Factors Influencing Microbial Growth and Manganese Oxidation

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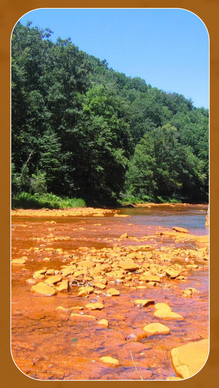
Introduction



Worldwide, and especially in Appalachia, abandoned mines contribute to major pollution of soil and sources of water. Manganese (Mn) is one of the most common metals found in polluted runoff, called acid mine drainage (AMD), from abandoned mines[1]. Numerous species of bacteria and fungi have been described from diverse microbial communities found in the treatment systems of AMD[3,4]. Manganese oxides are minerals produced by an oxidation reaction that converts soluble Mn^{2+} to insoluble Mn^{4+}. Formation and precipitation of Mn oxides has been found to be catalyzed by bacteria and fungi found in AMD sites[3,4]. Factors controlling the growth and oxidative metabolisms of these microbes is poorly understood. Further knowledge of ideal conditions for Mn-oxidizing microbes can improve the effectiveness of AMD treatment systems.

In this study, we examined whether the type and availability of nutrients in the environment affect the growth and oxidation of Mn-oxidizing microbes. Four fungal species and an unidentified strain of bacteria were grown on solid and liquid growth media that contained varying concentrations of different sources of carbon and nitrogen. The identity of the unknown bacteria isolate was confirmed through BLAST and phylogenetic placement. We recorded growth and Mn oxide precipitation of the fungi and bacteria over a set period of time. Comparisons were made between the five organisms to formulate trends regarding growth and Mn oxidation.

Materials & Methods

•Fungal species used were: *Plectosphaerella cucumerina, Pyrenochaeta* sp., *Stagonospora* sp., and *Acremonium strictum*

Figure 1. Fungal species of the study. Image A shows an AMD treatment site in central Pennsylvania from which the fungi were obtained. Image B shows the fungi as they grow on solid AY media with Mn^{2+}.

•Bacterial isolate used: AST2-a2AYBbac

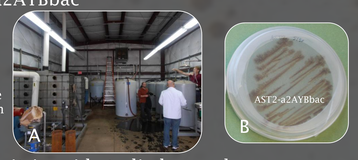


Figure 2. Bacterial isolate of the study. Image A shows the water treatment facility at North Carolina State University from which the bacterial isolate was obtained. Image B shows the bacterium as it grows on solid AY media with Mn^{2+}.

- Glucose, sodium acetate and citric acid studied as carbon sources
  - 5.0mM potassium nitrate used as standard nitrogen source
- Ammonium sulfate and potassium nitrate studied as nitrogen sources
  - 5.0mM sodium acetate used as standard carbon source
- C and N sources studied at 0.5mM, 5.0mM and 50.0mM concentrations
- Fungi and bacteria grown on modified Media 3[2]
  - Fungi grown on solid media (with agar) for 14 days
  - Bacteria grown in liquid media (no agar) for 3 days on
- Growth rate = Diameter (in mm) / Days of growth
- Oxidation recorded with 1.0M LBB (leucoberbelin blue) solution
  - Intensity of blue color corresponds to concentration of Mn oxides
- Total DNA extracted from AST2-a2AYBbac culture
  - 16S rRNA gene amplified using PCR
  - Sequence cleanup performed in Sequencher®
  - Phylogeny determined using maximum likelihood model with bootstrapping in Geneious®
- *Plectosphaerella* grown in 0.5mM, 2.0mM, 5.0mM and 10.0mM citric acid liquid media to investigate pH tolerance
  - Separate experiment examined why only *P. cucumerina* was able to grow and oxidize in 5.0mM citric acid media
  - pH, growth and oxidation recorded every 2 to 4 days for 14 days

Results

Bacterial Phylogenetics and Growth

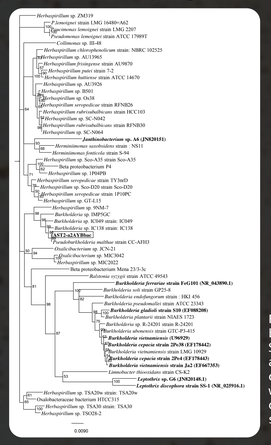


Figure 3. Phylogenetic placement of bacterial isolate AST2-a2AYBbac. Sequence data was obtained via BLAST and MUSCLE aligned. The tree was constructed using maximum likelihood with bootstrapping at 100. Known Mn-oxidizing strains are in bold text and the strain from the study is boxed.

- Little growth observed in nearly all media
- No growth in 50.0mM citric acid medium
- No oxidation observed in any media
- Most closely-related BLAST hits were *Pseudoburkholderia* and *Burkholderia* species
  - Bootstrapping supports relationship phylogenetically

Figure 4. Growth experiments with bacterial strain AST2-a2AYBbac. Clockwise from top right: ammonium sulfate, potassium nitrate, citric acid, sodium acetate and glucose growth experiments are shown. In each image, concentration of the nutrient is 0.5mM, 5.0mM and 50.0mM from left to right. Some growth is evident in cloudy flasks but no oxidation is present in any flask.

Fungal Growth

- Highly variable results for each species
- *Acremonium strictum* grew and oxidized in every media except 0.5mM, 5.0mM and 50.0mM citric acid
- *Plectosphaerella cucumerina* oxidized on relatively few media, despite high growth rates
- Ammonium sulfate 5.0mM media was only media in which all 4 species of fungi grew and oxidized
- No growth on 50.0mM citric acid media

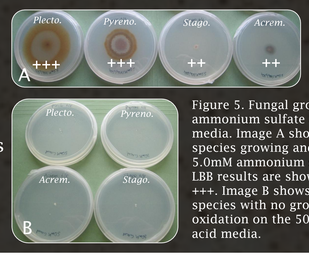


Figure 5. Fungal growth on ammonium sulfate and citric acid media. Image A shows the four species growing and oxidizing on 5.0mM ammonium sulfate media. LBB results are shown by ++ and +++. Image B shows the four species with no growth or oxidation on the 50.0mM citric acid media.

Reagent	Growth Rate (mm/day) *Stagonospora*	*Acremonium*	*Plectosphaerella*	*Pyrenochaeta*
Glucose, 0.5mM	1.29	2.93	5.36	3.82
Glucose, 5.0mM	1.86	2.68	4.79	3.39
Glucose, 50.0mM	1.39	2.61	4.21	3.54
Sodium acetate, 0.5mM	1.14	2.96	5.21	4.14
Sodium acetate, 5.0mM	1.86	2.64	5.36	3.79
Sodium acetate, 50mM	1.14	2.79	5.71	3.18
Citric acid, 0.5mM	2.36	3.04	5.14	3.73
Citric acid, 5.0mM	0.36	1.18	3.45	2.18
Citric acid, 50.0mM	0.00	0.00	0.00	0.00
Ammonium sulfate, 0.5mM	1.81	1.38	5.19	3.62
Ammonium sulfate, 5.0mM	1.92	1.54	5.38	3.54
Ammonium sulfate, 50.0mM	1.00	1.38	2.23	2.96
Potassium nitrate, 0.5mM	2.27	2.64	4.55	3.36
Potassium nitrate, 5.0mM	2.27	2.91	4.64	3.41
Potassium nitrate, 50.0mM	1.50	3.36	5.77	3.82

Figure 6. Final growth rate and LBB results of fungal growth experiments. Mean growth rates were determined from duplicate growth plates for each media and for each species. A piece of agar was removed from the growth plate, crushed and mixed with 150µL of 1.0M LBB solution. A result of –, meaning no Mn oxides were present, is represented as white; ++, meaning some Mn oxides, is represented as light blue or light orange; +++, meaning many Mn oxides is represented as dark blue or dark orange. Blue boxes indicate media examining carbon sources and orange boxes indicate media examining nitrogen sources.

Plectosphaerella Citric Acid Experiment

- Mn oxides present at 0.5mM, 2.0mM and 5.0mM citric acid concentrations
- pH of all solutions increased over time
  - Most significant change with 5.0mM and 10.0mM citric acid
  - 67.25% and 31.23% increase, respectively

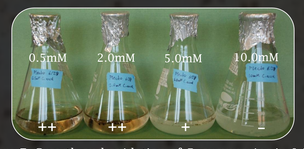


Figure 7. Growth and oxidation of *P. cucumerina* in 0.5mM, 2.0mM, 5.0mM and 10.0mM citric acid media. Oxidation is apparent in 0.5mM, 2.0mM and 5.0mM citric acid Media 3 and growth is visible in all flasks. LBB results are indicated by – (no Mn oxides), + (some Mn oxides) and ++ (many Mn oxides).

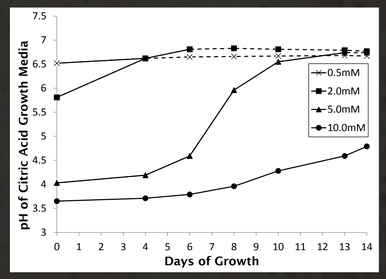


Figure 8. pH of citric acid media containing *P. cucumerina* during experimental period. Significant increases in pH are apparent in 5.0mM and 10.0mM citric acid media. Solid lines indicate growth without oxidation and dashed lines indicate growth with oxidation.

Discussion

- Bacteria strain AST2-a2AYBbac is most likely a *Burkholderia* species
  - Not directly related to other known Mn-oxidizing *Burkholderia* species
- AST2-a2AYBbac grew significantly less than any of the 4 fungi species and did not oxidize at all
  - Bacteria is able to grow and oxidize Mn on nutrient rich plate containing yeast extract
  - Possible that AST2-a2AYBbac is missing a vital nutrient that facilitates Mn oxidation
  - Also possible that the bacteria possesses a different metabolic pathway for oxidizing Mn

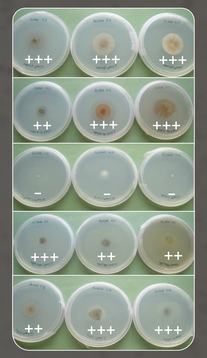


Figure 9. Results of *Acremonium strictum* growth experiments. Growth and oxidation are present in every media type except citric acid. LBB results indicated by –, ++ and +++.

- Variable growth across all species indicates each species has its own ideal growth conditions
  - Plays a role in management strategies for water treatment systems
  - Some species more adaptable than others
- *Acremonium strictum's* ability to grow in nearly all media suggests it is tolerant of many different conditions and is a generalist regarding nutrition
- No growth on 50.0mM citric acid media was due to a very low pH of 2.75

- *Plectosphaerella cucumerina* is able to alter the pH of its surroundings
  - Growth is possible at pH <5.5 but not precipitation of Mn oxides
  - Highlights importance of raising pH in water treatment systems
  - Possible that oxidation is occurring but Mn^{3+} is immediately reduced back to Mn^{2+}
- Observing when oxidation begins allows us to close in on determining the mechanisms for oxidizing Mn

Future Work

- Test for missing nutrient that will allow bacteria to grow and oxidize
- Investigate exact nutritional preferences of each fungal species
  - Find optimal conditions for maximum Mn oxidation to improve effectiveness of AMD treatment systems
- Determine whether oxidation and immediate reduction is occurring while fungi are growing at pH lower than 5.5
  - Examine if Mn^{3+} is present prior to precipitation of Mn oxides
- Identify "on-off switch" for Mn oxidation by fungi and bacteria
  - Once identified, we can investigate mechanisms by which microbes oxidize Mn

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[1] Banks, D., Younger P.L., Arnesen, R., Iverson, E.R., and Banks, S.B. 1997. Mine-water chemistry: the good, the bad and the ugly. Environmental Geology 32(3): 157-174.

[2] de la Torre, M.A. and Gomez-Alarcon, G. 1994. Manganese and iron oxidation by fungi isolated from building stone. Microbial Ecology 27(2): 177-188.

[3] Hallberg, K.B and Johnson, D.B. 2005. Biological manganese removal from acid mine drainage in constructed wetlands and prototype bioreactors. Science of the Total Environment 338: 115-124.

[4] Santelli, C.M., Pfister, D.H., Lazarus, D., Sun, L., Burgos, W.D., and Hansel, C.M. 2010. Promotion of Mn(II) oxidation and remediation of coal mine drainage in passive treatment systems by diverse fungal and bacterial communities. Applied and Environmental Microbiology 76(14): 4871-4875.