Student Checklist (1A)

This form is required for ALL projects.

1. a. Student/Team Leader: Blake Lippman Grade: 11

   Email: b.lippman03@gmail.com Phone: 516-532-6949

   b. Team Member: Tyler Bissoondial c. Team Member: Blake Lippman

2. Title of Project:

   Investigating the Use of Ceratopteris richardii as a Model Plant for the Phytoremediation of Cadmium

3. School: G.W. Hewlett High School School Phone: 516-792-8004

   School Address: 60 Everit Avenue
   Hewlett, NY, 11557

4. Adult Sponsor: Dr. Terrence Bissoondial Phone/Email: 516-425-9991

5. Does this project need SRC/IRB/IACUC or other pre-approval? ☐ Yes ☒ No Tentative start date: 06/03/2019

6. Is this a continuation/progression from a previous year? ☐ Yes ☒ No
   If Yes:
   a. Attach the previous year's ☐ Abstract **and** ☐ Research Plan/Project Summary
   b. Explain how this project is new and different from previous years on
   ☐ Continuation/Research Progression Form (7)

7. This year's laboratory experiment/data collection:

   06/03/2019 (Actual Start Date: (mm/dd/yy)) 10/15/2019 (End Date: (mm/dd/yy))

8. Where will you conduct your experimentation? (check all that apply)
   ☐ Research Institution ■ School ☐ Field ☐ Home ☐ Other: _____

9. List name and address of all non-home and non-school work site(s):

   Name: _____ _____

   Address: _____ _____

   _____ _____

   Phone/ email _____ _____

10. **Complete a Research Plan/Project Summary following the Research Plan/Project Summary instructions and attach to this form.**
    – See Attached

11. **An abstract is required for all projects after experimentation.**

Research Plan/Project Summary Instructions

A complete Research Plan/Project Summary is required for ALL projects and must accompany Student Checklist (1A).

1. All projects must have a Research Plan/Project Summary
   a. Written prior to experimentation following the instructions below to detail the rationale, research question(s), methodology, and risk assessment of the proposed research.
   b. If changes are made during the research, such changes can be added to the original research plan as an addendum, recognizing that some changes may require returning to the IRB or SRC for appropriate review and approvals. If no additional approvals are required, this addendum serves as a project summary to explain research that was conducted.
   c. If no changes are made from the original research plan, no project summary is required.

2. Some studies, such as an engineering design or mathematics projects, will be less detailed in the initial project plan and will change through the course of research. If such changes occur, a project summary that explains what was done is required and can be appended to the original research plan.

3. The Research Plan/Project Summary should include the following:
   a. **RATIONALE:** Include a brief synopsis of the background that supports your research problem and explain why this research is important and if applicable, explain any societal impact of your research.
   b. **RESEARCH QUESTION(S), HYPOTHESIS(ES), ENGINEERING GOAL(S), EXPECTED OUTCOMES:** How is this based on the rationale described above?
   c. Describe the following in detail:
      - **Procedures:** Detail all procedures and experimental design including methods for data collection. Describe only your project. Do not include work done by mentor or others.
      - **Risk and Safety:** Identify any potential risks and safety precautions needed.
      - **Data Analysis:** Describe the procedures you will use to analyze the data/results.
   d. **BIBLIOGRAPHY:** List major references (e.g. science journal articles, books, internet sites) from your literature review. If you plan to use vertebrate animals, one of these references must be an animal care reference.

Items 1–4 below are subject-specific guidelines for additional items to be included in your research plan/project summary as applicable.

1. **Human participants research:**
   a. **Participants:** Describe age range, gender, racial/ethnic composition of participants. Identify vulnerable populations (minors, pregnant women, prisoners, mentally disabled or economically disadvantaged).
   b. **Recruitment:** Where will you find your participants? How will they be invited to participate?
   c. **Methods:** What will participants be asked to do? Will you use any surveys, questionnaires or tests? If yes and not your own, how did you obtain? Did it require permissions? If so, explain. What is the frequency and length of time involved for each subject?
   d. **Risk Assessment:** What are the risks or potential discomforts (physical, psychological, time involved, social, legal, etc.) to participants? How will you minimize risks? List any benefits to society or participants.
   e. **Protection of Privacy:** Will identifiable information (e.g., names, telephone numbers, birth dates, email addresses) be collected? Will data be confidential/anonymous? If anonymous, describe how the data will be collected. If not anonymous, what procedures are in place for safeguarding confidentiality? Where will data be stored? Who will have access to the data? What will you do with the data after the study?
   f. **Informed Consent Process:** Describe how you will inform participants about the purpose of the study, what they will be asked to do, that their participation is voluntary and they have the right to stop at any time.

2. **Vertebrate animal research:**
   a. Discuss potential ALTERNATIVES to vertebrate animal use and present justification for use of vertebrates.
   b. Explain potential impact or contribution of this research.
   c. Detail all procedures to be used, including methods used to minimize potential discomfort, distress, pain and injury to the animals and detailed chemical concentrations and drug dosages.
   d. Detail animal numbers, species, strain, sex, age, source, etc., include justification of the numbers planned.
   e. Describe housing and oversight of daily care
   f. Discuss disposition of the animals at the termination of the study.

3. **Potentially hazardous biological agents research:**
   a. Give source of the organism and describe BSL assessment process and BSL determination.
   b. Detail safety precautions and discuss methods of disposal.

4. **Hazardous chemicals, activities & devices:**
   - Describe Risk Assessment process, supervision, safety precautions and methods of disposal.
   - Material Safety Data Sheets are not necessary to submit with paperwork.

Investigating the Use of *Ceratopteris richardii* as a Model Plant for the Phytoremediation of Cadmium

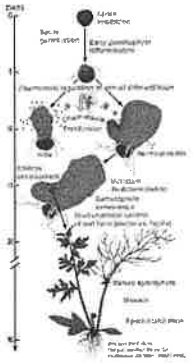
RESEARCH PLAN

A: Rationale

Background:

In many parts of the world, heavy metal contamination of soil and water is an emerging problem (Fritioff and Greger, 2006). Although some metals are required in small concentration in biological system, at high concentration, metals can be harmful by producing reactive species, displacing other metal ions, blocking essential functional groups or changing the conformation of biological molecules (Collin and Stotszky 1989).

Cadmium (Cd) is a widely used environmental pollutant (Li et al 2016). Pollution of the environment with Cd can occurs from natural and anthropogenic source. Cadmium can be found in many household appliances, automobiles and trucks, industrial tools, etc. Cd can pollute aquatic systems from agricultural runoff, industrial effluents, fossil fuel combustion, cement and steel production, and municipal solid waste incineration (Li et al., 2008; Sun et al., 2008). Since tobacco plant accumulates and concentrate Cd in the leaves, humans can be exposed to this metal through smoking. Cadmium is one of the most toxic metal for plants since it is highly soluble in water. Cadmium can cause a range of responses in plants leading to stunted growth and even plant death. Plants growing in polluted environment can bioaccumulate in plants, resulting in bio-concentrate in the food chain.



Life Cycle of *Ceratopteris richardii*

It is very difficult to remediate soil with metal-contaminated soil. Current technologies use soil excavation, landfilling or soil washing with physical or chemical separation of contaminants (Khalid et al, 2016). When large area are contaminated, removal of contaminant is difficult. Several plants have been proposed for clean-up (phytoremediation) of heavy metal from

the soil. Plants have evolved several mechanism that allowed them to cope with Cd stress. These mechanisms involved immobilization, exclusion, chelation, compartmentation of metal ions and the repair of damaged cell structures (di Toppi and Gabbrielli, 1999; Hall 2002). This study will explore if the semi-aquatic fern *Ceratopteris richardii* can use to model plant for the phytoremediation of cadmium.

Ceratopteris richardii is an aquatic fern grown in tropical and subtropical regions in the world (Chatterjee and Roux, 2000). Its biphasic life cycle (alternating between independent two-dimensional (2D) haploid gametophyte and 3D diploid sporophyte) makes for easy characterization of the effect of Cd at the cellular and organismal level without artificial tissue manipulation (Chatterjee and Roux, 2000).

Objectives:

- Determine the dosage of cadmium needed to induce cytotoxicity in the prothallial cells of wild type Ceratopteris
- Compare the tolerance of different mutant strains of Ceratopteris to identify possible strain resistant to cadmium
- Determine if exogenous application of melatonin can improve tolerance to cadmium in Ceratopteris
- Characterize the effect of cadmium stress on the N-acetylserotonin methytransferase (ASMT) gene in Ceratopteris

B: Research Question(s) Research Question(s), Hypothesis(es), Engineering Goal(s), Expected Outcomes:

Research Question:

How does cadmium affect the growth and development of gametophytes of Ceratopteris richardii?

Hypothesis/Engineering Goals:

As the concentration of cadmium increases, its cytotoxicity on prothallial cell will increase. Factors that stimulate the increase of melatonin will increase tolerance of prothallial cells to cadmium.

Description in detail of method or procedures:

A. Physiological characterization of gametophytes of Ceratopteris richardii

1. **Objective**: To determine the effect of cadmium on the gametophytes of *Ceratopteris richardii*

Expected Outcomes:

It is expected that exogenous melatonin will result in increased tolerance to Cadmium. It is also expected that the *pq45/glt1* mutant will be more tolerant to cadmium since the mutant is known to display increased tolerance to reactive oxygen species.

C:

Procedure:

Liquid C-Fern media (Catalogue# 156782) and C-Fern agar (Catalogue# 156782) will be purchased from Carolina Biological and prepared according to manufacturer's instructions. The pH of the media will be adjusted to approximately 6.0 with 1M NaOH. Pre-sterilized dry spores of *Ceratopteris richardii* will be purchased from Carolina Biological (Catalogue #156728).

Growth Conditions:

Prior to plating, spores will be imbibed with sterile distilled water in the dark for 7 days (for synchronization). Spores will then be plated on C-Fern agar and allowed to develop under continuous white light. Germination will be measured by the emergence of rhizoids through the spore-coat. Gametophytes will be allowed to develop for 7 days, then vary dosage of cadmium will be added to gametophytes:

Cadmium Concentrations:

25 μM $CdCl_2$
50 μM $CdCl_2$
100 μM $CdCl_2$

Control (RNW1)	25uM	50 uM	100 uM

All experiments will be performed in triplicates

Control (pq45/gtl1)	25uM	50 uM	100 uM

All experiments will be performed in triplicates

Data Analysis: Effects of Various Concentrations of Cd

Gametophytes will be allowed to develop in continuous white light and growth of the prothalli will be assessed.

Area of the prothalli will be calculated using ImageJ.

Cell division will be assessed by counting the number of cells of the prothalli of the gametophytes using a compound light microscope.

Cell death will be analyzed using Trypan Blue staining (Sigma Aldrich). Gametophytes will be incubated with Trypan Blue for 30 minutes. Gametophytes will then be rinsed thoroughly with distilled water. Under a compound microscope, cell death will be analyzed and recorded

2. **Objective: To determine if pretreatment of spores with melatonin will improve tolerance towards cadmium**
    a. Prepare the following concentrations:
    b. 100 uM of melatonin (Sigma Aldrich)
    c. Set-up the following conditions:

Control	Cd (50uM)	Cd (50 uM) + 100 uM of Melatonin

All experiments will be performed in triplicates

Data Analysis: Effects of Exogenous Melatonin

Gametophytes will be allowed to develop in continuous white light and growth of the prothalli will be assessed.

Area of the prothalli will be calculated using ImageJ.

Cell division will be assessed by counting the number of cells of the prothalli of the gametophytes using a compound light microscope.

Cell death will be analyzed using Trypan Blue staining (Sigma Aldrich). Gametophytes will be incubated with Trypan Blue for 30 minutes. Gametophytes will then be rinsed thoroughly with distilled water. Under a compound microscope, cell death will be analyzed and recorded

Tissue Analysis

To determine the amount of chlorophyll produced, gametophytes and sporophytes will be counted, and chlorophyll will be extracted for 24 hours at 4°C in 3ml of 80% acetone. Tissue will be pelleted at 14,000 g for 5 minutes and supernatants will be measured spectrophotometrically (670 nm).

Screening the Expressed Sequence Tag (EST) Library at the National Center for Biotechnology website and sequenced genomes available at the Phytozome website to identify putative ZIP genes

- Objective: To determine if ASMT gene family is present in *Ceratopteris richardii.*

Procedure for Molecular Characterization:

Run the Basic Local Alignment Search Tool (BLAST) to find similar genes in the Ceratopteris richardii EST library as well as selected genomes of plants available at: www. phytozome.net

Filter out identical genes and select unique sequences for further analysis.

Use the Open Reading Frame (ORF) Finder to translate the cDNA sequence into protein sequence.

Characterization of the ASMT gene sequences in silico

Objective: To determine if the isolated ASMT sequences contain structural motifs

Procedure:

DNA and translated sequences will be analyzed using various bioinformatics tools, including the Basic Local Alignment Search Tool (BLAST) which will be used to find similar genes in EST cDNA libraries (www.ncbi.nlm.nih.gov) or in sequenced genomes (www.phytozome.net). Phylogenetic trees will be constructed using the Molecular Evolutionary Genetics Analysis tool (http://www.megasoftware.net/). Further characterization of the identified genes will be done via the TOMTOM program and Jaspar database, the Plant Cis-acting Regulatory DNA Element (PLACE) database, the PROSITE database and the ExPASy server (http://web.expasy.org/compute_pi/).

Molecular characterization of the ASMT gene isolated in Ceratopteris richardii

Objective: To determine if the ASMT gene is affected by level of cadmium using RT-PCR.

Procedures: This procedure will be assisted by mentor in handling and disposing of hazardous chemicals.

Spores will be grown under two light conditions, low light at 100 μE m-2 s-1 and high light at 400 μE m-2 s-1. RNA will be isolated using the Ambion® RNAqueous®-4PCR Total RNA Isolation Kit and MSDS (Catalog # AM1914). The procedure will be followed as specified by manufacturer. RNA will be reverse transcribed using the Invitrogen SuperScript® III Platinum® One-Step RT-PCR Kit (Catalog #A11732-020). The procedure followed will be that specified by manufacturer. Amplification of the PCR products will be analyzed using Agarose Gel Electrophoresis. DNA will be visualized using 1% agarose gel in 1xTBE solution (89mM Tris Base, 89mM Boric Acid, 2mM EDTA) with SYBR Safe DNA stain (or ethidium bromide which will be handled and disposed of by Mentor) and UV light.

Procedure for RNA Isolation:

1. Start with 0.5-0.75 mg tissue
2. Disrupt samples in Lysis/Binding Solution
3. Add an equal volume of 64% Ethanol and mix
4. Draw the lysate/ethanol mixture through a Filter Cartridge
5. Wash with 700 μl Wash Solution #1
6. Wash with 2×500 μl Wash Solution #2/3
7. Elute RNA with 40-60μl preheated Elution Solution
8. Elute with a second 10-60 μl aliquot of Elution Solution

Procedure for RT-PCR:

RNA will be reverse transcribed using Invitrogen SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase from Thermo Fisher Scientific (Catalog # 12574-030). Procedures will be followed according to manufacturer's instructions. The thermal cycler will be programmed so that cDNA synthesis is automatically followed with PCR amplification.

Measuring Levels of Lipid Peroxidation:

Gametophytes will be treated with cadmium. Lipid peroxidation will be measured using the Sigma Aldrich Lipid Peroxidation (MDA) Assay Kit (Catalog #MAK085). 10 gametophytes will be homogenized in 300 µL of MDA Lysis Buffer containing 3 µL of Butylated hydroxytoluene (BHT). Samples will be centrifuged for 10 minutes at 13,000 g to remove insoluble substances. 200 µL of the supernatant from homogenized samples will be placed into a 1.5 ml Eppendorf tube. 600 µL of the TBA solution will be added into each tube containing the sample to create the MDA-TBA product. The sample will then incubated at 95 °C for 60 minutes and cooled to room temperature in an ice bath for 10 minutes. Mixtures will be placed in cuvettes and analyzed for lipid peroxidation.

ROS levels will be measured by treating gametophytes with 5 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 10 minutes. The gametophytes will be incubated for 1 hour in the dark at 37°C and then rinsed with distilled water. Gametophytes will be observed under a fluorescent microscope (Carl Zeiss-AX10) and images will be taken with the Cy5 filter set. They will be observed at 490-525 nm. DCFH-DA will be converted into DCFH intracellularly. When DCFH is converted into DCF, it fluoresces, indicating the presence of ROS. The integrated density, area of the cell, and the mean fluorescence of the background will be measured using ImageJ. The corrected total cell fluorescence (CTCF) will be calculated using the following formula: CTCF = integrated density - (area of selected cell × mean fluorescence of background readings).

Risk Assessment:

Risk and Safety:

Material Safety Data Sheets from US Research Nanomaterials, Cayman Chemicals, Sigma Aldrich, ThermoFisher Scientific, and Carolina will be used for the chemicals.

Cadmium Chloride: May cause cancer, respiratory inflammation, genitive defects, and may damage fertility of the unborn child due to prolonged or repeated exposure. Gloves, goggles, and lab coats will be worn wile handling for adequate protection. Will be disposed of according to official regulations by the chemical safety officer of G.W. Hewlett High School.

https://www.fishersci.com/store/msds?partNumber=C10100&productDescription=CADMIUM+CHLORIDE+ACS+100GM&vendorId=VN00033897&countryCode=US&language=en

DCFH-DA: Flammable liquid and vapor that is harmful if swallowed. Possible skin irritation and serious eye irritation may occur from direct contact. May cause drowsiness and dizziness. Goggles, gloves, and a lab coat will be worn for adequate protection when handling this compound. Will be disposed according to official regulations by the chemical safety officer of G.W. Hewlett High School.https://www.caymanchem.com/msdss/85155m.pdf

Trypan Blue - may cause cancer with prolonged exposure. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to official regulations by the chemical safety officer of G.W. Hewlett High School.

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=T0887&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Ft0887%3Flang%3Den

2,7-Dichloro-dihydro-fluorescein diacetate - causes skin irritation, eye irritation, and target organ systemic toxicity (single exposure). Material may be irritating to the mucous membranes and upper respiratory tract. It may be harmful by inhalation, ingestion, or skin absorption, and may cause respiratory system irritation. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to official regulations by the chemical safety officer of G.W. Hewlett High School.

https://www.caymanchem.com/msdss/85155m.pdf

Butylated hydroxytoluene (BHT) - a flammable liquid and vapour, is harmful if swallowed, causes skin irritation, causes serious eye irritation, and may cause respiratory irritation.

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=167215&brand=ALDRICH&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2F167215%3Flang%3Den

Acetone- highly flammable liquid and vapor; more than minimal risk may cause serious eye irritation; toxic to aquatic life; potentially harmful if inhaled or absorbed through skin; potentially harmful if swallowed. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to official regulations by the chemical safety officer of G.W. Hewlett High School.
https://www.fishersci.com/shop/msdsproxy?productName=AC177170010&productDescription=ACETONE

Invitrogen RNAqueous®-4PCR Total RNA Isolation Kit- more than minimal risk can potentially harmful if inhaled, absorbed through skin, or when in contact with eyes, causing irritation; potentially harmful if swallowed. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to manufacturer's instruction and according to official regulations by the chemical safety officer of G.W. Hewlett High School.
https://assets.fishersci.com/TFS-Assets/LSG/manuals/cms_059300.pdf

Invitrogen SuperScript® III Platinum® One-Step RT-PCR Kit- more than minimal risk can potentially harmful if inhaled, absorbed through skin, or when in contact with eyes, causing irritation; potentially harmful if swallowed. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to manufacturer's instruction and according to official regulations by the chemical safety officer of G.W. Hewlett High School.
https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/superscript_onestep_qrtpcr_man.pdf&title=Product%20Info%20Sheet:%20SuperScript%20III%20Platinum%20One-Step%20qRT-PCR%20Kit

SYBR Safe® DNA Stain- nonhazardous; nonetheless, gloves, goggles, labs coats will be worn when handling.
https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1290-PJ8342-CO128458-SYBRsafe-Green-Fact-Sheets-Corp-FHR.pdf

1xTBE has no health effects and is not flammable. Gloves, goggles, labs coats will be worn when handling.

https://www.fishersci.com/shop/msdsproxy?productName=BP24304&productDescription=1X+TRIS-BORATE-EDTA

Ethanol - more than minimal risk of eye, skin, gastrointestinal, and respiratory irritation, ignition, reaction with oxidizers, and formation of carbon monoxide and carbon dioxide via decomposition. Will be disposed according to official regulations by the chemical safety officer of G.W. Hewlett High School.
https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=E7023&brand=SIGALD&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigald%2Fe7023%3Flang%3Den

Agarose gel - more than minimal risk of skin, eye, respiratory, and gastric irritation. Gloves, goggles, labs coats will be worn when handling.
https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=P5472&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fp5472%3Flang%3Den

MDA Lysis/Binding Buffer - more than minimal risk of damage to eyes and respiratory tract, neuro- and nephrotoxic effects. Will be handled and disposed by mentor.
https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=MAK085&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fmak085%3Flang%3Den

Wash Solution #1 - more than minimal risk of skin, eye, respiratory, and gastrointestinal irritation, instability caused by excess heat and moisture, and reaction with oxidizing agents. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to manufacturer's instruction.
https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=W0263&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fw0263%3Flang%3Den

Wash Solution #2/3 - more than minimal risk of eye and gastric irritation. Gloves, goggles, labs coats will be worn when handling. Will be disposed according to manufacturer's instruction.
https://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=US&brand=SIGMA&productNumber=W4639&PageToGoToURL=https://www.sigmaaldrich.com/catalog/product/sigma/w4639?lang=en&region=US

Elution Buffer- more than minimal risk of eye and gastric irritation. Gloves, goggles, labs coats will be worn when handling.
https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=C0241&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fc0241%3Flang%3Den

Overall Data Analysis:

A one-way ANOVA test will be conducted using the PRISM Graphpad software whenever applicable in order to confirm the statistical significance of the data once it is obtained.

D. Bibliography:

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Liu, Y., Liu, K., Li, Y., Yang, W., Wu, F., Zhu, P., Zhang, J., Chen, L., Gao, S., Zhang, L., (2016). "Cadmium contamination of soil and crops is affected by intercropping and rotation systems in the lower reaches of the Minjiang River in south-western China." Environmental Geochemistry and Health, 38(3), pp. 811-820

Addendum:

No changes were made to the research plan.