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**TITLE:**

# High-throughput siderophore screening from environmental samples: plant tissues, bulk soils, and rhizosphere soils

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**KEYWORDS:**

Chrome azurol S (CAS); soil microbiology; metals biogeochemistry; microplate assay; ligands; iron limitation; wheat genotype

**SUMMARY:**

We present a protocol for rapid screening of environmental samples for siderophore potential contributing to micronutrient bioavailability and turnover in terrestrial systems.

**ABSTRACT:**

Siderophores (low-molecular weight metal chelating compounds) are important in various ecological phenomenon ranging from iron (Fe) biogeochemical cycling in soils, to pathogen competition, plant growth promotion, and cross-kingdom signaling. Furthermore, siderophores are also of commercial interest in bioleaching and bioweathering of metal-bearing minerals and ores. A rapid, cost effective, and robust means of quantitatively assessing siderophore production in complex samples is key to identifying important aspects of the ecological ramifications of siderophore activity, including, novel siderophore producing microbes. The method presented here was developed to assess siderophore activity of in-tact microbiome communities, in environmental samples, such as soil or plant tissues. The samples were homogenized and diluted in a modified M9 medium (without Fe), and enrichment cultures were incubated for 3 days. Siderophore production was assessed in samples at 24, 48, and 72 hours (h) using a novel 96-well microplate CAS (Chrome azurol sulphonate)-Fe agar assay, an adaptation of the traditionally tedious and time-consuming colorimetric method of assessing siderophore activity, performed on individual cultivated microbial isolates. We applied our method to 4 different genotypes/Lines of wheat (*Triticum aestivum* L.), including Lewjain, Madsen, and PI561725, and PI561727 commonly grown in the inland Pacific Northwest.

Siderophore production was clearly impacted by the genotype of wheat, and in the specific types of plant tissues observed. We successfully used our method to rapidly screen for the influence of plant genotype on siderophore production, a key function in terrestrial and aquatic ecosystems. We produced many technical replicates, yielding very reliable statistical differences in soils and within plant tissues. Importantly, the results show the proposed method can be used to rapidly examine siderophore production in complex samples with a high degree of reliability, in a manner that allows communities to be preserved for later work to identify taxa and functional genes.

**INTRODUCTION:**

Siderophores are important biomolecules involved primarily in iron-chelation for bioavailability, but with a wide array of additional purposes in terrestrial and aquatic ecosystems ranging from microbial quorum sensing, signaling to microbial plant-hosts, plant growth promotion, cooperation and competition within complex microbial communities[1,2]. Siderophores can be broadly classified according to their active sites and structural features, creating four basic types: carboxylate, hydroxamate, catecholate, and mixed types[3,4]. Many microorganisms are capable of excreting more than one type of siderophore[5] and in complex communities, a vast majority of organisms biosynthesize the membrane receptors to allow the uptake of an even wider variety of siderophores[1,6]. Recent work indicates that siderophores are particularly important at the community level, and even in inter-kingdom communications and biogeochemical transfers[7-11].

Chrome azurol sulphonate (CAS) has been used for over 30 years as a chelating agent to bind iron (Fe) in such a way that addition of ligands (i.e., siderophores) can result in dissociation of the CAS-Fe complex, creating an easily identifiable color change in the medium[12]. When the CAS is bound with Fe, the dye appears as a royal blue color, and as the CAS-Fe complex dissociates, the medium changes color according to the type of ligand used to scavenge the Fe[13]. The initial, liquid-based medium established by Schwyn and Neilands in 1987, has been modified in many ways to accommodate changing microbial targets[14], growth habits and limitations[15], as well as a variety of metals besides Fe, including aluminum, manganese, cobalt, cadmium nickel, lithium, zinc[16], copper[17], and even arsenic[18].

Many human pathogens, as well as plant growth promoting microorganisms (PGPM) have been identified as siderophore-producing organisms[3,19,20], and important rhizosphere and endophytic PGPM often test positive for siderophore-production[4]. The traditional Fe-based liquid method has been adapted to microtiter testing of isolates in cultivation for siderophore production[21]. However, these techniques fail to recognize the importance of the microbial community as a whole (the microbiome), in cooperation and potential regulation of siderophore production in soils and plant systems[22]. For that reason, we have developed a high-throughput community-level assessment of siderophore production from a given environment, based on the traditional CAS assay, but with replication, ease of measurement, reliability, and repeatability in a microplate assay.

In this study, a cost-effective, high-throughput CAS-Fe assay for detecting siderophore production is presented to assess the enrichment of siderophore production from complex samples (i.e., soil and plant tissue homogenates). Bulk, loosely-bound, and tightly-bound rhizosphere soil (in terms of how the soil was bound to the root) were obtained along with grain, shoot, and root tissues from four distinct wheat (*Triticum aestivum* L.) genotypes: Lewjain, Madsen, PI561725, and PI561727. It was hypothesized that fundamental differences in the wheat genotypes could result in differences in recruitment and selection of siderophore producing communities. Of particular interest is the difference between microbial communities associated with the PI561725 isogenic line, which is aluminum tolerant because it possesses *ALMT1* (Aluminum-activated Malate Transporter 1), compared with the aluminum sensitive PI561727 isogenic line, which possesses a non-aluminum responsive form of the gene, *almt1*[23-26]. The chief objective of the study was to develop a straightforward, rapid method of quantitatively assessing siderophore production in siderophore enrichment cultures of complex sample types while preserving the cultures for future work.

**PROTOCOL:**

Note: Location of Field Site: Washington State University, Plant Pathology Farm (46°46'38.0"N 117°04'57.4"W). Seeds were sown using a mechanical planter on October 19, 2017. Each wheat genotype was planted in headrows, approximately 1 meter apart to avoid overlapping of root system. Plant and soil samples were collected on August 9, 2018, when plants were ready for harvest. Samples were gathered from three replicates of four wheat genotypes: PI561727, PI561725, Madsen, Lewjain.

**1. Preparation of modified M9 medium**

1.1. Use $Na_2PO_4 \cdot 7H_2O$ (12.8 g/200 mL), $KH_2PO_4$ (0.3 g/200 mL), NaCl (0.5 g/200 mL), and $NH_4Cl$ (1 g/20 mL) reagents to prepare the M9 salt solution.

1.2. Use 18 g of $MgSO_4 \cdot 7H_2O$ in 100 mL of double deionized water ($ddH_2O$) to prepare 0.75 M $MgSO_4 \cdot 7H_2O$.

1.3. Make 1 M of $CaCl_2 \cdot 2H_2O$ by adding 14.7 g of $CaCl_2 \cdot 2H_2O$ with 100 mL of $ddH_2O$.

1.4. Prepare the buffer solution by dissolving 6.048 g of PIPES into 156 mL of $ddH_2O$ with stirring. Adjust the pH to 6.8 with 5 M NaOH.

1.5. Prepare 20% glucose (dextrose monohydrate) by dissolving 20 g of glucose into 100 mL of $ddH_2O$.

1.6. Prepare solutions and individually autoclave all but the glucose. Filter sterilize the glucose solution for addition to the M9 media after autoclaving (0.22 μm).

1.7. Prepare the modified M9 medium by mixing 156 mL of PIPES Buffer solution, 40 mL of M9 salts solution, 20 µL of $CaCl_2 \cdot 2H_2O$ solution, 266 µL of $MgSO_4 \cdot 7H_2O$, and 4 mL of 20% glucose solutions together in the biosafety cabinet, using sterile technique.

1.8. For preservation of the modified M9, seal the container, cover with aluminum foil to protect from UV, and place at 4 °C.

**2. Preparation of CAS-Fe-Agar medium**

2.1. Acid wash all glassware in 100 mM HCl/100 mM $HNO_3$ for a minimum of 2 h prior to utilization in the CAS assay.

2.2. Prepare an aluminum baking pan filled with laboratory grade sand and cover it with aluminum foil. Autoclave at 121 °C for 30 min and set aside.

2.3. Prepare HDTMA (hexadecyltrimethylammonium bromide) by adding 0.0365 g to 20 mL $ddH_2O$ and place the solution at 37 °C to promote solubilization.

2.4. Prepare 10 mM HCl and generate 1 mM $FeCl_3 \cdot 6H_2O$ using 10 mM HCl as the solvent. Add 0.0302 g of CAS to 25 mL of $ddH_2O$ while gently stirring with a sterile magnetic stir bar. Then add 5 mL of 1 mM $FeCl_3 \cdot 6H_2O$ (in 10 mM HCl) to the 25 mL of CAS solution while continuing to gently stir (the solution turns into dark reddish black color).

2.5. Slowly add the 20 mL of HDTMA solution while gently stirring, into the Fe-CAS solution (this yields a dark blue solution).

2.6. Prepare the buffer solution by dissolving 15.12 g of PIPES into 375 mL of $ddH_2O$, with gentle stirring. Adjust the pH to 6.8 with 5 M NaOH. Add water to bring the volume to 450 mL. Add 5 g of agarose to the solution. Autoclave the PIPES buffer solution and the CAS-Fe solution at 121 °C for 30 min.

2.7. Carefully add the entirety of the CAS-Fe solution to the entirety of the PIPES Buffer in the biosafety cabinet after each of them is autoclaved.

2.8. Place the mixed solution in a water bath at 50 °C.

NOTE: Freshly prepare all the reagents in the CAS-Fe-Agar medium before each assay, as long-term storage at 50 °C results in precipitation of the CAS-Fe complex, and cooling results in solidified medium.

2.9. Place a sterile reagent boat in the sterile sand in the biosafety cabinet and heat to 50 °C. Transfer the CAS-Fe-Agar to the boat, then quickly aliquot 100 µL to each well in a clear, flat-bottom, sterile 96-well microplate.

**3. Pyoverdine/EDTA standard preparation**

3.1. Pyoverdine preparation

3.1.1. Prepare 800 µM pyoverdine standard (mixture of succinic acid, 2-hydroxy glutaramide, and succinaminde forms of pyoverdine – see **Table of Materials**), in previously prepared modified M9 medium.

3.1.2. Dilute this solution into 400, 200, 100, 50, 25, 12.5, and 6.25 µM solutions.

3.2. EDTA standard preparation

3.2.1. Add 0.594 g of disodium ethylenediaminetetraacetic acid (EDTA: $C_{10}H_{14}N_2Na_2O_8.2H_2O$), to 500 mL of previously prepared modified M9 medium to prepare 3200 µM EDTA standard.

3.2.2. Dilute this solution into 1600, 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM solutions.

3.3. Standard curve generation

3.3.1. Add 100 µL of each concentration of pyoverdine and EDTA to separate wells of a 96-well microplate containing 100 µL of CAS-Fe Agar medium. Make duplicate technical replications of each concentration. Also, add blank wells with only M9 (no EDTA or pyoverdine).

3.3.2. Using a microplate reader, measure absorbance (at 420 nm and 665 nm) after 1, 6, and 24 h incubation at 22 °C, and use absorbance measurements to generate standard curves.

NOTE: For 420 nm measurements, subtract absorbance of blanks from the absorbance of pyoverdine or EDTA containing wells. For 665 nm, subtract absorbance of pyoverdine or EDTA containing wells from absorbance of blanks. Then, $\log_{10}$(µM pyoverdine or EDTA) is regressed against the absorbance measurements. For ease of interpreting sample results, use absorbance as the x-axis and $\log_{10}$(µM pyoverdine or EDTA) as the y-axis.

**4. Collection of environmental samples: soil and plant tissues**

4.1. Wash sampling equipment (shovels and scissors) with 0.22 µm filtered dd$H_2O$ followed by 70% ethanol, and wipe with paper towels before sampling and in between samples to maintain sterile technique and reduce cross-contamination.

4.2. Excise the plant tissues (grains and shoots) from plants in the field, and place them in a labeled plastic storage bag, leaving enough shoot stubble for ease in uprooting the desired soil and root samples.

4.3. Excavate a small root ball approximately 15 cm deep and 23 cm wide and place it in a separate, labeled plastic bag for sample preparation in the laboratory environment. This step is similar to the methods of McPherson et al.[27].

4.4. Place all samples (grains, shoots, bulk soil, and root balls in separate bags) directly on ice and keep at 4 °C until samples are processed for the siderophore production assay.

4.5. Separate root associated soil samples into bulk, loosely-bound rhizosphere soil, and tightly-bound rhizosphere soil.

4.5.1. Take the root balls out of the bags. Gently shake off soil from the root ball. Shaken off soil, along with the soil left in the bag comprises the “bulk” soil.

4.5.2. Use a rubber mallet to further remove soil from the root ball. This is the loosely-bound rhizosphere soil.

4.5.3. Generate tightly-bound rhizosphere soil by taking roots with the tightly-bound sample and putting them in a centrifuge tube. Add 30 mL of dd$H_2O$ and vortex it for 2-3 minutes. Remove roots to get the tightly-bound rhizosphere soil slurry dilution.

**5. Preparation of siderophore enrichment cultures and CAS-Fe siderophore production assay**

NOTE: All glassware should be acid washed prior to beginning the assays.

5.1. Soil sample preparation (for each of the three soil sample types)

5.1.1. Homogenize each soil sample within the sample bag, by mixing and turning the soil as much as possible without opening the bag.

NOTE: This helps reduce natural soil spatial variability and aligns with normal soil sampling procedures. Other methods may be utilized to homogenize environmental samples, as appropriate, and depending on the experimental design.

5.1.2. After each sample has been thoroughly mixed, aliquot and suspend 2.0 g of each soil in 20 mL of modified M9 medium in a sterile 50 mL centrifuge tube with a sterile foam plug to allow aeration.

5.1.3. For tightly-bound rhizosphere samples, add 2 mL of the rhizosphere soil slurry to 20 mL of modified M9 medium in a sterile 50 mL centrifuge tube with a sterile foam plug to allow aeration.

5.2. Tissue sample (root, shoot and grain) preparation

5.2.1. Surface sterilize the sample with 70% ethanol. Macerate 2.0 g of fresh tissue in 20 mL of modified M9 medium using a blender on high for 30 seconds. Transfer the sample to a sterile 50 mL centrifuge tube with a sterile foam plug to allow aeration.

5.3. Enrichment of Siderophore Production Through Fe Limitation

5.3.1. Incubate 50 mL centrifuge tubes at room temperature and shake at 160 rpm.

**6. CAS-Fe agar assays for detection of siderophore production in environmental samples**

6.1. At 24, 48, and 72 h after initiating the enrichment culture, remove 1 mL subsamples from the enrichment tubes using sterile technique and centrifuge at 10,000 x g for 1 min in 2 mL centrifuge tubes to pelletize the cells.

6.2. Collect the separated supernatant. Using sterile technique, add 100 µL of the supernatant to 100 µL solution of CAS-Fe-Agar in duplicate or triplicate in the microplate. Also add 100 µL of sterile M9 medium (as blanks). Then incubate the plate at 28 °C.

6.3. Suspend the remaining supernatant and pellet for each sample (not added to microtiter plate) into its own, sterile 2 mL centrifuge tube. Add 400 µL of sterile glycerol into each sample-supernatant tube and resuspend the pellet to create glycerol stocks. Freeze the stock at -80 °C for later analyses.

NOTE: This step can be modified to generate glycerol stocks according to any preferred in-house protocol.

6.4. Measure absorbance at 6, 24, 48, and 72 h, at 420 nm wavelength.

6.5. Use standard curves generated from pyoverdine or EDTA to interpret sample absorbance measurements in terms of pyoverdine equivalents.

NOTE: Pyoverdine was determined to be a superior standard compared with EDTA (vide infra), so EDTA was not used to interpret results in the current study.

**REPRESENTATIVE RESULTS:**

A pyoverdine mixture biosynthesized by *Pseudomonas fluorescens* was used as a standard to interpret and quantify absorbance (at 420 nm) of samples in terms of pyoverdine equivalents in µM. **Figure 1** shows the relationship between absorbance (420 nm) and starting concentration of pyoverdine ($Log_{10}$ molarity in µM). EDTA did not provide an adequate standard because samples exhibited greater absorbance measurements than were attainable with pyoverdine, and the $R^2$ was lower (**Figure 2**). While initial work using the CAS-Fe assay as a method of siderophore detection measured absorbance at 630 nm, in a related study using a very similar method (CAS-Fe-Agar was mixed 1:1 with modified M9 to generate a 200 µL column in the

microplate), it was observed that the peak absorbance was at 665 nm, but that 420 nm was more reproducible in terms of changes in absorbance induced by samples (**Figure 3**).

Siderophore production was observed in enrichment cultures of all tissue types after 72 h of Fe-deficit enrichment and siderophore activity appeared to stabilize after 48 h of incubation (**Supplementary Figure 1**). Thus, siderophore activity of the 72 h enrichment was assessed at 48 h incubation to determine the influence of genotype and sample type on siderophore isolation (**Figure 4**). Siderophore activity in bulk soil samples was relatively low and did not exhibit differences between the wheat genotype from which the bulk soil was sampled (**Figure 4A**). Enrichments of loosely bound soil isolated from the PI561725 genotype exhibited greater siderophore production compared with loosely bound soil from Madsen and PI561727, but not Lewjain (**Figure 4B**). Siderophore production in enrichments from tightly bound soil was not heavily influenced by genotype (**Figure 4C**).

Enrichment cultures of grain tissue yielded relatively low siderophore production regardless of genotype (**Figure 4D**). Enrichments of Lewjain shoot tissue had significantly lower siderophore production than the other genotypes, and PI561725 shoot tissue cultures resulted in more variable siderophore production (**Figure 4E**). Siderophore activity was more than 200% greater in in root tissue enrichment cultures of PI561725 compared with all other genotypes (**Figure 4F**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1**. **Absorbance at 420 nm and 655 nm regressed against the log10 concentration of pyoverdine.** (**A**) Absorbance at 420 nm regressed against the $\log_{10}$ concentration of pyoverdine in µM. A polynomial curve was fit to obtain an explanatory equation for interpreting absorbance in terms of pyoverdine equivalents. (**B**) Absorbance at 665 nm regressed against the $\text{Log}_{10}$ concentration of pyoverdine in µM. $R^2$ is the square of the Pearson correlation coefficient, and the equation explains the fitted curve. Points are duplicates of absorbance measurements at 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM pyoverdine after 6 h incubation at 28 °C.

**Figure 2. Absorbance at 420 nm and 655 nm regressed against the log10 concentration of EDTA.** (**A**) Absorbance at 420 nm regressed against the $\log_{10}$ concentration of EDTA in µM. A polynomial curve was fit to obtain an explanatory equation for interpreting absorbance in terms of pyoverdine equivalents. (**B**) Absorbance at 665 nm regressed against the $\text{Log}_{10}$ concentration of EDTA in µM. $R^2$ is the square of the Pearson correlation coefficient, and the equation explains the fitted curve. Points are duplicates of absorbance measurements at 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM EDTA after 6 h incubation at 28 °C, and error bars .

**Figure 3**. **Absorbance scans from 315−1000 nm of microplate wells containing 200 µL columns of 1:1 CAS-Fe-Agar and modified M9 or M9 medium with siderophore producing samples.** The plate was incubated at 28 °C for 72 hours before measuring absorbance in a microplate reader.

Absorbance scans show the three blanks containing no sample (black lines) yielded tightly clustered curves with a peak at 665 nm. Absorbance scans show the three blanks containing siderophore producing samples (gray lines) yielded curves with more variability, but with more consistent absorbance at 420 nm compared with 665 nm.

**Figure 4. Pyoverdine equivalents of siderophore enrichment cultures.** Pyoverdine equivalents of siderophore enrichment cultures associated with (**A**) bulk (**B**) loosely bound, and (**C**) tightly bound soil, and in tissue homogenates of wheat (**D**) grain (**E**) shoots, and (**F**) roots. Siderophore enrichment cultures were incubated for 72 h before transferring subsamples to a microplate and incubating at 28 °C. Siderophore production was assessed after 48 h of incubation with Chrome azurol S. Genotypes/Lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Asterisks represent significance at *alpha* = 0.008 (after Bonferroni correction). Bars are standard deviation.

**Supplementary Figure 1**. **Pyoverdine equivalents over time**. Pyoverdine equivalents of siderophore enrichment cultures assessed after 24, 48, and 72 h of incubation with Chrome azurol S. Sderophore enrichment cultures associated with (**A**) bulk (**B**) loosely bound, and (**C**) tightly bound soil, and in tissue homogenates of wheat (**D**) grain (**E**) shoots, and (**F**) roots. Siderophore enrichment cultures were incubated for 72 h before transferring subsamples to a microplate and incubating at 28 °C. and subsampled to assess siderophore production at each timepoint. Genotypes/Lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after, 24, 48, and 72 h of incubation. Bars are standard deviation.

**DISCUSSION:**

The primary result of this work is the production of a new methodology that can be used to rapidly enrich for siderophore producing microbes while quantitatively measuring siderophore production/activity in the environmental sample. The methodology is quick, simple, and cost-effective, and the results show how it can be used to detect siderophore activity from complex and novel sample types (e.g., soil and plant tissue). The protocol also results in the production of glycerol stocks of the enrichment cultures, which can easily be taken through time to accommodate studies of shifts in microbial community structure and function during Fe deficiency through DNA or RNA based techniques. Those interested in examining the kinetics of siderophore activity in ecological studies could also likely benefit from this method. The results also show that pyoverdine equivalents (pyoverdines are important siderophores in terms of the environment[28] and medicine[29]) provide a good method of quantitatively assessing siderophore production. An important finding is that absorbance measurements at 665 nm are inadequate for determining siderophore activity compared with those observed at 420 nm (**Figure 1**). Of particular importance was the finding that absorbance at 665 nm clustered across a broad range of pyoverdine concentrations (Pyoverdine µM = 50-800 µM, $\log_{10}$(µM pyoverdine) = 0.18-0.76), suggesting a detection ceiling at this wavelength (**Figure 1B**). It should be noted that while pyoverdine was a superior standard compared with EDTA, it is also costly, so it is suggested that preliminary work is performed with EDTA or other cost-effective chelators to ensure the methodology has been mastered before generating pyoverdine standards.

There are several critical steps throughout the protocol that require close attention. Firstly, it is important to maintain metal-free glassware and other wares wherever possible when working with metals, particularly those necessary in low concentrations, like Fe. Secondly, because cultures were enriched for siderophore production through Fe limitation, it is important to maintain aseptic conditions throughout the workflow to reduce the influence of environmental contaminants. Lastly, preparation of the CAS-Fe-agar requires careful attention to detail and should be prepared as closely to described as possible. For instance, if the CAS-Fe-agar solution is kept warm but is not used quickly, the CAS-Fe will precipitate. Additionally, it is essential to keep the CAS-Fe-Agar warm during transfer to the microplate. This was achieved by using heated, sterile sand and quickly transferring the medium to the microplate in a biosafety cabinet.

One limitation of the methodology is that because some plants also produce siderophores (phytosiderophores); these can contribute to measured siderophore activity in enrichment cultures of plant tissue homogenates. Also, there was relatively high variability in the results from field replicates, suggesting more replication could be beneficial in future studies. Another limitation of the technique is that while the microplate method is high-throughput, the sample gathering and preparation are time-consuming. Still, because a single microplate can be used for 96 samples (including standards), the time and cost inputs are much lower compared with existing techniques. This is primarily because other existing methods rely on performing the CAS-Fe assay in Petri dishes[30], which are inherently less time and cost efficient to prepare than a microplate. Additionally, because solubilized CAS-Fe complexes are prone to precipitation[12], the proposed method using CAS-Fe-Agar medium is superior to liquid-based methods, which have also be adapted to the 96-well format.

In terms of the reported findings, given that the primary difference between the PI561725 and PI561727 is the presence of *ALMT1* vs*. almt1*, respectively, the results suggest the presence of *ALMT1* likely results in the selection of microbial communities in both the plant and the soil which have a greater potential for siderophore production, as assessed *via* enrichment cultures. Future work should further investigate the phenomenon using a larger number of replicates, particularly to clarify if the presence of *ALMT1* specifically selects for enhanced siderophore activity.

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**DISCLOSURES:**
The authors have no conflicts of interest to disclose.

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Figure 1

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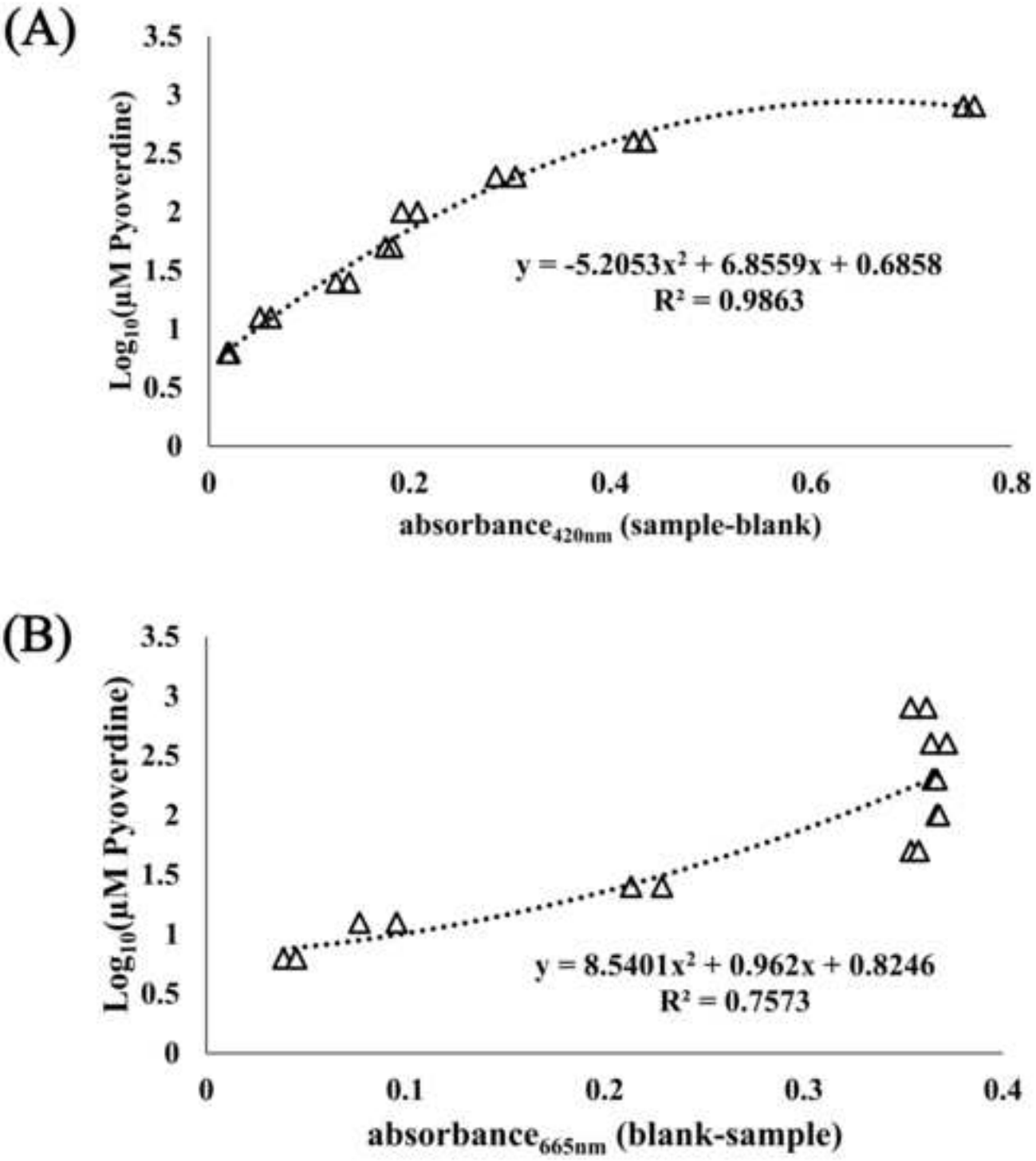


Figure 2



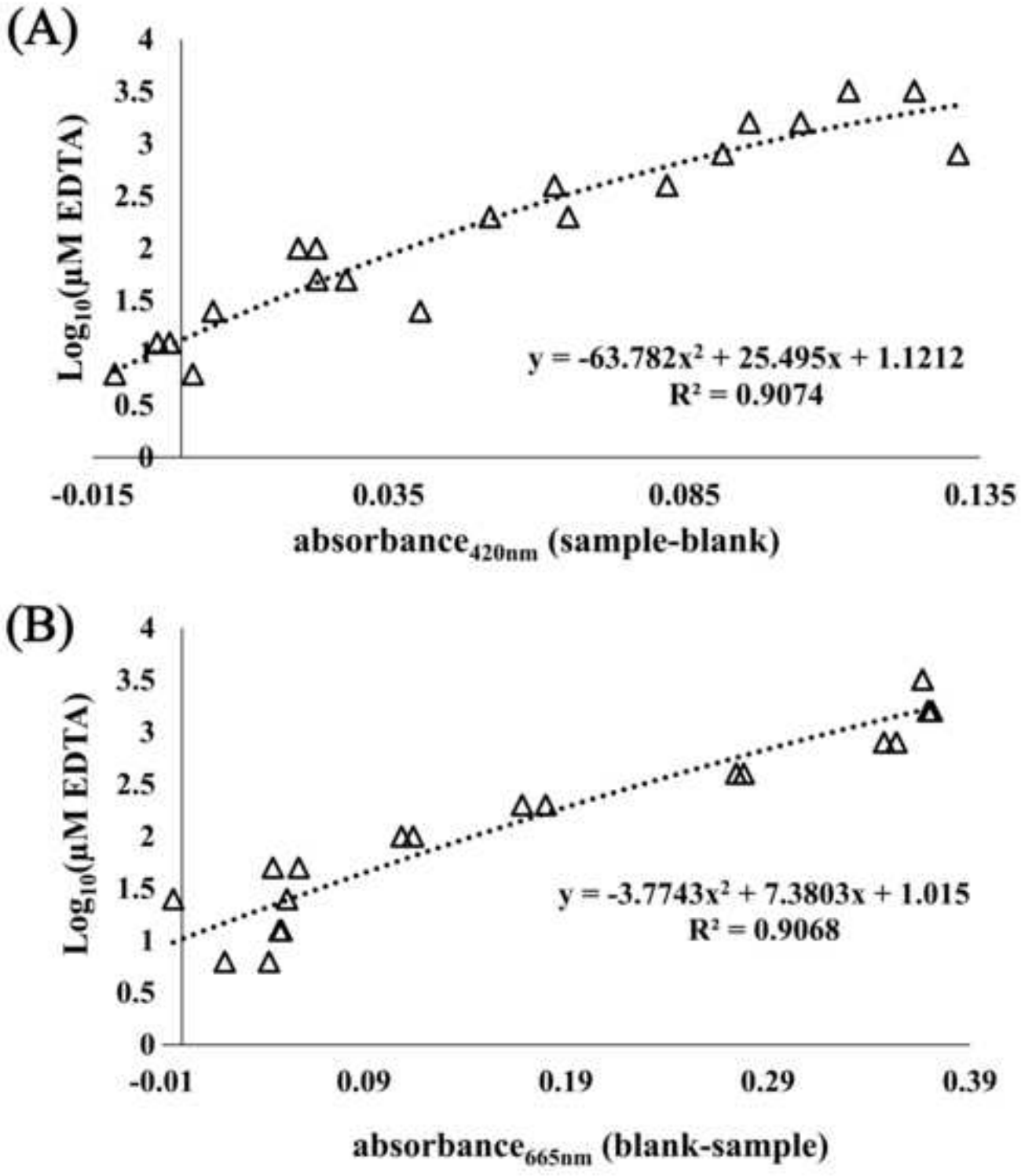


Figure 3



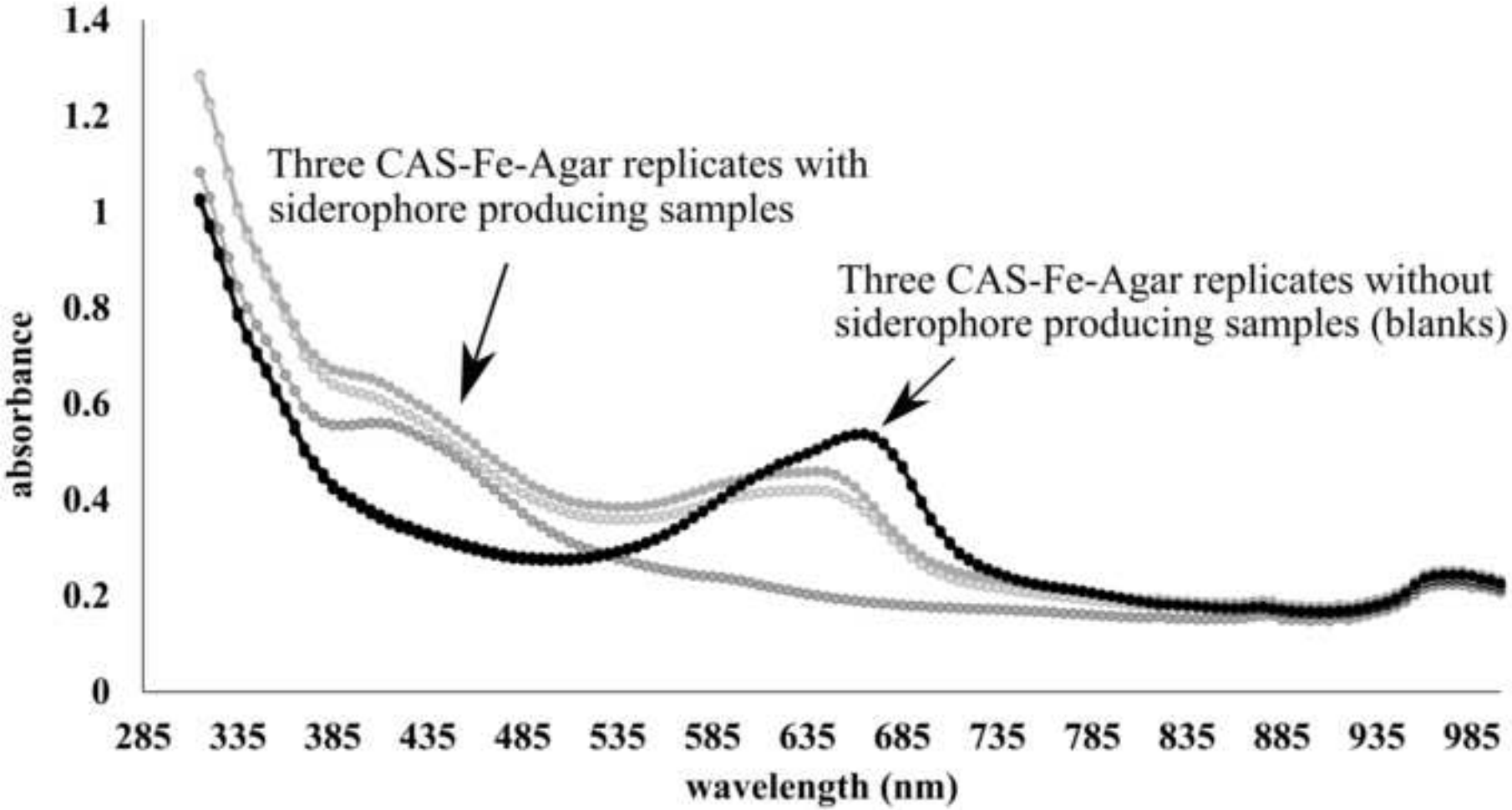
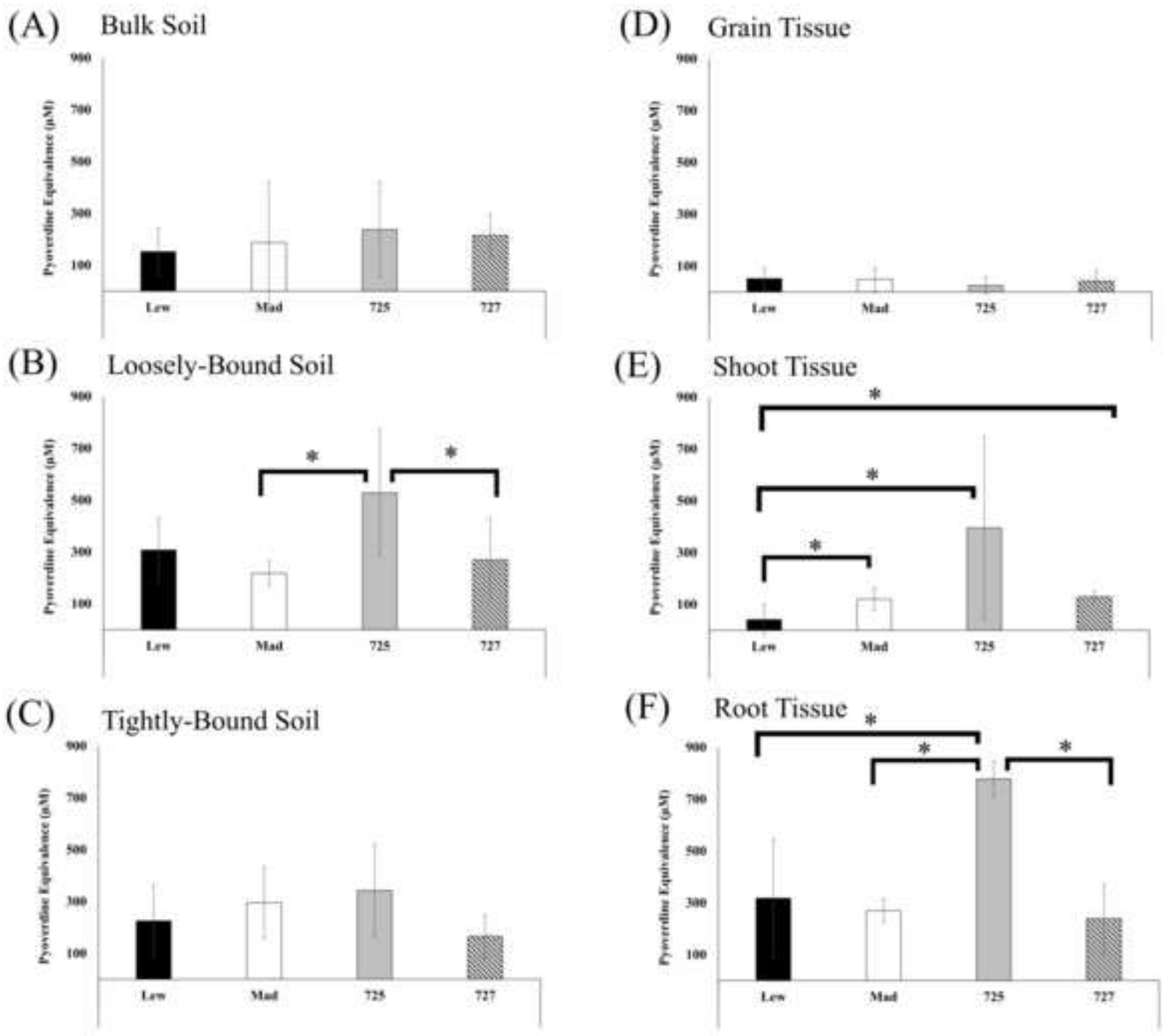


Figure 4





Supplemental Figure 1

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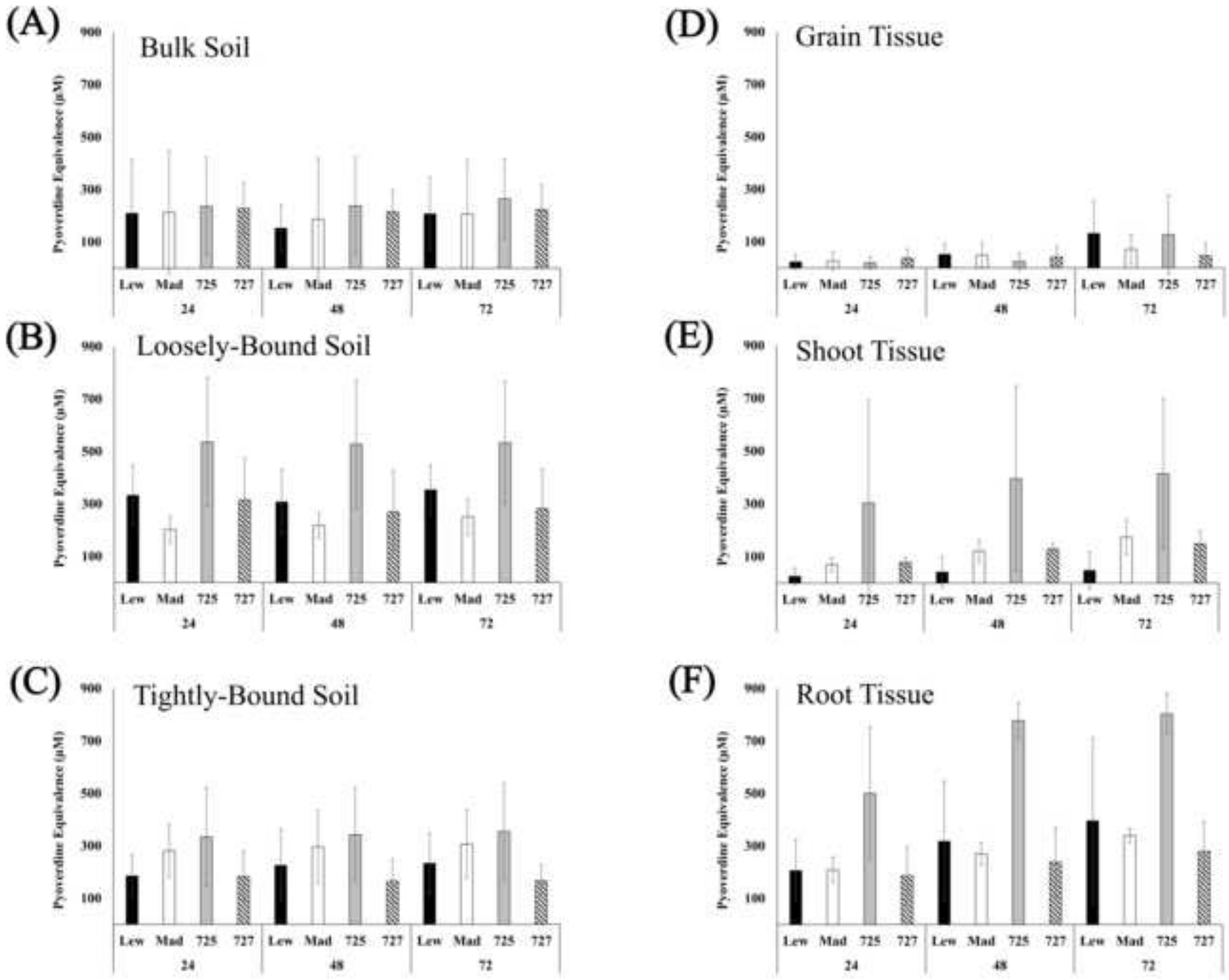

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| Name of Material/ Equipment | Company | LOT / Batch Number |
|---|---|---|
| Agarose | Apex | LF451320014 |
| Aluminum Baking Pan | | |
| Aluminum Foil | | |
| Ammonium chloride, granular | Fiesher Scientific | 152315A |
| Autoclave and Sterilizer | Thermo Scientific | |
| Calcium chloride dihydrate | Fiesher Scientific | 171428 |
| CAS (Chrome Azurol S) | Chem-Impex Int'l Inc) | 000331-27168 |
| Dextrose Monohydrate (glucose), crystalline | Fiesher Scientific | 1521754 |
| EDTA, disodium salt, dihydrate, Crystal | J.T.Baker | JI2476 |
| Glycerol, Anhydrous | Baker Analyzed | C22634 |
| HDTMA (Cetyltrimethylammomonium Bromide | Reagent World | FZ0941 |
| Hydrochloride acid | ACROS Organic | B0756767 |
| Infinite M200 PRO plate reader | TECAN | |
| Iron (III) chloride hexahydrate, 99% | ACROS Organic | A0342179 |
| Laboratory Fume Hood | Thermo Scientific | |
| Laboratory Incubator | VWR Scientific | |
| Magnesium Sulfate | Fiesher Scientific | 27855 |
| Niric Acid, (69-70)% | J.T.Baker | 72287 |
| PIPES buffer, 98.5% | ACROS Organic | A0338723 |
| Potassium phosphate, dibaisc,powder | J.T.Baker | J48594 |
| Pyoverdine | SIGMA-ALDRICH | 078M4094V |
| Sand | | |
| SI-600R Shaker | Lab Companion | |
| Sodium chloride, granular | Fiesher Scientific | 136539 |
| Sodium hydroxide, pellets | J.T.Baker | G48K53 |
| Sodium phosphate, dibasic heptahydrate, 99% | ACROS Organic | A0371705 |

**Comments/**
**Description**

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| | |
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| Title of Article: | High-throughput siderophore screening for communities of microorganisms from plant tissues, bulk soils, and rhizosphere soils. |
| Author(s): | Ricky W. Lewis, Anjuman Islam, Christine J. Dilla-Ermita, Scot H. Hulbert, and Tarah S. Sullivan |

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| Department: | Crop and Soil Sciences |
| Institution: | Washington State University |
| Title: | Assistant Professor |
| Signature: |  |
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**22 October 2018**

**Authors' Response to Review and Revisions**

The authors sincerely appreciate the considerable time and efforts taken for a thorough review and constructive input by the reviewers and editor. We have incorporated all the input and provided line-by-line responses to each concern below. Below you will find each comment individually addressed in the order each was received, with the reviewer comment in plain text, and the response in bold, blue font, directly below or beside the comment. Thank you for this opportunity to revise and resubmit for publication with *The Journal of Visualized Experiments*.

---

**Editorial Comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**The authors have done so and feel the manuscript is much improved.**

2. Keywords: Please provide at least 6 keywords or phrases.

**The authors have added keywords.**

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Milli-Q, Waring, Lab companion, etc.

**The authors have removed all instances of commercial language and replaced with generic terms with reference to the Table of Materials.**

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**The authors have revised to ensure conformation to the JoVE format and apologize for the previous mistakes.**

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

**The authors have revised the instructions to be in the imperative tense, and moved those relevant statements to the discussion section.**

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

**The authors have added more detail throughout, as well as the appropriate references.**

7. Line 120: Please specify the samples (grains, shoots, roots, or soil samples).

**The authors have reorganized and revised the manuscript so that Lines 220 through 297 all specify the different sample types and how each should be handled.**

8. Lines 122-123: Please describe the criteria for separating the soil samples.

**The authors have reorganized and revised the manuscript so that Lines 220 through 297 all specify the different sample types and how each should be handled.**

9. Line 130: How is the soil sample homogenized? Are loosely-bound and tightly-bound rhizosphere soil both homogenized?

**Lines 241 through 263 now give a better description of the process of handling these types of samples. Lines 257-262 now also describes how to homogenize a soil sample and that each type of environmental sample can be handled slightly differently, but that is beyond the scope of this particular article.**

10. Line 133: Do tissue samples refer to grains and shoots?

**The authors have reorganized and revised the manuscript so that Lines 268 through 272 now give more specifics on the types of tissues.**

11. Line 153: Do you mean measuring absorbance of pyoverdine standards at different time periods?

**The authors have reorganized and revised the manuscript so that the preparation of standards and curves and presented in Lines 188 through 218, and have clarified the time points for standards measurements.**

12. Lines 174-175: How about the rest of the supernatant? Is it discarded or left with the pellet?

**The authors have rewritten this step with better description of the process in Lines 290-293.**

13. Line 230: Please describe the specific actions being performed here.

**The authors have rewritten the manuscript in such a way that the actions previously described as "Lewis et al 2018" are described throughout the protocol, primarily Lines 278 - 300.**

14. Please include single-line spaces between all paragraphs, headings, steps, etc.

**Done**

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that shorter steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

**Done**

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Done.**

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are

given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Done**

18. Figure 2: Please define error bars in the figure legend.

**The figure has been revised so that there are no longer error bars in Figure 2, but all figure legends have been elaborated on.**

19. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
20. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

**The authors have revised and rewritten the discussion according to the guidelines provided and the new Discussion (with all the above items addressed) may be found in Lines 373 though 426.**

---

**Reviewer #1:**

1. The manuscript titled is not suitable with content because there are many reports which revealed wheat plant can able to sequestrates siderophore by itself. On this experiment they assess siderophore from tissue of the wheat plant. My opinion on that this siderophore may not be produce from microorganism and need this further confirmation.

**The authors gratefully acknowledge the reviewer's extent of knowledge in the area and have accordingly altered the manuscript title to address this concern and broaden the scope of the assay. The new title now reads, "High-throughput siderophore screening from environmental samples: plant tissues, bulk soils, and rhizosphere soils."**

2. The word use in the title should not be repeated in the key words.
**The authors have revised the list of keyword to a more appropriate set. (Lines 25-26)**

3. My recommendation the author need to isolate the microorganisms from the plant tissue separately and then to test their efficacy alone and along with plant tissues to underpin the actual consequence.

**The authors acknowledge this as a very useful follow-up study to the protocol as given. However, the objective of the current manuscript is to simply describe the protocol for detecting siderophores in environmental samples in a high-throughput, economical and robust manner. Because we also describe how to generate glycerol stocks, future researchers can use this method to isolate cells and communities capable of siderophore production and then perform a wide array of assays and further tests in the future.**

Minor Concerns: Some minor revision will be required.
**The authors feel the concerns of this reviewer were thoroughly addressed.**

---

**Reviewer #2:**

Major Concerns:
The manuscript would be greatly improved if the protocol, representative results, and discussion section were restructured, as indicated below. I'm not sure any of these items are major, but collectively indicate that the manuscript needs major revision.

Introduction section: This section is generally fine, and does a nice job of introducing the protocol. However, much of it is redundant (for example: Microbial siderophores are well recognized recently as important biomolecules.... could be streamlined to say: Siderophores are important biomolecules involved....). The introduction also talks about enrichment cultures, but does not explain what exactly is being enriched.

**The authors have revised and streamlined the lines 65-68, mentioned by the reviewer to:**

**"Siderophores are important biomolecules involved primarily in iron-chelation for bioavailability, but with a wide array of additional purposes in terrestrial ecosystems ranging from microbial quorum sensing, signaling to microbial plant-hosts, plant growth promotion, cooperation and competition within complex microbial communities[1,2]."**

Also, throughout the manuscript, the authors claim that the improved protocol is cost-effective and rapid but provide no evidence or reasoning for that claim.

**The authors have added language to support the point about cost in the conclusion:**

**Lines 412-418: "Still, because a single microplate can be used for 96 samples (including standards), the time and cost inputs are much lower compared with existing techniques. This is primarily because other existing methods rely on performing the CAS-Fe assay in Petri dishes[30], which inherently take more time and money to prepare than a microplate."**

Protocol: There are several confusing points in this section.
Section 1) How long were the plants grown? Were they harvested after maturation?

**The authors have addressed concerns in Lines 122-123 for the representative data: "Plant and soil samples were collected on August 9, 2018, when plants were ready for harvest."**

**However environmental sample collection can be varied according to experimental design and plant physiological stage can also be a variable at the experimenter's discretion.**

Section 2) What exactly is "loosely bound, tightly bound, and bulk soil"? Please provide your working definition.

**The authors have described how the samples were collected and operationally defined in Section 5 on sample collection and preparation, Lines 220-249.**

Section 3) Is it possible to put the recipes for the media first? I find it distracting that I haven't read the recipes before the protocol is described. Ideally I would like to see section 7 as section 3 or 4.

**The authors have reorganized and rewritten the protocol section to be less confusing, so that now the experimental design and site is described first. After that, the preparation of each medium is given, followed by standard curve generation, sample prep, and sample assessment.**

Section 3.4) I do not see how you are enriching siderophore producers. Can you explain?

**Siderophore production was enriched through Fe-deficit limitation. This is now emphasized in the heading for step 6.4.**

**Line 274: "6.4. Enrichment of Siderophore Production Through Fe Limitation"**

Section 3.5) The method indicates that 3 technical replicates are measured (presumably on a plate reader), but only one point on the graph is shown for each dilution. Also, I recommend that three stock solutions and separate dilutions prepared, instead of measuring replicates of a singular dilution. There is no mention at all in the entire protocol of how the reading are

measured. Is this a plate reader? Why are you measuring at 420 nm? Most other siderophore methods read at 630 nm...why the difference?

**The authors discovered in preliminary work, the peak absorbance in CAS-Fe medium at 665 nm. We also observed absorbance at 420 nm to be a sensitive and more accurate estimate of changes in the color of CAS-Fe in the presence of samples. In order to address this preliminary work and the rationale for the wavelength choice, we have now added the data and figures for the standard curves generated using absorbance at 420 nm (far better than those examining 665 nm). These points can be gleaned from examining Figures 1 and 3.**

Section 3.7) Was the glucose and other non-autoclaved components filter sterilized?

**These solutions were filter sterilized and the manuscript has been adjusted to reflect this (Lines 141-142).**

Section 3.7.2.4) Here and throughout the manuscript, you reference a "hood". DO you mean a laminar flow hood? Can you just say using sterile technique?

**The language was changed to “biosafety cabinet” and “sterile technique” where appropriate.**

Section 3.7.2.5 remove "hot" and just say "was placed in a 50 degrees C water bath"

**The authors have corrected this as suggested throughout.**

Section 3.7.3) What is described in Lewis et al??

**The authors have removed this reference. We are currently publishing a paper using a similar method with the same M9 formula. But as it is still in the revision process and we have fully described the solution, we felt it was appropriate to remove it from the manuscript and more thoroughly describe the preparation of the Fe-limited M9 medium.**

Representative results: There is no need to place quote marks around "pyoverdine equivalents". Why was pyoverdine chosen over other known siderophores?

The authors have removed the quotation marks, added some information on pyoverdines in Lines 303, and the following was added regarding the selection of pyoverdine:

Lines 384-385: “The results also show that pyoverdine equivalents (pyoverdines are important siderophores in terms of the environment[28] and medicine[29]) provide a good method of quantitatively assessing siderophore production.”

Figures and Table legends:

Figure 1) Please add error bars or indicate that the points shown are from a single experiment.

**The authors have revised the figure so that each replicate is now shown as a point and a new standard curve was generated. The new equation explaining the fitted curve was also generated so the data were all reanalyzed to reflect this. You will see there is basically no change in the results because the new formula is very similar to the old formula. We have also now shown the absorbance measurements at 665nm which we found to the the peak of CAS-Fe absorbance in another study (even though the convention is to measure at 630 nm).**

Figure 2 and Figure s1) These descriptions are pretty confusing. "Figure 2. Pyoverdine equivalents of 72 h enrichment cultures associated with bulk (A), loosely bound (B), and tightly bound (C) (in terms of roots) soil and tissue homogenates of wheat grain (D), shoots (E), roots (F). Genotypes/lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after 48 h of incubation. Asterisks 269 represent significance at alpha = 0.008 (after Bonferroni correction)." would be less confusing if written "Figure 2. Pyoverdine equivalents of enrichment cultures associated with (A) bulk ,(B) loosely bound , and (C) tightly bound soil and in tissue homogenates of wheat (D)grain , (E) shoots , and (F) roots were calculated after 72 h of enrichment. Genotypes/lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after 48 h of incubation with Chrome azurol S. Asterisks 269 represent significance at alpha = 0.008 (after Bonferroni correction)."

**The authors have altered figure descriptions to address concerns and they have been updated as such (Figure 1 is now Figure 4):**

"**Figure 4.** Pyoverdine equivalents of siderophore enrichment cultures associated with (A) bulk, (B) loosely bound, and (C) tightly bound soil, and in tissue homogenates of wheat (D) grain, (E) shoots, and (F) roots. Siderophore enrichment cultures were incubated for 72 h before transferring subsamples to a microplate and incubating at 28 °C. Siderophore production was assessed after 48 h of incubation with Chrome azurol S. Genotypes/lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Asterisks represent significance at alpha = 0.008 (after Bonferroni correction). Bars are standard deviation."

**"Figure S1**. Pyoverdine equivalents of siderophore enrichment cultures associated with (A) bulk, (B) loosely bound, and (C) tightly bound soil, and in tissue homogenates of wheat (D) grain, (E) shoots, and (F) roots. Siderophore enrichment cultures were incubated for 72 h before transferring subsamples to a microplate and incubating at 28 °C. Siderophore production was assessed after 24, 48, and 72 h of incubation with Chrome azurol S. Genotypes/lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after, 24, 48, and 72 h of incubation. Bars are standard deviation."

According to the author instructions, the discussion should be focused on:
* Critical steps in the protocol
* Modifications and troubleshooting of the method
* Limitations of the method
* The significance of the method with respect to existing/alternative methods
* Future applications or directions of the method

I'm not sure that the discussion addresses any of these points. Please rewrite and leave out any discussion of the representative results, as instructed.

**The discussion section has been re-written to address all these concerns as per our response to the editor above and reflected in Lines 373-426.**