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The influence of hydrogen peroxide on the enrichment of Fe(III) reducing bacteria from acid mine drainage

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Abstract

It is hypothesized that the ocean of Europa, a Jupiter moon, hosts bacteria on its oceanic floor. Understanding how Fe(III) reducing bacteria (FeRB) from AMD utilize organic materials within its surrounding environment outlines how FeRB could thrive and tolerate extreme conditions. FeRB are known to tolerate metals and highly reactive oxidants species (ROS), but in this experiment, H_2O_2 was the experimental factor to further test FeRB tolerance. H_2O_2 is a common ROS and is damaging to living material such as proteins, DNA, and RNA. A range of H_2O_2 concentrations were fed to the FeRB from AMD to measure their tolerance and see if their growth was inhibited. The FeRB was retrieved from the Friedline Mines acid mine drainage (AMD) of the Powdermill Nature Preserve in Pennsylvania. The FeRB were monitored over 0.9 mM, 0.45 mM, 0.23 mM, 0.12 mM, 0.012 mM, and 0.0 mM H_2O_2 concentrations. The FeRB could not survive within the medium with H_2O_2, but there were concentrations of H_2O_2 that tolerated the oxidant more than others. Bacteria could not tolerate 0.9 mM H_2O_2 but were able to tolerate 0.45 mM and 0.23 mM H_2O_2 within the anoxic FeRB media. Treatments with H_2O_2 had 0.46-11.3 ug/mL DNA in the original enrichment, but did not have any measurable DNA in the latest enrichment, after 5 transfers. The samples that had the strong oxidant added did not survive, but different concentrations had a higher tolerance than other H_2O_2 concentrations.

1.1. Search for Extraterrestrial Life

The only habitable world known to support life is Earth. However, lack of biological evidence on other worlds does not mean there is no life outside of Earth. There are many galaxies known to exist, which leads many to think there must be some form of life other than that sustained on Earth (Talbert, 2021; Garner, 2022). It is estimated with the Hubble Space Telescope that there are about two trillion galaxies within our observable universe.

When looking for life, there are a few necessities that are crucial. While biota is likely to function differently from most life on Earth, the building blocks of life should be similar. Habitability is dependent on liquid water, energy sources, and the necessary elements for the organism (Hand et al., 2009). Creating possible scenarios of how biologically useful energy is created and exchanged could give astrobiologists a grasp on how life functions on these worlds. Matter and energy are both part of a bigger process that allows life to process the molecules and elements in their surroundings. It is common for life on Earth to use nitrogen, phosphorus, sulfur, and other trace amounts of metals due to their abundance and significance for plant growth (Hand et al., 2009). To produce biomass, plants and bacteria must use primary production. Energy can be obtained through photosynthesis or chemosynthesis, dependent on what is available for the organism to biologically convert inorganic carbon, CO_2, to organic carbon. Plants and bacteria that have access to sunlight can use its composition to fix the CO_2 via photosynthesis. Solar energy has to be attainable for photosynthesis to occur. Not all primary producers utilize the sunlight because of limited access. Chemosynthesis-using plants and bacteria rely on redox reactions (Gaidos et al., 1999). These two processes are the major ways to produce biomass and having these components increase the likelihood of life in any given world.

1.2. Potential Life on Europa

On the list of the possible habitable worlds by NASA, icy moons are being considered (*Habitable Worlds*, NASA, 2022). From NASA's *Galileo* missions, Europa is known to host a salty liquid ocean beneath a layer of ice on the surface with a rocky oceanic floor (McKinnon et al., 2009; McCord et al., 1999). It is groundbreaking for Europa to contain liquid water, leading astrobiologists to consider this Jovian moon a possible habitable world. However, there are still many other factors to consider beyond the presence of liquid water. The habitability of Europa's oceans is a distinct issue from the ocean containing the building blocks for life (Hand et al., 2009). The energy that is readily available will affect the biota's productivity and metabolism, as well as the ocean's chemical composition. These chemical reactions would be reliant on the abundance of the oxidants and reductants in the system and the rate of the reaction occurring. To sustain chemotrophic life, energy is obtained using oxidation redox gradients to remove electrons from reactants. This process relies on the environment supplying these electron donors. Examples of oxidants include O_2 and H_2O_2 (Greenberg, 2010). Within Europa, it is likely this process occurs from the oxidized ice layer reacting with the bottom of the liquid water with the mafic or ultramafic rock bottom. Therefore, the thickness of the oceanic rock compared to the thickness of ice layer is crucial to the productivity of oxidation (Greenberg, 2010).

One possibility of life inhabiting Europa is by microbial life processing the ocean into meltwater on the surface. Radiation would be the driving force for surface microbial life. If biota were to exist within this ocean, life would need to thrive within the salty ocean's extreme conditions and need some other means besides sunlight to fix inorganic carbon to organic carbon. Due to Jupiter's magnetosphere and the charged particles, Europa's atmosphere can provide many organic and oxidant molecules (Chyba, 2000). There may be an abundance of

oxygen, hydrogen peroxide, and organics on Europa from the charged particles' interaction with the ocean water. Detected from the Galileo spacecraft, H_2O_2 is absorbed at concentrations of 0.13 % relative to the water ice (Chyba, 2000; Carlton et al., 1999). Another method is proposed by others that would support that microbial life is within the ocean, under the ice layer. The ice is considered relatively thick, which poses a problem with how much sunlight can penetrate the ocean. Though, researchers propose reduction-oxidation processes for the source of chemical energy (Gaidos et al. 1999; McCollum, 1999; Kargel et al., 2000; Zolotov and Shock, 2004; Zolotov and Kargel, 2009; Hand et al., 2009; Pasek and Greenberg, 2012; Vance et al., 2016; Russell et al., 2017). For reduction-oxidation to occur, there would need to be hydrothermal vents at the bottom of the ocean. Metabolites such as proteins, nucleic acids, and membranes would be synthesized through methanogenesis processes or the delivery of oxidants from the surface to the bottom of the ocean (McCollom, 1999; Huber and Wächtershäuser, 1997; Chyba and Phillips, 2002; Heinen and Lauwers, 1996; Miyakawa et al., 2002; Hudson and Moore, 1999; Dworkin et al., 2001).

The composition of Europan oceans remain unknown, but there are many hypotheses that give a framework of its chemical make-up. By inferring near infra-red mapping collected from the *Galileo spacecraft* mission, there may be hydrated salts of magnesium and sodium sulfates, and sodium carbonates within the ocean (McCord et al., 1999). Another study interpreted the magnetometer data of the magnetic fields of Europa and concluded that there are dissolved, conducting salts within the oceans (Khurana et al., 1998). A variation on this idea is a study that argues NaCl, KCl, and $MgCl_2$ are the salts in abundance in Europan oceans, rather than magnesium sulfate. Furthermore, the argument is that magnesium sulfate is not present throughout Europa and is a chemical product only in the trailing hemisphere (Brown and Hand,

2013). While there are debates on what salts are present, these authors agree that Europa has a salty ocean. The lower limit of the ocean is likely to resemble freshwater with ~3 ppt and the upper limit is close to or less than Earth's oceans with estimated levels of 100s of ppt of $MgSO_4$ (Zolotov and Shock, 2001; Zolotov and Kargel, 2009).

1.3. Acid Mine Drainage (AMD)

When discussing the delivery rates of reduction-oxidation chemical reactions, Europan oceans could either be highly reducing or highly oxidizing, or similar to the rate on Earth. Pasek and Greenberg argue that Europan oceans are highly oxidizing and highly acidic and have proposed a model. With this model, less than 10 km of the top oceanic rock is permeable and interacts with the ocean (Pasek and Greenberg, 2012). Furthermore, reactive oxygen species (ROS) would need to be present to be delivered to the bottom of the ocean, implying that biota could have an aerobic-like metabolism. There is a connection made with the characteristics of Pasek's model of Europan oceans and acid mine drainage (AMD). AMD is commonly found on Earth and are also high in acidity and behave similarly (Pasek and Greenberg, 2012).

Acid mine drainage is created when O_2-rich water percolates through mines that have exposed anoxic rock. These underground mines were previously undisturbed and are usually abandoned after metal extraction, leaving pockets of metal sulfides that become oxidized. The reaction that occurs is:

$$4FeS_{2(s)} + 14O_{2(g)} + 4H_2O_{(l)} \rightarrow 4{Fe^{2+}}_{(aq)} + 8{SO_4}^{2-}{}_{(aq)} + 8{H^+}_{(aq)} \qquad \text{(Equation 1)}$$

The O_2-rich water becomes high in trace metal content and yields acid-rich fluids, usually Fe(II) (Equation 1). When this acidic water eventually reemerges above surface, it enters circumneutral waters. Then, the water oxidizes due to exposure of the highly oxygenated atmosphere.

Following oxidation, the Fe(II) that is dissolved in the water oxidizes and precipitates out as Fe(III) in the water body or stream above ground.

For the oxidation reaction to occur, there needs to be a high concentration of O_2. This condition is met when the anoxic water meets the atmosphere that is rich in oxygen. Oxygen rapidly oxidizes the dissolved iron sulfide in the water and forces the metals to precipitate into Fe(III) and sulfate aqueous molecules (McKinnon et al., 2009). The pyritic sulfur reacts with high concentrations of O_2 which lowers the O_2 in the system and creates acidic levels. The acidity lowers the pH in AMD and does not support most biota. The water that came from the mine is now incorporated into the flowing stream water, increasing its acidity, lowering its pH, consisting of high amounts of dissolved metals, and changing the pre-existing conditions to the stream (Pomeranz et al. 2020; Abinandan et al., 2020).

The main issue with Pasek's model is the presence of the ROS molecules. These chemical species are unstable and make them react easily with other molecules. Their reactivity is detrimental to organic molecules such as DNA, RNA, and proteins, which are often targeted by these free radicals. O_2, H_2O_2, and OH are common ROS molecules and using Pasek's model, these ROS molecules would be derived by radiolysis of the surface ice (Pasek and Greenberg, 2012; Lobo et al., 2010). An issue arises with the delivering process of oxidants down to the bottom of the ocean.

1.4. Iron Snow Model

Considering how closely the two systems resemble one another, it is likely that the biota in AMD could simulate microbial metabolism in the oceans on Europa. AMD contain acidophilic microorganisms that can survive extreme conditions (McKinnon et al., 2009;

Abinandan et al., 2020). AMD systems are biologically and geochemically simple which makes analysis of microorganism ecology simple (McKinnon et al., 2009). There are limited metabolisms that can exist in these conditions. To ensure their survivability, a steady source of redox and proton gradients is needed for their metabolism to function (Merino et al., 2019). Terminal electron acceptors consists of CO_2, NO_3^-, SO_4^{2-}, Fe(III), O_2, H_2O_2, and ROS molecules that can make the delivery possible.

The Iron Snow model is a mechanism that can deliver oxidants while addressing the issues that Pasek's model had. Under the highly acidic and sulfate-rich conditions of the ocean, the Iron Snow model on using Fe to the organics on the oceanic floor (Figure 1). Due to ROS's highly reactivity and harmfulness to biota, there would need to be some process that separates the ROS from the organics. Fe is a major driver of redox reactions within AMD, which Pasek's model does not discuss. Aerobic ferrous iron oxidation and anaerobic ferric iron reduction occur together and are cycled in AMD (Brantner et al., 2014). Based off this process, the Iron Snow model incorporates the cycling of Fe(II) and Fe(III) in an equivalent exchange. In acidic bodies of liquid water, dissolved Fe(II) is oxidized near the surface. Resulting Fe(III) breaks down in the water and becomes insoluble Fe(III) hydroxides on top of the benthic sediment. Once the reaction occurs between Fe(II) and Fe(III) at the surface of the ocean, the insoluble $Fe(OH)_3$ will fall and settle on the benthic rock, to the bacteria and biota. $Fe(OH)_3$ acts as 'snow', hence the 'Iron Snow' name, and serve as electron acceptors during the oxidation process of sulfates. This model is closely modeled after how iron sulfide rich rocks in AMD react quickly with oxygen in an oxygenated atmosphere (Baker and Banfield, 2003). However, there is a lack of oxygen in Europa's ocean system.

It is energetically favorable for organisms respiring O_2 because oxygen yields the most energy, but when an environment lacks it, organisms move to the next readily available oxidant (Figure 1). Microorganisms in these oceans could respire Fe(II), using this chemical reaction:

$$Fe(OH)_3 + 3H^+ + e^- \rightarrow Fe^{2+} + 2H_2O \quad \text{(Equation 2)}$$

Using this reaction, microorganisms use iron hydroxide for respiration to break down their food. Based on equation 2 and the research approach conducted by Santangelo and Senko (2020), iron-reducing bacteria from acid mine drainages will be enriched. This research will test the tolerance of Fe(III) reducing bacteria (FeRB) to hydrogen peroxide. These microorganisms from AMD may tolerate strong oxidants. The presence of the iron snow, $Fe(OH)_3$, allows ROS molecules to react with it, keeping it away from organics:

$$Fe^{2+} + H_2O_2 = Fe^{3+} + \bullet OH + OH^- \quad \text{(Equation 3)}$$

$$Fe^{3+} + H_2O_2 = Fe^{2+} + \bullet OOH + H^+ \quad \text{(Equation 4)}$$

The Iron Snow Model produces insoluble $Fe(OH)_3$ that would oxidize reducing benthic sediments, which could prevent ROS molecules from damaging Europan benthos. Biota would be protected from the reactivity. Also, under this model, the whole ocean does not need to be oxidized since the Fe(III) hydrolysis occurs at the surface of the ice and water layer. The hydroxide is safely transported to the bottom of the ocean to organics that need oxidants.

Oxidants mentioned before were O_2 and H_2O_2. The presence of oxygen would be energetically favorable and allow bacteria to metabolize organic carbon, but the ice layer above the ocean stops the sunlight from penetrating into the ocean and allowing bacteria to photosynthesize. This impediment for photosynthesis increases the likelihood for chemosynthesis bacteria. Oxidants are the driving chemical species that allow biota to

metabolize inorganic carbon into organic carbon. ROS are oxidants, but due to their highly reactive nature, these chemical species are harmful. That is why the Iron Snow model is proposed because ROS are utilized into the system while factoring in how to separate the harmful ROS from living bacteria. The ROS would be kept near the surface of the ocean and react with other species, while $Fe(OH)_3$ would fall down to benthic biota.

Though AMD bacteria are resistant to high traces of metals and extreme living conditions, it is worth exploring if the FeRB are resistant to strong oxidants. If not, what concentration of the strong oxidant is considered tolerable for these bacteria that live in AMD conditions. Conducting this experiment will let us gauge how tolerant AMD microorganisms can be towards hydrogen peroxide (H_2O_2) under anaerobic conditions. It is hypothesized that a strong oxidant, H_2O_2 inhibits growth of FeRB, but some H_2O_2 concentrations may be tolerated by the organisms. The research will determine if, and what concentration of H_2O_2 inhibits FeRB growth.

2. Material and Methods

The source of the acid mine drainage acquired for this research was the Friedline Mines AMD treatment system at the Powdermill Nature Reserve in Pennsylvania, United States (Figure 2). To test the effect of H_2O_2 on the enrichment of FeRB, a medium composed of distilled water, 0.625 g/L $(NH_4)_2SO_4$, 0.496 g/L $MgSO_4$ * 8 H_2O, 0.125 g/L tryptic soy broth (without glucose), 0.45 g/L glucose, 5 g/L $Fe_2(SO_4)_3$, 2.5 ml/L Tanner's vitamins, and 2.5 mL/L Tanner's trace metals was incorporated with varying concentrations of hydrogen peroxide (0.0 – 0.9 mM) (Santangelo and Senko, 2020). After creating the medium, NaOH was added to bring the pH

down to 4.5, using a pH probe and meter used to read the pH. The media was placed into a flask and bubbled with N_2 for 15 minutes to create an anoxic media. The media was distributed into 18 test tubes, each with 10 mL and autoclaved to sterilize the media. All samples were consistently monitored under anaerobic conditions.

3% H_2O_2 was used to achieve concentrations of 0.9 mM, 0.45 mM, 0.23 mM, 0.12 mM, and 0.0 mM in the complete media (Table 1). The first 18 test tubes with the respective H_2O_2 concentrations were used as the original enrichment. For each concentration of H_2O_2, two test tubes were inoculated with 1 mL of the added Powdermill Nature Preserve AMD sample, and one test tube stayed uninoculated for a control group. There were 5 total enrichments made by removing 1 mL medium from the previous enrichment and transferred to the new medium to a new set of 18 test tubes.

Periodically, 0.2 mL was taken out of each sample to measure the Fe(II) concentrations. Active, growing FeRB microorganisms respired Fe(III) hydroxides and produced Fe(II). The collected media were placed into a 1.5 mL centrifuge tube with 0.2 mL HCl (0.5 M) and centrifuged at 12000 rpm for 5 minutes (Figure 4). The added HCl solubilized any solid-associated Fe(II), allowing all Fe(II) to be measured. The samples were centrifuged and the supernatant was collected, and the Fe(II) concentration was measured with a spectrophotometer using ferrozine assay (Table 5-8; Stookey, 1970).

The microorganism's growth was also assessed by measuring the DNA concentrations in each medium. DNA samples from the original enrichment and the 5^{th} enrichment were extracted using the DNeasy® PowerSoil® Pro Kit by QIAGEN. 0.5 mL was extracted from each of the 38 samples and added to the solutions provided in the kit. A homogenizer, vortex machine, and the centrifuge were used to extract the DNA from each medium. A Quibit® 3.0 Flurometer by

Thermo Fisher Scientific was used to measure the DNA concentrations of each sample following DNA extraction.

3. Results

The 18 samples' Fe(II) concentrations were monitored over the course of 225 days and transferred to new FeRB medium five times. The mean and the standard deviations of duplicate samples were calculated. Within all the samples, the Fe(II) accumulated at different rates (Figure 5-7).

An accumulation graph of Fe(II) was made using the measured concentrations within the first 38 days of the experiment, with each condition graphed over time (Figure 5). Looking at the first enrichment (R1), the Fe(II) concentration increased, decreased, and then increased again (Figure 5). It was not until day 7 that the samples increased in Fe(II) concentration and the distinction between the inoculated and uninoculated samples was noted. The inoculated samples continued to increase in Fe(II) up to day 14, and then decreased in Fe(II) concentration afterwards. Samples with 0.12 mM H_2O_2 had a standard deviation of 0.968 and behaved differently than the other samples (Table 5). Day 38 had high Fe(II) compared to the low Fe(II) concentrations on day 20.

After the microorganisms had been subjected to the same medium for a month, they were transferred to the second enrichment. Since there was Fe(II) being produced in R1, it was expected the Fe(II) on day 0 in R2 to be higher than day 0 of R1 (Figure 5-6). All the inoculated samples increased at the same rate, but some Fe(II) concentrations were higher than others. The

culture with 0.43 mM H_2O_2 had the most accumulation of Fe(II) and 0.12 mM H_2O_2 had the least accumulation of Fe(II).

After examining the Fe(II) concentration in the third enrichment over the 5 days, 0.23 mM H_2O_2 began showing more activity than the 0.45 mM H_2O_2 sample (Figure 7). These two samples tolerated the H_2O_2 the most, compared to the 0.9 mM and 0.01 mM H_2O_2 samples that tolerated H_2O_2 the least.

In the three Fe(II) concentrations graphs, 0.9 mM H_2O_2 showed the lowest Fe(II) concentration on the first few days when extractions were taken (Figure 5-7). These lower Fe(II) concentrations showed that high concentration of H_2O_2 influenced the growth of FeRB. To compare, 0.0 mM H_2O_2 began with the greatest increase in Fe(II) concentration, but in the later samples, 0.0 mM H_2O_2 did not have the highest concentration of Fe(II). Samples containing 0.9 mM and 0.012 mM H_2O_2 consistently had less Fe(II) than 0.0 mM H_2O_2. Out of all the samples, the medians of 0.45 mM and 0.23 mM H_2O_2 had the highest Fe(II) concentrations every enrichment.

There was a distinct difference between the R1 and R5 DNA yields (Figure 9). As expected, the controls did not have any detected DNA. In the inoculated samples, DNA concentrations were lowest in the 0.9 mM H_2O_2 and 0.0 mM H_2O_2, and highest in 0.23 mM H_2O_2. In R5, DNA concentrations were too low to be detected in all the samples, except in the innoculated 0.0 mM H_2O_2 sample. Since there was a DNA change from R1 to R5, there was a decrease in bacteria over the enrichments. Coupled with the high Fe(II) concentrations seen in R5 and increased Fe(II) concentrations over the enrichments, H_2O_2 had an effect inhibiting the growth of FeRB.

4. Discussion

The Fe(II) accumulation tell a lot about the how each FeRB sample was behaving under the given conditions. When the FeRB were put into an iron-rich and anaerobic media, they were expected to grow. Though, with the addition of the highly reactive oxidant, H_2O_2, to the system, growth had been affected. To understand if the Iron Snow Model is plausible, the Fenton Reaction of Fe(III) with H_2O_2 had created Fe(II) and insoluble hydroxides (Equation 4). As the the Fe(II) accumulation was being produced by the FeRB microorganisms within the inoculated samples, the higher accumulation showed growth within the medium (Figure 5-7). The uninoculated samples remained constant and did not show any growth because there was no inoculum and served as a control of the changing Fe(II).

Samples were given a range of H_2O_2 concentrations, between 0.9 mM to 0.0 mM and each condition behaved differently within the experiment. The less Fe(II) measured, the less tolerant the bacteria were to that concentration of H_2O_2. Overall, each sample accumulated Fe(II) at a similar rate but some samples had higher Fe(II) concentrations (Figure 5-7). Though, in R1, day 38 had an increase after day 21 had a decrease in Fe(II) accumulation. The decreased and then increased Fe(II) concentrations were likely due to an error during the HCl dilution. As the Fe(II) concentration increased and deviated from the standard readings, more HCl was needed to dilute the sample.

The highest concentration of H_2O_2 given was 0.9 mM, which was the most inhibited by H_2O_2. The Fe(II) concentrations increased the least with 0.9 mM H_2O_2 at the start of the enrichments and the accumulate Fe(II) continued to be lower than 0.45 mM and 0.23 mM H_2O_2. The strong oxidant damaged the bacteria and prevented the Fe(II) from being produced when there is a high concentration of the H_2O_2.

The bacteria in the sample with 0.0 mM added H_2O_2 did not have the most Fe(II) but was the only sample with DNA after the experiment period. However, the DNA concentrations may have been affected by the interference of H_2O_2 with the DNA extraction kit. Within the R1 DNA concentrations, the DNA accumulation decreased with the addition of H_2O_2. Due to the strong oxidant properties, it is also likely that H_2O_2 immediately damaged the DNA in the samples when the medium was extracted, which affected how the DNA was quantified (Figure 9). Either way, the measured DNA concentration may not correlate with the FeRB bacterial growth. H_2O_2 had a high reactivity with the DNA from the extraction kit.

Within the Fe(II) graph of R5, the 0.0 mM H_2O_2 showed a lower Fe(II) than 0.43 mM and 0.23 mM H_2O_2 (Figure 8). However, the values represented how productive the bacteria were at that time, not necessarily if the bacteria were growing. The Fenton reaction was a contributor to producing more Fe(II) within the experiment's given conditions and made the samples with added H_2O_2 comparable, excluding the samples with no added H_2O_2. The bacteria survived when H_2O_2 was not present in the sample.

The two H_2O_2 concentrations that were shown to have the highest tolerance was 0.45 mM and 0.23 mM H_2O_2. These samples did not survive in the fifth enrichment, but they had produced the most Fe(II) and exceeded the sample with 0.0 mM H_2O_2 (Figure 9). It is likely that the two samples produced more Fe(II) because H_2O_2 was used to produce the Fe(II) (Figure 5-8, Equation 4). Despite higher Fe(II) found in 0.45 mM and 0.23 mM than 0.0 mM H_2O_2, the 0.45 mM and 0.23 mM sample had less FeRB activity. When looking at the DNA concentrations after five enrichments, all the samples with H_2O_2 did not survive (Figure 9). The only sample to have contained DNA was the inoculated 0.0 mM H_2O_2 sample. H_2O_2 affected the FeRB growth and

inhibited the growth of the bacteria, but it is important to note that there was a tolerance of H_2O_2 among the samples.

5. Conclusions

Exposing microorganisms from AMD to H_2O_2 in an anaerobic environment helped to test the microbial tolerance of strong oxidants. From interpreting the produced Fe(II) concentrations within each sample, it can be stated Fe(II) reducing bacteria from AMD tolerate highly reactive oxidants, such as H_2O_2. Though, 0.9 mM of H2O2 was too high a concentration for AMD FeRB to tolerate. Knowing that H_2O_2 could be tolerated by FeRB can advocate the Iron Snow method. While ROS would be reacting away from the FeRB, a tolerance to the H_2O_2 would increase the survival of FeRB.

The higher Fe(II) accumulation within the H_2O_2 samples meant there was more activity from the bacteria. Within the enrichments, the inoculated 0.23 mM and 0.45 mM H_2O_2 samples showed more microbial activity than 0.0 mM in the earlier enrichments. The Fenton reaction with Fe(III) and H_2O_2 yielded more Fe(II) than the inoculated samples with no added H_2O_2. Also, the FeRB had a tolerance to the 0.23 mM and 0.45 mM H_2O_2 concentrations because the most Fe(II) was accumulated within these samples with added H_2O_2.

Fe(II) accumulation should not be correlated with the DNA concentrations within the samples. Interpreting the DNA, the 0.0 mM H_2O_2 sample was the only medium to contain DNA in the fifth enrichment, at the end of the experiment. FeRB samples with H_2O_2 did not contain extractable DNA in the fifth enrichment. It is likely the H_2O_2 interferred with the DNA extraction kit.

Despite the fifth enrichment showing that there was no DNA within the test tubes, the accumulated Fe(II) had been increasing over time. This indicated that the organisms may have been growing, but the DNA was not recovered from them. Overall, Fe(II) production was not inhibited in the presence of H_2O_2. FeRB cannot tolerate H_2O_2 within anaerobic, acidic environments if the concentration of H_2O_2 is greater than 0.45 mM H_2O_2. This may be important for future research when considering the Iron Snow model. Within this experiment, there was not an ice surface and a layer of bed sediment within the media to test the iron snow processes.

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Table 1. FeRB enrichment conditions in these experiments.

Sample	H_2O_2 Added (mL)	H_2O_2 Concentration (mM)	Treatment
1	1.0	0.90	Inoculated
2	1.0	0.90	Inoculated
3	1.0	0.90	Uninoculated
4	0.5	0.45	Inoculated
5	0.5	0.45	Inoculated
6	0.5	0.45	Uninoculated
7	0.25	0.23	Inoculated
8	0.25	0.23	Inoculated
9	0.25	0.23	Uninoculated
10	0.1	0.12	Inoculated
11	0.1	0.12	Inoculated
12	0.1	0.12	Uninoculated
13	0.01	0.012	Inoculated
14	0.01	0.012	Inoculated
15	0.01	0.012	Uninoculated
16	0.0	0.00	Inoculated
17	0.0	0.00	Inoculated
18	0.0	0.00	Uninoculated

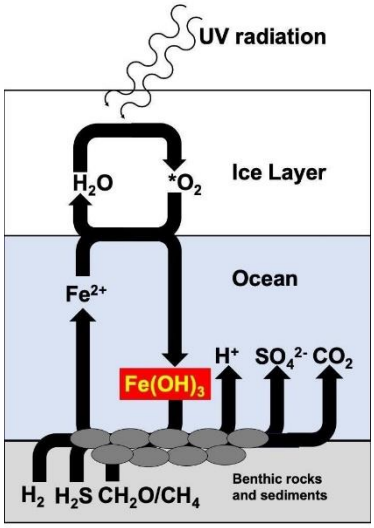

Figure 1. Hypothesized metabolisms in Europan ocean, where benthic rock-derived electron donors support Fe(III) reducing bacterial (ovals) activities. The Fe(III) is produced when Fe(II) is oxized by radiolytic ROS.

Figure 2. (top) Aerial photo with Friedline Mines circled in relation to the Pennsylvania Turnpike and (bottom) larger scale aerial view of acid mine drainage sampling site (GoogleMaps, 2022).

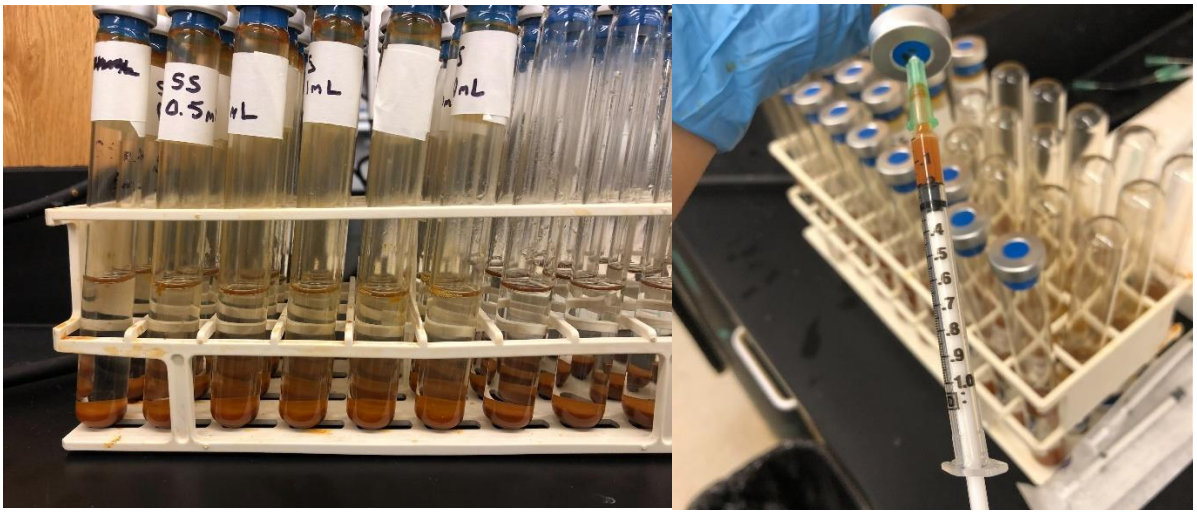

Figure 3. Picture of 0.2 mL of medium being extracted from the 18 samples tubes of the original media to measure Fe(II) concentration.

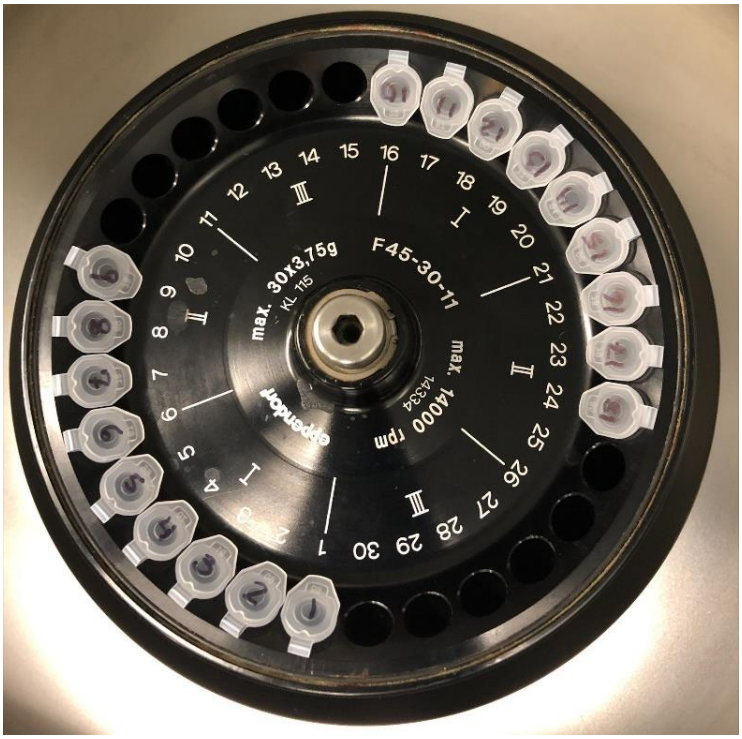

Figure 4. The 18 samples of an enrichment were placed evenly into a centrifuge to separate the respired solubilized Fe(II) in the supernatant from any solids.

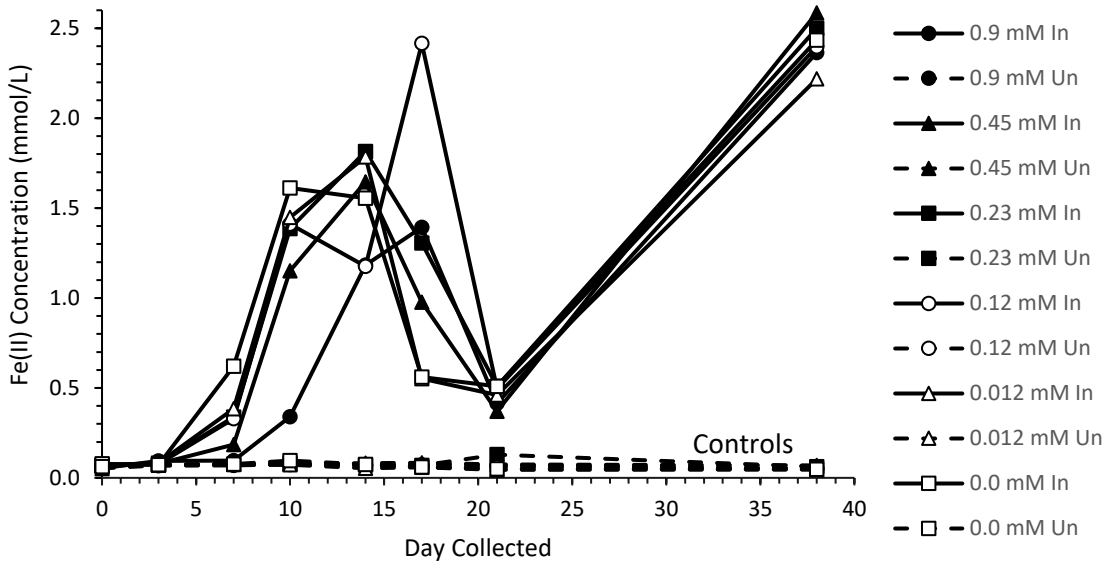

Figure 5. The Fe(II) accumulation with different amounts of added H_2O_2 found in the original enrichment.

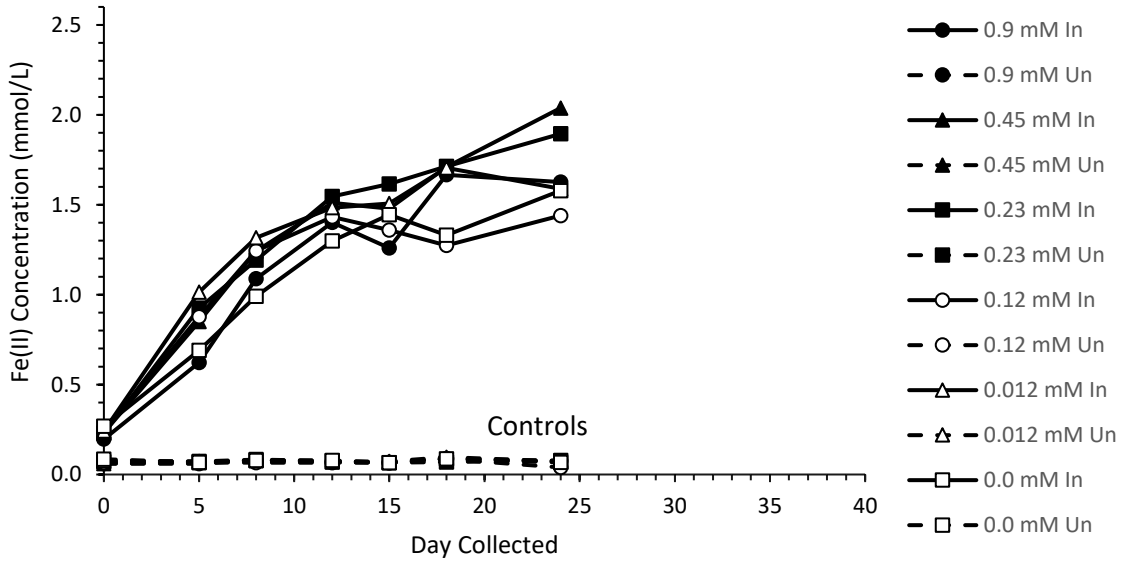

Figure 6. The Fe(II) accumulation with different amounts of added H_2O_2 found in the second enrichment.

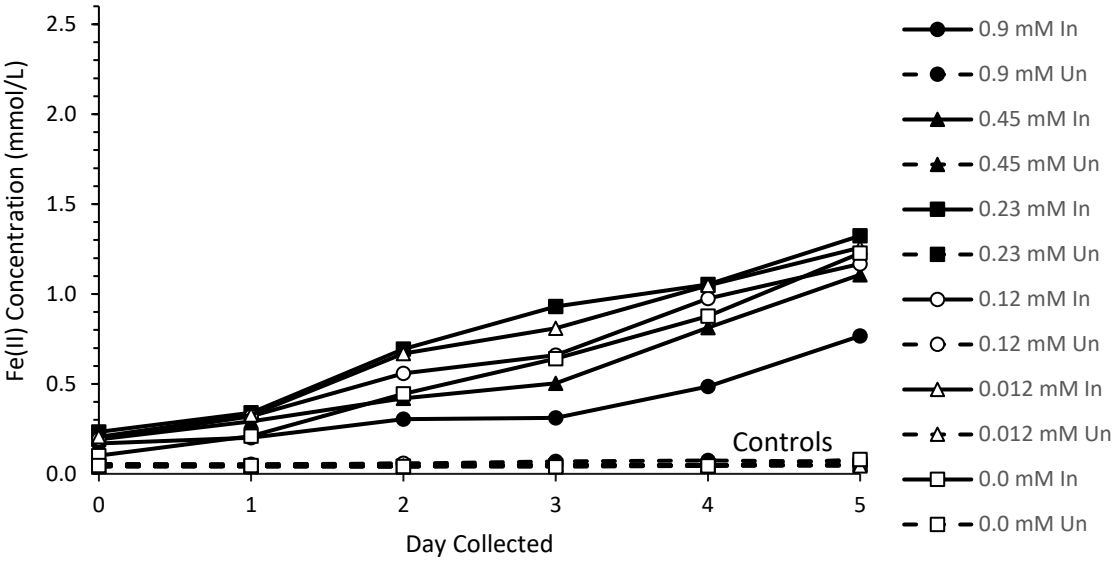

Figure 7. The Fe(II) accumulation with different amounts of added H_2O_2 found in the third enrichment, with day to day measurements.

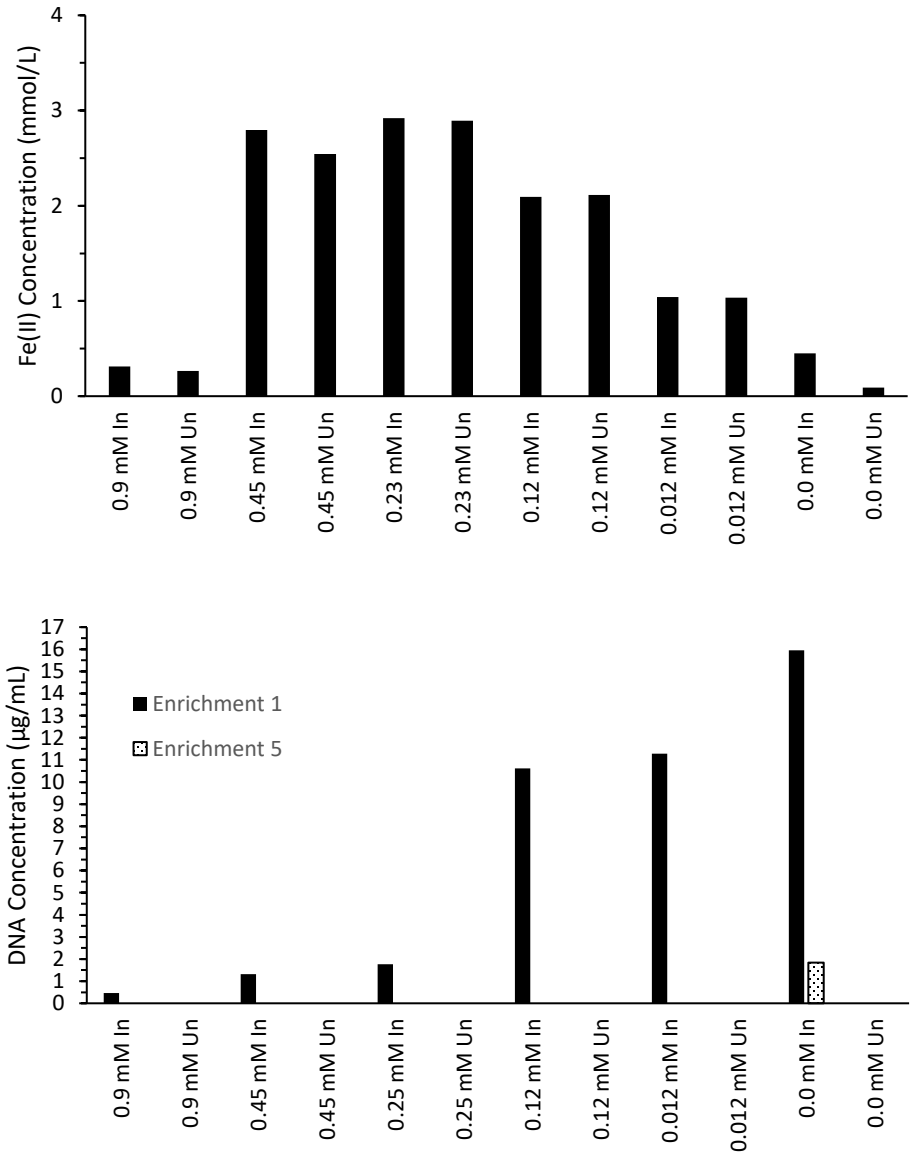

Table 8 and 9. (top) The Fe(II) accumulation within the fifth enrichment collected on day 21 (bottom) DNA concentrations of the 38 samples of all the media in both the 1st and 5th enrichment.