IRON BIOACCUMULATION IN DUCKWEED (*LEMNA MINOR*) AND OYSTER MUSHROOM (*PLEUROTUS OSTREATUS*)

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DEDICATED TO

I would like to dedicate this thesis to my late father, Robert Fechner. I would also like to dedicate this thesis to my son, Aiden Fechner, who drives me to work my hardest and be the best person I can be every day.

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ABSTRACT

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Over 3,000,000 gallons of contaminated acid mine tailings were released into the Animas and San Juan watersheds on August 15, 2015 leaving behind high concentrations of iron hydroxides. The orange water raised concerns for local farmers who utilized these watersheds for agricultural irrigation. Iron can complex with other metals and can also form hard precipitates out of solution ("ferricrete"), which can affect both aquatic and terrestrial life. Some plants, such as *Lemna minor* (duckweed), can sorb certain contaminants at high concentrations. Two nine-day experiments under controlled conditions were conducted with duckweed to assess its effectiveness in removing iron from solution. Duckweed (0.10 kg) was added to 2.9 kg H_2O and 1 g fertilizer (NPK: 20-20-20) in plastic containers with three treatment levels of an iron supplement (0, 20 g, and 40 g). The 0 g iron additive or, control, consisted of water, duckweed,

and fertilizer only; separate controls were established to assess if iron was binding to the fertilizer or precipitating out of solution. Water samples analyzed for total iron using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) decreased in iron concentration over time. Removal of iron from the water may have been by precipitation (reddish stains were evident on the containers), binding to fertilizer, or sorption onto or into duckweed. No differences in water iron concentrations were detected with the presence of duckweed or fertilizer ($p > 0.05$) indicating that neither of these factors had significant effect on iron removal when compared to iron precipitation. Duckweed was collected and oven-dried at 65 ^{o}C, microwave digested using EPA Method 3052, and analyzed for total iron content via ICP-OES. The highest amount of iron sorbed by duckweed was about 17,000 parts per million (ppm) with an average sorption of 11,271 to 14,434 (± 861) ppm in the two experiments. Oyster mushroom (*Pleurotus ostreatus*) mycelium was inoculated with harvested duckweed and iron transfer into the fruiting bodies (basidiocarps) was then analyzed. The degradation ability of the iron-enriched duckweed using oyster mushroom mycelium was assessed. Mycelium was recovered from 100% of the duckweed.

Understanding the behavior of high iron concentrations in watersheds will allow us to better understand how other transition metals may behave. This research will provide an understanding of duckweed use in phytoremediation of high iron concentrations while assessing the potential in the utilization of contaminated duckweed within the agricultural sector. This research will also provide a fundamental understanding of the role oyster mushrooms play in the degradation of phytoremediators and in the iron transfer between organisms.

Keywords: duckweed, oyster mushrooms, remediation, degradation, iron

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CHAPTER 1

1.0 INTRODUCTION

1.1 Mining History along the Animas River

Mining in the southern Colorado and northern New Mexico regions started in the late 1800s, with gold development starting at the Little Giant Mine in the San Juan Mountains. In 1886, the Gold King Mine was developed and primarily used for gold mining, as well as for silver, lead, and copper (U.S. Department of the Interior, 2015) and operated until 1922 (Chief et al., 2016). For many years, the Gold King Mine drained into Cement Creek and subsequently into the Animas River north of Durango, CO. The Animas River flows south towards northwestern New Mexico and creates the Animas watershed.

The watershed can be divided into three different geochemical areas based on parent rock material and land use. These three areas consist of the Upper Animas River watershed (upstream of Silverton, CO), the Animas River between Silverton, CO and Farmington, NM, and the Animas River near Farmington, NM before joining the San Juan River. The Upper Animas River watershed is heavily mineralized with high concentrations of iron (Fe). The area between Silverton and Farmington has a lower concentration of iron than the Upper Animas River and the area near Farmington is generally low in iron and other metals, but has more nutrients due to heavy agriculture in the area (Church et al., 2007; U.S. Department of Energy, 2015; Church et al., 2000). Natural iron-bearing minerals such as jarosite, which is a metal sink, and goethite have been reported in the Upper Animas River (Bove et al., 2007; Dalton et al., 2007; Dutrizac and Dinardo, 1983). The bioavailability of these metal-bearing minerals increases through

geochemical weathering allowing the iron within these minerals greater potential to be taken up by plants irrigated with the river water (Kimball et al., 2016; Hayes et al., 2012; Schaider et al., 2007; Hayes et al., 2009).

Iron is a metal that is naturally present in the Animas River watershed. Two major iron-bearing minerals found in sediment samples collected along the watershed and analyzed were illite and chlorite (Rodriguez-Freire et al., 2016). These minerals contribute to the iron load naturally present in the Animas River.

1.2 Interaction between Iron and Water

Iron is found in water supplies due to natural processes. It is found most often in the form of heme or iron-sulfur complexes (Philpott, 2006). Iron in sea water ranges from 1-3 parts per billion (ppb), while rivers and groundwater usually contain 0.5-1 parts per million (ppm) and 100 ppm of iron, respectively. Humans should not consume more than 200 ppb of iron in their drinking water (Lenntech, n.d.). Water soluble iron compounds are more dangerous than elemental, or metallic iron, which does not dissolve readily in non-acidified water. If ferrous iron (Fe^{2+}) binds with chlorine or sulfate, creating iron(II) chloride ($FeCl_2$) or iron(II) sulfate ($FeSO_4$), respectively, it can be lethal for humans if consumed at 10-50 g (Lenntech, n.d.).

Iron-containing minerals such as magnetite, hematite, goethite, and siderite can be altered through natural weathering processes when exposed to air and water. Ferrous iron (Fe^{2+}), or the reduced form, occurs under anaerobic conditions while ferric iron (Fe^{3+}), or the oxidized form, occurs in environments with readily available oxygen and is mainly found in the form of iron

hydroxide ($Fe(OH)_2)^+_{(aq)}$) (Lenntech, n.d.). Results from Hopkins et al. (1944) and Somers and Shive (1942) suggest that manganese (Mn) can use up oxygen before iron does, thus reducing Fe^{3+} to Fe^{2+} and increasing iron solubility. Ferric iron is insoluble at neutral pH (pH = 7) and is the most stable and most common iron species (Kosman, 2003; Bauer and Knölker, 2008). Plants are known to favor the ferrous form over the ferric form of iron, except at extremely low pH, because ferrous iron is more water soluble and bioavailable (Jeong and Connolly, 2009).

1.2.1. Iron Sorption Chemistry

There are two main mechanisms involved in plant-iron interactions. These two mechanisms are iron adsorption and iron absorption. Adsorption refers to iron binding to the outer surface area of the plant. This includes iron coating of root, shoot, and leaf surface area. Absorption refers to the uptake of iron into the plant, as opposed to on the surface, through the roots or leaves.

Ferric iron's low water solubility is mediated by the formation of soluble complexes with fulvates, organic acids, and siderophores, as these have the ability to effectively extract iron from soluble iron-bearing minerals. Goethite, hematite, and ferrihydrite are examples of secondary ferric iron precipitates with highly reactive surface hydroxyl (OH) functional groups that can participate in surface complexation (adsorption) reactions. The charge on these functional groups are pH dependent and provide cation or anion exchange sites depending on the solution pH. Additionally, iron oxyhydroxides can scavenge for metals and ligands that can form strong surface complexes via ligand exchange or inner-sphere complexation (Essington, 2015).

Pyrite (FeS_2) is a common reduced iron mineral associated with metal ore bodies that can undergo oxidative dissolution (Evangelou, 1998). Iron also has the ability to form hard precipitates out of solution through a process called oxidative precipitation. Precipitation reactions are chemical reactions that occur in solutions to form solids. Oxidative precipitation refers to the addition of oxygen in solution which reacts with the iron to form a solid outside of solution (ferricrete).

1.3 A General Overview of Acid Mine Tailings

The Animas and San Juan watersheds have been subject to natural mine drainage since mining in the San Juan Mountains began. Acid mine drainage, also known as acid rock drainage, is defined as water that leaches through sulfur-bearing rocks such as iron sulfide (pyrite or fool's gold) that are common ore-bearing minerals in mining districts. It is characterized as having a low pH, high specific conductivity, and a high concentration of metals and potentially toxic elements (Akcil and Koldas, 2005). When pyrite is exposed to air and water it oxidizes, forming sulfuric acid (H_2SO_4) and mobilizing heavy metals (Akcil and Koldas, 2005). Since 2005, acid mine drainage has been conventionally treated by adding lime (usually calcium carbonate) to neutralize the solution and precipitate metals as hydroxides (Akcil and Koldas, 2005). The high pH associated with the lime leads to iron precipitation (Lenntech, n.d.). Containment of contaminants by flooding to prevent oxidation has also been attempted but has failed due to difficulties in managing water levels in certain areas (Akcil and Koldas, 2005).

Accidental mine blowouts and the threat to the environment has resulted in water quality standard initiatives set in the upper Animas River by the State of Colorado Water Quality Control Division. The three ideas behind these initiatives were to (1) Identify environmental problems surrounding abandoned mine sites, (2) Determine how much unnatural acid drainage was contributing to the metal load in the waterways, and (3) Improve water quality at affected sites (Russell, 2000).

Weathering and oxidation of pyrite can lead to the formation of acid drainage resulting in iron and other metals naturally leaching into waterways. Mining activities increase the amount of acid drainage into local waterways. Two of the tributaries to the Animas River, Mineral Creek and Cement Creek, are where the highest iron loads accumulate within the watershed (Church et al., 2007). Cement Creek alone accumulates almost 50% of its iron load from mining activities (Church et al., 2007).

Iron is a transition metal and these metals can be divided into two groups: One necessary for plant metabolism and the other not necessary for, or even toxic to, plants. The first group includes iron, copper, manganese, and molybdenum while the second group includes cadmium, mercury, and lead (Siedlecka, 1995). Metals necessary for plant metabolism can be further divided into three subgroups related to their accumulation in plant roots, shoots, or both (Hara et al., 1976 a, b; Jastrow and Koeppe, 1980). Iron generally accumulates in the roots with an intermediate amount in the shoots (Siedlecka, 1995). Iron is an element essential for plant and animal life and is generally not categorized as a contaminant, although it can be toxic in high concentrations.

Iron is used in various plant processes such as photosynthesis, respiration, nitrogen fixation, DNA synthesis, and is a co-factor of many hormone-synthesizing enzymes (Briat et al., 1995; Connolly and Guerinot, 2002; Kobayashi and Nishizawa, 2012). High levels of iron can be toxic for biological organisms by reacting with oxygen (Fenton reaction) and accelerating the formation of free radical iron species (Connolly and Guerinot, 2002; Kobayashi and Nishizawa, 2012). Free radical iron species such as ferritin or aconitase (McCord, 2004) can have devastating cytotoxic effects on plants such as membrane disintegration and cell death due to oxidative stress (Briat et al., 1995, 2010; Almeida et al., 2015). Free iron also produces hydroxyl radicals from the Fenton Reaction, which are toxic to cells (Jeong and Connolly, 2009).

Metals have the potential to inhibit the uptake of other nutrients through competition and root damage. Damage to proteins, decreased active transport of nutrients, or inhibited root growth may all be caused by excess iron in plant cells (Siedlecka, 1995). Iron can complex with other metals and compete with them for various pathways into plants. The binding of transition metals is partially due to the insertion reaction, where one molecule or molecular fragment inserts itself into an existing chemical bond (Bauer and Knölker, 2008). When iron binds with another metal they can compete for transport proteins or interfere with other uptake mechanisms. Iron and other metals can also compete for different chelating molecules that may inhibit or retard the absorption capability of the biological system (Sandström, 2001). For instance, iron is taken into cells in its ferrous (Fe^{2+}) form by metal ion transporters that can also transport Mn^{2+}, Ni^{2+}, and Cu^{2+} (Kosman, 2003). These metal transporters generally recognize single valence states and cannot transport metals with other valences (Philpott, 2006). For this reason, ferric

iron must be reduced to ferrous iron before it is able to be taken up via ferrous iron transporters. Jeong and Connolly (2009) describe the movement of iron in plants using ferric iron reductases. These reductases play an important role in the translocation of iron within subcellular compartments of the plant.

Iron has been shown to decrease the absorption of elements such as zinc and calcium and lower their bioavailability, especially in aqueous solutions (Sandström et al., 1985). Zinc toxicity has also been linked with iron deficiency in plants due to competition and interaction (Misra and Ramani, 1991). Prior studies looking at heavy metal and plant mineral nutrient interactions suggest that iron has inverse relationships with copper, cadmium, zinc and lead in root, cotyledon, and shoot accumulation (Hunter and Vergnano, 1953; Walker et al., 1987; Terry, 1981; Khan and Khan, 1983; Greger and Lindberg, 1987; Misra and Ramani, 1991; Siedlecka and BaszyńAski, 1993). Available phosphorus and manganese in plants have also been shown to decrease with excess iron (Singh and Dahiya, 1976; Tanaka and Navasero, 1966).

1.4 The Gold King Mine Spill

On August 5, 2015 the Environmental Protection Agency (EPA) was inspecting an abandoned mine site near Silverton, Colorado when a natural soil plug was disturbed and knocked loose by machinery. The disruption of this plug led to roughly 3,000,000 gallons of contaminated wastewater uncontrollably flowing from the site into the Animas River and was referred to as a “mine blowout” (U.S. Department of the Interior, 2015). Roughly 250,000 pounds of iron leaked into the river, turning it a yellow-orange color (from iron-bearing minerals

known as “yellow boy”), along with other metals such as copper, arsenic, and lead (Chief et al., 2016). Sediments of iron oxyhydroxides formed and deposited on the inner surface of the mine combining with heavy metals that were released into the river as a result of the “blowout.” The spill affected six states and 12 Native American tribes including Colorado, Utah, New Mexico, Arizona, Nevada, California, and the Navajo Nation (Chief et al., 2016). The Animas and San Juan Rivers continued to carry metal-laden water downstream until settling at Lake Powell in Utah (U.S. Department of the Interior, 2015).

The spill affected local area farmers and tribes that use the river water for irrigation, recreation, or cultural purposes. The contaminants introduced to the river from the Gold King Mine spill have the potential to accumulate in crops and become dangerous for human consumption if they are present above recommended values provided by the World Health Organization (WHO) and the U.S. Environmental Protection Agency (EPA).

The San Juan River serves as the main irrigation source for the Navajo Agricultural Products Industry (NAPI), the largest farming operation on Navajo land. As of 2019, local farmers wishing to use the river for irrigation still questioned whether the water was safe.

A technical evaluation of the Gold King Mine spill, requested by the EPA, found that mining spills occur more often than previously thought (U.S. Department of the Interior, 2015). This is partly due to a lack of consistent governmental requirements in mandating the re-opening of abandoned mine sites. Guidelines for re-opening abandoned mine sites tend to change between agencies (U.S. Department of the Interior, 2015).

1.5 Clean-Up Efforts

There are different methods used to remediate water affected by a mine spill. One of the remediation approaches after the Gold King Mine spill was the establishment of settling ponds to divert the acid mine drainage away from the main riverbank and treat the water afterwards (US EPAe, 2015). Due to freezing temperatures in Colorado, it was unsafe to collect and treat the acid mine drainage in settling ponds (Chief et al., 2016).

The mine spill also impacted the economy in the areas affected. Claims from farmers who lost harvest receipts due to the spill were filed, as well as claims from farmers who had to haul clean water to their crops to replace the unusable river water. Because of the recreation services the Animas River provides, businesses like rafting and other water-related companies lost profits as well. Some homeowners were also affected due to the metal-laden water contaminating their wells.

In late 2015 the EPA issued a statement saying that the water quality had returned to the level it was at prior to the spill but still designated the Gold King Mine a Superfund site to help prevent future blowouts from happening and to provide long-term monitoring of the watershed. Water quality along all waterways, except Cement Creek, have returned to pre-spill levels and can be used for irrigation but there is still ongoing concern regarding the effects the metals may have on plant and aquatic ecosystems (US EPAa, 2015; CDPHEa, 2015; CDPHEb, 2015). It is important then to explore alternative approaches to mine spill remediation.

1.6 Use of (*Lemna minor*) Duckweed to Help Clean Contaminated Waterways

Phytoremediation refers to the ability of certain plants to remove or stabilize environmental pollutants or alter them to less harmful forms (Raskin et al., 1997). Lesser duckweed (*Lemna minor*) is a non-graminaceous (non-grass) macrophytic plant species (Figure 1) that has been previously studied for ecotoxicological purposes and is considered a model remediation tool for different metals, metalloids, and excess nutrients (Harvey and Fox, 1973; Teixeira et al., 2014; Singh et. al., 2012).

Figure 1. Individual duckweed plantlets.

Macrophytes are defined as plants that can be found in nearly any type of aquatic habitat (Anawar et al., 2011; Rahman and Hasegawa, 2011; Mudgal et al., 2010; Kheir et al., 2007). Duckweed is cold-hardy, vegetates (produces clones) at 1-3 ^{o}C, and has been found to double its biomass every four days when grown in wastewater effluent (Harvey and Fox, 1973). Duckweed

is composed of two modified stems and two roots and is valued for its high nitrogen content and hardiness (Leng et al., 1995). These properties make it suitable for use in remediation of contaminated waterways (Kostecka and Kaniuczak, 2008). Duckweed has also been identified as a phytoaccumulator of many different nutrients, metals, and metalloids, surpassing the uptake and accumulation abilities of algae and other aquatic macrophytes (Vidakovic-Citrek et al., 1999; Anawar et al., 2008). Metals, metalloids, and nutrients such as lead, nitrogen, phosphorus, iron, nickel, copper, calcium, zinc, manganese, boron, uranium, and arsenic may accumulate in duckweed (Singh et al., 2012; Harvey and Fox, 1973; Jain et al., 1988, Mkandawire and Dudel, 2005; Demirezen et al., 2007; Hou et al., 2007; Alvarado et al., 2008; Uysal and Taner, 2010; Böcük et al., 2013). One mechanism behind this accumulation may be the reduction of immobile metal ions by specific plasma membrane bound metal reductases, thus increasing the bioavailability of some metals. Chemical reduction works particularly well to convert Fe^{3+} and Cu^{2+} to forms that are more bioavailable (Raskin et al., 1997).

Iron is taken up by non-graminaceous plants by releasing root exudates called phytosiderophores to assist in acidification, reduction, and transport of the metal (Jeong and Connolly, 2009). Acidification occurs by excretion of hydrogen (H^+) ions in the root epidermis by H^+-ATPases (González-Guerrero et al., 2016), mobilizing Fe^{3+} by increasing its solubility.

A hyperaccumulator is a plant that can accumulate metals, metalloids, or nutrients at a concentration of 1-5% of its dry weight, which is an order of magnitude greater than non-accumulators growing in similar environments (Raskin et al., 1997). Previous studies have looked at the bioaccumulation of various elements by duckweed under specific parameters such

as pH, light exposure, temperature, and other environmental conditions to determine the optimal condition for removal or remediation of metals from contaminated water.

1.7 Use of Pearl Oyster Mushroom (*Pleurotus ostreatus*) in Duckweed Recycling and Iron Transfer between Organisms

Oyster mushrooms (*Pleurotus ostreatus*) are members of the sub-phylum of Basidiomycota in the fungal phylum Dikarya. They are of economic importance being the third largest commercially grown edible mushroom in the world (Obodai et al., 2003) and are valued for their nutritional benefits including high amounts of vitamin D, protein, and fiber.

Basidiomycotan fungi utilize different strategies to uptake iron. One strategy is the ability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) using an iron reductase enzyme. This strategy provides protection against free radicals produced during oxidative processes, making these fungi ideal for iron remediation (Almeida et al., 2015). Basidiomycotan fungi may also excrete siderophores that are ferric iron-specific to facilitate iron uptake by mobilizing the metal and increasing its solubility (Haas, 2003). Fungal siderophores serve the same function as plant-induced phytosiderophores but are structured differently. Iron is stored in structures of the mushrooms using intracellular siderophores as iron storage compounds and recovered by cells using specific uptake mechanisms (Almeida et al., 2015; Haas, 2003; Winkelmann 2001, 2002; Philpott, 2006). This stored iron can then be released through a reduction of the iron-siderophore chelate (Ardon et al., 1998). Almost all fungi can uptake iron through siderophore excretion (Hsiang and Baillie, 2005).

Fungal siderophores have been shown to bind more iron than other biological ligands, making them extremely important in iron uptake mechanisms of fungi (Philpott, 2006). It has been reported that increased concentrations of iron in culture media positively correlates with increasing concentrations absorbed by oyster mushroom mycelium (Almeida et al., 2015), with mycelium growth inhibited at 300 mg Fe L^{-1} (Almeida et al., 2015). Dunn et al. (2007) reported that high iron levels created oxidative stress and caused cell damage in mushrooms.

Oyster mushrooms were chosen for this study because of their previously reported ability to bioaccumulate iron and other metals, such as silver and cadmium (Almeida et al., 2015; Favero et al., 1990; Bressa et al., 1988; Philpott, 2006; Haas, 2003). It has been shown that the combination of iron acquisition strategies employed by fungal species allows fungus to uptake iron from virtually any environmental source (Philpott, 2006).

Oyster mushrooms were also chosen for this study because they grow quickly, aggressively, and can be cultivated on a wide variety of media or substrates (Almeida et al., 2015). They are saprophytic (grow on dead and decaying organic matter) in nature (Almeida et al., 2015; Gern et al., 2008; Urban et al., 2005) and have been shown to grow on sawdust (both fresh and composted), banana leaves, maize stover, corn husks, and rice husks (Obodai et al., 2003). Oyster mushrooms are highly dependent on cellulose, lignin, and fiber contents of the growing medium to produce a maximum yield (Obodai et al., 2003). They have also been shown to tolerate high nitrogen environments (Almeida et al., 2015), increasing in biomass when grown on substrates high in nitrogen (Gern et al., 2008).

Oyster mushroom fruiting bodies have been shown to bioaccumulate different metals such as lead, copper, nickel, cadmium, chromium, and silver from natural substrates (Favero et al., 1990; Isildak et al., 2004; Bressa et al., 1988; Garcia et al., 2009). They are reported to be of low risk to public health, aside from those with weakened immune systems, due to low measured metal concentrations on a dry weight basis (Almeida et al., 2015). Iron has been reported to naturally occur in fruiting bodies (basidiocarps) of *P. ostreatus* at 48 to 280 mg kg^{-1} (Vetter, 1994; Tüzen et al., 1998; Demirbas, 2001; Isildak et al., 2004; Genccelep et al., 2009; Patil et al., 2010).

Iron is one of the most abundant naturally occurring elements but has limited bioavailability (Neilands et al., 1987) due to its dissolution properties at different oxidation states. The low bioavailability of iron results in iron deficiency in humans, also known as anemia. If iron is able to transfer from decaying duckweed to oyster mushroom fruiting bodies, there may be potential in exploring the use of oyster mushrooms to fight anemia. If, on the other hand, oyster mushroom fruiting bodies do not accumulate iron from decaying duckweed there is potential that other, more dangerous metals will also not transfer. If oyster mushroom mycelium can actively degrade iron-enriched duckweed, then there is potential in its use as an environmentally friendly means of disposing bioremediating plant material.

Remediation of waters potentially hazardous to human health and crop development remains a top priority. Using aquatic plants for remediation efforts may be a cost-effective method of metal removal but environmentally friendly disposal methods after site remediation are not well-studied (Raskin et al., 1997). Generally, phytoaccumulating plants are incinerated

after harvest and the metals are extracted from the ash using various processes. Incineration ultimately adds to the atmospheric carbon load. Plants may also be composted and metals extracted after degradation, however composting can take several months. For example, a composting experiment was set up in this study but was unsuccessful due to the slow degradation of duckweed as a sole component. If deemed non-toxic, some plants may be considered edible after remediation as well.

Prior studies have examined duckweed and oyster mushrooms separately for their abilities to remove iron from their growth environments but have not yet linked the two organisms together. Previous studies have also investigated the bioremediation potential of plants but often overlooked disposal afterwards.

1.8 Objectives

The objectives of this study are:

1. Assess duckweed's ability to remove iron from water source.
2. Assess whether iron can be directly transferred from decaying duckweed into oyster mushroom fruiting bodies.
3. Assess the ability of oyster mushrooms to colonize iron-enriched duckweed.
4. Combine objectives to propose an alternate method for remediation and disposal.

2.0 MATERIALS AND METHODS

2.1 Duckweed and Iron Interaction

To assess the ability of duckweed to remediate iron-enriched water, plants were grown in a series of plastic containers having high, low or no additional chelated iron. The experiment was conducted in a classroom located at Fabian Garcia Science Center during May-August 2019 and was set up twice. Live duckweed was purchased from Pond Plants Online (Gainesville, Florida). Contents of the plastic containers (34.6 cm length x 21 cm width x 12.4 cm height) used in the experiment are presented in Table 1. Containers having no duckweed served as a separate set of controls to observe other mechanisms that could result in lower iron concentrations in the water including precipitation and fertilizer binding.

Table 1. Contents of the plastic containers used for each treatment in the duckweed experiment.

Treatment	Tap water (kg)	Duckweed (kg)	Grow More, Inc. 6.0% Fe EDDHA chelate (mol/L)	Jack's Classic 20-20-20 Fertilizer (g)
Control	2.90	0.10	0.00	1.0
Low iron	2.90	0.10	0.123	1.0
Control 2 (Low)	2.90	0.00	0.123	1.0
Control 3 (Low)	2.90	0.00	0.123	0.0
High iron	2.90	0.10	0.247	1.0
Control 2 (High)	2.90	0.00	0.247	1.0
Control 3 (High)	2.90	0.00	0.247	0.0

* Each treatment had five replicates (n = 5) per experiment

Iron EDDHA [ethylenediamine (di o-hydroxyphenaylacetic acid)] has been widely evaluated and was used in this study because it is the preferred iron chelate for research in calcareous soils, which are common in New Mexico (Goos and Germain, 2001). Additionally, it has been reported that iron EDDHA stays in solution for a longer period of time and is more stable across a wider range of pH values compared to other commercially available iron chelates (Goos and Germain, 2001; Mengel and Kirkby, 1982).

2.1.1 Experimental Design

Plastic containers were filled with 2.9 kg of Las Cruces city tap water and zero, low, or high amounts of commercial 6% EDDHA iron chelate. Chelated iron powder was added at a rate of 20 g Fe chelate powder per 2.9 L H_2O (1.2 g available iron) and 40 g Fe chelate powder per 2.9 L H_2O (2.4 g available iron) for the low and high treatments, respectively. To determine the amount of available iron, the following equation was used:

Grams of EDDHA iron chelate powder used x 0.06 = grams of available iron (assuming full dissolution)

To determine the concentration of EDDHA iron chelate in moles per liter, the following equation was used:

(Grams of EDDHA iron chelate used x (1 mol Fe/56 g))/(2.90 L) = iron concentration (mol/L)(assuming full dissolution; although the molarity of Fe will be ≤6% of the EDDHA)

All containers were aerated using two 32 W, 950 GPH aquarium aerators (950 GPH, Vivosun, City of Industry, California) for one week to dissolve the iron compound prior to introducing duckweed. Control containers were also aerated for one week. Once duckweed was added, aerators were removed. Temperature (Celsius) and pH of each container were measured once daily at approximately the same time over the course of nine days. Water pH was measured using a portable pH meter (Multi-Parameter Testr™ 35 Series, Wilmington, NC). The pH meter was calibrated before use with pH buffer solutions of 4.0, 7.0, and 10.0 (Grainger, Lake Forest, Illinois). Standard deviations and means of pH and water temperature were calculated using Microsoft Excel (2002) and separated by experiment.

Water samples (20 mL) were collected from each container initially then every three days for nine days. All water samples were acidified with 0.25 mL of 1% nitric acid (HNO_3, trace metal grade) per 20 mL sample. Water lost by evaporation and sampling was replenished daily by refilling to the original volume with tap water.

Duckweed (~11 g wet weight) was harvested by skimming from the surface of each container initially (within 15-20 minutes) then every three days after for nine days. The plants were placed on aluminum pie tins lined with butcher paper and oven dried at 65 ^oC for two days. The first (initial) and fourth (final) duckweed harvest were analyzed for total iron concentration; the second and third harvest were reserved for additional analysis if needed.

2.1.2 Laboratory Analyses

All plant iron concentrations were analyzed using EPA Method 3052 without HF (Microwave Assisted Acid Digestion of Siliceous and Organically Based Matrices). Dried duckweed (and later the oyster mushroom fruiting bodies), were weighed out to 0.5 g and mixed with 5 mL HNO_3 (nitric acid trace metal grade) plus 3 mL H_2O_2 (hydrogen peroxide) in Teflon microwave vessels before being put into a Milestone Ethos Up Microwave Digestion System (Shelton, CT). Temperature in the digestion system was approximately 210 ^oC and pressure was 80 bar (roughly 1160 psi) for 40 minutes. The vessels were removed after 12-18 hours and the remaining solution was filtered, then brought to a volume of 100 mL to be analyzed via inductively coupled plasma-optical emission spectroscopy (ICP-OES). Acid-preserved water samples were also analyzed using ICP-OES.

For each digestion run, two quality control standards with known concentrations and one blank containing only the acids were included. The quality control standards were alfalfa (grown in Las Cruces) and SRM 1575a Pine Needle NIST. During the ICP-OES analysis, there were quality control samples to confirm before proceeding with the digested samples and water samples. After the calibration, an ICV (Initial Calibration Verification) standard was analyzed to validate the calibration and a LFB (Laboratory Fortified Blank) to check stability at low concentrations. Throughout the analysis, a LRB (Laboratory Reagent Blank) and a CCV (Continuing Calibration Verification) standard were analyzed every 10 samples to ensure accuracy and precision. A spike of one sample per batch was analyzed to confirm there was no matrix interference. The acceptable percent recoveries for these ICP-OES quality control samples were calculated following EPA protocol. The ICP-OES method is set up to read three replicate readings for each sample and the average of these readings were the reported result. The ICP-OES print-out determines the SD (Standard Deviation) and RSD (Relative Standard Deviation), which were calculated from these three replicate readings in order to ensure stability.

2.1.3 Statistical Analyses

Iron concentrations in duckweed were statistically analyzed using a mixed model approach with fixed effects for the first and second experiment, iron treatment (0, low, and high iron), duckweed sampling day, and all interactions among these three factors. Separate compound symmetric covariance structures were fit to each treatment level to account for repeated measures from experimental units and nonconstant variance among treatment levels.

Means separation was conducted to determine the nature of detected differences among iron treatment levels. Results show significance ($p < 0.05$) in experiment by treatment and treatment by sampling day (day 0 and day 9), so a post-hoc analysis was conducted to further analyze interactions among the three factors. Analyses were conducted using SAS version 9.4 software (SAS Institute Inc., 2016) and significance was defined at $\alpha = 0.05$.

Iron concentrations in water samples were analyzed separately by iron treatment level (0, low, or high iron). For controls with duckweed, the model had fixed effects for experiment, sampling day, and interaction among these two factors. For low and high iron treatments the model had fixed effects for experiment, duckweed/fertilizer presence (levels: no duckweed, no duckweed + fertilizer, duckweed + fertilizer), sampling day, and all interactions among these three factors. No significance was found at $\alpha = 0.05$. A compound symmetric covariance structure was used to account for repeated measures on controls and a heterogeneous compound symmetric covariance structure was used to account for repeated measures on low and high iron levels.

2.2 Oyster Mushroom Iron Transfer Experiment

Commercial mushroom-growing kits (Back to the Roots Inc., Oakland, CA), consisting of solid blocks (30.48 cm length x 12.7 cm width x 10.16 cm height) containing spent coffee grounds as a substrate and mycelium of oyster mushroom (*P. ostreatus*), were used to conduct this portion of the study (Figure 2) in a laboratory with an average temperature range of 19-22 ^{o}C under fluorescent lighting at New Mexico State University (NMSU) main campus in Las Cruces.

To initiate the growth of the mushroom, the plastic wrap around the substrate was cut open, and the surface of the substrate was scored and roughened using a fork (Figure 2) following the instructions on the package provided by the supplier. The block of substrate was then soaked in tap water overnight. Moist paper towels were placed on the mushroom kits to prevent desiccation.

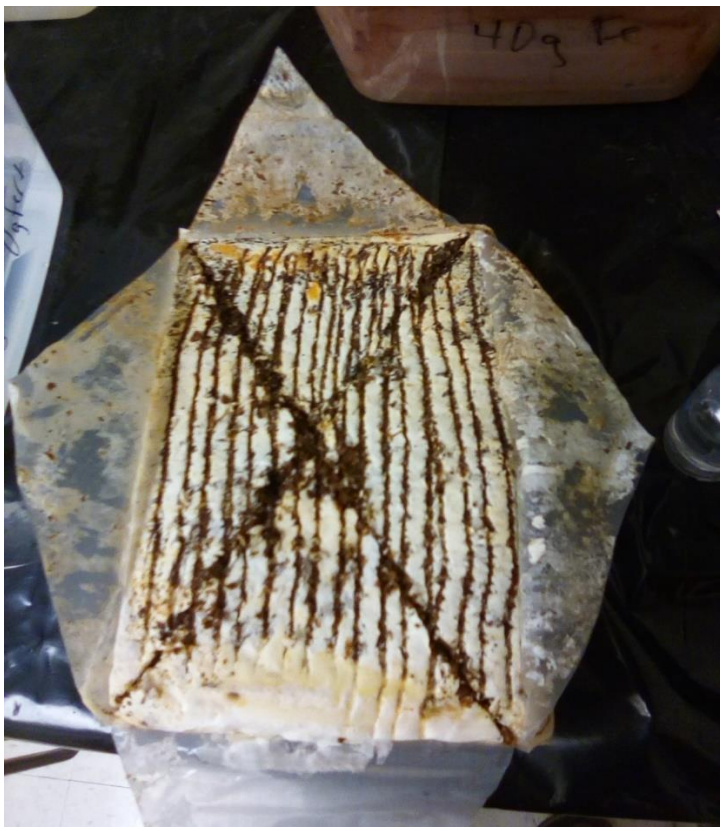

Figure 2. A commercial kit for growing oyster mushroom. Mycelium is visible on the block which was scored with a fork and soaked in water overnight. (30.48 cm length x 12.7 cm width x 10.16 cm height)

The mycelia on the mushroom kits were allowed to grow until they reached maturity, which was defined as the moment fruiting bodies (Figure 3) began to produce spores. The fruiting bodies were then harvested before the mushroom senesced and potentially released any iron it may have taken up. Harvesting also allowed room for new mushroom growth and subsequent harvests.

Figure 3. Fruiting bodies of oyster mushroom.

The harvested fruiting bodies were dried at 65 ^{o}C in an oven and the dry weights were recorded. Dried fruiting bodies were stored at room temperature (~21 °C) in paper bags to avoid mold contamination and were ground using a kitchen spice grinder (Waring Commercial 3 Cup Electric Wet/Dry Power Grinder, model number WSG60, Torrington, CT) before microwave-assisted digestion and ICP-OES analysis.

2.2.1 Experimental Design

The study of transferrable iron from duckweed to oyster mushroom fruiting bodies was set up using a completely randomized design with two iron treatments (low and high), a control with no iron, and a second control with no duckweed. Duckweed from experiment one was used in a composting attempt. Only duckweed from the second experiment was used on the mushroom growing kits. The no-iron control and low iron-treated mushrooms had five replicates (n = 5). The high iron-treated oyster mushrooms only had four replicates (n = 4) because there was not enough harvested duckweed from the high iron treatments. The no-duckweed control also had four replicates due to lack of mushroom kits (n = 4).

Each growing kit was placed laterally into plastic containers, and a thin layer of oven-dried duckweed was placed on top of the kits corresponding to their equivalent treatment (i.e., low iron duckweed used for low mushroom experimental unit, etc.). They were misted daily with tap water using a hand-held spray bottle and the containers were covered with clear plastic wrap to keep humidity high and to reduce contamination from airborne spores. Placement of the grow

kits was determined by assigning each kit a number and placing that kit in a location via random number generation.

Final harvest of fruiting bodies was concluded after five weeks due to contamination by other microbes and their potential to spread throughout the oyster mushroom kits and increase variation in the results.

2.2.2 Statistical Analyses

Total yield on a dry weight basis across all mushroom harvests was statistically analyzed using a one-way analysis of variance (ANOVA) test (SAS version 9.4). Significance was set at $\alpha = 0.05$. A contrast was used to detect differences between harvest means of three duckweed treated and one non-duckweed treated mushroom kits.

The first harvest across all treatments was statistically analyzed for iron concentration using one-way ANOVA and significance was set at $\alpha = 0.05$ (SAS version 9.4). Second and third harvests were omitted from formal analysis due to inadequate harvests within replicates of each treatment.

2.3 Degradation of Duckweed by Oyster Mushroom Mycelium

Spore prints were made using deep dish petri plates (25 x 100 mm depth) placed over potato dextrose agar (PDA) to obtain the mycelium in pure, uncontaminated culture. The cap of the fruiting body was affixed onto the lid of a petri plate using petroleum jelly and placed over PDA medium contained in the base of the petri plate. The fruiting body was affixed in such a

way the hymenium faced the surface of the PDA medium. Spores were discharged from the cap onto the medium and were allowed to germinate into mycelial colonies. A few colonies were transferred to acidified potato dextrose agar (APDA) to limit bacterial contamination and to allow further mycelial growth.

To assess the ability of the oyster mushroom to degrade duckweed, one plug (~8 mm diam.) of mycelium from the APDA used for the spore print was removed and transferred to another APDA plate containing three surface-sterilized duckweed samples (~0.2 g) placed around the edge of the petri plates. To determine if the duckweed was penetrated by the oyster mushroom mycelium, the duckweed was removed from the APDA medium once colonized, surface sterilized for a varying amount of time (15, 30, or 45 s), and re-plated onto a new APDA plate. The protocol for this procedure is represented by Figure 4.

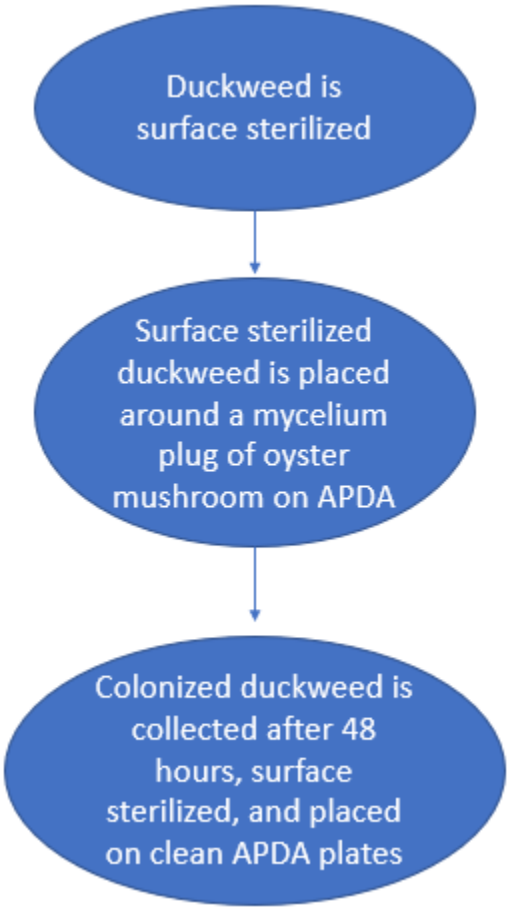

Figure 4. Procedure for evaluating colonization of duckweed by mycelium of oyster mushroom

3.0 Results and Discussion

3.1 Water Results

Daily temperatures of waters for each container were taken and averaged across the duration of the study (nine days) for both experiments. The average temperatures in the first experiment were 19.07 ^{o}C (min) to 19.69 ^{o}C (max), with standard deviations of 0.65 and 0.76, respectively. Average water temperatures across all treatments in the second experiment were 20.19 ^{o}C (min) to 20.29 ^{o}C (max), with standard deviations of 0.47 and 0.46, respectively. Figure 5 shows daily water temperature averages across nine sampling days.

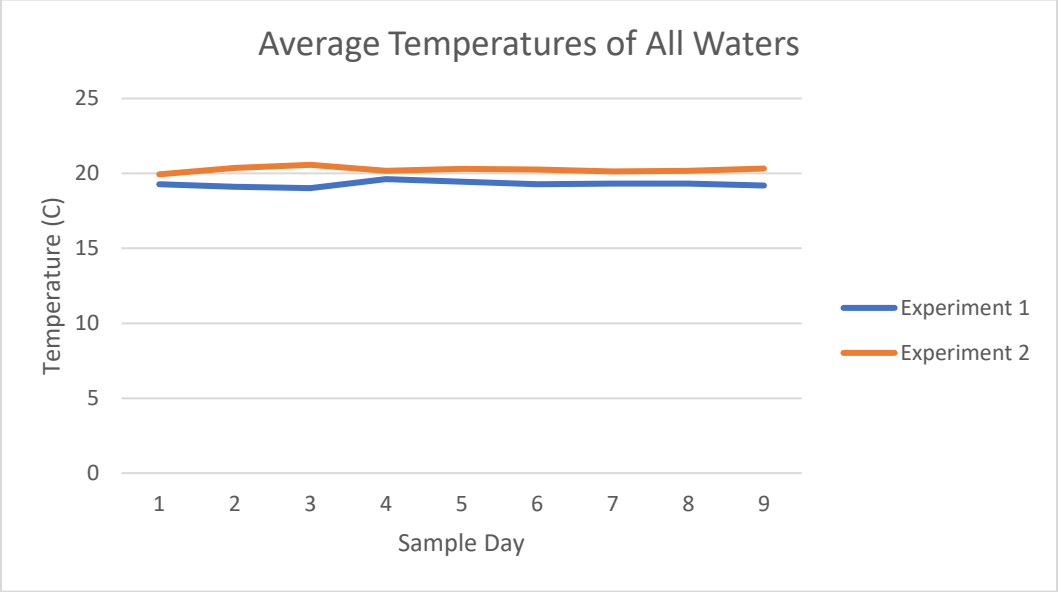

Figure 5. Average water temperatures of both experiments across nine days (n = 315 from 7 treatments x 5 reps x 9 days). Experiment 2 has slightly higher average temperatures than experiment 1.

Daily pH of waters in each container were also taken, and minimum and maximum pHs were averaged across the duration of the study. Average pH of waters across all treatments in the first experiment were 8.09 (min) to 8.38 (max), with standard deviations on 0.22 for both. Average pH of waters across all treatments in the second experiment were 7.83 (min) to 8.37 (max), with standard deviations of 0.54 and 0.26, respectively. Climate controlled conditions

were most likely responsible for the reduced variability in temperature and pH values across both experiments. Figure 6 shows daily average pH values across nine sampling days.

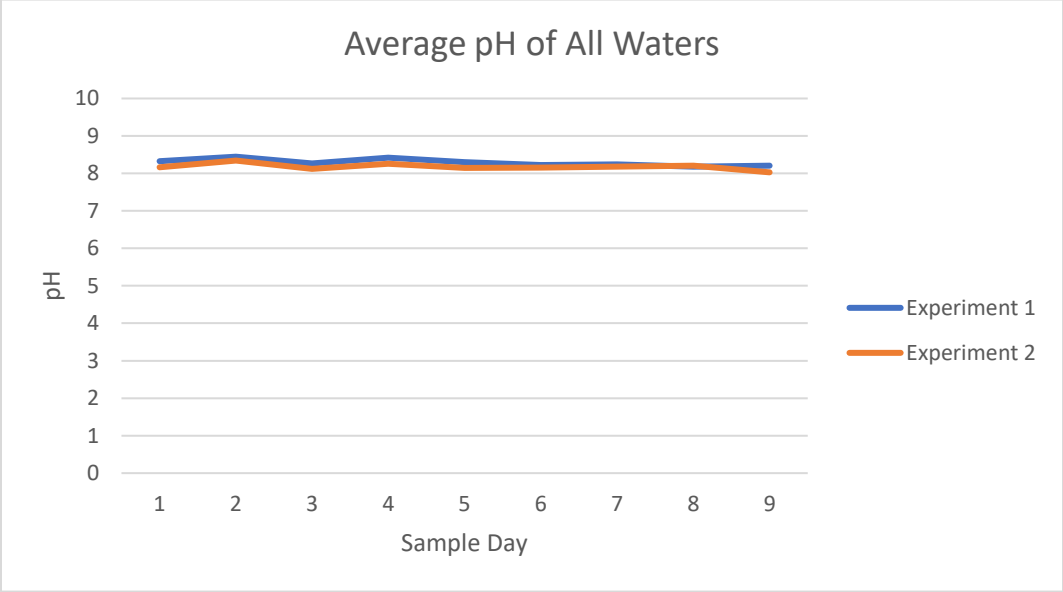

Figure 6. Average pH of all waters across duration of study (n = 315 from 7 treatments x 5 reps x 9 days). Experiment 1 is slightly higher than experiment 2 initially but is consistent with experiment 2 towards the end of the study.

The ICP-OES results indicate that not quite 6% of the iron EDDHA dissolved when added to tap water and aerated for one week. Additionally, thick reddish-brown stains were observed on the walls of each container that had iron included and appeared above the water line after evaporation. These stains did not disappear after refilling with water.

No differences in water iron concentrations were detected with presence of duckweed or fertilizer ($p > 0.05$) indicating that neither of these factors had significant effect on iron removal when compared to iron precipitation. Iron levels in waters of control were significantly different between experiments ($p = 0.0042$) with experiment two having higher iron than experiment one (0.24 (± 0.11 SE) ppm for experiment one and 0.87 (± 0.11 SE) ppm for experiment two). Both low and high iron treated containers showed significance ($p < 0.0001$) in experiment by sampling day but no effects including duckweed or fertilizer presence were significant. Two separate analyses were conducted for iron levels in water, one analysis for low treated containers and one for high treated containers. Iron levels in the waters of both low and high treatments dropped relatively consistent from one sampling day to the next in the first experiment. In the second experiment, iron levels in the low and high treated waters stayed relatively consistent until dropping more on the fourth sampling day. Both low and high iron treated waters in both experiments show similar levels of iron decrease for each treatment across four sampling days (Figures 7 and 8). Lower means were calculated for control in the first experiment and iron treated containers in the second experiment (Tables 2 and 3). This may be attributed to sampling error.

Table 2. Average iron concentrations in water sampled during the first trial in 2019.

Treatment	Duckweed (Y/N)	Iron (g)	Fertilizer (g)	Day 0 Avg Fe Concentration (mg L^{-1})	Day 0 [1]SD	Day 3 Avg Fe Concentration (mg L^{-1})	Day 3 [1]SD	Day 6 Avg Fe Concentration (mg L^{-1})	Day 6 [1]SD	Day 9 Avg Fe Concentration (mg L^{-1})	Day 9 [1]SD
Control	Y	0	1	0.43	0.16	0.17	0.04	0.11	0.06	0.26	0.22
Low Fe	Y	20	1	324.70	75.67	308.30	61.46	204.96	59.60	210.20	60.35
Control 2 (Low)	N	20	1	351.86	12.79	264.52	48.66	219.18	31.65	226.42	42.44
Control 3 (Low)	N	20	0	352.62	33.00	240.41	83.34	221.52	41.94	252.96	26.95
High Fe	Y	40	1	670.70	40.89	425.12	86.03	447.16	69.52	460.06	118.2
Control 2 (High)	N	40	1	710.58	46.86	532.04	155.52	410.60	86.22	440.30	76.70
Control 3 (High)	N	40	0	722.40	50.00	566.10	102.17	379.12	60.34	405.84	101.98

[1]Standard Deviation

Table 3. Average iron concentrations in water sampled during the second trial in 2019.

Treatment	Duck weed (Y/N)	Iron (g)	Ferti lizer (g)	Day 0 Avg Fe Concentr ation (mg L^{-1})	Day 0 [1]SD	Day 3 Avg Fe Concentrat ion (mg L^{-1})	Day 3 [1]SD	Day 6 Avg Fe Concent ration (mg L^{-1})	Day 6 [1]SD	Day 9 Avg Fe Concen tration (mg L^{-1})	Day 9 [1]SD
Control	Y	0	1	0.85	0.36	0.75	0.45	0.96	0.40	0.94	0.40
Low Fe	Y	20	1	306.14	65.59	277.08	31.26	291.50	42.16	199.80	44.19
Control 2 (Low)	N	20	1	278.62	69.91	271.14	78.17	338.48	33.10	183.78	33.45
Control 3 (Low)	N	20	0	259.28	77.94	256.18	53.75	275.58	45.81	143.16	33.58
High Fe	Y	40	1	451.14	67.30	467.88	90.28	500.80	125.14	341.50	111.62
Control 2 (High)	N	40	1	510.04	117.60	484.40	104.56	642.56	35.04	346.14	85.61
Control 3 (High)	N	40	0	482.42	95.83	412.98	69.72	491.76	74.62	276.66	85.44

[1]Standard Deviation

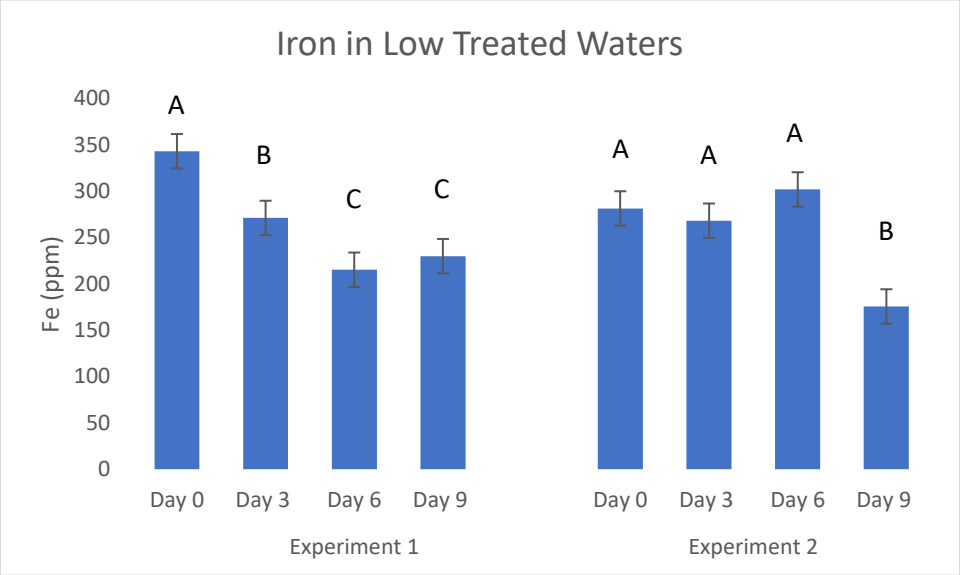

Figure 7. Iron levels in low treated waters (20 g EDDHA/2.90 L) on day 0, 3, 6, and 9 for each experiment. Within experiment, bars with the same letter are not significantly different ($p > 0.05$) according to the Least Squares Means.

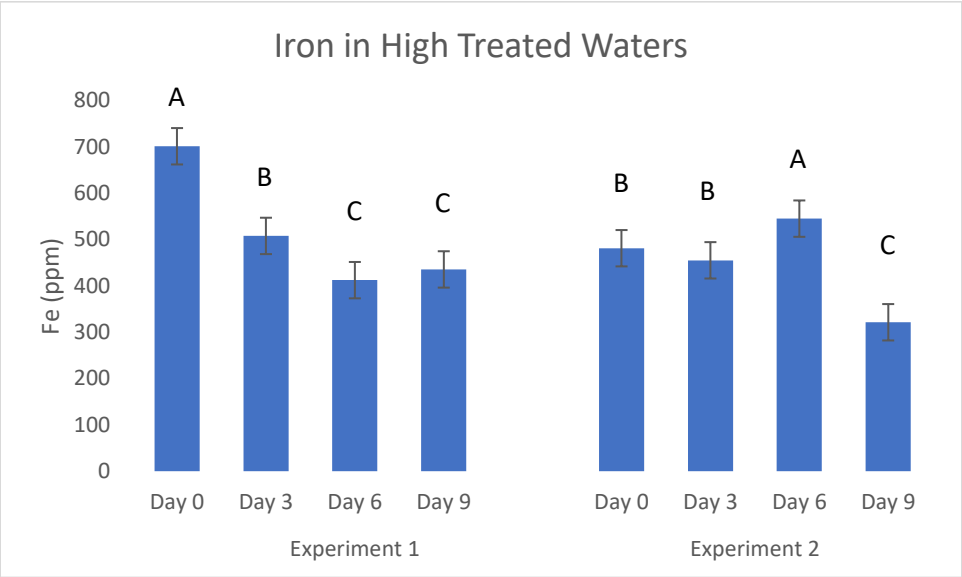

Figure 8. Iron levels in high treated waters (40 g EDDHA/2.90 L) on day 0, 3, 6, and 9 for each experiment. Within experiment, bars with the same letters are not significantly different ($p > 0.05$) according to the Least Squares Means.

Spikes in the water concentration showing iron increase for the high treatment were not significantly different but may have been the result of sampling error or precipitated iron moving back into solution after tap water was replenished to account for evaporation. The mechanism(s) of iron removal were not assessed in this study.

3.2 Iron Removal Via Duckweed

Results indicate no significant difference in iron levels in water with the presence or absence of duckweed when formally analyzed and compared separately by experiment or combined ($p > 0.05$). This result suggests that a similar amount of iron may be precipitating out or binding to the fertilizer, as is sorbed by duckweed.

When formally analyzing and comparing iron sorption between duckweed treatments, there was shown to be significance ($p < 0.05$) between low and high iron treatments and control over time in both experiments. The highest reported iron concentrations were in the high iron treatment and the lowest iron concentrations were in the control, indicating an interaction between the duckweed and iron in solution. Average duckweed iron levels in the first experiment increase between initial and final sampling day across all treatments (Table 4). In the second experiment, average duckweed iron levels decrease for control, but increase for low and high iron treatments, over time (Table 5).

Table 4. Average iron concentrations in (or on) duckweed harvested during first experiment in 2019.

Treatment	Initial Avg Fe Concentration (mg L^{-1})	Final Avg Fe Concentration (mg L^{-1})	Standard Error
*Control	301.20	470.06	85.22
*Low Fe	8150.60	10227.00	506.03
*High Fe	12778.00	14434.00	861.08

*Number of samples (n) = 5 for all occasions. Averages and SEs were obtained from the mixed model analysis.

Table 5. Average iron concentrations in (or on) duckweed harvested during second experiment in 2019.

Treatment	Initial Avg Fe Concentration ($mg\ L^{-1}$)	Final Avg Fe Concentration ($mg\ L^{-1}$)	Standard Error
*Control	988.30	964.66	85.22
*Low Fe	4695.80	8506.20	506.03
*High Fe	8234.00	11271.00	861.08

*Number of samples (n) = 5 for all occasions. Averages and SEs were obtained from the mixed model analysis.

When combining both experiments, significance was found ($p < 0.05$) between low and high iron treatments and control when formally compared. There was also a significant difference ($p < 0.05$) when comparing low and high iron treatments by sampling day over time (Figure 9). For both low and high iron treatments, there was no significance ($p > 0.05$) found when formally compared with iron levels in water. These results suggest an interaction between iron and duckweed, iron and water (precipitation), and iron and fertilizer (binding), although interaction among all three factors was not shown to be significant at $\alpha = 0.05$.

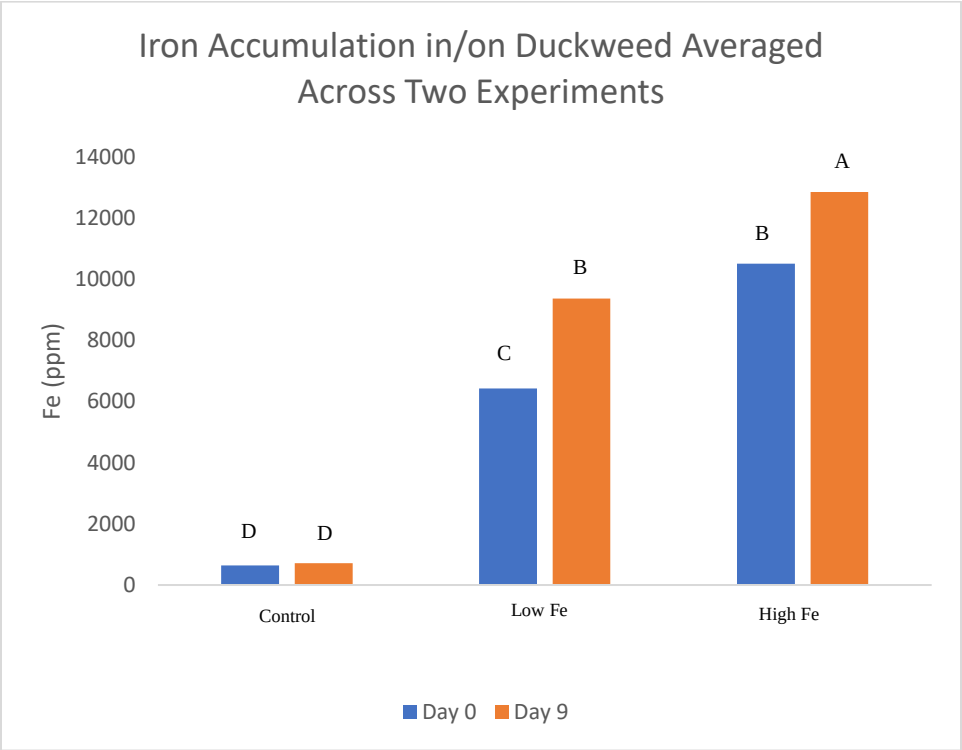

Figure 9. Iron concentration of duckweed combined across both experiments (n = 10 from 5 reps x 2 expts). Bars with the same letters are not significantly different ($p > 0.05$) according to the Least Squares Means.

Oxidative precipitation may have occurred due to the aeration of the water at the beginning of the study and fertilizer binding may have happened due to chemical attraction of the iron to another metal in the fertilizer.

Two potential plant sorption mechanisms related to iron removal from the water via duckweed may be at work – adsorption onto the surface of the duckweed or absorption into the

duckweed. Both mechanisms acting together may also contribute to the iron removal, although this was not examined in this study.

Iron is a transition metal and, for the purpose of this study, was used as an analogue to speculate how other transition metals, such as lead (Pb) may interact with the plant. Singh et al. (2012) explored the potential use of duckweed to remove lead from wastewater effluent. Differences in pH and temperature were shown to influence in the effectiveness of lead removal via duckweed. Duckweed was reported to remove 69% of lead from the wastewater at 20 ^{o}C and pH = 8. The temperatures recorded for our study averaged around 20 ^{o}C with pH = 8 indicating the proper temperature and pH range for potentially effective metal removal.

Teixeira et al. (2014) reported on the use of duckweed as a bioremediating agent for an iron-rich mine effluent. This study found that the mechanisms behind iron removal were most likely due to both absorption and adsorption, as the roots turned an orange-brown color because of the iron that accumulated on them. The study by Teixeira et al. (2014) resulted in the maximum amount of iron removal to be within the first seven days of a 21-day experiment.

3.3 Iron Transfer from Duckweed to Oyster Mushrooms

Average dry weights and standard deviation of fruiting bodies of oyster mushroom harvested under different treatments are shown in Table 6. When comparing overall means, the mushroom kits with duckweed yielded higher on average than the kits without duckweed, although not significantly different (p = 0.4592). This result suggests that duckweed may have assisted in the growth of the mushrooms.

Every replicate for each treatment produced a first harvest but not a second or third harvest so iron concentration was formally analyzed in the first harvest only (Table 7). No significant differences ($p = 0.2549$) were found at $\alpha = 0.05$ in iron measured in the mushroom fruiting bodies when formally comparing first mushroom harvest data across all treatments (Figure 10). This result suggests that iron was not transferred from the decaying duckweed into the mushroom fruiting bodies.

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Table 6. Average dried oyster mushroom total yield across all available harvests.

Treatment	Duckweed (Y/N)	No. of samples (n)	Average Harvest Weight (g)*	Standard Deviation
Control	Y	5	11.15	5.07
Control 2	N	4	6.86	8.78
Low Fe	Y	5	7.93	6.77
High Fe	Y	4	9.23	2.65

Table 7. Iron accumulation (ppm) in oyster mushroom fruiting bodies from first harvest*

Treatment	Harvest 1 Average Fe Concentration (mg kg^{-1})	No. of samples (n)	Standard Deviation
Control	67.65	5	11.19
Control 2	76.63	4	11.65
Low Fe	62.04	5	4.92
High Fe	64.73	4	13.90

*Dates of harvest not included due to variations in harvest dates within replicates of treatments

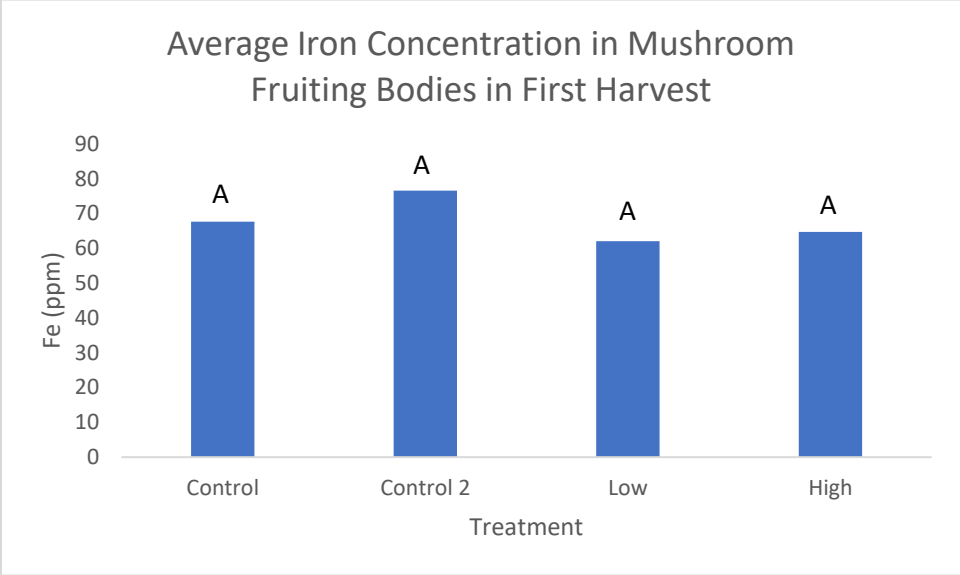

Figure 10. Average iron concentrations in mushroom fruiting bodies in first harvest. Sample size varies by treatment (n = 5 for control and low; n = 4 for control 2 and high). Bars with the same letters are not significantly different ($p > 0.05$) according to the Least Squares Means.

Iron's role as a transition metal makes it suitable for our study as an analogue for other transition metals and how they may act in a similar environment. Iron was not found to be transferred from duckweed to mushrooms so it can be speculated that other transition metals would also be unable to transfer, although more research must be done to confirm this. If this holds true, oyster mushrooms may have the potential to break down plants containing toxic transition metals such as cadmium or mercury and remain a viable, edible food source if below recommended values for metal(s) consumption provided by the WHO and U.S. EPA.

In our study, some of the mushroom grow kits experienced a pink growth which was then isolated on APDA (acidified potato dextrose agar) and viewed under a compound microscope to determine if it was of an outside origin or if it may be identical to the oyster mushroom mycelium (Figure 11). When observed under the microscope the pink material did not contain fungal hyphae and was amorphous. The pink growth may be due to a secondary metabolite produced by the oyster mushroom, although we lack evidence to prove this. *Pencillium* sp., a ubiquitous fungus, contaminated some of the kits (Figure 12). This fungus was mechanically removed as much as possible using a sterilized razor blade before a serious spread occurred.

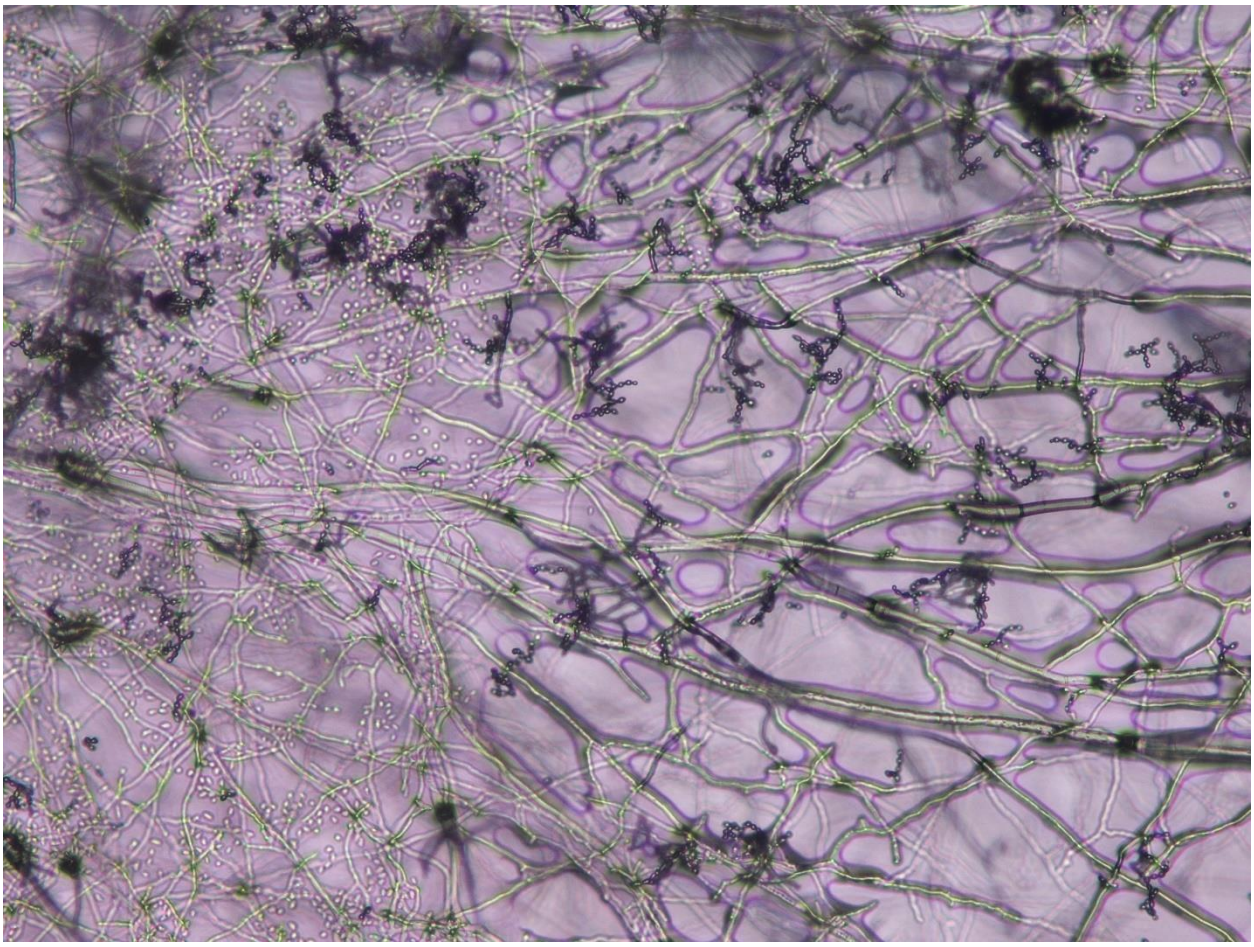

Figure 11. Septate mycelium from oyster mushroom.

Figure 12. *Penicillium* sp. (green) growing on mushroom grow kit.

The iron concentrations found in the mushroom fruiting bodies from our study correspond with the iron concentrations reportedly found naturally in oyster mushrooms. Many authors (Vetter, 1994; Tüzen et al., 1998; Demirbas, 2001; Isildak et al., 2004; Gençcelep et al., 2009; Patil et al., 2010) have reported natural iron concentrations in fruiting bodies of oyster mushrooms to be at 48 to 280 mg kg^{-1} on a dry weight basis. This coincides with our findings.

Mushrooms analyzed in the controls and low and high iron treatments in our study all retained values within 48 to 280 mg kg^{-1} (Figure 10). This result suggests that iron did not directly transfer from the duckweed to the mushroom fruiting bodies following colonization of the duckweed. This may be explained by way of intracellular siderophores, which are iron storage compounds within the fungi. If iron is not needed by the fruiting bodies, it can be retained within the intracellular siderophores and stored until needed (Winkelmann 2001, 2002). A study done by Almeida et al. (2015) demonstrated the ability of oyster mushrooms to grow in an enriched iron substrate. Their study found that mycelium was able to grow in a high iron substrate and bioaccumulate some of the iron in its mycelium. The study done by Almeida et al. (2015) did not analyze the fruiting bodies of the mushrooms.

The oyster mushrooms colonized the duckweed at 100% in all three treatments, signifying its ability to grow and survive on iron-enriched duckweed. Since reports of iron-extracting mycelium exist, mycelium in our study was not analyzed for iron content.

3.4 Degrading Duckweed with Oyster Mushroom Mycelium

Using mushroom mycelium as a disposal avenue for iron-enriched duckweed was the final phase of this study. Oyster mushrooms are a primary decomposer and a naturally saprophytic species (Stamets, 2005) used to degrade plant and animal tissue. Our study showed that mycelium penetrated and was retained within the duckweed across all treatments. The mycelium colonized the duckweed within two days of being transferred (Figures 13 and 14). Results showed mycelium starting to emerge from the duckweed within two days of the second

plating and colonized the petri plate within one week (Figures 15 and 16). This result speculates the use of oyster mushroom mycelium as a disposal avenue for iron-enriched duckweed. Using oyster mushroom mycelium as a means of degrading contaminated plant material, however important, remains largely unexplored in previous studies.

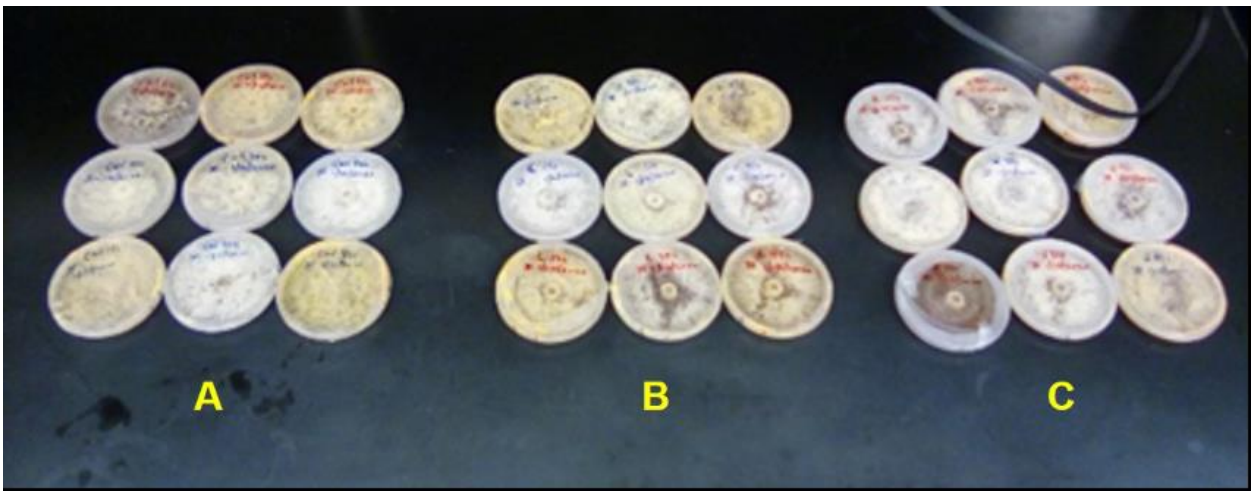

Figure 13. Colonization of duckweed by mycelium of oyster mushroom on APDA plates under three treatments: (A) Control, (B) Low Iron Treatment (20 g Fe), and (C) High Iron Treatment (40 g Fe)

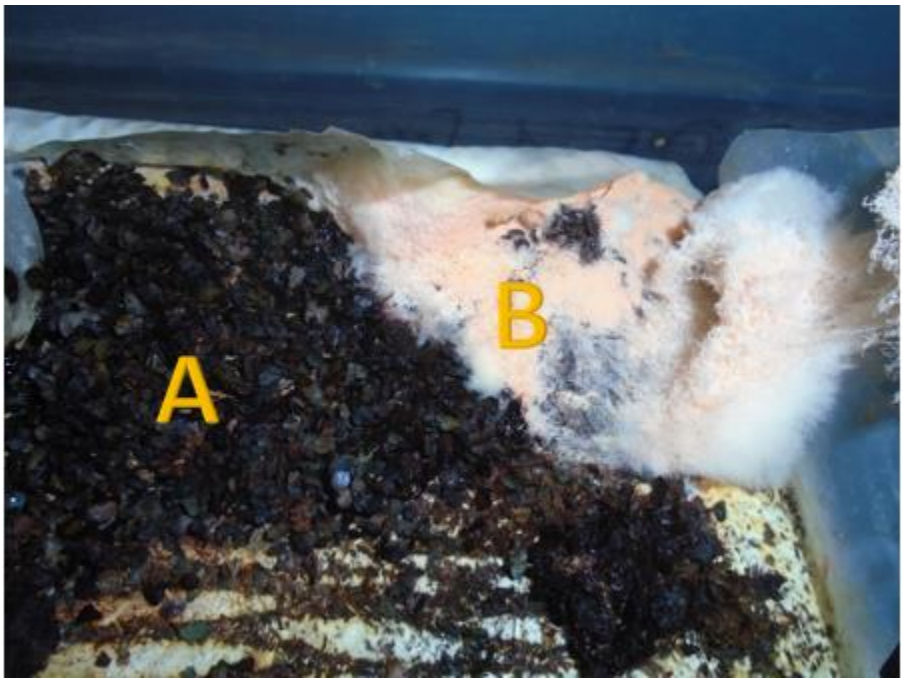

Figure 14. Colonization of duckweed (A) by mycelium (B) of oyster mushroom in high iron treatment

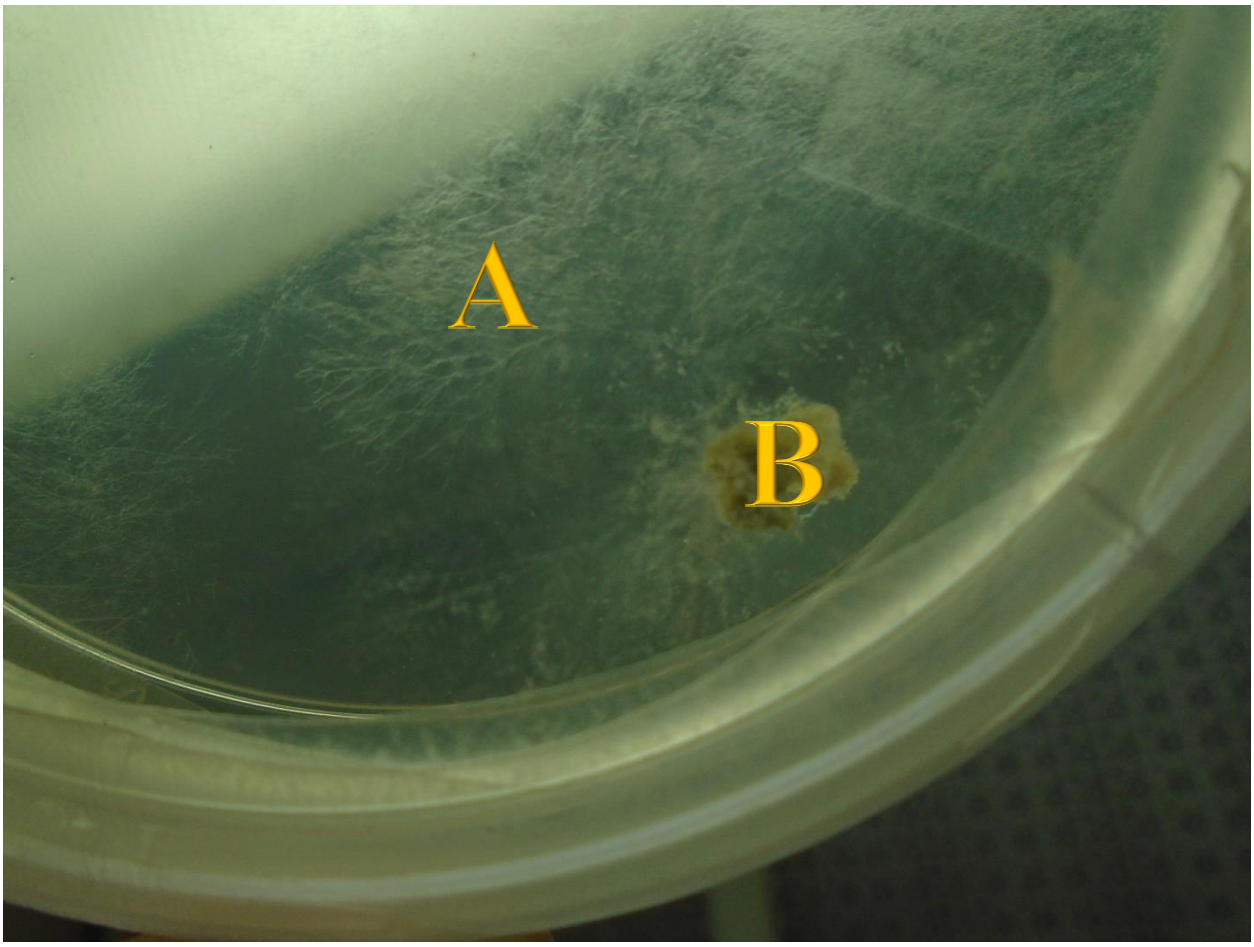

Figure 15**. Close-up of mycelium (A) recovered from duckweed (B).**

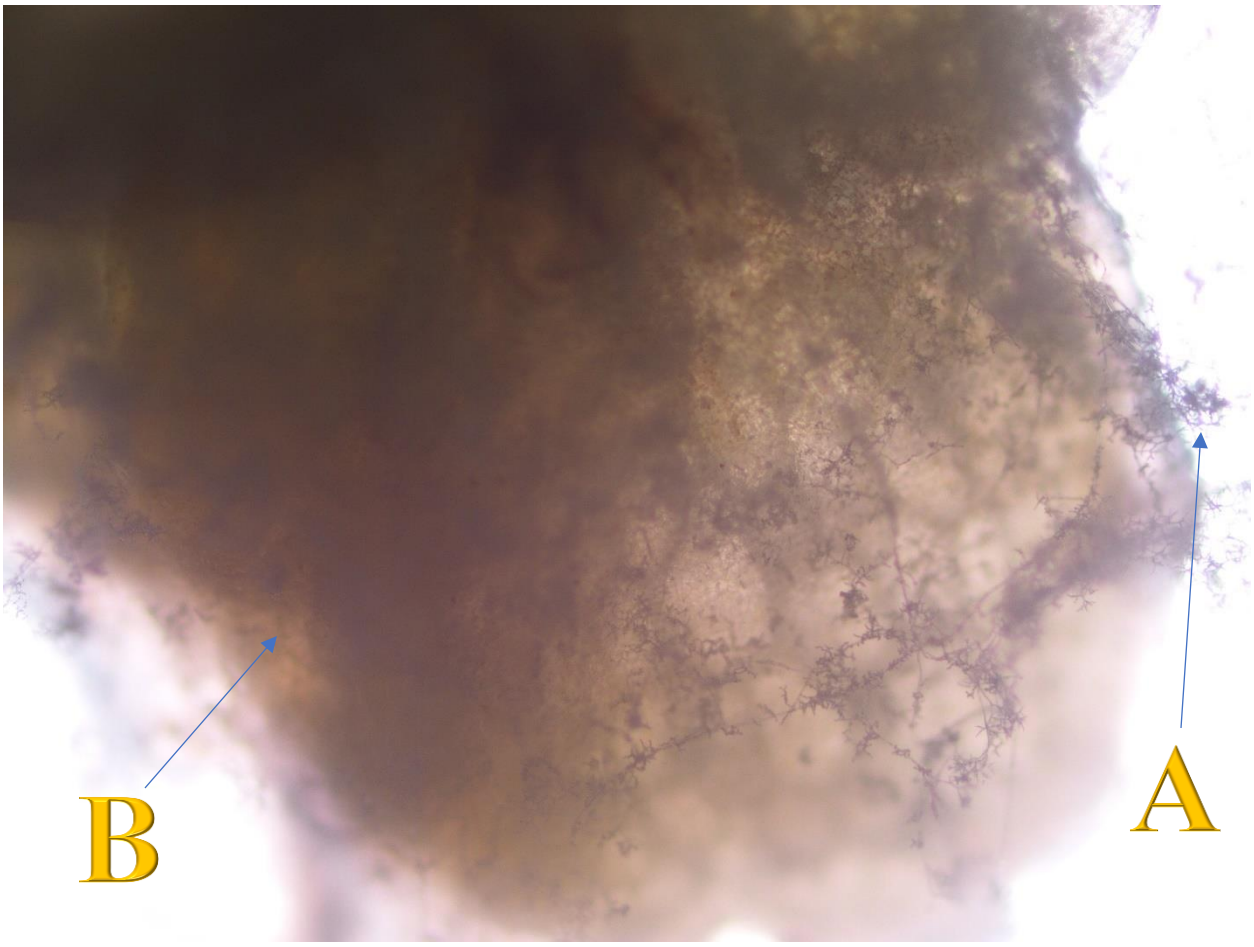

Figure 16. Oyster mushroom mycelium (A) recovered from duckweed (B) at 100% frequency.

Oyster mushroom mycelium grew out of plated duckweed with a recovery rate of 100% so no formal analysis was conducted. This shows that mycelium was retained within all duckweed samples across all treatment levels.

Oyster mushrooms have been reported to grow on a wide array of substrates, including substrates with high levels of nitrogen. Gern et al. (2008) found that nitrogen is the primary

nutrient that yields a greater harvest of oyster mushroom fruiting bodies. An increase in nitrogen was shown to increase maximum yield in their study. Due to duckweed's high nitrogen content and studies showing oyster mushrooms' ability to grow in high nitrogen environments (Obodai et al., 2003; Almeida et al. 2015), it is implied that oyster mushrooms can successfully colonize and degrade duckweed and this study has assessed that implication.

The degradation of metal-laden plant material is important to address after using plants in phytoremediation efforts, so the potentially toxic metals do not get released back into the system they are being removed from. Many studies have assessed the ability of oyster mushrooms to grow on and degrade various materials, such as wood and straw, but have not looked at the mushrooms' ability to degrade enriched iron plant material. This study was done to assess the use of oyster mushroom mycelium as an environmentally friendly disposal method of contaminated plant material.

To date, degradation of contaminated plant material is often carried out by way of composting or incineration. A composting experiment was explored in our study as a means of degrading the duckweed. Restriction in time along with the slow decay of duckweed as the sole component of a compost pile discouraged this approach.

A study done by Kostecka and Kaniuczak (2008) recognized the slow decay of duckweed and set up experiments using vermicomposting (composting with the addition of earthworms). Their study resulted in a faster degradation time of the duckweed as well as a compost with good granular structure, an absence of odor, and low levels of toxic microelements such as cadmium

and lead. Vermicomposting was not attempted in our study due to difficulties in tracking the location of the iron within the compost with the addition of worms.

Duckweed tends to survive on small amounts of moisture, which is another disadvantage of the sole decomposition of duckweed. The optimum moisture range for a compost pile, as reported by Dehghani et al. (2012), is 40% - 60%. Dehghani et al. (2012) also reported health issues in individuals due to some primary fungal species found in compost, such as *Aspergillus* spp. It is therefore pertinent that alternatives to the degradation of metal-enriched duckweed be explored.

4.0 Conclusions and Future Directions

Iron levels in water dropped consistently in all containers in the experiment one and showed similar levels of decrease in experiment two until the final sampling day. On the final sampling day for experiment two, iron levels in the water showed a sharper decline when compared to experiment one. Duckweed and fertilizer presence showed no significant effect on the removal of iron. Results suggest that iron removal from water may be due to iron precipitation, and minimal removal via fertilizer binding or duckweed, although no significant effect among these three factors was found at $\alpha = 0.05$. Hard precipitates were observed and oxidation of the solution to help with dissolution most likely increased the rate of precipitation.

Iron levels were similar in the mushroom fruiting bodies first harvest and remained at their natural level (48 to 280 mg kg^{-1}) across all treatments in the first harvest. This indicates that

iron from the decaying duckweed may not be transferring into the mushroom fruiting bodies. The mycelium was not tested for iron concentration in this study.

Oyster mushroom mycelium was shown to colonize the duckweed. Recovery rates showed that the mycelium penetrates the duckweed and is speculated to break it down in the process due to its saprophytic nature. Amount of iron in or on the duckweed showed no effect on the growth and colonization of the mycelium. Inclusion of duckweed, when compared to exclusion of duckweed, resulted in higher total yield of mushroom fruiting bodies, suggesting that duckweed may assist in the growth of the mushroom.

Future directions include conducting another compost experiment due to the unsuccessful attempt with this study. Culturing different microorganisms found in the compost would be applied to the duckweed to help accelerate the rate of decomposition. Employment of oyster mushrooms may assist in active degradation in compost as well. Compost has been shown to be a sustainable way to dispose of biological remediators in an environmentally friendly manner. Vermicomposting, defined as composting using worms, has been shown to be the best method of composting when decomposing duckweed (Kostecka and Kaniuczak, 2008). Duckweed has a very low decomposition rate when it's the sole component, so adding worms may increase the rate of decomposition (Kostecka and Kaniuczak, 2008). This is a method that may be explored in future studies.

If the study were to be repeated, a protocol for when, where, and how to sample would be included to eliminate or minimize sampling error and variance. Repetition of the mushroom portion of the study would also be employed to match the repetition in the duckweed portion and

conclude our findings. Mycelium would be tested to account for its potential role in iron uptake and transfer and individual duckweed plantlets would be analyzed via electron microscopy to assess the mechanism responsible for iron sorption in the plant. Roots and shoots would be analyzed separately to assess where iron is accumulating on or within the plant tissue.

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