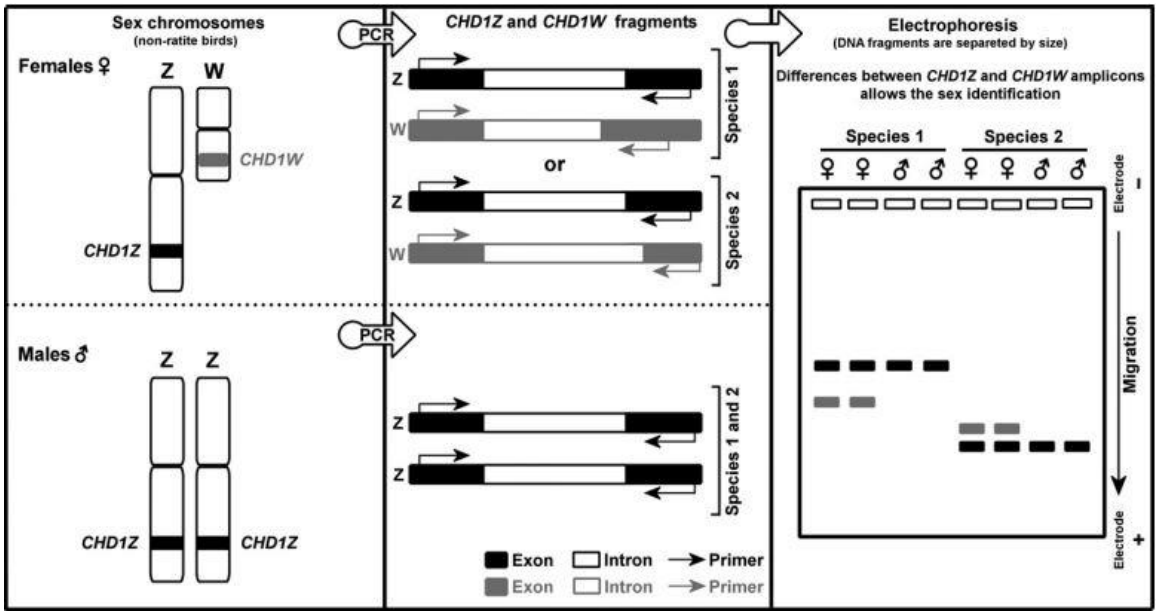

Figure 3. (Morinha et al. 2012) Diagram of the basic principles of avian molecular sexing. Males have two copies of CHD1Z allele, while females have one copy of CHD1Z and one of CHD1W. The primers will amplify these regions enabling the sex identification. The CHD1Z allele will always be the same between both sex and species, but CHD1W will vary in size between species. The separation of the fragments allows the genotyping of males (ZZ) and females (ZW).

Statistical Analyses

Sex identification success rate: After PCR, the PCR product was examined for accuracy. If the correct number of bands for showed up then we counted it as a success, but if the incorrect number showed up we marked it as a failure.

Results

Two DNA extraction runs were performed, each which included all three of the extraction protocols, but due to a malfunction in a machine we were only able to use the results of one. With the single DNA extraction run, we ran a total of 72 PCRs which included three different samples, three different extraction methods, four different primers, and two different PCR programs. The quantification values (Table 2) and quality values (Table 3) given by both the Thermo Fisher Scientific Nanodrop 2000 and BIOtek Synergy H1 Hybrid Reader proved to be not significantly different so we proceeded to combine the values given by both machines to get better mean quantifications.

Table 2. ANOVA Table testing the difference between the quantity readings of DNA samples read by two different machines (Nanodrop & H1 Hybrid Reader).

Source Table	SS	df	MS	Fstat	Fcrit	P-value
Sstotal	614.18	44				
Sstreatment	4.65	1	4.65	0.33	4.08	> 0.20
Sserror	609.53	43	14.2			

Table 3. ANOVA Table testing the difference between the quality readings of DNA samples read by two different machines (Nanodrop & H1 Hybrid Reader).

Source Table	SS	df	MS	Fstat	Fcrit	P-value
Sstotal	80.81	38				
Sstreatment	5.64	1	5.64	2.78	4.08	0.10 > P > 0.05
Sserror	75.17	37	2.0			

DNA Extraction Kit Comparison

The largest quantity and the most pure quality were found both using the Omega kit (Table 4, Figures 4&5). The Omega kit consistently produced large readings, but the single largest quantity (9.06) was made when using the Qiagen Kit. With that, closely following the Omega kit in both quantity and quality was the Qiagen kit, with the Tiangen kit producing no DNA; it only produced either protein or RNA. There was a significant difference in the amount of DNA (F=20.80, df=44, P-value=5.27E-07) based on the extraction method, but there was no significant difference in the quality (F=0.039, df=38, P-value=0.96) of DNA based on the kit

used (Table 5). The amount of DNA extracted using the Tiangen kit was significantly less than that of the Omega and Qiagen kits.

Through electrophoresis we were also able to examine and compare the successful sex identification rate based on the DNA extraction method used. Both the Qiagen and Omega kits yielded DNA that had the highest successful identification rate at 47% (Table 6). Based on the kit alone, Tiagen only correctly identified 7 of the 24 samples.

Table 4. Mean and standard deviation for quantity and quality measurements of DNA extracted using three different DNA extraction kits.

		Qiagen	Tiangen	Omega
Black-throated Tit 8 yrs				
Quantity	Mean	9.06	0.22	6.96
	St. Dev.	0.33	0.43	3.84
Quality	Mean	1.88	-0.31	1.82
	St. Dev.	0.13	2.31	0.15
Long-tailed Tit 5 yrs				
Quantity	Mean	3.70	-0.24	7.58
	St. Dev.	1.60	0.37	0.72
Quality	Mean	1.70	4.15	1.88
	St. Dev.	0.30	4.03	0.25
Long-tailed Tit 4 yrs				
Quantity	Mean	1.80	-0.71	2.36
	St. Dev.	0.89	1.00	0.84
Quality	Mean	1.53	3.94	1.70
	St. Dev.	0.31	0.29	0.23

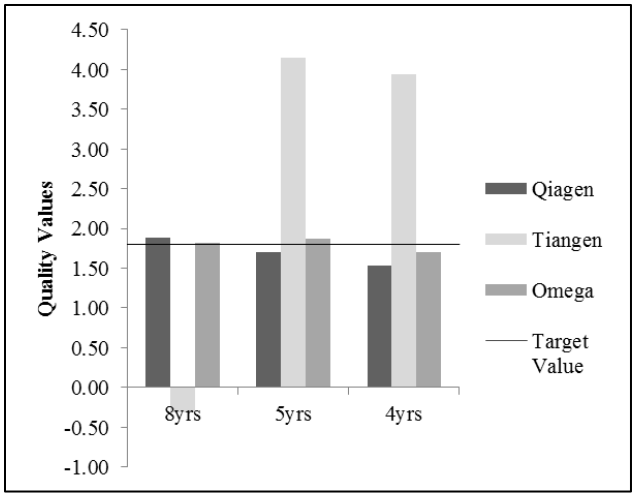

Figure 4. The quality values of DNA from three feather samples based on DNA extraction kit. Target value for pure DNA is 1.80 .

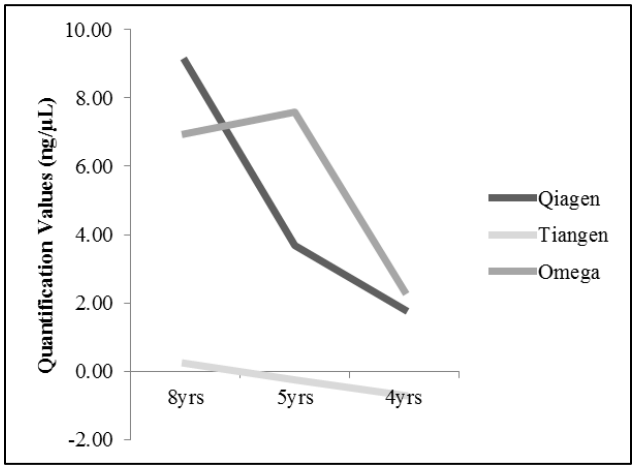

Figure 5. The quantity values (ng/µL) of DNA from three feather samples based on DNA extraction kit.

Table 5. Tukey Test analyzing the significant differences between the quantity readings of DNA samples with DNA extracted using three different extraction kits.

Comparison	Difference	HSD	Significance
Omega-Qiagen	0.78	1.44	No
Omega-Tiangen	5.88	1.44	**Yes**
Qiagen-Tiangen	5.10	1.44	**Yes**

Table 6. Sex identification results of PCR products from DNA extracted from three different DNA extraction kits.

	Total no.	Failed no.	Success (in %)
Qiagen	24	14	**42**
Tiangen	24	17	29
Omega	24	14	**42**

Feather Age

For the feather analysis, we decided to exclude all results given by samples produced using Tiagen kit due to most quantities being outliers. There was a significant difference in the amounts of DNA extracted (F=18.91, df=29, P-value=7.33E-06) from feathers of different ages, but the quality of DNA extracted (F=2.62, df=29, P-value=0.09) from those feathers was not significantly different (Table 7). The feathers taken from a specimen in 2006 had the largest quantity (8.01) and best quality, while the feathers taken from the most recently collected specimen, 2010, had the smallest quantity (2.08) (Figure 6). On the contrary, DNA from all three ages produced close to pure DNA (Table 8). DNA extracted from the feather of the specimen collected in 2009 had the most pure DNA, while the other two closely followed.

Table 7. Tukey Test analyzing the significant differences between the quantity readings of DNA samples extracted from feathers varying in age (8 years, 5 years, 4 years).

Comparison	**Difference**	**HSD**	**Significance**
8yrs-5yrs	2.38	1.44	**Yes**
8yrs-4yrs	5.93	1.44	**Yes**
5yrs-4yrs	3.56	1.44	**Yes**

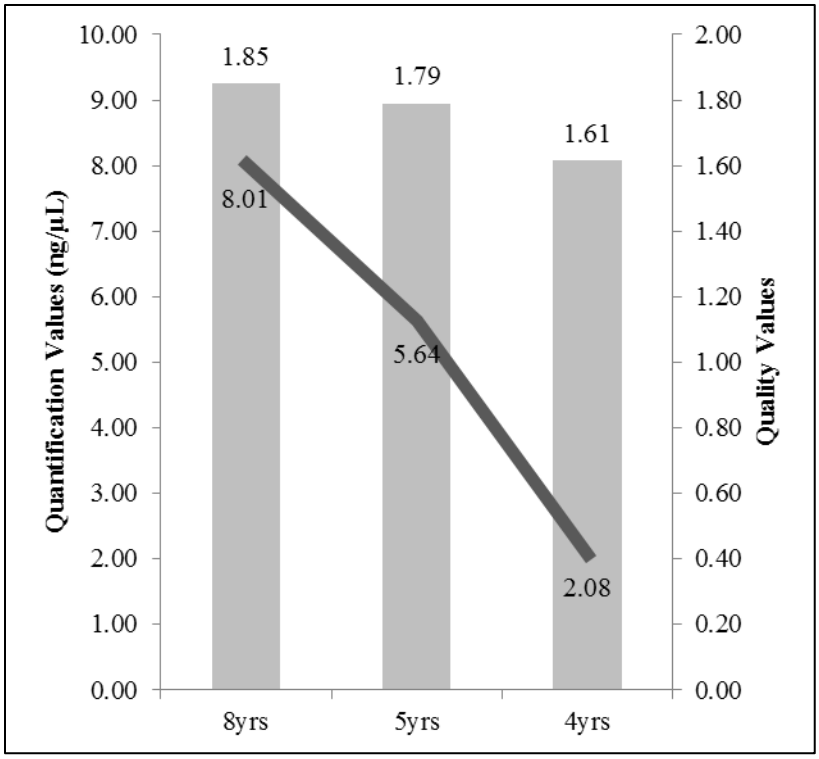

Figure 6. The quantity and quality values of DNA extracted from feathers 8 years, 5 years, and 4 years old. The line represents the quantity values using the primary (left) axis, and the bars represent the quality values using the secondary (right) axis.

Table 8. Mean and standard deviation for quantity and quality measurements of DNA extracted from feathers of different ages.

		8 yrs	5 yrs	4 yrs
Quantity	Mean	8.01	5.64	2.08
	St. Dev.	2.80	2.36	0.86
Quality	Mean	1.85	1.79	1.61
	St. Dev.	0.14	0.28	0.27

DNA Extraction Kit & Feather Age Interaction

The ANOVA test was positive for an interaction affect (Quantity – $F=9.46$, $df=44$, P-value<0.0001). The interaction is evident when looking at the quantity of DNA extracted from feathers eight and five years old using the Qiagen and Omega kits (Figure 4). The quantity of extracted DNA using the Qiagen kit consistently decreases as the age decreases. The Omega kit, on the contrary, produced more DNA as the feather age decreases from 8 years to 5 years, and then decreases significantly from 5 years to 4 years old feathers.

Primers

Through electrophoresis we were able to examine and compare the successful sex identification rate based on the primers used (Table 9). Primers P2/P8 failed the most frequently when correctly identifying the sex, while primer 2718R/2550F was the most successful with a 33% success rate. Based on a survey, primers 2718R/2550F and sex1'/sex2 had the most visible bands and were the most distinguishable for sex identification (Figure 7&8).

Table 9. Sex identification results of PCR products using four different primers.

	Total no.	Failed no.	Success (in %)
P2/P8	24	21	13
2718R/2550F	24	16	**33**
Sex1'/Sex2	24	17	29
2718R/2376F	24	17	29

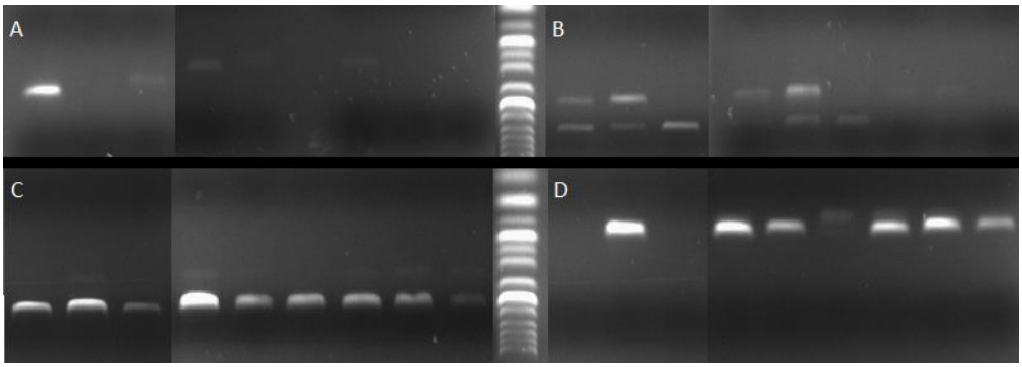

Figure 7. Sex identification of Long-tailed Tit (wells 2 & 3) and Black-throated Tit (well 1) using different primer pairs using PCR-1. (A) Primers P2/P8, (B) 2718R/2550F, (C) Sex1'/Sex2, (D) 2718R/2376F. A DL1000 marker was used.

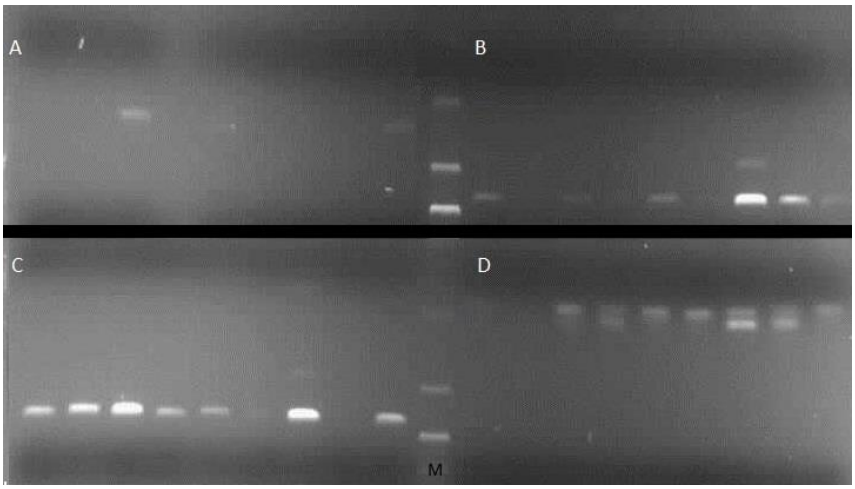

Figure 8. Sex identification of Long-tailed Tit (wells 2 & 3) and Black-throated Tit (well 1) using different primer pairs using PCR-2. (A) Primers P2/P8, (B) 2718R/2550F, (C) 2718R/2376F (D) Sex1'/Sex2. A DL2000 marker was used.

Discussion

With well-known DNA extraction kits and primers, both of which are backed up by literature, it is difficult to choose which kit and primers will produce the best results – especially when dealing with DNA from feathers. Unlike DNA from blood, the DNA from feathers is more scares and is not as easy to obtain. With limited sources it is important to not waste any DNA to troubleshooting. The ANOVA test showed there was a significant difference between the amounts of DNA based on the kit used and the feather age, and there was also an evident interaction between the kits and the feather age (Figure 4). Interestingly enough, feather age had a reverse effect than what we hypothesized. Feathers that were taken from the specimen that was collected in 2006 produced a significantly larger amount of DNA than feathers taken from the more recent specimen collected in 2010. In our study it shows that feather age does have an impact on the amount of DNA collected, but overall it suggests that older feather age does not influence the amount or the purity of DNA extracted as much as one would expect. These results compliment those that Bayard de Vollo et al. (2008) discovered as she was able to successfully extract DNA from feathers ranging from 1 to 10 years old. We also found that between the Qiagen and Omega kit, feathers extracted from the specimen collected in 2009 had opposite effects on each kit. Despite the conflicting results shown by the Qiagen and Omega kits, the Tiangen kit was the worst of the three kits producing very few positive results. Overall, the Omega kit was the most dependable when it came to producing pure DNA.

Despite the little to no DNA that the Tiangen kit produced, the right primers enabled it to correctly sex seven of 24 individuals. This once again suggests the importance of a primer. For our analysis we tested four primers. We found primer 2718R/2550F had the highest success rate with a 33% success rate. Although 33% seems like a low number, we must be reminded that this percentage includes all kits and all ages, even the low quality and seemingly unsuccessful ones. To mitigate these low and misleading percentages, we created a matrix (Table 10) that shows the probability of a primer working based on each individual kit. When using the Qiagen kit and primer 2718R/2550F there was a 4/6 success rate, and when using the Omega kit and primer sex1'/sex2 there was a 5/6 success rate. These two primers had the highest success rate relative to the correct kit used. With these results, we conclude that primers 2718R/2550F and sex1'/sex2 are the best primers for sex identification.

Table 10. Sex identification results of each DNA extraction kit and primer combination

	Qiagen	Tiangen	Omega
P2/P8	2/6	0/6	1/6
2718R/2550F	**4/6**	2/6	2/6
Sex1'/Sex2	1/6	3/6	**5/6**
2718R/2376F	3/6	2/6	2/6

Primers and their success rate can differ based on the species. With that being said, although these primers worked best for our specimens it does not mean that they will work best for all species. We recommend that once DNA is successfully extracted to run a PCR using primers 2718R/2550F and sex1'/sex2 to test which primer works best for that specific species. Further research should be done testing all four primers on more species, containing a larger age range, using a single DNA extraction kit.

Acknowledgements

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Appendix A

DNA Extraction Methodology

Qiagen – DNeasy Blood & Tissue Kit Modifications

1. Begin by adding 180 of Buffer ATL to a 1.5ml microcentrifuge tube, then cut whole feather calamus into tube
    a. Leave the scissors used to cut the calamus in the tube – new scissors for each sample
    b. Cut the calamus into small pieces inside the buffer using the same scissors for initial cut
2. Add 30µL of Proteinase K to the bottom of each tube
    a. Do NOT vortex
    b. Incubate overnight at 56°C in shaking water bath (200-300rpm)

Right before beginning step 3, place Buffer AE into incubator at 70°C

3. Add 200µL Buffer AL and incubate at 70°C at 50 rpm for 10 minutes, then add 200µL of ethanol and INVERT 12 times; Centrifuge at 4°C for 1 min at 9000rpm
    a. While the sample is in the incubator, check it every 5 minutes and slowly invert to check for oil or it being gelatinous. After 10 minutes, if it's still gelatinous, leave it in the incubator and check every 5 minutes until it is no longer gelatinous.
4. Transfer mixture from step 3 into spin column WITHOUT dislodging the pellet. Centrifuge at 4°C for 1 min at 9000rpm
    a. After first centrifuge DO NOT discard the flow through. Instead place the spin column into new 2ml collection tube and add flow through to new tube and re-centrifuge at 4°C for 1 min at 9000rpm
5. Same except centrifuge at 4°C for 1 min at 6000rpm
6. Same except centrifuge at 4°C for 4 min at 14000rpm
7. Transfer spin column to a new 1.5ml microcentrifuge tube and let it air dry for 5 min; After 5min, add 50µL of Buffer AE directly onto the spin column membrane and let it sit for another 5 min with the top closed (place Buffer AE back in incubator during this time); Centrifuge at 4°C for 2 min at 12000rpm; Add 50µL more of Buffer AE and let sit for 5 more min with the top closed; Centrifuge at 4°C for 2 min at 12000rpm
    a. Discard spin column and store at 4°C

Tiangen – TIANamp Genomic DNA Kit Modifications

Note: Not all steps listed in this section are modification. This list contains all steps taken for DNA extraction. We were unable to find an English version of the Tiangen protocol to differentiate which steps were modifications or original steps.

1. Cut full feather calamus into 1.5 microcentrifuge tube – leave the scissors used to cut the calamus in the tube (new scissors for each sample)
2. Add 200µL of Buffer GA to tubes containing feathers then cut the calamus into small pieces inside the buffer using the same scissors for initial cu
3. Add 20µL of Proteinase K to the bottom of each tube
    a. Incubate overnight at 56°C in shaking water bath (200-300rpm)

Next Morning

4. Add 200µL Buffer TB and incubate at 70°C at 50 rpm for 10 minutes
5. Invert, and then add 200µL of ethanol and invert for 20 seconds
6. Centrifuge at 4°C for 1 min at 12000rpm
7. Transfer mixture from step 6 into spin column WITHOUT dislodging the pellet. Centrifuge at 4°C for 2 min at 12000rpm
    a. After first centrifuge DO NOT discard the flow through. Instead place the spin column into new 2ml collection tube and add flow through to new tube and re-centrifuge at 4°C for 2 min at 12000rpm
8. Transfer spin column to a new tube (discard flow through)
9. Add 500µL of Buffer GD then centrifuge at 4°C for 1 min at 12000rpm
10. Transfer spin column to new tube (discard flow through) and add700µLof Buffer PW
11. Centrifuge for 4°C for 1 min at 12000rpm
12. Repeat steps 10-11
13. Transfer spin column to new tube then centrifuge for 4°C for 2 min at 12000rpm
14. Transfer spin column to a new 1.5ml microcentrifuge tube and let it air dry for 5 min
15. After 5min, add 50µL of Buffer TE directly onto the spin column membrane and let it sit for another 5 min with the top closed
16. Centrifuge at 4°C for 2 min at 12000rpm
17. Add 50µL more of Buffer TE and let sit for 5 more min with the top closed
18. Centrifuge at 4°C for 2 min at 12000rpm
19. KEEP flow through, but discard spin column
    a. Store at 4°C

Omega – MicroElute Genomic DNA Kit Modifications

1. Cut full feather calamus into 1.5 microcentrifuge tube – leave the scissors used to cut the calamus in the tube (new scissors for each sample)
2. Add 200µL of TL Buffer then cut the calamus into small pieces inside the buffer using the same scissors for initial cut

3. Step is the same except do NOT vortex
4. Incubate overnight at 56°C in shaking water bath

Next Morning

5. Skip
6. Skip
7. Add 200µL BL Buffer. INVERT to mix thoroughly
8. Same
9. Add 200µL 100% ethanol. INVERT to mix
10. Centrifuge for 2 min at 12000rpm
11. Same
12. Same
13. Centrifuge for 1 min at 9000rpm
14. Same
15. Same
16. Same
17. Centrifuge for 1 min at 9000rpm
18. Same
19. Add 100µL DNA wash buffer
20. Centrifuge for 4 min at 14000rpm
21. Skip
22. Skip
23. Skip
24. Transfer the spin column to a 1.5mL microcentrifuge tube and let it air dry for 5 min with the cap open
25. Same (50µL Elution Buffer)
26. Let it sit for 5 min
27. Centrifuge for 2 min at 12000rpm
    a. Add another 50µL of Elution Buffer and let sit for another 5min
    b. Centrifuge for 2 min at 12000rpm
28. Same

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Cultural Experiences

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Trip and Cultural Report
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Have you ever questioned whether or not you made the right decision? Well that was my exact thought when I arrived in China on May 23, 2014. This would be my first time out of the United States and I was clueless on what to expect. I kept asking myself "Rosie, what are you doing? You don't even speak Chinese, how are you going to survive seven weeks here?" I was scared out of my mind, but it was too late to turn back. I was here and a life changing journey was about to begin.

Shanghai

Our first stop was Shanghai. The city was absolutely beautiful. The buildings were stunning at night, reminding me of New York City, but more exquisite. I was surprised to find that most of the buildings were fairly old, but appeared to be relatively new. My favorite building was the Pearl Tower. The building stood about 468 meters tall and was a superlative tourist attraction. All of us seemed to be amazed by the city, but the people from Shanghai were more fascinated with us. They stared, wave, and took pictures. Many of them were shy at first and then they would try to talk to us. We hardly knew what they were saying, but it shocked me that we had everyone's attention. They were especially fond of the people with darker skin. Our braids seemed to capture their attention most. People would run to touch my face and run their fingers through our braids. They would often ask "How do you wash your hair?" I was surprised that most of them were unaware that my hair was not mine.

When we able to have our first banquet-style, I was amazed. In America when you order a meal, it's for you personally. Here, everyone shared each dish. This was one of the things I grew to love about China. The first time I ate with chop sticks I was horrible. I remember dropping a piece of chicken in a class. Everyone found that hilarious. We all had to learn to eat with these new instruments. It was exciting to see everyone get the hang of it.

Nanjing

Our next stop was Nanjing, where we would spend majority of our time. This became our home away from home. I remember my first day in the café the most. I remember trying to order a dish that consisted of rice and chicken, but the café worker had the slightest idea about what I was saying. Later, she and I formed a relationship. The language barrier was difficult, but a smile can utter so many words. I also distinctly remember my first night sleeping in my new bed. I could not believe how solid the bed was. The beds in America are so nice and comfortable. I could not believe that people were able to receive a good night's rest here. I often had cases of insomnia. I would toss and turn for hours before I could finally drift off to sleep.

Our next week in Nanjing, we were able to explore. The place I remember most was the Nanjing Memorial. I was unaware that this event happened prior to my visit. It shocked me mostly the amount of people that was killed in such a short time. I was even more surprised that Japan tried to cover the fact that they committed this horrific crime. I know that Jewish Holocaust and slavery was wrong but I can at least give credit to the people for owning up and admitting that what they did was wrong. It came as no surprise to me that there are places in China that will not allow people of Japanese descent into them.

My favorite part of Nanjing was trying to find my way back to campus. I cannot begin to count the many times I became lost. My research required me to do a lot of traveling, mainly at night. With very few people knowing how to speak English, I was always in for a game of Charades. I was grateful that the natives of Nanjing would try to help. They would often times take time out their schedule to make sure I knew exactly what all the "hand signals" meant.

Wuxi

Our first trip was to the city of Wuxi. Here we were given the opportunity to learn about bamboo. I had no idea that there were so many species of bamboo. Not only were there over a 1000 species of bamboo but a million ways that you could use bamboo as well. People in Wuxi cultivated bamboo to make items such as hard wood floors, towels, clothes, and even keyboards. I was astounded by all the items that you could make from bamboo. It was even more intriguing that bamboo could be used as food as well.

My favorite part of Wuxi was the ballet. When the ballet first began, I was oblivious to what the concept of the play was because the narratives were all spoken in Chinese. However, the amazing movements of the dancers captured my attention. After paying close attention, I learned that the story-line told of an all women military during WWII.

Shiyang and Yangzhou

Our last trip was to Shiyang and Yangzhou. This was my absolute favorite trip. In Shiyang, we visited the Poplar tree research and productions sites. At the research center, we learned how the Poplar tree was cultivated and its importance to the Chinese culture. In Yangzhou, we visited the Milu Deer Reserve. Before visiting the reserve, we were told all these bizarre stories of how the deer contain the head of a horse, the hoofs of a cow, the antlers of a deer and, a body of a donkey. I could not wait to see this genetically-mutated animal in person. How wrong was I? This was creature was magnificent, not genetically-mutated at all. The Milu Deer is an endangered species of China perished from China during the early 1900s. If Pere David, a naturalist from France, had not notice the declining population and sent approximately 20 of the Milu Deer to Europe, the deer may have become extinct and robbing China of a very unique species of deer. With years of effort, the Milu Deer returned to its native land, China, in the late 1980s to the Milu Deer Reserve. An animal that was almost extinct now has a population of almost two thousand at the Milu Deer Reserve.

College Students

When it came to college, a college student is a college student. When conversations would arrive, the Chinese students would be ecstatic to learn about college in America and I was equally eager to learn about their college life. Most of them would always discuss how difficult it was to earn a spot in college in China. When most of the students would tell me of their college entrance test, they would often mention how tough the test was. They also would discuss how hard many of them "fought" to earn their spot in one of the institutions. I then would then realize that sometimes I can take the smallest things for granted. I also was very appreciative and felt blessed to be a United States citizen.

Food and Beverages

Adjusting to the food in China was a challenge for me. I often found myself eating rice or vegetables. However, towards the middle, I became adventurous. I promised myself I would try one new dish at each banquet style dinner. The other students, who loved the Chinese cuisine, had no problem holding me to this promise. By the end of the summer I had tried all types of new dishes. Some of them were quiet good but others I did not like very much. My favorite was the bar-b-que lamb. It was absolutely delicious. However, none thing compared to the flaming cabbage. That was my favorite dish of all.

In China, many people prefer not to drink cold drinks. Many of them believe that it was not healthy to consume cold beverages. Often times they would serve us hot water for dinner. I would often joke about the drinks being hot and Cicely would say, "No. It's warm Rosie." She always found it amazing that I like my drinks cold. I quickly learned how to ask for "bing shui", cold water. Chinese people also loved tea, hot of course. I liked tea too, but mine is usually cold with sugar. Yet, I like the different kinds of tea in China. My favorite was the green tea. My least favorite was the milk tea. Although, I was happy I tried the milk tea because it was Cicely's favorite drink. I wanted to show her that I was just as appreciative to embrace her culture as she was to accept mine.

Although I was trying new foods and drinks, I often times missed American food. We would often time make trips to KFC and McDonald's. My favorite restaurant was Pizza Hut. Although at home I rarely eat there. Pizza Hut in America cannot compare to the Pizza Hut in China. Pizza Hut in China reminds me of Chill's or Applebee's in America. My favorite meal at Pizza Hut was their breakfast. I know, Pizza Hut serves breakfast and it is scrumptious. I will definitely miss those Saturday morning trips to Pizza Hut.

Never in a million years would I have imagined spending seven weeks or my 21st birthday in China. I am so delighted that I was given this opportunity. I have learned so much about myself over these last couple of weeks. Most importantly, I have learned that taking risk and stepping outside your comfort zone teaches you who you really are. Never prejudge a situation until you are actually in it. I am also happy that Wi-Fi was very limited. This gave me the opportunity to learn more about the culture and explore. I never realized how much I used the social media until I could not use it all the time. When I look over my experience in China, I realize that no matter where you are in the world people have some of the same goals. Some of these goals consist of being successful or simply being accepted. If given the opportunity I would love to come back in China. There is not a single moment I did not cherish about this experience.

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Trip and Cultural Report
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This year's traveling in China as part of the REU-China program was a deeply enriching experience. I ventured to Shanghai, Nanjing, Beijing, Chengdu, Wuyishan, and finally Shennongjia, with Wuyishan and Shennongjia being my main study sites. In the larger cities, and in transit between them, I found many new sights and opportunities that I had not in previous travels. In the cities I learned about how modern Chinese herpetological research is conducted, made new colleagues and friends, and sampled cultural staples of these different regions. During the weeks spent conducting my research in the more remote locations, I was able to glimpse an entirely new side of Chinese life. There we interacted with farmers, villagers, and their local customs on a daily basis. I spoke to local people about our research and the animals that live around them, and I got to witness first-hand the impact of local management decisions on Chinese wildlife. Being able to interact with the people of China in both urban and rural settings was invaluable for informing my understanding of Chinese cultural and perspective for my future research.

As of this summer, I have studied the Mandarin Chinese language off and on for a total of seven years. It began with an introductory course during my freshman year of high school and continued into my undergraduate career. My level of fluency increases dramatically with every visit to China. This year, I was able to put that knowledge and experience to use translating for colleagues as I learned more with each interaction. For example, as my mentor, Kevin Messenger, and I were walking a remote trail looking for snakes we came across a farmer and his herd of goats. I was able to communicate with the farmer that we were there to study the snakes on his land. We had a short conversation with him and then went on our way. As we parted, I told the farmer "我们走." (pronounced "wǒ mén zǒu" translating to "we'll go") and he responded "慢走." (pronounced "màn zǒu" meaning "go slowly"). His response struck me as very kind and reflected the generally open and courteous sentiment we had been experiencing with people all over China.

Beijing

Kevin and I visited the capital city of China before our major field research sites in order to allow me the chance to train in safely working with snakes in the field. While there, we stayed at the home of a friend of Kevin's, Scott Lupien. Scott has been conducting business in China for nearly a decade. From him I gleaned much about the struggles of living and conducting business in China as a foreigner. Scott introduced me to his client Wulong. Wulong had met Kevin in previous years and had been particularly impressed with Kevin and his research. Wulong treated us to several extravagant Beijing-style meals including Peking duck and fish hot-pot. At meals with Wulong, we toasted each other in typical Chinese fashion and Wulong expressed his eagerness to see Kevin and my research progress. He even went as far as saying that, after learning about Kevin's research and his appreciation of reptiles and amphibians, Wulong will no longer eat snakes. This is despite snakes being, as he said, Wulong's "favorite animal to eat." I found this change of heart to be very encouraging.

My training in Beijing consisted of searching for and capturing *Gloydius brevicaudus* on a remote, ruined section of the Great Wall of China. We took all day to make the strenuous hike up and down steep, crumbling sections of the wall. We safely caught five G. brevicaudus that day. The snakes were photographed and then released where we had found them on the wall. Finding that many snakes in a relatively small area in one day speaks volumes about their high abundance in the area. That and the ruins of the wall led me to consider the deep roots of Chinese culture and its interactions with the wildlife of China. I wondered how the laborers building the wall dealt with the venomous snakes.

Catching snakes on the Great Wall of China – a section of the Wall that has not been restored, and thus is out of reach from most tourists.

Chengdu

After leaving Beijing, Kevin and I visited Chengdu for one week so that I could take morphological measurements from the specimens at Chengdu's Museum of Herpetology. Chengdu is widely known as the birthplace of modern Chinese herpetology. We were hosted there by another friend of Kevin's, Wang Xiaohe. Xiaohe is a graduate biology student at the Chengdu Institute of Herpetology. We had a wonderful time in Chengdu thanks to her hospitality. Xiaohe showed us where she works in the institute and helped us gain access to the samples we needed to measure. She also took us out to try the Sichuan-style cuisine of Chengdu.

I spoke with Xiaohe at length about what it's like to conduct graduate research in China. She told me about various aspects of collecting data, writing, and publishing papers in China that I had never heard of before. Wang Xiaohe leant me a new perspective on academia in China and how it is changing.

Nanjing

While in Nanjing I experienced another new facet of China: student life. I lived in a student dorm on the Nanjing Forestry University campus for many weeks while going to classes, eating at the cafeterias, and exploring the city. One evening I was even able to visit Purple Mountain near campus to collect toads for my research. I met many students at NFU from all over the world.

Wuyishan National Nature Reserve

In Wuyishan, as in most places, we were often treated with enthusiastic hospitality, often being invited into peoples' homes for tea and conversation. The villagers were very curious about us and our purpose for being there. I was able to communicate well enough to satisfy some of their curiosity and learn a bit myself. A common first question was "What country are you from?" to which I usually answered "Take a guess." in a joking manner. Top guesses for our homeland were Australia, France, and Canada. I had no doubt that we were the first Americans that many people we'd met had encountered.

We visited the home of a tea seller in a village known as Seven Li. I ended up buying three canisters of it to bring home. The tea was grown in Wuyishan (probably in the same fields I was surveying for research) and was dried and oxidized over an open fire that gave the tea a strong smoky smell and flavor. I'm a fan of black tea and this is by far the favorite of my collection.

In that same home of the tea seller, I was surprised to see an image of Jesus on the rear wall. Later I noticed more and more Christian imagery in the area. I'm told that Wuyishan was the origin of tea for the western world. That is, the first tea brought to Europe was gathered in Wuyishan. I wonder now if it was done by missionaries.

Shennongjia National Nature Reserve

It was very interesting to hear what the villagers had to say about their local wildlife and its management with regard to their own rapidly-expanding construction projects in Shennongjia. There Kevin and I witnessed a slightly unsettling instance of local management decisions affecting that wildlife. As part of my research, we spent many evenings surveying one particular icy-cold stream in search of a certain species of frog. During these surveys we also commonly

encountered fish, toads, arthropods, small mammals, and (a personal favorite) numerous salamanders in and around the rushing water. On our last evening at that location, Kevin and I arrived at the stream to find these same fish, toads, arthropods, and salamanders either dead or exhibiting convulsions. We gathered up and photographed each species we found affected (apparently only those of small body size that had been in the water). As for the cause, there was a road near the stream that was being expanded at the time. Three hours before we had arrived at the stream, Kevin and I had heard an explosion from the construction site where they were clearing away a small section of the mountain. The choice to build that road so near to the stream had resulted in the devastation of that stream's inhabitants that day. It was unclear to us how many times that had happened or how capable the stream's ecosystem was in recovering from these events, but the simple destruction was plainly evident. While this was unfortunate, I'm very grateful to have been present to witness and help record the event for science.

While that event illustrates part of the negative aspect of studying wildlife near remote villages, in Shennongjia, and elsewhere in my travels, we did encounter what I would consider positive changes. This was especially evident in the mindsets of those who had met Kevin in previous years. Kevin was instantly recognizable to many of the communities who had heard about him visiting every year and heard that he is interested in snakes, but specifically wanted to see them alive and in their natural setting. I believe that Kevin's demonstration of valuing wildlife in situ has gone a long way in instilling that sentiment among the villagers.

To witness both the good and the bad in small Chinese villages interacting with local wildlife was a treasured privilege for me. I value those conversations I was able to have with them very highly because, from a conservation perspective, it is these villagers that it's most important to understand. The people that are going to have the most dramatic impact on the wildlife we study are those that live there. I believe this experience will dramatically affect my future career as a biologist.

Future directions

My time in China this year has exposed me to so many new facets of the country and its people that I can't help but want to return to see more. I hope to continue my research on the wildlife of China into the future, interacting with local friends and villagers along the way. I am both

excited and nervous for what China's future might look like. As the needs of the country's economy grow and standards of living rise, more and more pressure will undoubtedly be placed on its natural resources.

However, being able to make friends and interact with people in China has boosted my optimism. I look forward to seeing what solutions the researchers of China develop for these issues and to seeing how the minds of smaller communities might be swayed towards a more sustainable path.

Hollis posing for a photo over the glass lookout at the top floor of the Pearl Tower in Shanghai, May 2014.

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Trip and Cultural Report
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Initial Thoughts

The anticipation was immense. My opportunity to study abroad has finally presented itself, and I was selected to go to China. I knew I was in for a treat. My imagination was running rampant. I began diligently researching the country to get a clearer perspective of what was to come. After all, China is an overpopulated communist society, right? However, this is a once in a lifetime opportunity, and I am not going to let it pass me by. I am grateful that our sponsors carved time for the students to interact prior to departure; after all we will be spending the next two months together. We were in for a long flight, but it allowed us to further bond and provided an opportunity for anxiety levels to subside. So Nanjing, China, make room for Andrew Lawhorn.

Taking it all in stride cameras flashing, natives genuinely excited to see us in their country— the complete opposite of what I expected based on my research. When we arrived in Shanghai, we were treated like royalty. Upon checking into our hotel, we were guided to a nearby restaurant. No need to place orders; our meal was already on the table. I have not seen that much food since Thanksgiving. It was such a treat to indulge in native cuisine of the land.

Now it is time to pack up and head to Nanjing. Traveling from Shanghai to Nanjing gave me a deeper understanding of China's infrastructure. There were miles upon miles of tall buildings. People were everywhere. The traffic seemed to have a mind of its own, and it seemed as if the rules of the road did not apply. Everyone shared the road, be it fast moving cars, scooters, bikes or pedestrians walking about their day. Much to my surprise, what appeared to be complete chaos was an organized, harmonious flow.

University Life

We received a warm welcome from the faculty and students at Nanjing Forestry University (NFU). The welcoming committee helped the REU students and faculty get settled by giving us our meal cards, providing extension plugs, and ensuring that we safely arrived at our dorm

rooms. During this time, students also had the opportunity to meet their mentor(s). Over the next couple of days, we were given a brief introduction to Chinese culture and language. At first glance, the language seemed challenging. However, our teacher was phenomenal; she gingerly corrected our errors and provided copies of her lesson plan. In additions to the language and culture classes, we had classes that discussed effective ways to apply the scientific method when conducting proper research.

The next week I met my Chinese mentor, Dr. Yulong Ding. He gave me instructions for a research project and information about our location site, Nanjing Forestry University Research Station in Xiashu. Over a period of five days we collected data from plots located on the site. The accommodations were nice. We stayed in a two-bedroom apartment with access to a cafeteria; the only drawback was no Internet service. As a result, there was time to relax after a hard day's work.

The work we performed on twelve bamboo plots consisted of measuring height, weight, and diameter of culm at ground level. We used GPS equipment, scales, measuring tapes, calipers, handsaws, hatchets, spades, and soil extraction plunges. The days were long when we performed fieldwork; however, it did not to matter because I was surrounded by great people. On the fourth day, a few of us left for NFU earlier then our original return date.

Andrew posing with a piece of bamboo during one of his field data collecting outings.

Cultural Attractions and Experiences

We set out on a journey to explore Shanghai. Our first stop was at the Oriental Pearl Tower. This tower is well constructed—nineteen stories and six observation decks. The transparent observation deck was my favorite. It is over 270 meters above ground and has some of the thickest glass I have ever walked on thus far. The views from this deck that overlook the city's riverfront are simply amazing. Once we departed the Oriental Pearl Tower, we went on a riverboat ride and walked down to the shopping district.

During our first week in Nanjing we went to Mochou Lake Park to watch the Dragon Boat races and to the Nanjing Massacre Memorial Hall. The Nanjing Massacre Memorial Hall explicitly displays how Japanese forces massacred people in Nanjing. I wish we had more time to soak in the history. In fact, I would have paid extra for an English translator so that I could get a full understanding of the recorded survivor interview footage. Due to inclement weather, we postponed our Presidential Palace tour to the next day. We stayed at the palace for about two hours, but again, I would have liked to stay longer. The architecture is amazing, as is the art that was on display. Overall, I really enjoyed our outings and the time we spent learning more about historical places and events from Nanjing.

Our next weekend trip was to Yixing Bamboo Forest Research and Production Sites. There we saw how bamboo is transported, manufactured, and retailed to the public. Afterwards, we had lunch at a restaurant that was close to our farm. We witnessed a vast area of moso bamboo marked with numbers that displayed their age so harvesters could pick them at the appropriate time. Also, we were allowed to pick berries from a bayberry plantation. Then, we traveled towards Wuxi for dinner and a ballet performance. Understanding the Chinese language was not necessary because the Chinese ballet was performed without dialogue. The following day we took a canal boat tour and did some shopping in the area.

We took our final scheduled weekend trip a few days prior to our presentation. This was a three-day trip to Sheyang, Yancheng, Dafeng, and Yangzhou. In Sheyang, we viewed an array of tree species at the arboretum. The sight and sound was breathtaking. Afterwards, we traveled to a poplar processing factory where we saw some of their production lines. Next, we ate dinner at our hotel in Yancheng. When dinner was over, we went for an evening stroll. The place looked

very desolate. It reminded me of something out of an apocalyptic movie. I was told how in some places in China there are ghost towns or ghost cities where a multitude of structures exist but there is a very small population living in that area.

The next day, after breakfast, we visited the Red-Crowned Crane Preserve. At first glance, I was disappointed to see them in captivity but learning how the cranes get out to practice and perform for crowds slightly eased my despair. We traveled in carts to view additional wildlife species. We ventured inside of a man-constructed camouflaged tunnel that had small windows looking out at more wildlife native to the area. Thereafter, we went to a building on site to have lunch and prepare for travel to Dafeng David's Deer Preserve.

As we arrived, what looked to be a zoo, to my surprise, was a Pere David's Deer (also known as the Milu Deer) sanctuary. This animal looked like a mixed breed of a deer and horse. In fact, it turned out to be a species containing characteristics of a horse, cow, deer and donkey. As we traveled through the preserve, we learned that they are listed as extinct in the wild, and all populations are under captive management. In China, the captive population in recent years has increased, and there is hope that they will be reintroduced into the wild in the near future.

When I went to the lab one day, I was shocked and surprised to see that I received an invitation to attend a graduation dinner. It was for graduate students from the forestry department. I felt very privileged to be invited to such a special occasion. The forestry department over these weeks has made me feel very appreciative. I look forward to a continual relationship with all of those I have met during my visit here.

In Beijing, our stay was brief; however, our schedule included many places we visited. Unlike, the previous places we traveled to, the Beijing population was diverse. I enjoyed being in the "big city" but having heavy traffic on a daily basis would get old quickly. Having the opportunity to live in a country so different from my own has given me a new perspective. There are some things that I really like. For example, I like the way Chinese treat their elderly. On the other hand, there are some things I can do without, such as social acceptance of cigarette smoking. I was very disappointed to learn how this is a way of life almost everywhere we traveled. Nevertheless, I am truly honored that I was afforded the opportunity to study abroad. I

enjoyed the overall cultural experience in China and look forward to taking it to my next endeavor.

Andrew (right) with REU students Angelica Durrah and Linzi Thompson (left to right), and a friend in Shanghai, along the Huangpu River, May 2014.

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Trip and Cultural Report
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REUG China 2014

As a former English teacher who lived and worked in Hunan province for one year, writing a paper on my cultural experience was somewhat of a challenge for me. Cultural practices that have surprised, baffled, and even shocked other interns were as normal to me as apple pie is for Americans. Foods that most interns considered odd, weird, or just plain non-edible was one aspect of China I couldn't wait for. The staring eyes of individuals who have only seen foreigners through media did not faze me as others experienced this phenomenon for the first time. With this in mind, I decided to instead focus on new experiences and differences I noticed since the last time I was in China.

The easiest and most prominent new experience for me in China was conducting research. Right from doing field work, to conducting experiments in the laboratory, all aspects of the entire process was in at least one way different from my understanding of research as I had learned in the states.

Of all aspects of research, what I most noticed to be different was field research. My study site was a 3 hour drive from the university, located on a predominantly poplar plantation farm on the northwest shore of Lake Taihu. Before leaving I was informed by the graduate students I was working with that we would be collecting samples and taking measurements for 3-4 days, and as such I packed accordingly. When we first left, I already knew I was anticipating too much; both of my grad students brought only a small backpack while I had brought my camping backpack and was dressed in full field gear ready to begin work when we arrived.

Upon arriving, we were all treated to lunch by the owner of the plantation followed by a tour of the plantation and study site rather than doing any field work. It is quite normal for a host to treat his or her guests to dinner, and so this didn't come to me as much of a surprise even though we were there for field work. It was quite interesting to gain a glimpse into plantation forestry occurring in China, but I was a little disappointed I couldn't begin field work that day.

The next day we finally were able to start doing field research! However, I learned very quickly the definition of field work is very different in China compared to America. Instead of collecting soil, water and tree samples ourselves, the grad students enlisted the help of two employees who worked and lived on the plantation. Although I am not against extra help, I genuinely felt bad letting these older men do all the physically demanding aspects of sample collections, such as using the soil auger. Perhaps it is because I am accustomed to crew leaders and professors always assigning me the back-breaking work in the field because I am young, but I couldn't stand by and watch as these men labored for our research. I soon grabbed the soil auger and vacuum pump for water samples from these gentlemen so they didn't have to bear the burden alone.

With their help we were able to collect all the samples on the same day, and realized the initial three days of field work my graduate students had quoted me on was actually one day of traveling to the study site, one day of field work, and one day of traveling back to Nanjing Forestry University. Again I attribute this to different definitions of field work to Americans and Chinese, but also partially to the language barrier. After all, I feel grateful for the experience and the newfound knowledge, because it has helped prepare me for any future research and field work I will conduct in China in the near future.

Lab work was also different for me in a variety of ways. In most of my prior experience, I have dealt with the field measurements, and subsequent analysis of measurements through statistics and calculus. Thus, using a mixture of chemicals to isolate both nitrogen and phosphorus in all of our samples was both exciting and a learning experience. I actually felt like the stereotypical, nerdy scientist in a lab coat I had imagined when I was a child growing up! I also became good friends with the graduate students that I had collect samples with as we worked side by side in the laboratory. The only difference I could explain from my own experience in America was a loss of some samples. It is no reflection on my lab crew or school, but the university was unable to provide transportation both to and from the study site, thus some samples were lost as we had to mail most of our samples back to the university and carry the rest back by bus and taxi.

Besides my research experience, undoubtedly my most favorite memory from this trip will be exploring nature and bird watching. When I first lived in China, I virtually had no contact with

the natural world. Returning to China with the ability to identify trees and bird species was the most rewarding experience. It was akin to sliced bread; a new take on an old favorite.

Places I have seen and visited previously was like discovering it for the first time again! I could now identify trees and bird species I had never seen in my life before, or had seen but never appreciated and understood until this internship. This new-found understanding also gave me a newfound appreciation and insight into the health of the ecosystems in cities and forests we had visited during this trip. This has helped to expand my general knowledge of China overall, which was previously confined to the history, cultural practices, and good travel sites.

My appreciation and knowledge of nature has also led me to explore areas I would have never thought of going when I previously lived in China. Whether it be hiking to the top of Purple Mountain looking for rare plants or an elusive bird species, walking along Xuanwu Lake for the waterfowl, or even taking a stroll through the NFU campus admiring their vast collection of native tree species is an opportunity I will always appreciate this internship for providing.

In conclusion, I can summarize my cultural experience of this internship as an opportunity of new and old experiences. It has reinforced my conviction to conduct research within this highly biodiverse country after I graduate from Humboldt State University, but has also given direction for me on how I will conduct this kind of research in China. It has provided insight on new ecoregions and places to explore for flora and fauna when I return to China that I would have never considered previously. Most importantly, this internship has let me fall in love with this country all over again, and I can truthfully call China a second home.

Michael climbing trees and birding at the Ming Xiaoling Mausoleum, after a day of touring on Purple Mountain in Nanjing, China, June 2014.

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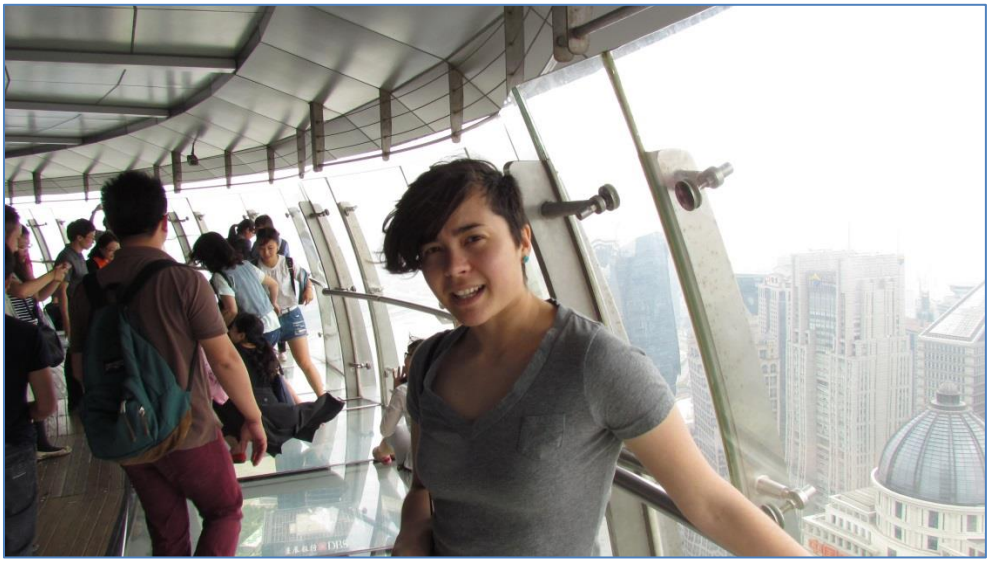

Trip and Cultural Report
Nicole Mihelich
REUG China 2014

Introduction

When I was applying to internships for this summer, I would not have guessed that I would be spending two months doing research in China. I love to travel, so the opportunity to do research *and* be surrounded by a new culture was an opportunity I could not pass up. Needless to say, I was ecstatic when I found out I was accepted into this REU-China program. This was my second time in Asia, but my first time in China. Even though I have been to India before, I still had no idea what to expect of the culture of China. I was prepared for the possibility of things like squat toilets and haggling, but other than that, I was going in blind. There is a big difference between going to a place for vacation and actually living somewhere for an extended period of time. As a student researcher during the week and a tourist on the weekends, I got a very interesting mix of cultural experiences during my seven week stay. The following is not a series of chronological stories of the sights we saw on our weekend field trips, but rather a compilation a few cultural topics that I knitted together in my mind over the course of the trip: Chinese language, Chinese food, and Chinese spirits.

Part 1. Language

After a few days of settling at Nanjing Forestry University, I met my Chinese Principle Investigator whose lab I would be working in for the entirety of my stay. He seemed very happy to meet me, though I soon found out he spoke almost no English. He actually knew French very well, but that was not helpful for our communication either. That was the first of many moments where I felt irresponsible as a citizen of the world for only knowing one language. So many people around the world learn at least a second language starting very early in their education. Sure, I dabbled in French in grade school and got to Spanish IV in high school, but I would not even be able to have a simple conversation in either of those languages if the situation presented itself.

After these rather guilty thoughts, I was glad to learn that the REU group was scheduled to take a week-long crash-course in Chinese Language and Culture. We started out learning the basics of Pinyin, the official phonetic system for transcribing written Mandarin Chinese into the Latin alphabet. This was much less daunting than trying to delve into Chinese characters right away. The first things we learned in pinyin were the basic sounds of consonants and vowels. As we went around the classroom reciting each of the sounds to get used to them, it sounded like we were in music class. Totally unlike the English language, along with each syllable of a word, Chinese words are often accompanied by one of four tones: 1.) a straight tone (like singing a note "Ahh"), 2.) an ascending tone (like asking a question "what?"), 3.) a tone that dips down and then ascends (like saying "well…"), and 4.) a descending tone (like ending a sentence "no!"). Trying to remember to not end a string of sounds with a descending tone was the hardest because that is what English speakers are accustomed to, lest the sentence sound incomplete.

In the Chinese Language class, we also got to try learning some Chinese characters and calligraphy. Looking at Chinese characters, it is hard for my brain to register that they contain information since they are completely different from the English alphabet and any other Latin-based language. We started out by looking at a small percentage of characters that used to be based on pictograms, and can still be recognized as such. We were also shown how these characters have evolved over the course of hundreds of years to become the modernly recognized characters they are today. Characters for words such as "net," "gate," "man," and "forest" still held this pictogram origin. After the lesson on the characters, we got to do some calligraphy with the special paper and brushes and everything! I tried in vain to replicate the beautiful characters of poetry, which ended up reading as nonsense, I'm sure. It was still a very hands-on and entertaining way to learn about the culture. Prior to this language lesson, I thought of the Chinese language as overly complicated and verbose. However, comparing direct translations between English and Chinese, I noticed that often the Chinese sentence was shorter than the same sentence in English, albeit more compact. Counting up the strokes involved in both languages for the same sentence, the time taken to write out the same message actually seemed roughly even.

Obviously this was not enough to make me even remotely able to communicate with Chinese speakers, but it deepened my respect and appreciation for anyone who has ever learned a foreign language, especially in an academic and professional setting. For instance, international students

who take classes that are not in their native language are real troopers. For me, college classes at times are difficult, confusing and barely understandable even in my native language. I couldn't fathom having to add a language barrier on top of that. Being introduced to the basic phonetics of Chinese also helped me understand why native Chinese and other related Asian language speakers have particular accents when speaking English. For instance, there do not seem to ever be instances in Chinese where a word will end with an "L" sound, so Chinese people often have trouble with English words like that. For instance, when I introduced myself as Nicole, saying my name back seemed difficult. Luckily I often go by Nikki, which eliminates the ending "L" sound and helped some of my Chinese associates be more comfortable with addressing me. People who make an effort to learn a second language may not have perfect pronunciation or grammar, but at the end of the day I have learned to better respect the hard work that people put in to learn another language. The language classes got me excited and inspired to try to pick up a little bit of Chinese. By the end of the trip I was able to communicate very rudimentary things to people in Chinese mainly when ordering food, saying I where I was from, and haggling. In the future, I hope to maybe one day learn the language more fully, as it is becoming more and more sought after to know as the world becomes more globalized and China further increases their world influence.

Part 2. Food

There is a saying that the Chinese eat everything with legs but the table itself. I realized that this is basically true in the best way possible. I greatly enjoyed and respected the huge variety of different meats, vegetables, and other ingredients in the cuisine. In restaurants and cafeteria alike, I never had to have the same dish twice, and there was always something new available for sampling. During meals, it became commonplace for me to pop an unknown morsel into my mouth, enjoy it, and inquire to my fellow diners, "Mmm, what is this?" No one would know, so I would just shrug and say, "Well, it tastes really good, so I'm going to keep eating it!" If there were any Chinese people dining at the table, I would consult them about the identities of any mystery foods. However, I eventually realized that many of the answers they gave were not accurate despite their confident responses. My suspicions for this were formally validated when I was told that chicken was duck despite later noticing that the chicken's head was present and clearly identifiable on the plate. This was further driven home when I was told that pieces of

some kind of avian or reptilian skin (most likely chicken) was sea cucumber. I didn't really mind, though. Overall, I tried everything and did not let the dish's specific contents make me biased to the taste. It was part of the fun to investigate and debate what food was what. The Chinese people that I talked to about this didn't seem too concerned with what was what, maybe because at the end of the day and it's all food and it all tastes good! Here are a few mini adventures of experiencing Chinese food:

Stinky Tofu

It is interesting how when you go to a foreign country, people are either advised to stay away from street food or are told that it is even safer than restaurant food! The good thing about it is that you can see the food prepared right before your eyes. The bad thing is that if you come up to a stall with piles of food already out, you don't know how long it's been there. I couldn't bear to miss out on the opportunity to absorb the spectacle with local grub, so I decided to go for it, but kept a vigilant eye to ensure freshness. One of the more curious morsels I had was of chòu dòufu, aka stinky tofu. It is a common street food consisting of fermented tofu, and from a few stands down we knew we were approaching it as an odor of garbage and rottenish meat filled our nostrils. It was fried up right on the spot and served in the form of a greenish black square. The cook walked over to us from the stall and handed me a bowl of rice with the square on top, which was good because had I gotten any closer to the grill, my eyebrows would've burned off from the cumulative stench! However, legend has it that the stinkier the odor of the chòu dòufu, the better the actual flavor. Well, it seemed to hold true as the first bite filled my mouth with the familiar texture of tofu and a fermented flavor that bit right back at me. It was tasty though! Disclaimer: As a cheese lover and student in Wisconsin, I can generally appreciate the delicious and exhilarating pungency of a properly aged food.

"Ginkgo Fruit"

I am always willing to try new foods, because I never know if a dish may become my new favorite. I will try anything at least once. At one of our group lunches during a field trip weekend, a standard Chinese vegetable dish was placed on the table, containing foods such as celery and red peppers. However, these familiar vegetables were sitting beside a bright yellow food about the size and shape of an olive. Upon consulting the China natives at the table, I was

told that this peculiar food was ginkgo fruit. I happily accepted this identification and popped a fruit into my mouth. What happened next took me by surprise. As I bit down on the morsel, the fruit mushed between my teeth similarly to the texture of a potato and flooded my mouth and olfactory system with an overwhelmingly pungent flavor. I like to think I have a pretty robust palate, but in that moment I found myself fighting back the urge to gag. My brain frantically groped for the words to describe the taste, but to no avail. It seemed so familiar yet managed to escape me. After a few minutes of recovery and palate cleansing, curiosity and determination overshadowed my initial revulsion in efforts to more accurately describe what the ginkgo tasted like. I sampled two more fruits before it hit me. It somehow smelled and tasted like my grandparents' basement: old and musty. Not exactly my ideal adjectives for food. Once this association dawned on me, there was no hope of unthinking it. Curiously enough, after the fact I did some background research, and it does not seem like those musty yellow foods were actually from ginkgo like I was told, so what I actually consumed on that fateful day is still a mystery. I will periodically give foods another chance if I tried them and did not like them before. Maybe the dish was not prepared well or some other factor caused me to not enjoy it. However, if I ever see those yellow "fruits" again, I admit I will be hesitant to give it another shot.

Fish Eyes

One thing I really appreciated about Chinese food was how often most or all parts of an animal are utilized in a dish. In the United States, most people prefer the animals we eat to be reduced to the choice parts, without bones, and cut into bite sized pieces, to the point where recognition of the animal is impossible. This may be because subconsciously Americans do not like to be reminded that they are eating an animal. In China however, it is a different story. Fish are usually served whole, including the head and eyes staring up at you. According to the Chinese, the meat behind the eye of the fish is the best on the whole animal, so Americans who can't stomach to have the fish's head included in the dish are really missing out! Personally, my favorite parts of the fish were the eyes. Eating the eyes is a fun experience from beginning to end. First of all, getting the eye out of the socket with chopsticks as the Lazy Susan is on the move makes for a very challenging time trying to get them onto the plate. The taste is much like the flavor of the rest of the fish, but has a uniquely harder and almost crunchy texture that is just so satisfying to me. Finally, eating the eyes was also great because it often came with the amusement of other

people's bewilderment upon watching me specifically select and consume this part of the fish. Many other Chinese dishes also utilized many parts of animal. Another delicious part of fish that I had never tried before was the fish bladder. It was less fishy than the meat and had the texture of wide flat noodles that are often used in Thai cuisine. Also, often when a chicken was served, the whole chicken was present. Because of this I had the opportunity to try chicken brain, which tasted a lot like liver but much richer. All of it was delicious! Other examples of animal parts uncommonly served in America that I tasted or at least saw in China were chicken feet, chicken organs, beef tendon, cow stomach, pig ears, pig feet, pig tail, duck head, and duck tongue.

Overall, I greatly respected the immense diversity and resourcefulness of Chinese cuisine. I have never had the opportunity to try such a huge amount of new foods either. As a person who will try just about anything at least once, China presented my most vigorous, challenging, and exciting experiences with food yet. This experience helped me more fully realize that food is a tool to connect with people, cultures, and ideas. Being in China and any other foreign place, experiencing the food is an integral part of experiencing the culture. Not everyone can speak to locals in the native tongue, but just about everyone can shove food in their mouth and use their tongue to taste it. And Chinese people love to see when we foreigners enjoyed their food and were super impressed when could use chopsticks. Tasting all this new and sometimes crazy different food played a big part for me in forging a connection with China to understand other cultural aspects.

Part 3. Chinese Spirits

Chinese banquets were very interesting and amusing to me. First of all, I found that often when people refer to "dinner" in China, they do not just mean the evening meal. They are actually referring to a banquet or dinner *party*. I learned this after a while when I would get ready to leave the lab and Wang, my graduate student mentor, would often ask me if I was going to dinner, and I would say yes. I found out much later that he thought that I was going to dinner parties almost every night! He probably thought I was super popular and a party animal, when really I was usually just eating alone in the cafeteria and then going back to my room.

Anyway, Chinese banquets were very fun. For our internship welcome dinner, all of the interns and American professors dined with some of the coordinators and department heads of Nanjing

Forestry University. Before the dinner, our American professors gave us a friendly reminder to dress nice, to be polite, professional, and to be respectful of our Chinese hosts, implying that if we were offered alcohol, it would not reflect well if we got so much as a buzz. Upon arriving, we came upon the usual round tables with big Lazy Susans in the middle. The peculiar thing I noticed right away was that along with a wine glass at each table setting was this tiny four inch high glass pitcher with a tiny wine glass to match, with the fluid capacity of a bottle cap. I quickly learned their purpose as my little pitcher and nostrils were filled with the pungent presence of báijiǔ. Báijiǔ is a clear liquor often made of rice but also can be derived from sorghum, wheat, barley, or millet. One of the Chinese faculty proceeded to pour from their pitcher into the tiny glass and raised it to wish us all a good stay at China and NFU. We all rose with our little glasses and exclaimed "gān bēi!" which I assumed to mean "cheers!" It does indeed mean that, but the literal translation is "dry cup," so a more accurate meaning might be "bottoms up!" Some people would even hold the upturned glass above their head cheerfully to prove that the cup is indeed dry, lest they let any remaining liquor drip on to their head. Upon my first "gān bēi," I promptly learned that báijiǔ is the worst kind of hard alcohol I have ever had the displeasure to slither down my throat. The smell and taste is so strong and vile, and stuck with me for such a long time afterward. In my opinion, out of all the other less revolting liquors, I could not understand why anyone would choose to drink báijiǔ for enjoyment, outside of tradition. To be polite I decided to keep persevering through each toast as a kind of extreme baptism into Chinese culture. I also quickly learned that it is considered good hospitality in China if you get your guests drunk. So naturally all of the Chinese faculty proposed many toasts, and some even went around to each table and toasted with every single person. Before long, the volume of conversation and laughter raised as more glasses and spirits were raised. It was a very merry atmosphere and a great welcome to the country and campus.

We had another event like this for our farewell dinner, where the merriment was somehow even more fervent than the welcome. Many more speeches, toasts, and jokes me were made, and of course, báijiǔ was greatly consumed. Luckily, I was much better prepared for the dinner this time. After being in China for seven weeks and experiencing the hospitality of many Chinese people, I had developed the strength to better tolerate the stuff. Given the opportunity, I would still choose any other hard alcohol over báijiǔ, as the taste never grew on me. However, I will

forever positively associate it with the wonderful joyous spirit and hospitality of the Chinese people that I was fortunate to experience during my stay.

Conclusion

After a whirlwind research project and touring of China, I fell in love with the country. The trip made me seriously consider living in China for a year or two if the opportunity ever presented itself and if the Chinese air ever gets any cleaner. The language, meals, and toasts were parts of the trip that I especially cherished. However, in terms of my whole personal experience, these things are only scratching the surface. I could probably write a whole book about it if I had the time, and that is only after being there for less than two months! I am very grateful to have gotten the opportunity to live in China for that short time and be exposed to a totally new culture. It was also wonderful to live among other American students from all over the country. Even though we shared a lot of the same experiences, everyone experienced them in a way that was unique to each person, which further enriched the overall diversity of views and opinions. Learning about the culture of China in such a setting has been immensely instrumental in helping me become a more open-minded as well as globally-minded person, and I wouldn't trade it for anything.

Gān bēi!

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Trip and Cultural Report
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REUG China 2014

If you were to ask me what my plans for the summer were, traveling to China for seven weeks and doing research on soil at Nanjing Forestry University would've never come up. Around late April, early May, I was given the opportunity to participate in Alabama A&M University's (AAMU) joint Research Experience for Undergraduates (REU) Program with Nanjing Forestry University (NFU) in China. Not fully knowing what I was getting myself into, I arrived in Huntsville, Alabama on May 18, 2014 ready for this new adventure. While at AAMU, we spent three days preparing for this journey across the world by having prep classes and panel discussions from previous participants. Being my first time really away from home, and my first time out of the country I was nervous and eager for a new experience. The first night in Shanghai when I was unable to connect to the Wi-Fi successfully, it hit me; I was away from the comforts of home.

Shanghai

Shanghai was where I experienced my first "bite of China". On our flight, there was a group of students from Southern University, but after getting off of the airplane, that was our last time seeing a large number of black people for the rest of the summer. Riding through Shanghai to get to our hotel, I was able to see the big city all lit up with numerous high rises. After arriving at our hotel in Shanghai, I quickly questioned myself, "What did you get yourself into Morgan?" but I knew there was no turning back. Our first dinner was at a restaurant near the hotel where we got to experience some Chinese cuisines and a "Lazy Susan" style meal,which was just a large spinning disk on a circular table that made sharing food easy, practically the whole menu was ordered and put on the disk. The food at dinner was quite interesting to say the least, the dishes varied from duck to a plate of egg, which I took to liking. At this dinner, I soon realized rice was going to be my best friend for the next seven weeks. While in Shanghai, we got to visit the Shanghai Oriental Pearl Tower, which is the 3rd tallest building in Shanghai, reaching 1,535 ft. We also got a chance to take a boat tour on the Huangpu River. On the boat ride, we got to see

various buildings from old and "modern" sides of Shanghai lit up, which was very beautiful and a wonderful experience.

Nanjing

Nanjing was where we spent majority of our time here in China, Nanjing Forestry University (NFU) to be more specific, and it became my home away from home. When we first arrived at NFU, we were escorted to the back of campus where the international dorms or apartments are located, which are better than some of the housing at my university. The first few days at NFU were spent getting adjusted, language and cultural classes, and more prep classes to help us with our research. Nanjing used to be the capital of China, and has so much rich history, so while in Nanjing we got to visit many historical and cultural places. The first weekend in Nanjing, we got to attend the Dragon Boat Festival, which is a 3-day holiday that includes many boat races. In Montgomery, Alabama, my hometown, we have a Dragon Boat Festival, and they both share many similarities. We also visited the Nanjing Massacre Museum, which was very saddening. During World War 2 and when Nanjing was the capital, Japanese soldiers came in and killed about 300,000 people. Confucius Temple and Presidential Palace were next on our places to visit. With Nanjing being once the capital, the president had a palace in Nanjing; it was very exciting to see how the president used to live. Out of all the places we visited and toured, Purple Mountain was my favorite. At Purple Mountain, we visited Yat Sen Mausoleum and Ming Xialoing Tombs. At Yat Sen Mausoleum, we got a chance to see a beautiful view of China. While searching for Ming Xialong Tombs, we climbed what seemed like a million stairs, just to go in a circle. I named this adventure, "The Stairs to Nowhere". But, I do believe this was my favorite experience because I got a chance to learn more about the historic city and I was able to appreciate the history of Nanjing.

Campus Life at NFU

Life at NFU often reminded me of being at my own university, which I greatly appreciated. Knowing the short cuts to get to the building where I spent most of my time, the school store being stocked with my favorites, and the cafeteria worker knowing exactly what I wanted made my summer here an easy adjustment. However, it was extremely common to see girls holding hands, this was just their way to show their friendship, would be very uncommon at my

university. One thing that was a little uneasy for me to get used to was the beds on campus. They were simply wooden beds, with a “mattress” that was the equivalent to a thin pillow, and a comforter. I encountered many restless nights and insomnia, but it was just something I had to get used to.

The best experience I had here would have to be working in the lab at NFU. The graduate student I worked with, Yang Jingyu, or “Young”, and everybody in the lab were so welcoming and eager to learn about my life and America, just as much as I was eager to learn about their life and China. The graduates students in the lab used me as I way to practice their English, they were always excited to talk to me and learn more. Some days, all of the students and I would go out to eat, they would make sure it was to a restaurant that I would be comfortable eating at, or sometimes would let me pick! Every Friday, we would have lab discussions, topics ranged from Food in China vs. America, Movies and Music, Campus life, and Hometown, Family, and Friends. These discussion allowed me see their ways of life and culture, while they got a chance to view mine. I enjoyed the daily conversations that were held in the lab from asking about my family, college life, and my hair. Whenever I showed them pictures of family, friends, and my first year in college, they would say how my life was so “colorful”; colorful was their way of saying exciting. At times, the language barrier would get in the way, but that didn’t stop them, they would just whip out their phones and begin translating and spelling the words out in their hands.

Nanjing>Yi Xing City> Wuxi>Nanjing

We got a chance to travel outside of Nanjing, and one weekend we traveled to Yi Xi City, Tai Hua Town, and Wuxi. In Yi Xing City we had the opportunity to visit a bamboo processing factory and a bamboo cultivation base. Yi Xing was a poor city but the locals planted the bamboo, and began getting some profit off of it. Bamboo in used to almost everything imaginable, desks, beds, flooring, and it is even a common dish. At the bamboo cultivation base we got a chance to pick some bayberries, which are really sour when they become ripe! We traveled to Wuxi, where we got a chance to go to a ballet, which was very exciting experience! The ballet was about the Chinese Women Red Army.

Nanjing>Siyang>Yancheng>Sheyang>Dafeng>Yangzhou>Nanjing

We also got a chance to visit five different cities, in one weekend! The first stop on our weekend long road trip was Siyang; here we visited a poplar tree plantation, processing factory, and nursery. The next stop was Sheyang, where we visited the Red-Crowned Crane Preserve and had to fight off the many vicious mosquitoes. After Sheyang, we traveled to Dafeng. In Dafeng, we got a chance to see the Milu deer at Dafeng David's Deer Preserve. This was very interesting to me, one because they were almost extinct, however a wealthy British man brought them back to China and two, they are thought to have many parts from different animals. The deer is thought to have "hoofs like an ox, the head of a horse, the antlers of a deer, and the body of a donkey." Our final destination before heading back to Nanjing was Yangzhou, I was really excited about this stop because they are famous for their fried rice and I couldn't wait to try! At dinner in Yangzhou, not only was I able to taste the famous fried rice but also got to try this delicious desert that was almost like a doughnut hole with icing you could dip them in!

One, Two, Three, Cheese!!

Before arriving in China, people warned me that people would be taking pictures of me because they've never seen a black person before, but its totally different when you experience it for yourself. Our first day in Shanghai, was hard to get used to not only because I was experiencing a cultural shock, but also because people were taking pictures of me! Sometimes people were kind enough to ask before taking the picture, by pointing to the camera and smiling, however, others would sneak which was a fail! Whenever we went outside of campus, we gradually became prepared for people to stop and stare and take pictures. I found it very funny when people would take pictures with us and then switch with the person who took the picture. Not only would they take pictures but also touch my braids, and begin talking to me and smiling at me, I would just smile back and nod, not knowing what they really said.

Posing with Angelica Durrah (right) and three Chinese children (at the behest of their parents) at the Forbidden City in Beijing.

The "Chinese Way of Life"

Having to adapt to the Chinese food, language, and culture was a big adjustment for me. Before arriving to China, one of my biggest fears was the food and what I was going to eat. Back at home, I am known for liking foods that most of my friends wouldn't dare trying, but that changed in China. I became very hesitant to try unusual foods that were presented to us whenever we had a "Lazy Susan" style meal. I stuck to dishes that looked somewhat familiar such as bell peppers with eggs, fish, and rice. Fast food in China was almost like a delicacy for me, with occasional trips to KFC, Pizza Hut, and McDonald's, which I never eat in America but wouldn't question it in China. However, the fast food restaurants are very different in China than America. The KFC and McDonald's serve similar items from America, but then again still offer Chinese style foods, the Pizza Hut offers a variety of pizzas and breakfast! Yes, Pizza Hut in China offers breakfast, which is very delicious! However, I did find some Chinese restaurants that I liked very much such, as the restaurant that sold the Chinese BBQ and a restaurant on "back street" that sold chicken and rice.

Whenever I mentioned to someone that I was going to China for the summer, the first question they asked was, "Do you even speak Chinese?" Not knowing a single Chinese word, I quickly

learned how to say some vital words such as: the greetings, bottled water, fried rice, and chicken. The language barrier was very difficult at times like trying to order food, asking for directions, or just asking questions period. Majority of the time, playing charades and pointing would help when trying to get the point across. Ordering food in the cafeteria was a tedious task because of the language barrier since most of the workers did not speak English. Sometimes there were students who were willing to help order our foods, other times showing pictures or previous recipes of food we ordered previously worked. At one station, the worker got so used to me ordering the same thing, she knew exactly what I wanted before even ordering it, "chaofan" (fried rice with eggs and cabbage).

The Chinese culture differs greatly from American culture. From family being very important in the Chinese culture, considering that families can only have one child, to traffic being a chaotic mess with mopeds, buses, cars, taxis, and bicycles all on one street with no one really caring about traffic laws. And seeing children with no diapers, with slits in the front and back of their pants and peeing in the middle of the street. At times, I would constantly think to myself, "This would never happen in America", but I had to remind myself that this is "The Chinese way of life".

It is one thing to hear and read about someone's cultural experience, but it is another to actually experience it, and I am grateful that I got the opportunity to experience it. The culture, food, people, and history truly made this summer an amazing one, to say the least. Not only did I learn about China, but I also got to learn a lot about myself. Numerous things in China have inspired me, which will have a role in impacting my future

Morgan posing with labmates where she conducted most of her research and analyses, at Nanjing Forestry University, 2014.

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Trip and Cultural Report
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The Initial Shock

As I'm sitting here reading through my journal to recount my first few days in China, I'm laughing at my initial "culture shock" notes: "*a ton of dishes on a spinny thing*," "*I keep dropping my chopsticks*," *"I've had to take so many pictures with random strangers today*," and "*Never thought I'd eat that*" just to name a few. Now, at the end of our trip, these things that were initially part of the shock are now everyday norms that I will greatly miss back in America. For example, I can already tell that I will be craving the endless "buffets" of new and unknown foods that I was so used to eating every time we left campus. Additionally, we've all gotten *so* good at chopsticks to the point that we shoo people away when they try to hand us forks. This report will give a brief recount of my time in China – including both the interesting and the strange!

The Food

Having traveled to over a dozen countries around the world, I have definitely tried some new and "strange" foods. However, China wins. At every meal I had during my stay, there had been a minimum of at least 5-10 new foods that I had never seen or tasted before (many of which I had no idea were even edible). A few of the strangest foods I tried in China include:

- Crunchy jellyfish
- Duck tongue
- Fish buoyancy bladder
- Pig belly, ear, and intestines
- Soft-shelled turtle and river eel soup
- Chicken brain
- Gingko fruits

- Fermented stinky tofu
- Full, unchopped squid tentacle
- Duck blood

However, I never found one thing that I couldn't try at least once. There were so many incredible tasting foods that every day was a new experience.

(Left) A street vendor cooks up and sells a variety of stinky tofu in Shanghai. (Right) Linzi and others trying Chinese street food for the first time (right).

Our daily food experience, however, was eating in the university's cafeteria. These cafeterias are much different from home; in fact, at NFU there are three levels in each cafeteria, and each has at least seven different "shops" to choose from. Some were buffet style, a few were a "build-your-own-soup" with fresh meats and veggies set out, and many served a rice, noodle, or dumpling dish that would be cooked right in front of you (if you could speak Chinese!). Additionally, popsicles are extremely popular, and you can get dozens of flavors from strawberry and vanilla to peas and corn. However, the sudden change from American to Chinese food left us a little homesick. Because of this, we would occasionally find ourselves at the "Imported Food" section of Walmart or Carrefour to get "uncommon" foods like peanut butter, cereals, cheese, and ham. Wandering around these 2-story tall grocery stores, however, led us to many surprises

such as a “pick your own live turtles, frogs, and eels” section similar to the live lobster tanks at home. We also saw a cart selling every part of a pig imaginable as well as an ice bin stacked with uncovered chicken pieces that you could choose and bag up as if picking out apples. Of course, the rest of the store was just like at home with kind sample ladies throughout and aisles of other goodies.

Some evenings, especially during our weekly group dinners, we would wander the roads of the backstreet searching for new local restaurants to try. Of these “hole-in-the-wall” restaurants, our favorite was the tiny Chinese BBQ where they cooked many kinds of foods with very hot spices. In fact, we could feed 7 people for only $35! And, when we needed a break from rice and noodles every day, Pizza Hut and KFC were just a short bus ride away. The poor waiters had to struggle to understand our orders, yet they wouldn’t accept our tips for their hard work! However, even when craving American food, KFC does have its Chinese attributes such as serving rice with a meal platter and putting red beans and tapioca pearls in ice cream. Did I mention that they put red beans in everything, especially for dessert? I could go on and on about the food in China!

Campus Life

My favorite part about this REU was getting the chance to live on campus as an International Student. In Oklahoma, I work closely with our campus’s International Office and the majority of my friends at ECU are from around the world. Thus, living on campus in China helped me put things in perspective for how life must be like for my international friends. Living in the international dorms was a great experience because students from all over the world, including Mexico, Cameroon, Britain, and Spain, to name a few, lived in our same building. A few times when I ran into my friend from Mexico, he would jokingly throw his hands in the air and say “How did we all end up in China?!”

According to my local Chinese friends, our international dorms were extremely nice and roomy compared to what the local students live in. In our dorms, I stayed with two of my friends from the REU program. We each got our own private room, a shared kitchen, and a bathroom (that even had a Western style toilet!) complete with a shower. However, as my friends and family are now very aware, the beds were my only complaint (largely because I don’t consider a one-inch

thick pad on a wooden bed a "mattress"). But of course, you forget that quickly as you're typically exhausted from the daily research and traveling! Additionally, this was also my first time to do all my laundry in a bucket in the shower. Regardless, I enjoyed life in our dorms. However, we were told that the Chinese-student dorms are quite the opposite. They are typically packed with four to six students in one room with the bathroom being shared by the entire floor. While this doesn't sound too different from traditional dorms at home, the only showers on campus were on the 4th floor of the cafeterias.

Weekend Trips

***Shanghai*:** Before beginning our weeks of research, we first got to enjoy the initial few days in Shanghai. On the Saturday after 15 hours in the air, we visited Pearl Tower – a famous site in the Shanghai skyline. The view from the top was incredible, but we quickly noticed people staring at us. This was our first experience with the Chinese tourists taking our pictures – as if we were a part of the attraction! Typically they would ask before taking a picture with us, but occasionally we'd catch them taking a selfie with us without asking. Even more common was the staring, which we got used to over our 8-week stay. We called it our "celebrity treatment" and thought it was kind of neat!

Next in Shanghai we took a cruise on the Huangpu River (even getting admitted into the VIP section, which had chairs). From there, we visited the Night Market where I got my first whiff of fermented stinky tofu. I will probably never forget that smell.

***Nanjing*:** During our first weekend in Nanjing, we watched the Dragon Boat Festival – a very famous Chinese holiday in which boats shaped like dragons are raced across lakes all over the country. Afterwards we visited the Nanjing Massacre Museum in which hardly anyone could walk through without getting teary-eyed (not even me, who's supposed to be tough!). The next day we visited the Presidential Palace – a huge garden palace where the first President of the Republic of China was housed when the Republic was first established in 1927. As we walked in a shop within the palace walls, we started giggling as we noticed Christmas music was playing in June. However, the biggest culture "shock" happened when a very young child ran over to my friend and me, then decided it was time to use the restroom by our feet. With the surprised look still on our faces (which I still laugh about today), his grandmother curiously asked if that was

normal in the US. We politely told her it was uncommon, yet the surprise never ended as this happened *several* times during our stay. Afterwards, we visited Confucius Temple and took a boat ride along the river. It was surprising to visit the tourist shops near the temple, because the items appeared to be things that awe-struck Westerners would likely buy (i.e. traditional Chinese fans, dresses, umbrellas) yet the items were actually catered toward Chinese tourists. The next weekend, we visited Purple Mountain where Sun Yat-sen's (the founder of the Republic of China) Mausoleum and the Ming Tombs can be found.

During one Friday night, we visited an underground night club to see how it differed from home. Turns out it was *literally* an underground bar that had a live band and several pool tables. It felt like we had stepped back into America as the place was filled with Westerners, the band played American pop music, and nearly everyone spoke English. The only odd thing was the man playing pool in his boxers!

***Yixing and Wuxi*:** Because our REU is a forestry and environmentally focused program, we spent part of our time visiting bamboo plantations and a sustainable bamboo processing and flooring production site in Yixing. Afterwards, we stayed the night in Wuxi where we visited the opera house and watched an amazing ballet titled "Red Detachment of Women" about the female soldiers of the Chinese Red Army (ca. 1930s). The next day in Wuxi, we were led on a tour throughout the historic 16^{th}-19^{th} century downtown area where many old shops and buildings still stand and are open to the public. The only uncomfortable side was that our tour guide always held up a big flag and used a microphone so that we wouldn't get lost – we were the epitome of tourists!

***Siyang, Yancheng, Sheyang, Dafeng, and Yangzhou*:** During our three-day weekend trip to these cities, we visited China's only poplar museum, a poplar plantation, and a poplar manufacturing plant where wood floors are produced. We were also invited to a poplar nursery where we were surprised to learn many sustainable facts about this species, such as that the plants can grow 5m in their first year alone! Additionally, it was interesting to learn that all of the seedlings were a cloned, transgenic version and were all male-only; this was because female will cover and pollute the ground with their seeds. The next day, we visited a beautiful natural area where endangered red-crowned cranes are raised. Even though it was pouring rain, we

couldn't resist seeing all of the crane species that were being rehabilitated. Afterwards we visited the Dafeng Deer Preserve. These deer had gone extinct in China just one hundred years ago; luckily, however, a few had remained alive at a mansion in England. That last remaining herd was reintroduced to China just 30 years ago, and now there are hundreds on this preserve! The next day we had a traditional style Chinese breakfast that consisted of stacks and stacks of steamed dumplings filled with various meats. Afterwards we visited the Ge Botanical Gardens – one of the top four gardens in the country.

***Beijing*:** Once all of our work at NFU was complete, we spent our last few days in Beijing before returning home to America. During this time, we visited the Great Wall of China, Tiananmen Square, and the Forbidden City. It was a great "vacation" after weeks of hard work doing research!

Other Unexpected Things

Just to list a few other parts of China that were unexpected:

- Next to the sink in each bathroom, there was a bucket with a colander inside so that you can throw your tea leaf waste out without clogging up the sink
- Smoking is allowed in most of the buildings and restaurants
- Lots of people thought it was cool that Michael's last name is Kennedy – "Are you related to the President?!"
- It's near impossible to find cheese. When in the convenient store, I got excited when I found a bag of Cheetos, only to realize that they were green and pork flavored
- Many things were *very* inexpensive in China. For example, Mercedes got an eye exam and a new pair of glasses for only $15!
- We were asked by the University to be in commercial for the Nanjing Youth Olympics because we're foreigners – we wound up doing a photo shoot in their studio (aka a hotel room)

The Environment: Before we had even made it to our destination, I had heard many stories about the smog in China. It wasn't until our first day in the country that we noticed how thick the smog really is. Sometimes I couldn't tell if it was rainy or smoggy outside, and visibility was often less than a $1/4^{th}$ of a mile with PM 2.5 levels 5x higher than what the World Health Organization recommends for safe *short-term* inhalation. In fact, their weather reports even include what the day's visibility will be like. However, this is probably the price paid by the less-stringent air pollution laws well as the large amount of "Made in China" products that are produced and exported to the US each year.

However, efforts are still being made by the government to reverse pollution in the country. For example, while on a boat ride in Nanjing it was noted that China spends millions of dollars trying to clean the channel, but that it still remains grimy as people continue to dump trash into it. However, despite the air and water problems, every city that I visited in China was *full* of trees and foliage. The streets and highways were lined with plants and every possible space was thick with greenery. Additionally, another idea that I wish America would consider: China requires payment for plastic bags in order to deter their usage and to prevent unnecessary pollution.

Pictures with Strangers – "American Celebrities": As I mentioned earlier, the Chinese *love* taking pictures with us and of us. My favorite memory occurred within our first hour in Nanjing when we stopped at a local restaurant. As we walked to the second floor, *everyone* turned to stare at us as we entered (worse than when you walk into a small-town restaurant). As we sat at our table, some people started to go back to their meals, but not everyone. A man randomly walked up to our table, took a picture of us, and then walked back to his seat. A little while later, a different man walked up to Rosie, said "It's nice to meet you," then walked away. Everyone was staring again as we left the restaurant, so naturally I did the queen's wave as I walked down the stairs. Half of the staring people waved back in return. Probably what a celebrity feels like?

Chinese tourists staring at our group – we got used to it!

I'm also undoubtedly in dozens of strangers photographs. I'm not sure what they do with the pictures – maybe show them off to their friends to prove that they met an American? My favorite, though, was a guy around our age who ran up to us on the street. He said it was his first time to meet foreigners and wanted to have a picture with us. Back home I'll be just another boring American, so I will definitely miss that about China!

Chinese Wedding – A Night Filled with Luck: During my time in China, I was also fortunate enough to be invited to a wedding with my Chinese graduate student, Kyle. I wore my best dress to the event, but when picked up I was surprised to find him in shorts and a t-shirt! He informed me that at Chinese weddings no one cares what you wear (despite the weddings costing $20,000+). When we arrived at the wedding's location in the tallest building in Nanjing, we took an elevator to the 6th floor. But why the 6th floor? The numbers 6 and 8 are the two luckiest numbers in China. Because of this, the wedding was on June 8th (6/8) at 6:18pm (18:18). Additionally, every table had an 8 on it, and the meals (cold and hot) were served in sets of 8. As the elevator door opened, the bride and groom welcomed their guests by inviting them to take pictures with them on a stage outside the dining hall. Guests are also given many sweets and are invited to sign the wedding memory book (mine was the only English name!). We all sat in a beautiful dining room that featured a slideshow of the couple while American pop music was playing. Once the on screen timer hit 6:18pm, the MC came out to start the wedding. From here, the differences between American and Chinese weddings became extremely apparent. For one,

an MC was used instead of a priest. Additionally, after the groom got on stage and sat in a throne, the MC handed him a scepter. This scepter was then pointed into the air and lowered toward the doors at end of a runway where the bride then stepped out. I'm not sure if this is how most weddings are, but it was extremely entertaining. After this, the food was served and everyone began eating. During this time, stuffed animals and red envelopes of money were thrown to the crowd for good luck (rather than a bouquet). Next a magician performed on stage before volunteers were invited on stage to play Simon Says with the chance to win a huge teddy bear. I got pulled on stage to play, but me not knowing Chinese got me disqualified pretty quickly! Best of all, because so many people love the NBA in China, we had many toasts to the OKC Thunder!

An Unforgettable Experience

Overall, this incredible 7-week experience in China has left me with a better understanding of the culture of this great country. I hope to return soon to visit all of the wonderful friends I've made during this time and I know that I will never forget this amazing opportunity.

Linzi and her mentors enjoying a banquet style meal at a local restaurant near campus after a long day of field work.

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Trip and Cultural Report
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REUG China 2014

Cultural Experience Paper

I never thought in a million years that I would be given the opportunity to go to China and conduct research. That alone was enough of a shock, but then to actually get on the plane and get here was just life changing. Coming to China has been my first international experience so it was overwhelming at first. The sights, smells, and just different cultures just offered so much to see and take in with so little time to experience everything that makes China into the place that it is culturally and historically. Getting on the plane from Huntsville to San Francisco alone was enough because I've never been in California before but then to board a flight that was almost half a day long took a lot out of me. I slept off and on while reading, watching movies, listening to music, and eating the food that they brought us about every 4 hours. One of the things that I found truly breath taking was that during the entire flight from San Francisco to Shanghai was that the sun never set in the sky, it remained steadfast the entire duration of the flight and only started to go down once we'd left as if to say welcome to China the land of the rising sun.

The night that we arrived in Shanghai I was extremely tired; however, we still had to get to the hotel, check into our rooms, and go to eat. Checking in took a while because, for one we didn't speak Mandarin and they didn't speak English. The second factor was that the tour guide/translator had to check everyone in using their passports in groups of two. I ended up getting checked in last so I had my own room which was fine with me because after sleeping and being stuck between two complete strangers for hours on end I wanted some space of my own for at least a short time. I sprayed down the bed, wiped down the bathroom and processed to make the room a little more comfortable. I was happy to see that the bed was a king, which is full sized in America, but it wasn't as fluffy as it appeared so that was a downer. When we went to dinner that night and I saw all of the food sitting on the table I became nervous and overwhelmed again. Did they expect this small group to eat all of the food that was on the table? And how did we try food and decide we didn't like it without offending them? Was there a certain word we were supposed to say or something? Would my stomach be able to handle all of this after

spending an entire day on an airplane? I was completely clueless. However I did see some foods that looked familiar such as broccoli, rice, bell peppers, and eggs. Mostly everything else looked completely foreign. So I took it easy that night and only ate what looked familiar to me so that my stomach could adjust, this was extremely helpful. And it didn't hurt that we would eat at that same place for our duration in Shanghai so if we saw something we liked we could just request that it be brought to the table.

The next day would be the first full day in Shanghai and I must say I was just looking around like a curious new born. There was so much going on for it to be so early in the morning. I would know this because I only got about three hours of sleep that night and the horns didn't stop blowing until 3 a.m. but started back up again about two hours later. The streets seemed as if they were constantly filled with traffic no matter what time of day it was. The first place we went was the Pearl Oriental Tower. When we first got there I think the combination of all of the different smells upset my stomach but after I took some medicine I was fine. The view from the observation deck was amazing! I didn't want to stand on the glass at first but then figured why not because this very well could be my only time being there. I was putting something in my bag and a lady reached out and touched my hair. I thought it was so funny because she was really fascinated by the twists that I had and I guess was curious as to how they felt. We smiled at each other and she said some things that I didn't understand. There were also a bunch of people staring and taking pictures of some, some with and some without our consent. We had been told before at the school that there would be lots of people staring and taking pictures so I thought I would be ready; however, it's different when it's actually happening right there in your face. And then once one person does it everyone wants to join in and take pictures with you as well as of you. It turned into quite the event to say the least. Once we left the observation deck we went downstairs to the museum and got to see some of the things that were there about the history of Shanghai and China as a whole. It was a good way to try and understand why the city is the way that it is.

After a few more days in Shanghai we then left for our home city of Nanjing. The drive there was about four and a half hours long and pretty uneventful since most people slept all the way there. We did make a stop to get gas and I tried some Lay's chips that were lime flavored. I was nervous at first about them but they turned out to be really good. Too bad I never found them

anywhere else. Once we got to campus we had to walk what seemed like a mile and a half to get to the dorms where we would be staying for the next month and a half. At that point I was just hoping that they had western toilets in them. The rest of that day was uneventful; we all just unpacked and lay down so that we could rest. I didn't get much sleep because I was nowhere near adjusted to the time difference.

For the next week we settled into the routine of having classes all day. They we very interesting, I really enjoyed the interactive classes such as the Chinese language class. Because I am a graduate student I also had to teach a few classes along with Mercedes. Although sometimes we were unsure of what to say when we would it down and prepare everything, the classes went easier than we expected and after speaking with some of the other participants about how we did we could tell that what we were trying to say came across very easily. That was a great thing to hear because that was our intended goal with the topics we were given. That Friday night we attended the welcome dinner from our hosts at the hotel on campus. This was the first big dinner that we'd have since our arrival in China so I was nervous to see how that would go. There were different foods, some that we'd seen since being here and others that we hadn't seen however I told myself that I would try a little bit of everything. The most memorable new food that I had that night would have to have been the cow's stomach. It was very chewy and didn't really have a taste. The next day we took our first trip to Purple Mountain in order to see the Ming Dynasty tomb as well as the tomb of Sun Yat Sen. I had been told about the "Tomb of a thousand steps" but didn't quite understand the name until I saw it for myself. Although I was intimidated at first because it was hot and I had a backpack on that weighed at least 10 pounds, I trudged my way up the stairs flight by flight until I reached the top. I can say that I was proud of myself because when I first saw them and Ms. Lisa said that we had to climb them to get to the tomb I heard a resounding "Heck no!!!" inside my head.

The next stop on the Purple Mountain trip was the tombs of the Ming Dynasty. The old palaces were breath taking; the attention to detail in every aspect was simply astounding and is something that isn't seen so much in today's architectural structures. The most memorable part of these tombs would probably be when we climbed the stairs trying to find the Emperor's tomb and ended up going in a huge circle through the woods. I think after we realized that we'd just walked in one of the biggest circles ever, we were all ready to just sit down for a while and rest

our feet. While we rested we met some people who were from Germany. I thought that was very interesting because at first I thought they were a couple touring but as it turned out they both teach in China, in two different locations actually. So I was wondering if they knew each other before they got here, met by chance, etc. but thought it best that I didn't allow my nosey side to show too much. After the fifteen minute break we went to see the stone animals. It was a row full of stone animals but what I found to be so neat was that you could see what the people during those times believed these animals to look like. Some were mythical while others were real and for each group of animals there was an explanation of what they meant to the culture. I believe a lot of times we just think of animals as just being there but how we treat them, care for them etc. says a great deal about the culture and society that we live in.

The next activity for the weekend was a visit to the Nanjing Massacre Museum. Since I love history I was very excited because I'd never heard of any Nanjing Massacre, but then again before being accepted into the program I'd never heard of Nanjing. To say the least that it was a very humbling experience would be an understatement. Because in primary school we are only taught about certain events such as the Holocaust or the African diaspora doesn't mean that other races and cultures haven't gone through some experiences of their own that are horrific. It also shed some light as to why there is such tension between the two cultures. There was a holiday on Monday, Dragon Boat Festival, so the school was going to be closed meaning we had a three day weekend. This was exciting because the Monday before was Memorial Day and we didn't get to celebrate so at least we would have some type of celebratory activities during this week. It was nice to see something that is a part of the heritage and culture that takes place every year, just like the holidays that we have at home. The next week went by the same as the first for the most part, classes, meetings, lab tours and campus tours. I did meet my Chinese mentor Dr. Zhuge for the first time. When we had our first meeting, I thought that we were going to sit down and discuss what I would be working on for the duration of my stay; however, from my understanding I was just going to be watching and learning techniques. We were not able to get this straightened out until the next week on a Thursday morning so I was really behind as far as lab went. But as long as I had a lab I was happy.

The lab environment is almost completely different from what I'm used to back at home. If we use something then we clean it and put it away. The same thing goes for equipment, kits,

chemicals, beakers, etc. So to go from that to being in a lab where they eat and do work in the same office was a complete shock. No one had a set work space or time, everyone worked where they could around the beakers and instruments. I knew that in South America it's a common practice to have a siesta; I had no clue that the Chinese have a similar practice as well. I don't believe they have a set name for it other than going to lunch and then taking a rest. So when I first got into the lab I would go eat lunch and come straight back since I didn't have an office key just to find that everyone was still gone. So that was very interesting to adjust to.

That weekend we took our first weekend trip to Wuxi were our main purpose was to see the bamboo manufacturing plant, bamboo plantation, and a play about the women of the Red Army. My favorite part of the weekend was the play. I thought that for the two cultures to be perceived as being so different there were so many similarities. The play was a wonderful experience because I've been to a play in New York before so I was comparing what I could which was just the visual aspects since I don't understand Mandarin.

Out of all of the different foods that I tried I would have to say that my favorite was the chicken and rice that we would get from a vendor on the back street behind the school. When Rosie, Morgan, and I were back there walking one day we realized that we'd missed the cafeteria. So Rosie and I decided to try the food since we could see them prepare it and it smelled good. I can honestly say that was one of the best decisions that we made as far as food because whenever we wanted something that we knew we'd eat that became our go to. My favorite food place that we went to would probably be the barbeque place, as we called it, on the back street also. It was like getting a small taste of home to just hold you over when you wanted anything but Chinese food because we were eating it every day for lunch and most times dinner as well.

Never in million years did I think that my first international experience would be China of all places but I'm so happy that it was. I feel like if I could handle China and all of the curve balls that it had such as the squatting toilet, then I can almost handle just about anything. I found it strange how that at the beginning of the trip we were all counting down the days until we were going to get back on the plane to go home. However, the closer we got towards the end we slowly stopped doing that and started to open ourselves up to the culture more. It's not that we didn't miss our homes anymore, because we did. It's just that we adjusted to the lifestyles that

we were living while being here and I never thought that I would get adjusted to the time difference let alone get adjusted to the lifestyle. I can honestly say that they are some parts of China that I will miss when I get back home, and one of them will be the wonderful group of people that I was able to share this experience with. Because of them the entire process was a lot easier and no one lost their minds the entire time thank goodness. Overall, my experience in coming to China for this period of time has been so wonderful and I will forever be grateful for the opportunity.

Angelica and Nikki on the bus heading up Purple Mountain, Nanjing, 2014.

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Trip and Cultural Report
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REUG China 2014

Dear American Friends,

So you want to visit China. I personally think it's a great idea and I support you 100%! There's nothing better than exploring a new country and learning a new culture. With that being said, there are probably a few things you should know about China just to prepare you for the experience of a lifetime:

- The second you sit in a cab in China you will be scared for your life, but don't worry, that fear can all be blocked if you just close your eyes.
- The food is… different, but stay strong! Try new food every day, that's the only way you'd ever find out you don't like cooked ducks blood.
- The more you drink, the cooler you are. Period.
- You are a celebrity to these people, so eventually you'll get use to the stares and pictures being taken; And if you really want to get the people going shoot them a smile and a big wave.
- There are beautiful blue skies in China despite popular belief.
- Lastly, the people are some of the sweetest and best people you will ever meet. The language barrier may be intimidating at first, but most young people know some English and are actually eager to learn more. Seriously though, they are some of the best people.

You're probably wondering how on earth I know this. I'm just a 20-something year old trying to make her way through grad school without keeling over. Did I look it up on google? No. Through Wikipedia? No.

I went there. I experienced it. I lived it.

I was one of two graduate students chosen to travel along with nine undergrads from all over the country. We were part of an REU (Research Experience for Undergraduates) Program through Alabama A&M University to do research in collaboration with Nanjing Forestry University. With that being said, let me give you a taste of some of my most memorable moments in China.

I got to China a little later than the rest of the group, I travelled with three other professors who had to leave later too, so my experience in Shanghai was different than the rest of the groups. I was lucky and travelled over with Dr. Yong Wang, who grew up in Shanghai, so he knew exactly what he was doing. We got off the plane and next thing I knew I was sitting a cab staring up at all the lights and tall buildings. I remember just being so overwhelmed, the kind where all you can say is, "holy crap I'm in China!" Riding in the cab is where I got my first Chinese driving experience, and it was terrifying. See I'm use to the driving in America, where people stay in their lanes, where people only honk in the case of an emergency (or unless they're a jerk), and when the only time you run into a scooter is if you're on an island or in a college town. In China though, the driving is totally different. The lines on the road were interpreted as a simple suggestion that no one followed, people were honking left and right, and just when you thought you were in the clear five scooters would come whipping past you. Speaking of scooters, in America scooters (and bikers too), must obey the same traffic laws as a motor vehicle, but in China scooters pay no mind to any traffic laws. They would just blow right through a red light and it was the driver's job to avoid them. The only way I can think to describe what it's like to sit shotgun in a China taxi cab is like this – you know that racecar game at the arcade where you're going as fast as you can, swerving in and out of cars and trying not to hit pedestrians, with music blaring and you're seat leaned back. Well that's it. That's China driving for ya. What is so surprising though is that I only saw one accident throughout my whole time in China, and it was a simple fender-bender at that. I saw more pedestrians almost get hit by the people on scooters than anything. The scooters here can ride up on sidewalks and basically just go wherever their heart desires. If you're in their way they'll just honk and continue to honk until you move. You'll never quite get use to the honking, or the resisting the urge to raise that middle finger the good LORD gave you, but just remember that they mean well. To them, we're the weird ones.

Like I said before, I travelled to China with an expert of Shanghai so after we set all of our stuff in our hotel rooms he took us around town. As we were walking around, we realized we were hungry so we stopped at this restaurant with crawfish statues out front. It reminded me of my time in Louisiana so I was excited to try the food. We started off eating some vegetables, and then they brought out my highlight of the night, lamb. They were on skewers and were seasoned and spiced just right. They were delicious! Hands down some of the best lamb that I have ever

had. After that night I thought that I didn't have anything to worry about when it came to food in China, but I quickly had a wake-up call as the trip continued. Throughout the rest of the trip I had a bunch of different foods, some of which I was glad I tried and other that I will be perfectly happy never seeing again. Some of the odd dishes included: fish eyes, duck blood, jellyfish, fish bladder, cow intestines, chicken brain, Gingko fruit, donkey and eel. The last two might not be odd to some people, but it was a first for me! Out of that list the ones that I will try again include donkey and eel, everything else I could do without. Most of those dishes I had at a group dinner, where there is a ton of food so you have plenty of other foods to choose from. When I say a ton of food I really mean a TON. For larger group dinners everyone sits at a round table with a giant Lazy Susan in the center and the waitress bring out dish after dish and just sets it on the Lazy Susan and you just pick off the dishes they bring out. One thing that I learned early is that you must pace yourself or else you're going to be full very quickly. I began to only take a bite or two from every dish they brought out, and I had to resist pigging out on the one or two dishes I really liked. One thing that you never had to worry about though was whether it was fresh or not. Every animal that they sell at the market is alive. You can hand pick your favorite chicken, dove, frog, duck, eel, quail, fish, etc., and then take it home and kill it when you're ready. One morning in Nanjing we saw a woman walking down the street carrying a chicken by the wings just like it no big deal. She was probably on her way home to cook breakfast or prepare lunch for her family. Oh and speaking of breakfast, they have none in China. Or I guess they technically have breakfast, but it's not like an American breakfast. In America we have specific breakfast foods like bacon, eggs, cereal, waffles, pancakes, hashbrowns, etc., but in China they just eat the same thing for breakfast, lunch and dinner. There was one instant when we did have these pastry dumblings with red bean in them, and that was kind of breakfast like. Most other times it was simply rice, fish, soup, dumplings and boiled eggs. Whether it was breakfast, lunch or dinner though, they would always end a meal with rice and watermelon. Every single time they brought out rice and watermelon you would know that the meal was over, and a part of you would be thankful because you couldn't imagine eating anymore. The one good thing about eating so much is that it enabled you to drink more, which is a positive thing in China.

Oh boy, where do I even start with this? I was told before I left the States that in Chinese culture, the more you drink the smarter they think you are. Never did I imagine that it was true.

Remember that restaurant I mentioned before that had the crawfish out front and the great spicy lamb? Well that same night I had my first Chinese beer. The waiter came out with a beer bottle that was as big as a wine bottle back home! Of course being from America, I just slipped my UGA koozie on the bottle and started drinking straight from the bottle. It turns out though that in China they pour the beer into small glasses. Who would have thought. Anyway, the beer tasted like water. It was very weak compared to any American beer. After that night I thought I had it in the bag that the people here would think I'm a genius because I knew I could drink a bunch of those beers without a problem – that is until I tried rice liquor.

The first time I tried rice liquor was at the group dinner. The waitress came and placed what looked like small (2-3in tall) wine glass with an equally small pitcher next to it filled with some clear liquid. A gentleman at our table told me that the most important thing was to ALWAYS keep your glass full – I immediately thought this is my kind of dinner. After we were all seated one of the professors stood up to make a toast and ended it with, "Gan Bei!" (pronounced Gon-bay). In Chinese that means "bottoms up" and everyone finishes their glass no matter how much liquor, beer or wine they have in their glass. The rice liquor was *pretty* strong. It was kind of like a mixture of Vodka and Tequila, which is any person's worst nightmare. As I began to fill my glass up again (because like they said, your glass should never be empty), another professor stood up and gave a toast and ended it with "Gan Bei!" Before I was even able to sit down, we were taking another shot of this vodka/tequila mixture. After a couple more of those, I decided that I should probably switch to wine since we were at a school/business related dinner. Not soon after I made that decision and returned my "shot glass," I turned to the professors table and they had moved past gan bei-ing with the glasses and were gan bei-ing with the pitchers. The best part of that night was when we were leaving and the president of the school walked up to a few of us and said "Hello! I am drunk," and then walked (almost skipped) away. It was hilarious. I also learned that once someone finds out you are from America, they automatically think you can drink an extensive amount of beer. While I was in China, I got to work at a lab in Beijing so I spent about 10 days there. On my last night in Beijing after doing research all week, a couple of the grad student I worked with took me to dinner where we ate a little and drank a lot. One kept on saying to me, "I say you can drink 8 beers! And your boyfriend, he can drink 15 beers!" I

laughed thinking he was joking, but he was actually serious. I never lived up to his expectation because I still wanted to be professional, but it was a good laugh.

Another benefit with being a foreigner in China is that people treat you like a celebrity. It is once in a blue moon that they see anyone who is not Chinese, so they fully take advantage of the opportunity and snap some pictures with you. At first I didn't understand why everyone was staring. Of course my first instinct was that I had something on my face or I had a clothing malfunction, but it was just because I looked different. I would actually confuse people being born in South America but being raised in America. They would approach me and ask where I was from and I would tell them America, then they would look at another girl from America and look back at me giving me a confused look. The best "celebrity" experience I had was after I was chosen to be in a commercial for the Nanjing Youth Olympics. Of course with it being a commercial, there were cameras everywhere and people doing hair and make-up (which was awesome by the way), but there was a particular time that I really felt special. It was after I had my hair done and I was waiting to do my segment of the commercial, a girl approached me and asked me where I was from. I told her I was from America and then she smiled and timidly walked away. She continued to look at me from afar as she giggled with her friend, and then she approached me again and this time she looked at me with big eyes and a huge smile on her face and asked, "Are you an actress?" What I should have said was yes, I am Angelina Jolie, but instead I told the truth and had to disappoint her with a no. She still asked for my name though and she asked me to write it down for her so I did that and I gave her a business card which lit her up even more. Having that celebrity status is something that I miss from China, because in America you're just another face in a crowd (unless you really are Angelina Jolie).

I mentioned before that I spent some time in Beijing, and in Beijing they had blue skies almost every day. They also had sunsets with all the blues, oranges, and reds. Yes, in most of China the air quality is very bad, but there are some parts of China that aren't as bad. So let me reiterate this to make sure it's fully understood. There are blue skies in China, and they are breathtaking. One day, after leaving Beijing and being back in Nanjing, I was talking with my grad student, Pengcheng Wang, and I said something about the smog. Then he said these words I will never forget, "After the rain, the sky will become blue, wait some time, it will become better." Although when he said this he was really just talking about the sky, they had such a deeper

meaning to me. I mean, how often is the chaos, pain, and confusion just so overwhelming that we can't breathe. That the rain is coming down so hard that we see no way out. But if we just wait, if we just wait some time the sky will become blue and it will all be better. Gosh, y'all! Pengcheng just spoke some awesome wisdom to us all and he didn't even realize it. Okay I'll get off my soapbox, but I'm going to continue talk about how cool Pengcheng is.

There is a reason that I saved this part for last because I want you to know that even if you don't think anything else that I said was true, this part is. The people here are incredible. Yes, they can be intimidating and sometime their resting face make it look like they're mad, but it you take the time to talk to them you will realize they are some of the most genuine people out there. I learned most of this from the grad student that I worked with in Beijing, Pengcheng. After my advisor left me in Beijing by myself, I was scared as all get out. I didn't think there was any way I was going to make it there by myself, and then Pengcheng came in and just made it all worthwhile. He had broken English, but it was very easy to understand what he was saying and despite what he would say, I think he had very good English. I spent six days with him, working long hours in the lab and just getting to know each other more. I was able to help him practice his English (which was perfect because he had his oral English exam while I was there) and he was able to teach me in the lab. He introduced me to his beautiful girlfriend who was equally as nice as can be. On my last full day in Beijing we went out to "play" as Pengcheng calls it – we went to the Great Wall. Pengcheng is now one of my dearest friends, and we are already beginning to plan his trip to America.

During my trip to China I met many awesome people like Pengcheng. People who genuinely want to learn more about you and your life. People who care about you after only knowing you for a few minutes. Just good people. Maybe I was just lucky and had the experience of a lifetime, but I honestly think that if you decide to travel to China, you'll have just as great of an experience. There is so much history, culture, and life in China, and I highly recommend you experience all of it.

God Bless and Go Dawgs,

Mercedes Bartkovich

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