The Effect of Low pH and Nitrate on Microbial Growth and Community Interactions

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ABSTRACT

Bioremediation with microbial communities is considered an efficient and natural strategy for the removal of nitrogenous waste and acidity in soil. This ability has been demonstrated by microbes isolated from the ORFRC site in Oak Ridge, Tennessee. Removing nitrogenous waste is important as it is toxic to organisms and increases the risk of toxins leaching into groundwater. To implement bioremediation as effectively as possible, we need to know which microbes can tolerate and remediate stressful conditions, which we investigated by asking: how does low pH and nitrate affect microbial growth and interactions? To answer this, a microbial community isolated from the ORFRC site was grown in three conditions: (1) low pH, (2) high nitrate, and (3) low pH + high nitrate. The microbes were grown individually and in pairs. Low pH and high nitrate were found to have a significant effect on the growth dynamics of the microbes, where low pH + high nitrate had the lowest growth rate (14%) and high nitrate had the highest growth rate (20%). low pH had the lowest diversity of microbes (Shannon Diversity = 1.75) while high nitrate had the highest diversity (2.23). Pseudomonas strains grew well in the low pH, and Micrococcales strains grew well in the nitrate condition. Paired growth was much higher than individual growth in low pH (63% vs 19%), whereas paired growth was much closer to individual growth in high nitrate (30% vs 20%).

KEYWORDS

Bioremediation, denitrifying bacteria, paired synthetic communities, predictive modeling, selective pressure

INTRODUCTION

Microbial communities can shift and change to thrive in many kinds of situations, from pristine and nutrient-rich environments to desolate and highly toxic conditions. They can adapt to these differing environments by changing their community composition to one that favors the conditions they are in: for example, if an environment becomes very acidic, then the more acid-tolerant microbes will thrive and become abundant, while the acid-sensitive microbes will die out and decrease in number (Venturelli et al. 2018). They can also adapt to differing conditions by changing their community dynamics: for example, in the same acidic environment, some microbes may secrete metabolites to increase the pH of the soil and make it it less acidic so that other acid-sensitive microbes, which provide beneficial metabolites, can survive (Ratzke et al. 2018).

These characteristics of microbes are important for many reasons, one being it can be used to understand and possibly change an ecological environment that is stressful and toxic. An example of this is a nuclear waste site, a toxic environment that is manually hard to clean and remediate, but is still tolerable and remediable to some organisms who can adapt to the conditions by shifting their community composition, adapting, and metabolizing toxic substances into something less harmful (De et al. 2008). By studying how microbial communities can adapt to different environments, we can apply similar concepts and findings to various 'bioremediation' strategies to make them more efficient and productive.

Bioremediation is the process of using biological activity, such as that of microbes, to make dangerous areas full of contaminants and toxins more ecologically viable and safe. With proper planning and implementation, bioremediation is a low-cost, low-risk and natural method to conducting remediation (Vidali, 2001). However, bioremediation is limited by the site it is applied to, depending on what environmental conditions are present and what microbes may or may not be best suited to those conditions. For example, certain compounds like highly chlorinated or aromatic hydrocarbons are resistant to microbes – so trying to use bioremediation for that kind of problem wouldn't be very effective (Jansen et al. 1994). Another thing to consider is the types of microbes that can work and grow together. Growth patterns of two microbes grown together can be different from the growth of those microbes grown individually (Williams et al. 2014). If microbe A competes with microbe B for resources and causes it to die out, then those two microbes in a community may not be a good idea. Whereas, if microbe A and microbe B can coexist and

supplement each other's growth, then it might be a good idea to grow them together (Friedman et al. 2017). With the right plan and implementation, bioremediation can be used to remediate a wide range of scenarios, from pesticides to high acidity to nuclear waste.

Nuclear waste sites can produce a lot of nitrogenous waste, which not only makes it hard for plants and other sensitive microbes to grow in the soil, but it also increases the risk of nitrogenous waste and toxins leaching into groundwater reserves, making water toxic, and increasing the probability of eutrophication (Ghaly et al. 2015). To restore areas affected by toxicity and nitrogenous waste to an ecologically safe state, bioremediation measures can be implemented at such contamination sites. One particular contamination site of study is the ORFRC site in Oak Ridge, Tennessee. This area was originally a place for nuclear material to be processed for the making of the World War 2 atomic bomb. The nuclear waste that came as a result also produced secondary contaminants such as high levels of nitrogenous waste, metal contamination and low pH (Carlson et al. 2019). Despite the harsh conditions caused by the nuclear waste, it was found that there were thriving microbial communities at this site that were also remediating it by reducing nitrogenous waste. This reduction of nitrogenous waste was also correlated with increased pH levels and helped to make the area less toxic (Carlson et al. 2020). By further studying how the microbes in this area were able to survive and remediate contaminated areas, we can learn and apply similar bioremediation strategies to other areas with high acidity and nitrogenous waste to help remediate the toxic conditions.

To design optimal bioremediation strategies and create a predictive model that can predict what microbes are needed to remediate certain environmental conditions, we first need to know how the microbial communities respond to these stressful conditions. From there, we can also begin to understand how these microbes are reducing nitrogenous waste and functioning in such low pH as well. There are studies showing that low pH may influence a microbial community's growth and ability to reduce nitrate (Kim et al. 2017). What we don't know is whether low pH increases or decreases this specific microbial community's ability to tolerate high nitrate conditions. We also know that when all the ORFRC microbes are grown together, they can tolerate low-pH-high-nitrate conditions very well (de Raad et al. 2022), but we don't know which specific microbial strains survive and die out as pH and nitrate concentrations change. To create an accurate model, we need to also understand how each microbe's growth may change when grown with other microbes. While it has been shown that some microbes conduct metabolic processes to make

the conditions less toxic (Carlson et al. 2020), we don't know which microbes are doing what, and whether these responses change when grown with other microbes. To answer these questions, we aim to investigate the following:

The central research question we are trying to answer is: what is the effect of low pH and nitrate on microbial growth and community interactions? To answer these questions, we aim to investigate three sub-questions: (1) How do microbial communities respond to changes in low pH? (2) How do microbial communities respond to changes in high nitrate? (3) How do microbial communities respond to changes in low pH and high nitrate? We hypothesize that community dynamics will shift in low pH to favor the more acid-tolerant species and to exclude the acid-sensitive species. We will investigate this by growing microbes in three types of media: low pH media, high nitrate media, and low pH + high nitrate media. We will also graph microbial growth curves over time in the three conditions to see how the microbes grow in each condition. We will grow the microbes individually in each condition to observe their individual responses, then finally pair the microbes and see how the paired growth changes their growth responses.

METHODS

Study site

When the atomic bomb was created, the nuclear waste that was generated from the project remained at the Oak Ridge site in Tennessee (He et al. 2018). Over the years, the nuclear waste has led to heavy metals and high nitrate conditions in the soil. These toxic contaminants have also been found to leach into the surrounding Y12 watershed. However, it was found that the soil's nitrate and heavy metal content was stable and remediated in some cases. This was being done by the nitrate-reducing microbial communities living in the watershed (Liu et al. 2019). These microbial communities were isolated from the Y12 watershed site at Oak Ridge, Tennessee. I worked with 96 species (Appendix A) that were determined by the Arkin Lab to be the best representatives of all the microbes growing in the site.

Data collection methods

Media for three conditions

To determine the response of microbial growth dynamics when grown in low pH and high nitrate, me and my mentor, Dr. Bradley Biggs, grew microbes in four types of media: (a) 500 mM pH~6 sodium nitrate, (b) 160 mM nitric acid pH~4, (c) 3.14 mM Hydrochloric Acid (HCl) pH~4, and (d) control pH 7 media. To make the different-condition media, I first made 250 mL of the control media, pH 7 NLDM, and then I added different compounds to it to create the conditions desired.

NLDM base media preparation. To make NLDM media, I obtained a 250 mL autoclaved flask to place NLDM solution in. Then, I added 25 mL of NLDM Solution 1 - 'Sugars' (room temperature), 25 mL of NLDM Solution 2 - 'Organic_Acids' (room temperature), 25 mL of NLDM Solution3 - 'Amino_Acid' (room temperature), 5 mL of NLDM Solution 4 - 'Nucleobase /Nucleoside' (-20 C storage), 5 mL of NLDM Solution 5 - 'Other_Cpd' (-20 C storage), 5 mL of NLDM Salt solution 1 - 'MgSO4' (4 C storage), 5 mL of NLDM Salt solution 2 - 'NH4Cl' (4 C storage), 5 mL of NLDM Salt solution 3 - 'CaCl2' (4 C storage), 5 mL of NLDM Salt solution 4 - 'PO4' (4 C storage), 2.5 mL of *Wolfe's Minerals* (4 C storage), 2.5 mL of *Wolfe's Vitamins* (-20 C storage), and 140 mL of milli water. I then took the pH of the media using a *SevenExcellence pH/Ion meter S500-Std-K Mettler-Toledo* digital pH meter to make sure the pH was approximately 7. At this stage, the base media was done, and the amendments for selective pressures like nitrate and low pH could be added.

High nitrate media preparation - sodium nitrate. To make the high nitrate media (sodium nitrate), 10.6 grams of sodium nitrate (molecular weight = 84.99 g) was added to 250 mL of NLDM media. This amount was determined by adding sodium nitrate until the pH dropped to ~6.

High nitrate + low pH media preparation - nitric acid. To make the high nitrate + low pH media (nitric acid), 160 uL of nitric acid was added to 250 mL of NLDM media. This amount was determined by adding nitric acid until the pH dropped to ~4.

Low pH media preparation – HCl. To make the low pH media (HCl), 1570 uL of 0.1M Hydrochloric Acid (HCl) was added to 250 mL of NLDM media. This amount was determined by adding HCl until the pH dropped to ~4.

Preculture plate

Before putting the microbes in the media with selective pressures (low pH, nitrate), I inoculated them into the control media (NLDM pH 7) from glycerol stocks to make sure they were growing before they were transferred to a media with harsher conditions. To grow the pre-culture plate, I used a 20-200 uL multichannel micropipette to pipette 200 uL of NLDM into each well of a 96-well plate. Then, using a 2-20 uL multichannel micropipette, I scraped a little of the frozen glycerol culture stock into each corresponding well (refer to Appendix A for which strains were put in which well). Then an *EXCEL Scientific AeraSeal* was placed over the pre-culture plate and the plate was put into the *Labnet Vortemp 56 Incubator* at 30°C for 2-3 days, until most of the strains were visibly growing. Growth was characterized by the plate wells showing cloudiness or color (Figure 1). If some wells didn't grow, those wells were taken note of and inoculated with extra culture the next time.

Figure 1. Basic flow of data collection method. Step 1- make a pre-culture plate. Step 2- inoculate pre-culture plate into growth plate with selective-pressure media. Step 3- place inoculated growth plate in plate reader for 96 hours.

Growth plate for individual (monoculture) microbial growth

To see how individual (monoculture) microbial growth was affected by the different selective pressures (low pH, nitrate), I transferred the grown pre-culture microbes to another 96-well growth plate with media containing those different conditions (NLDM + condition). Once the

pre-culture plate was grown, I used a 20-200 uL multichannel micropipette to pipette 200 uL of NLDM + condition into each well of a new 96-well plate (growth plate). Then, using a 2-200 uL multichannel micropipette, I pipetted 2 uL of each well from the pre-culture plate into the corresponding well on the growth plate, until all 96 strains were pipetted into the corresponding well on the growth plate (pipette 2 uL of well A-1 (preculture plate) into well A-1 (growth plate), then pipette 2 uL of well A-2 (preculture plate) into well A-2 (growth plate), etc.). Once all the wells were transferred, I placed a *Breath-easy clear seal* on top of the plate, rolled over it and placed it in the *800TS microplate reader* on the following settings in the *Gen5 sequence* application: 96 hours total read time, reading taken every 15 mins, OD 600. This data would then be saved on an excel file and used later to make growth curves for data analysis.

Growth plate for paired microbial growth

To determine if the conditions had an effect on microbial interactions with each other, through their growth rates and survival outcomes, I grew the strains using the exact same procedure as the monoculture plate, but also added another strain to each well so that there were two strains in each well. For this, I did one-down pairings. To do this, once the first round of transferring was done (end of monoculture procedure), I pipetted 2 uL of each well from the pre-culture plate directly into the well below on the growth plate, until all 96 strains were pipetted into the corresponding one-down-well on the growth plate (pipetted 2 uL of well A-1 (preculture plate) into well B-1 (growth plate), then pipetted 2 uL of well A-2 (preculture plate) into well B-2 (growth plate), etc) (Figure 2). Once all the wells were transferred, I placed a *Breath-easy clear seal* on top of the plate, rolled over it and placed it in the *800TS microplate reader* on the following settings in the *Gen5 sequence* application: 96 hours total read time, reading taken every 15 mins, OD 600. This data would then be saved on an excel file and used later to make growth curves for data analysis.

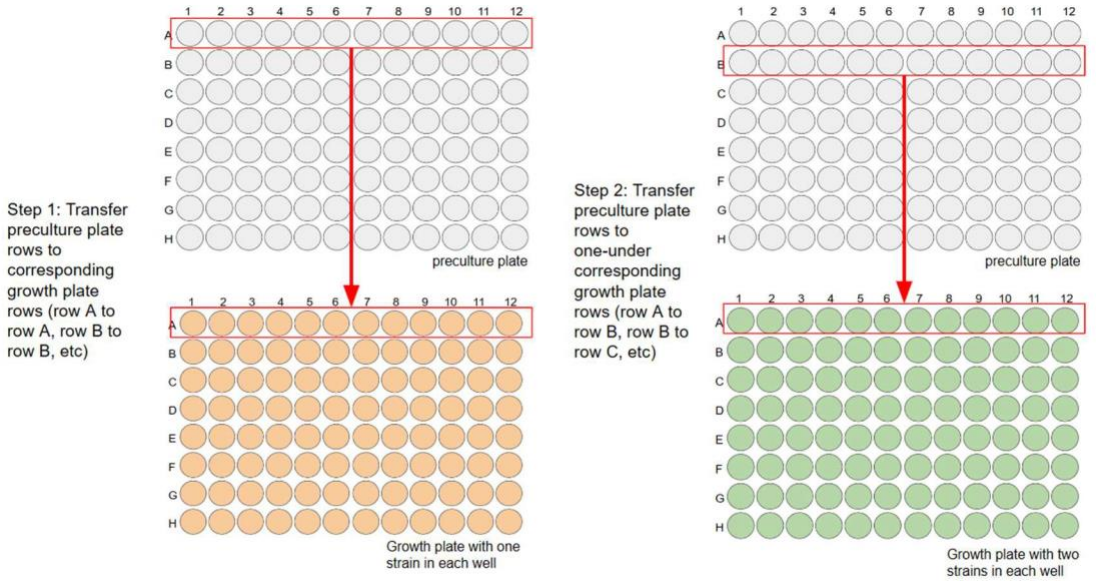

Figure 2. Transferring pre-culture plate to growth plate for paired growth plate.

Once the 96 hours ended, I spun down the wells in an *Eppendorf Centrifuge 5810 R 15 amp version* on 4000 rpm for 10 minutes to obtain a DNA pellet, and sent it to be prepped for 16S Illumina sequencing to measure the cell count of the strains in each well to see how much of each strain grew compared to the other it was paired with.

Data validation methods (for individual strain growth)

To check that the strain growing in each well was the strain I thought it was (and there was no contamination), I conducted PCR and 16S Sanger sequencing following the Takara Primestar Protocol (listed in Appendix B below). Briefly outlining the process, I extracted 200 uL from the wells that I wanted to check on the growth plate, spun the cultures down to obtain a pellet, and performed PCR with a bit of the pellet. After that, I took 5 uL of the PCR and did gel electrophoresis to check the DNA band size was ~1.5 kbp (Figure 3). If the gel band sizes were correct, the rest of the PCR was prepped for gene sequencing with ExoCIP and sent for Sanger Sequencing. Looking at one strain at a time, the sequences were then compared with reference sequences from our reference genome library to make sure each well matched the expected strain. If there were any double peaks in the sequencing, or there were a lot of red lines and mismatches between the sequences, then contamination likely occurred and I would have to re-do the trial (re-

grow pre-culture plate, re-do growth plate, etc.)

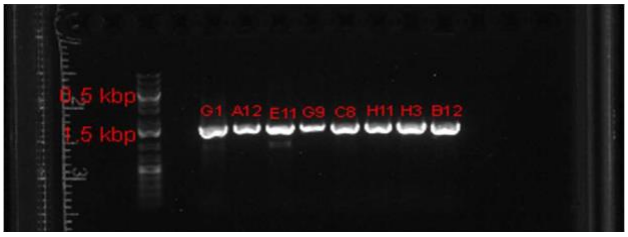

Figure 3. Successful PCR (Polymerase Chain Reaction) with correct DNA band size.

Data Analysis Methods

Growth Curves

To assess the effects of the conditions on the growth of the microbes, the plate reader data (OD vs time) was plotted to characterize how the microbes grew in that condition over time. This was done by transferring the growth plate data to an Excel Sheet, with the OD readings recorded for each well recorded. OD readings were taken in 15-minute intervals, leading to a total of 385 OD readings for each well. The data was plotted with the x-axis as time (in minutes), and the y-axis as OD reading (Figure 4). This was done for each well, so that there would be a total of 96 growth curves.

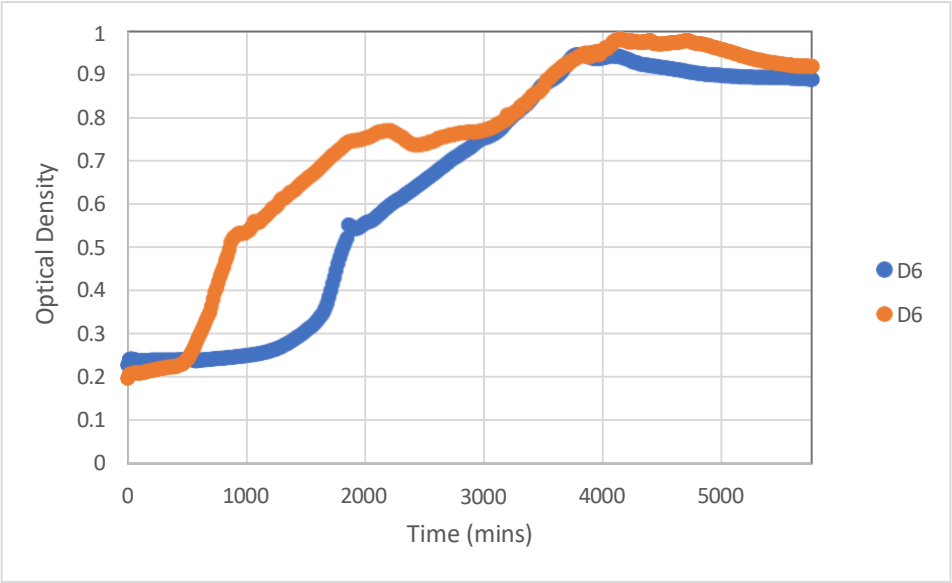

Figure 2. OD data plotted onto growth curve (OD reading on y-axis, time (mins) on x-axis). Different colored

lines indicate different trials for the same well.

These graphs were then visually compared and analyzed to determine which wells and strains did grow in the treatment conditions, by assessing if the shape and size of curves of each of the trials was the same. If the shape was the same and curve was different, then growth could be similar but at a different magnitude. I considered the microbe to be growing if the OD was above 0.35.

For the paired growth curves, I also visually compared and analyzed the growth curves to determine which paired strains grew in the treatment conditions in the same way as the individual growth curves. I considered the microbes to be growing if the OD was above 0.35 for this as well. This data would later be coupled with the Illumina 16S sequencing data to see the cell counts for each strain in each well to see which strains outgrew the others and were more competitive or mutualistic with each other.

Alpha Diversity Metrics

To determine how diverse the sample microbial communities were for each condition, I listed the microbes that individually grew well in the condition and looked up what order it was based on the strain it was (Appendix A). To determine richness, based on the number of unique orders present in that sample, I counted how many distinct orders were found growing in that condition. To determine evenness, based on the relative abundance of each order in the sample, and the Shannon Diversity Index, based on the overall diversity of orders within the sample, a Shannon Diversity index calculator was used (Rain, 2022).

Beta Diversity Metrics

To determine how the diversity of the sample microbial communities compared between the three conditions, I listed the orders that grew in each condition and created a Venn diagram to see what orders each condition had in common.

RESULTS

Effect of three conditions on individual microbial growth

Table 1: Effect of three conditions on individual microbial growth. Represented by percentage of 96 strains that grew in each condition, and z-score. All graphs can be found in Appendix C,D,E.

	Low pH	High Nitrate	Low pH + High Nitrate
% of strains that grew	19%	20%	14%
z-score	20.23	19.60	24.28

To test how low pH influenced individual microbial growth, I grew the microbes individually in pH 4 HCl media and measured the OD over time (Appendix C). ~19% of the 96 microbes grew in the low pH media (Table 1). The z-score between the low pH and control conditions was statistically significant (z = 20.23), indicating that the low pH did have a significant effect on individual microbial growth dynamics.

To test how high nitrate influenced individual microbial growth, I grew the microbes individually in pH 6 sodium nitrate media (Appendix D). ~20% of the 96 microbes grew in the high nitrate media (Table 1). Out of the three conditions, microbes grew in this condition (high nitrate) the most. The z-score between the high nitrate and control conditions was statistically significant (z = 19.60), indicating that the high nitrate did have a significant effect on individual microbial growth dynamics.

To test how low pH + high nitrate influenced individual microbial growth, I grew the microbes individually in pH 4 nitric acid media (Appendix E). ~14% of the 96 microbes grew in the low pH + high nitrate media (Table 1). The least number of microbes grew in this media (low pH + high nitrate). The z-score between the low pH + high nitrate and control conditions was statistically significant (z = 24.28), indicating that the low pH + high nitrate conditions resulted in the largest deviation of microbial growth from the control.

Effect of three conditions on diversity of microbial communities when grown individually

Adding up all the orders of bacteria that grew in each condition, 12 total orders of bacteria were found to grow over the three conditions (Figure 3). Looking at the Alpha diversity metrics, 7 strain orders grew in the low pH media, and had a Shannon Diversity score of 1.75, making this the least diverse community out of all three conditions. 8 strain orders grew in the low pH + high nitrate media, and had a Shannon Diversity score of 1.97, making this a similarly low diversity, but not as low, community as the low pH alone. 11 strain orders grew in the high nitrate media, and had a Shannon Diversity score of 2.23, making this the most diverse community (Table 2).

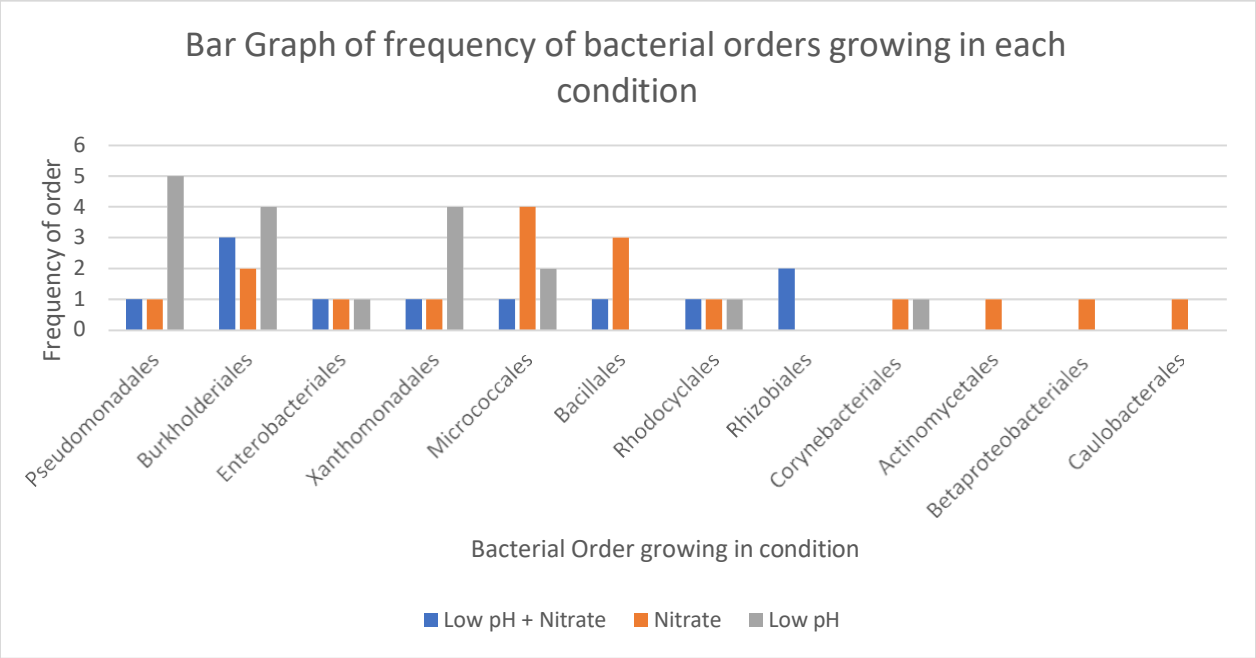

Figure 3. Bar graph of frequency of growing bacterial orders in each condition.

Table 2. Alpha-diversity metrics between all three conditions.

	Low pH	High Nitrate	Low pH + High Nitrate
Shannon Diversity Index	1.75	2.23	1.97
Evenness	0.899	0.931	0.948
Richness	7	11	8

Looking at the Beta diversity metrics, out of all 12 orders that grew in any of the three conditions, 6 orders grew in all three conditions (Figure 4). Most microbes that did grow, grew in all three conditions. 3 of the orders grew only in the high nitrate media, 1 order grew only in the low pH + high nitrate media, and no orders grew in the low pH alone. Between the three conditions, high nitrate was the most favorable condition, and low pH was the least favorable condition for microbes to grow in.

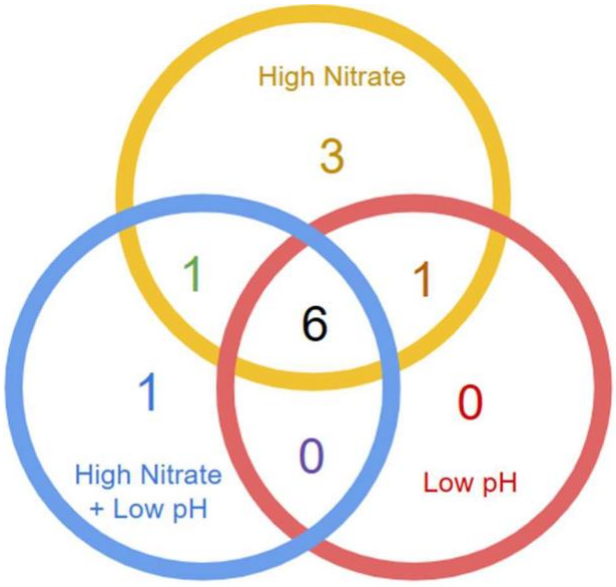

Figure 4. Venn diagram showing Beta-diversity metrics across all three conditions.

Effect of low pH and high nitrate conditions on paired microbial growth

To test how low pH influenced paired microbial growth, I grew the microbes in pairs in pH 4 HCl media (Appendix F). ~67% of the 96 microbe pairs grew in the low pH media (Table 3). Out of the two paired conditions, microbes grew in this condition (high nitrate) the most. The z-score between the low pH and control conditions was statistically significant (z = 6.876), indicating that the low pH did have a significant effect on paired microbial growth dynamics.

To test how high nitrate influenced paired microbial growth, I grew the microbes individually in pH 6 sodium nitrate media (Appendix G). ~30% of the 96 pairs grew in the high nitrate media (Table 3). Out of the two paired conditions, microbes grew in this condition (high nitrate) the least. The z-score between the high nitrate and control conditions was statistically significant (z = 14.97), indicating that the high nitrate did have a significant effect on paired microbial growth dynamics.

Table 3. Frequency of paired strains that grew in 3 conditions. There is no data for the low pH + high nitrate condition because there wasn't enough time to collect data for that.

	Low pH	High Nitrate	Low pH + High Nitrate
% of strain pairs that grew	~67%	~30%	N/A

Comparing the paired strain growth with the individual strain growth, some paired strains seemed to grow even though neither strain grew individually. In the low pH condition, there were approximately 32 wells that grew as paired strains that didn't grow as individual microbes (Appendix H). Considering that each microbe can be found in two wells (its corresponding well and the well below it), approximately 16 microbes grew in the paired strains that didn't grow individually.

Some paired strains also seemed to not grow, even though one of the strains grew individually. In the high nitrate condition, there were approximately 6 wells that grew better as individual microbes than paired strains (Appendix I). In the high nitrate condition, if it didn't grow as individually, it didn't grow as a paired strain either.

DISCUSSION

To answer the central question, pH and nitrate were found to have significant effects on the growth dynamics and composition of microbial communities isolated from the ORFRC site in Oak Ridge, Tennessee. The three conditions tested did not affect all bacteria in the same way though, indicating that low pH, high nitrate, and low pH + high nitrate conditions have different effects on microbial communities. From this, we can also assume that high nitrate concentration cannot be correlated with the results of low pH in a predictive microbial model. Looking at the results of paired strain versus individual strain growth, it is also indicated that microbes in this community can have mutualistic and inhibitive interactions in the different conditions. This provides more evidence for what kind of interactions can be expected of these bacteria when they are applied to bioremediation models, based on what tolerance and resistance they have to these extreme conditions.

Low-pH and high nitrate as selective pressures

Most strains favored the low pH media over the low pH + high nitrate media, although one more order preferred the low pH + high nitrate media than the low pH media. Overall growth in the low pH + high nitrate could have been lower than the low pH media because of the added stress of low pH weakening the microbes' abilities to reduce nitrate effectively. Conversely, although both were pH 4, the presence of nitrate in the low pH + high nitrate media may have been the reason why more orders were able to grow there. Other studies have found that while low pH is a selective pressure due to toxicity on microbial communities like this one, nitrate was not a selective pressure (Carlson et al, 2019). In addition to this, the highest survival rate was in the high nitrate condition, indicating this was the most favorable condition for microbes to grow in. This finding indicates that nitrate may not have been the problem in the nitric-acid media, but something else was contributing to the low microbial growth.

Microbial tolerance mechanisms to low-pH and high nitrate

When grown individually, microbial tolerance and growth in these conditions can be heavily influenced by the types of metabolites and physical characteristics they have that protect them against these harsh conditions. One of the highest-growing orders in the community, Pseudomonadales, has a species called Pseudomonas Aeruginosa that has been found to grow well in low pH by inducing genes for the production of extracellular DNA, which could be a possible tolerance mechanism (Lewenza et al. 2019). Rhodanobacter, one of the high-growing Pseudomonadales strains, has been found to grow abundantly in low-pH settings in other studies as well. In a study where microbes were grown in heavy metals, toxic ions, and low pH, Rhodanobacter abundance was positively correlated with low pH, whereas other non-Rhodanobacter isolates were inhibited by low pH (Carlson et al, 2018). The ability of Rhodanobacter and Pseudomonas aeruginosa to grow in these low pH conditions indicates that other Pseudomonadales strains that grew well in the low pH may use similar mechanisms to tolerate the acidity.

There were some strains that grew well in the low pH and low pH + high nitrate media, but many of the orders that grew in both conditions did not grow at the same rate: most microbial growth was higher in low pH than in low pH + high nitrate. There was only one bacterium that grew very well in the low pH + high nitrate, Burkholderia Rhynchosiae (Rhizobiales order strain) (Appendix A). The reasoning for this ‘double tolerance’ could be because of how Burkholderia Rhynchosiae (1) has nod and nif genes, which are associated with the ability to fixate nitrogen, and (2) has been found to grow as root nodules of Rhynchosiae plants that are tolerant to acidic and infertile soils, so they could infer similar tolerance mechanisms to their host (De Meyer et al. 2013).

Micrococcales was the most abundant growing order in the high nitrate condition, specifically of the genus Arthrobacter. The reason for this order’s high tolerance to nitrate is potentially due to the universal stress-related proteins (USPs) encoded in its genome, which contains many proteins related to metabolizing nitrogenous compounds (Mongodin et al. 2006).

Paired community tolerance to low pH and high nitrate

Data from microbial interactions in paired communities can be used to improve a predictive microbial model meant to show how the presence of certain microbes in a site can influence the ecological functions of that environment. Microbes that grow individually in a condition will not always exhibit the same growth patterns when grown with other microbes, so having data on paired interactions coupled with individual growth patterns can help to create a more accurate microbial model. This was indicated in my results, as many microbial orders that didn't grow individually, seemed to grow much better (and sometimes worse) when grown with other microbes.

In many cases, the paired-microbes that grew well in low pH seemed to have Pseudomonadales as a partner-species. Rhodanobacter, a strain of Pseudomonadales, that seemed to grow well in our experimental conditions, has also been found to grow well in other microbial communities as potential denitrifying or pH-increasing bacteria (Van Den Huevel, 2010). This could be attributed to how Pseudomonadales strains, like Rhodanobacter, provide some role in secreting metabolites or reducing compounds to make the surrounding environment more favorable to other strains that would otherwise not survive those conditions by themselves.

Many of the strains that did grow better as pairs in the low-pH condition had a Pseudomonas strain as one of the pairs. The reason why there could be higher growth when the two microbes were grown together could be because Pseudomonas strains, like Pseudomonas veronii, might be increasing the pH of the medium slightly, which could provide a positive feedback and increase the growth of the other paired microbe (Ratzke and Gore 2018). The higher growth of the bacteria could also lead to one providing metabolites to the other microbes as a result, improving growth in the well. Other pairs in this condition could have followed similar trends to achieve higher growth.

There was not much of an increase in growth from individual strain to paired strain growth in nitrate as many bacteria, such as Pseudomonas, may have been competing to use the nitrate as an electron-acceptor for themselves (Park and Yoo 2009). This could be a reason for the lower growth amongst paired strains in the nitrate condition. However, it is important to note that the paired-microbe interactions are only inferences from the growth curve trends and the microbes that are typically thought to grow in those conditions – we don't know for certain which microbes

are thriving in the pairs (nor can we fully infer how they are thriving) without the 16S Illumina sequencing cell counts.

What results indicate for bioremediation

As the paired microbes indicate that many microbial communities grow better together versus individually, this could mean that many strains provide metabolites and conduct metabolic processes, such as denitrification and detoxification, that help make conditions more favorable. Rhodanobacter is thought to help with nitric oxide detoxification in communities where Rhodanobacter and Gallionellaceae are grown together, and the addition of the Rhodanobacter is correlated with the survival of Gallionellaceae (Huang et al, 2021). This indicates that Rhodanobacter has some kind of detoxification mechanism that other microbial species can also benefit from. Another study shows that Rhodanobacter made up a large part of bacterial communities grown in high-nitrate conditions, which indicates that they have the ability to denitrify or tolerate the nitrate conditions around them (Van Den Huevel, 2010). The ability of Rhodanobacter and potentially other Pseudomonadales species to tolerate and rescue microbial communities in these harsh conditions implies that they may have the ability to remediate these harsh conditions to more ecologically favorable conditions as well.

Limitations

My study was limited by a few aspects that limited the scope of my results. My list only contained 96 strains, but there are still many unidentified bacterial isolates at the site that could be contributing to the denitrifying conditions of the on-site community. In addition to that, due to time constraints we couldn't get our Illumina Sequencing data back in time to see how cell counts vary in paired communities, so we don't for certain know which microbes were growing better in the paired conditions (if it was both or just one). Another fallback was that we only conducted experiments in aerobic conditions, but many microbes can function anaerobically and denitrification usually happens in anaerobic settings, so our implications of these findings for bioremediation are limited to aerobic conditions. Also, the use of nitric acid may have been a bad way to look at the effects of nitrate in microbial communities, because while HNO3 (nitric acid)

does dissociate into nitrate, the effects of the nitric acid were unexpectedly much more detrimental to microbial communities than we were expecting, indicating something else must have been happening besides pH and nitrate.

Future Directions

In the future, it would be good to try taking a more quasi-experimental approach instead of an experimental approach to see how results may differ. A quasi-experimental approach would allow us to mimic the complex on-site conditions better, and would provide more external validity for our findings. Another further step to take would be to start growing and replicating the methods in anaerobic conditions to see how that affects microbial growth and interactions, as many bacteria are known to grow and denitrify anaerobically. Also getting back Illumina sequencing data and looking at how cell counts change with paired microbes in different conditions. It would also be better to re-make the media, where the low pH + high nitrate media is made with a combination of the amendments used for the other two (HCl + sodium nitrate) as opposed to a completely different compound (nitric acid) and to rerun experiments and see if results change.

Broader Implications

Looking at how pH does impact the growth of microbial communities and their interactions, we can use these results to inform our predictive microbial model. There are certain microbial orders that grow better in low pH than others, but there are also microbial orders that are capable of rescuing other microbes and forming symbiotic relations that allow them all to grow in harsh conditions. This survival ability can be attributed to secreted metabolites or denitrifying mechanisms, but to know what specifically happens is a next step in this field of research. We can use these findings for bioremediation purposes, to rescue heavily contaminated ecological communities and make soil conditions favorable for ecosystem function again.

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APPENDIX A

96 Microbial Strains Organized by Well Number, ENIGMA Strain ID, and Strain Name

A1	FW510-R10	Rhodanobacter fulvus
A2	GW456-R20	Delftia acidovorans
A3	FW507-12TSA	Pseudomonas sp. str. HMPB1
A4	FW305-3-2-15-E-R2A2	Cupriavidus basilensis
A5	FW306-1B-D06B	Lysobacter soli strain DCY21
A6	GW460-4	Acinetobacter soli str. B1
A7	FW305-3-2-15-E-TSA4	Pseudomonas sp.
A8	GW101-3F08	Hydrogenophaga taeniospiralis strain 2K1
A9	FW306-05-D	Methylobacterium goesingense strain iEll3
A10	FW300-N2E2	Pseudomonas fluorescens
A11	GW460-12-1-14-LB5	Janthinobacterium sp.
A12	MPR-TSA4	Azospira oryzae strain 6a3
B1	GW823-FHT05D11	Acidovorax radicis N35
B2	FW104-10B01	Rhodanobacter sp.
B3	GW456-E5	Delftia acidovorans
B4	FW305-3-2-15-F-TSA7	Sphingomonas sp.
B5	FW305-3-2-15-C-LB1	Pseudomonas extremorientalis
B6	FW305-C-10-9	Ensifer adhaerens strain NBRC 100388
B7	FW305-70	Pseudomonas corrugata
B8	FW305-53	Pseudomonas frederiksbergensis strain DSM 13022
B9	FW305-3-2-15-C-TSA3	Pseudomonas extremorientalis
B10	GW822-FHT02A01	Rhodoferax saidenbachensis strain ED16
B11	GW458-12-2-14-TSB2	Arcicella aquatica
B12	FW306-1B-G06A	Arthrobacter bambusae strain THG-GM18
C1	FW305-3-2-15-A-LB1	Hydrogenophaga sp.

C2	GW456-L13	Pseudomonas jessenii
C3	FW104-T7	Rhodanobacter thiooxydans
C4	GW456-A8	Acidovorax delafildii
C5	FW507-4G11	Cupriavidus basilensis strain DSM 11853
C6	FW306-02-D09B	Pseudomonas rhodesiae strain CIP 104664
C7	FW306-07-L	Mucilaginibacter defluvii strain A5
C8	FW215-T2	Pseudomonas jessenii strain CIP 105274
C9	FW305-F6	Bradyrhizobium japonicum strain 3I1b6
C10	GW247-5R2A	Lysinibacillus fusiformis strain NBRC15717
C11	GW460-C3	Pseudomonas mandelii
C12	GW460-8	Pseudomonas mandelii str. CIP 105273
D1	FW507-14D01	Bacillus megaterium strain NBRC 15308
D2	FW305-3-2-15-E-LB1	Stenotrophomonas maltophilia R551-3
D3	GW822-FHT05D05	Delftia acidovorans strain NBRC 14950
D4	FW104-R5	Rhodanobacter sp.
D5	GW460-11-11-14-LB4	Acidovorax sp.
D6	FW305-17	Pseudomonas mosselii strain CFML 90-83
D7	FW305-E2	Pseudomonas putida strain KT2440
D8	FW305-127	Pseudomonas mosselii strain CFML 90-83
D9	MPBC4-4	Cupriavidus basilensis strain DSM 11853
D10	GW247-6R2A	Lysinibacillus fusiformis strain NBRC15717
D11	CPT56D-587-MTF	Bacillus cereus
D12	GW822-FHT05C07	Zoogloea resiniphila strain DhA-35
E1	FW507-F1	Chromobacterium sp. str. 2002
E2	GW821-FHT01B05	Xylophilus ampelinus strain BPIC 48
E3	FW104-16D08	Castellaniella hirudinis strain E103
E4	GW101-3H11	Acidovorax soli strain BL21
E5	FW510-T8	Rhodanobacter sp.
E6	GW101-20D03	Acidovorax soli strain BL21
E7	GW460-12-1-14-LB1	Variovorax paradoxus

E8	GW460-11-11-14-TSB4	Pedobacter soli strain 15-51
E9	GW247-4R2A	Simplicispira sp. str. R-22835
E10	MLSD5-FHT05B12	Afipia broomeae strain F186
E11	FW305-123	Arthrobacter aurescens TC1
E12	MPR-R2A5	Pseudomonas marginalis strain ICMP 3553
F1	GW823-FHT05C09	Acidovorax facilis strain CCUG 2113
F2	FW305-104	Rhodococcus erythropolis PR4
F3	MPR-LB4	Dechlorosoma suillum PS
F4	GW456-11-11-14-TSB4	Pedobacter koreensis
F5	FW507-14D01	Bacillus megaterium strain NBRC 15308
F6	FW104-R3	Rhodanobacter sp.
F7	GW458-11-26-14-TSB4	Acidovorax sp.
F8	FW306-1C-G01B	Burkholderia ambifaira strain AMMD
F9	GW101-20A05	Ensifer adhaerens
F10	EB106-09-02-XG168	Lysinibacillus sp. strain Brt-P
F11	FW305-3	Pseudomonas chlororaphis strain NBRC 3904
F12	FW305-C-30-35	Novosphingobium sp. strain FW305-C-240A
G1	FW305-C-30-S16	Caulobacter sp. ECN-2008
G2	GW823-FHT01D03	Pseudomonas koreensis strain Ps 9-14
G3	FW305-F13	Streptomyces globisporus strain KCTC 9026
G4	FW306-05-A	Sphingomonas glacialis strain C16y
G5	FW305-107	Undibacterium pigrum strain CCUG 49009
G6	GW460-12-10-14-LB3	Methylobacterium fujisawaense
G7	FW510-R12	Rhodanobacter thiooxydans
G8	GW101-11A03	Acidovorax delafieldii strain 133
G9	EB106-03-01-XG64	Microbacterium sp. HP8G
G10	EB271-A4-7B	Rhizobium selenitireducens strain B1
G11	EB106-07-01-XG149	Bacillus thuringiensis strain EI-18
G12	EB106-05-01-XG147	Ralstonia sp. SK1
H1	EB106-06-01-XG185	Bacillus cereus strain ANP221

H2	FW306-02-B	Burkholderia rhynchosiae strain WSM3937
H3	MLSD5-FHT05C12	Staphylococcus warneri strain AW 25
H4	GW821-FHT05A03	Clavibacter michiganensis subsp. phaseoli strain LPPA 982
H5	GW456-11-11-14-LB4	Flavobacterium sp.
H6	GW821-FHT05B06	Erwinia billingiae strain Billing E63
H7	GW456-L13	Pseudomonas jessenii
H8	FW300-N1A1	Pseudomonas migulae str. T19
H9	MLSD5-FHT05A06	Curvibacter delicatus strain NBRC 14919
H10	GW821-FHT02G11	Pseudomonas veronii strain CIP 104663
H11	FW306-05-C	Arthrobacter liuii strain DSXY973
H12	GW821-FHT04F04	Ferribacterium limneticum strain cda-1

APPENDIX B

PrimeSTAR Colony PCR protocol for 16S sequencing

1. Streak out desired strain on a plate.
2. Once clear colonies have formed, pick a single colony with a small pipette tip and pipette up and down into 20 uL of nuclease free water to remove the majority of the colony.
    a. If you want to make a glycerol stock of the strain, inoculate a culture with the pipette tip before discarding.
3. Use this "colony water" as template for PrimeSTAR Max 16S PCR.

1. Alternatively - if you have a liquid culture you can spin the liquid culture down and decant.
2. Touch your pipette tip to the culture so as to grab approximately 0.1-0.2 uL of the cell pellet. You don't need to pipette up to get a sufficient amount to act as a template for the 16S PCR.
3. Use this "colony water" as template for PrimeSTAR Max 16S PCR.

*Note: A critical component of this being successful is the "cloudiness" of the colony water. You want it to have some cloudiness but not to become opaque. If it becomes too opaque just add nuclease free water to the 20 uL until the opaqueness dissipates. Too high of a concentration of cells/previous cell culture in the template water will interfere with the PCR. PrimeSTAR Max is fairly robust to this type of contamination, other PCR products are less so.

For the PCR:

15 uL PCR (5 uL will be used for a gel, 10 uL for Exo-CIP and Sanger Sequencing)

1. 7.5 uL of 2x PrimeSTAR MM
2. 5.5 uL of DNAse free water
3. 1.5 uL of 10x primer mix (16S combined mix of 27F and 1492R)
4. 0.5 uL of "colony water"

If you create a mastermix, take the number of reactions and multiply by 1.1 to have some extra

volume, and add 14.5 uL of the master mix into each PCR tube. Then, add 0.5 uL of the colony water.

Thermocycler protocol (volume 15 uL)

1. 98 C for 5 min (boil to break open cells)
2. 30x cycles of
    a. 98 C, 10 sec
    b. 55 C, 15 seconds (if primers have annealing temperature above 55 for both primers this step can be 5 seconds, for the 27F and 1492R I use 15 s)
    c. 72 C, 5 seconds/kB (I do a minimum of 25 s typically, and have been doing 45 s for 16S)
3. 72 C for 5 min (extension)
4. 12 C for forever

4. Take 4.5-5 uL of PCR, add 1 uL of loading dye and run on 1% agarose with SybrSafe.

5. Image to confirm successful PCR (clean ~1600 bp band)

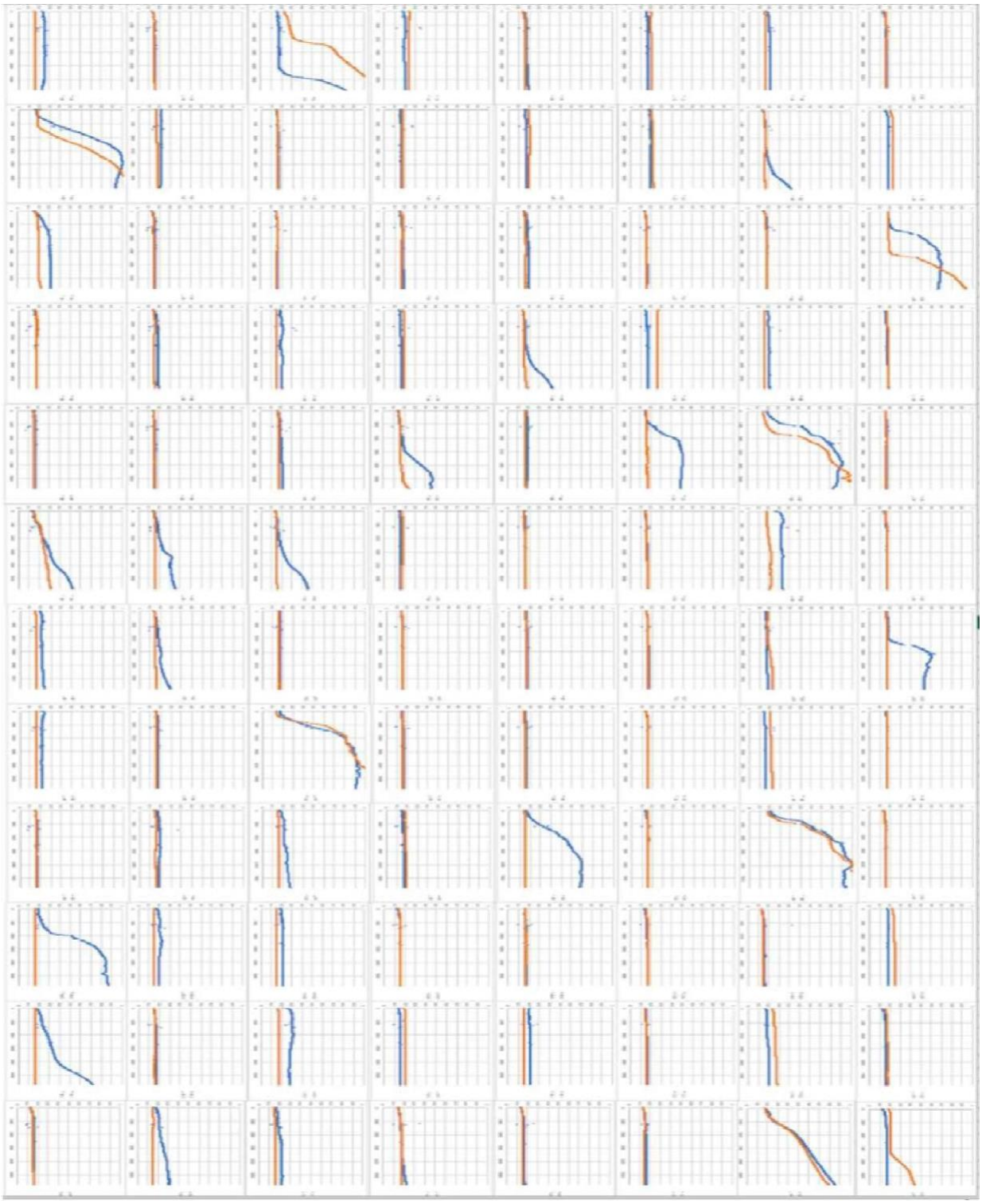
6. For successful PCRs, add 2 uL of Exo-CIP A solution and 2 uL Exo-CIP B solution (14 uL total volume now)

7. Mix and briefly spin down

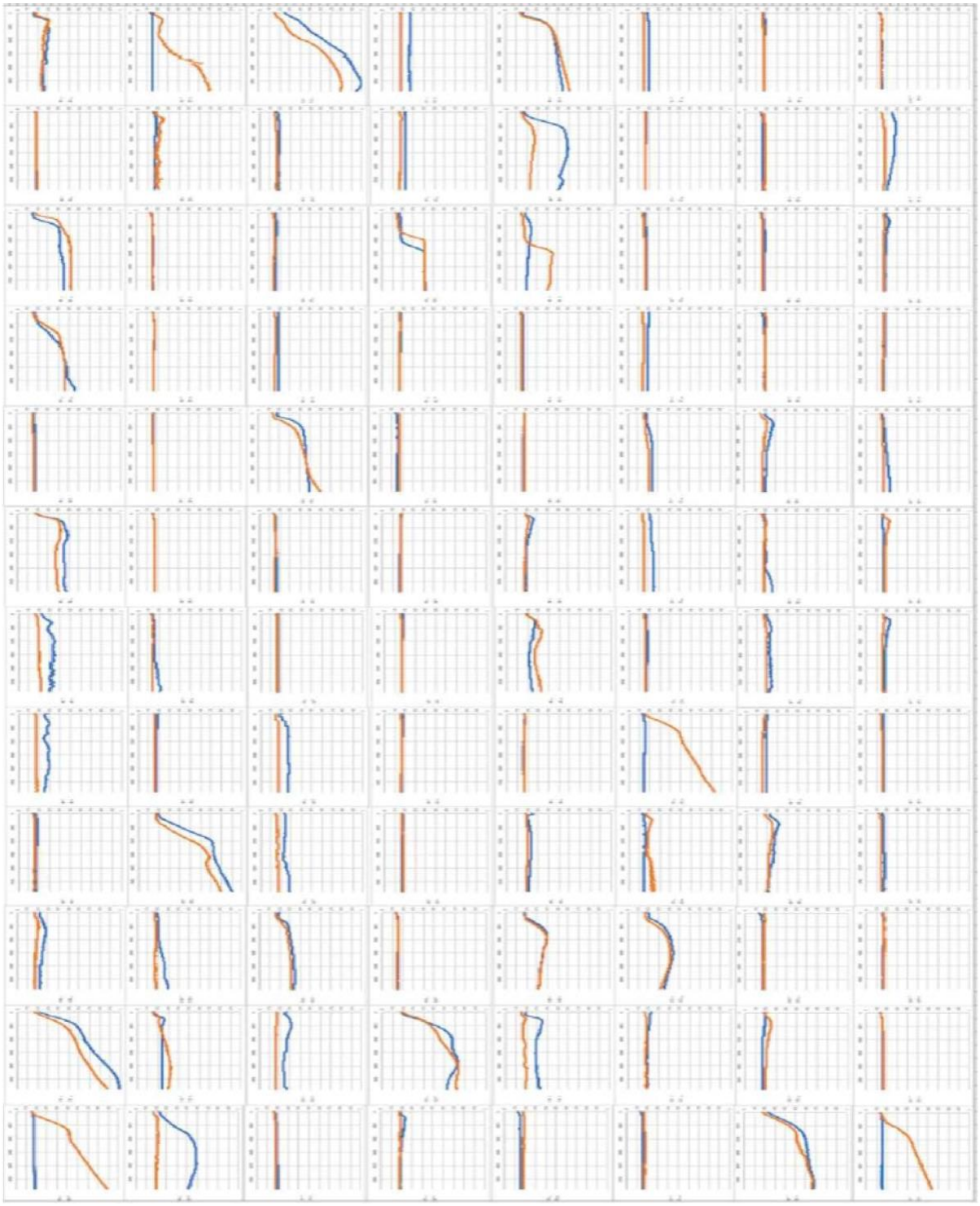
8. Run on the thermocycler at 37 C for 4 minutes and then 80 C for 1 min.

9. Send for sequencing.

APPENDIX C

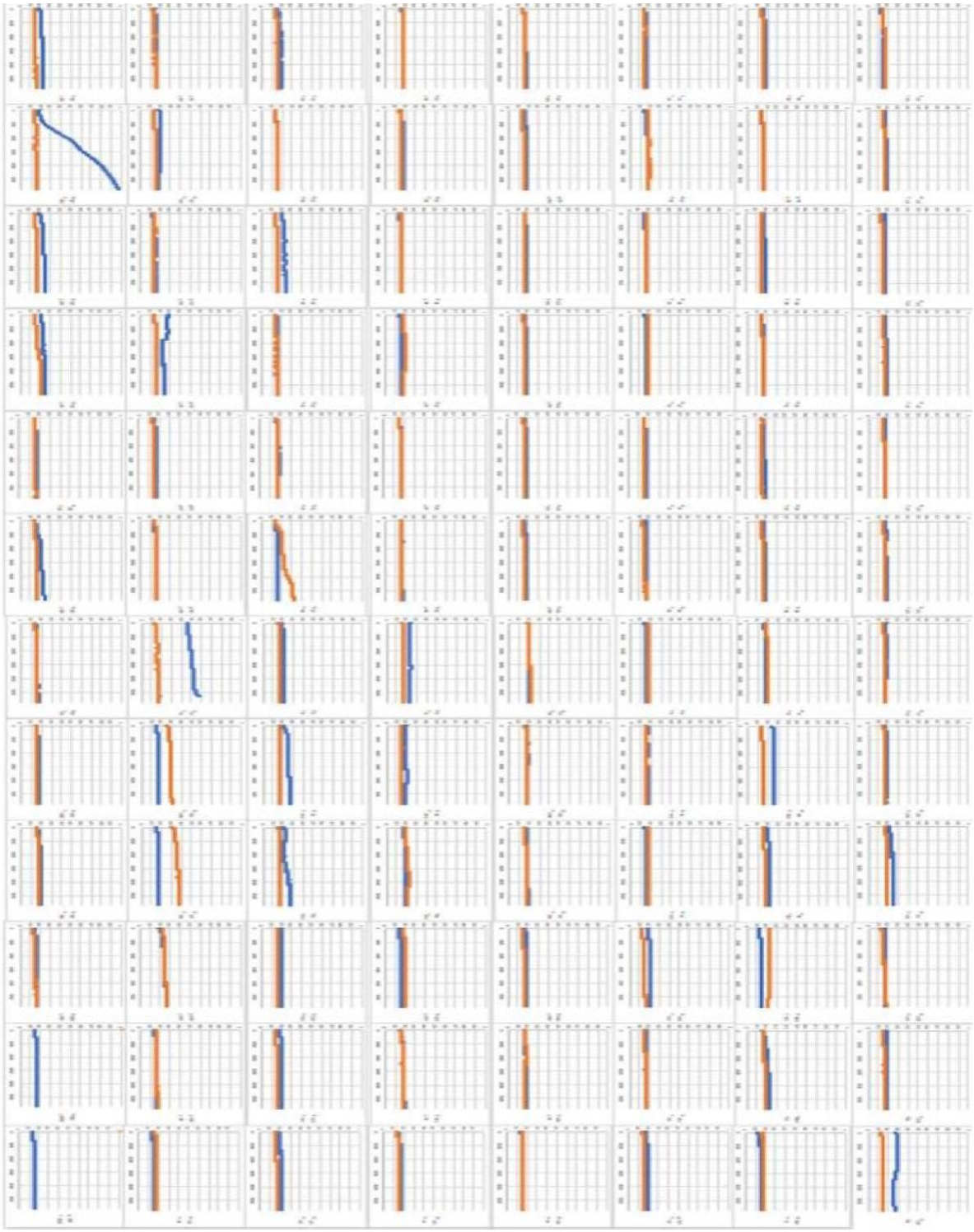


APPENDIX D



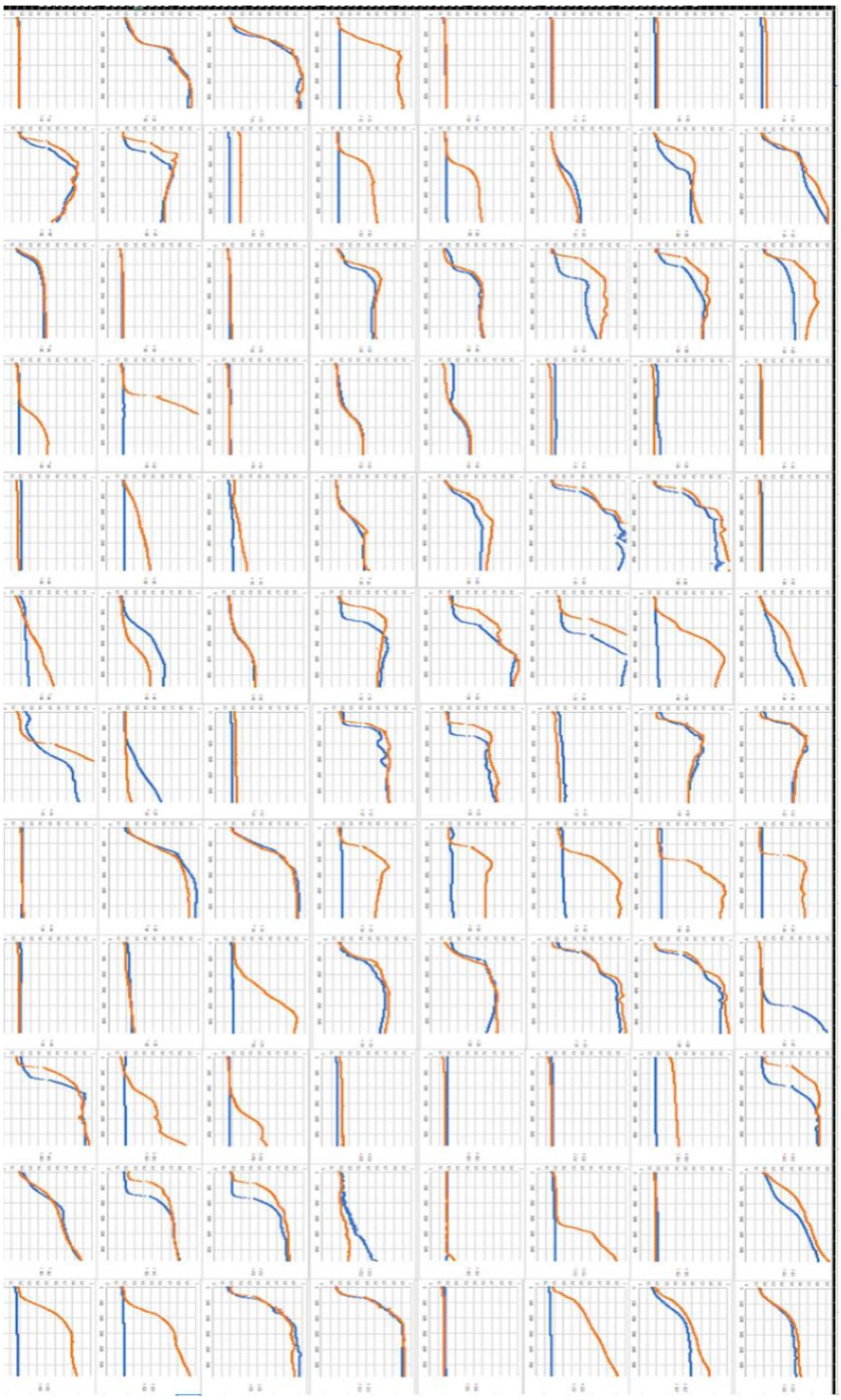
APPENDIX E

Individual growth curves for low pH + high nitrate condition. The orange and blue line on the graphs represent two different trials.



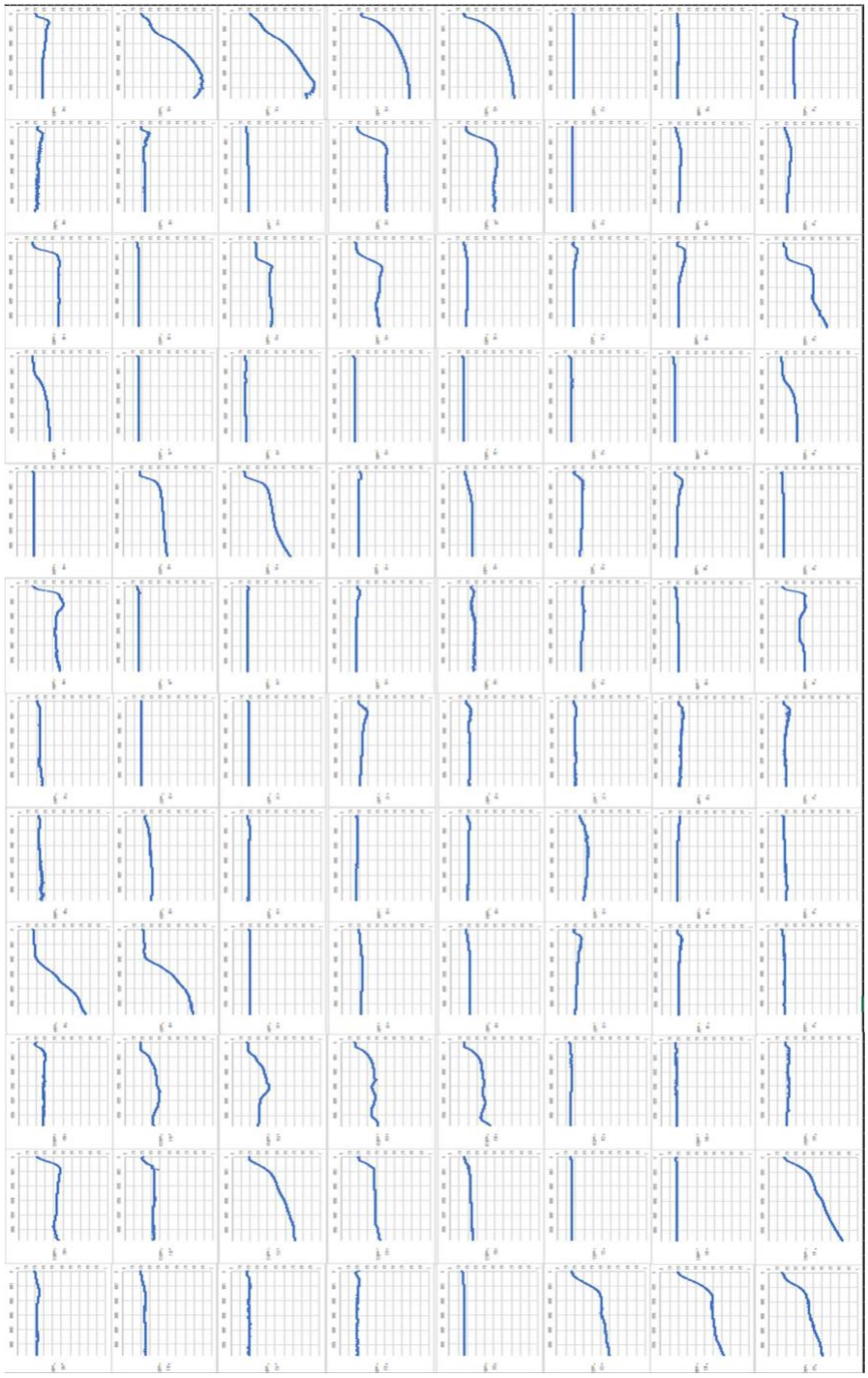
APPENDIX F

Paired growth curves for low pH condition. The orange and blue line on the graphs represent two different trials.



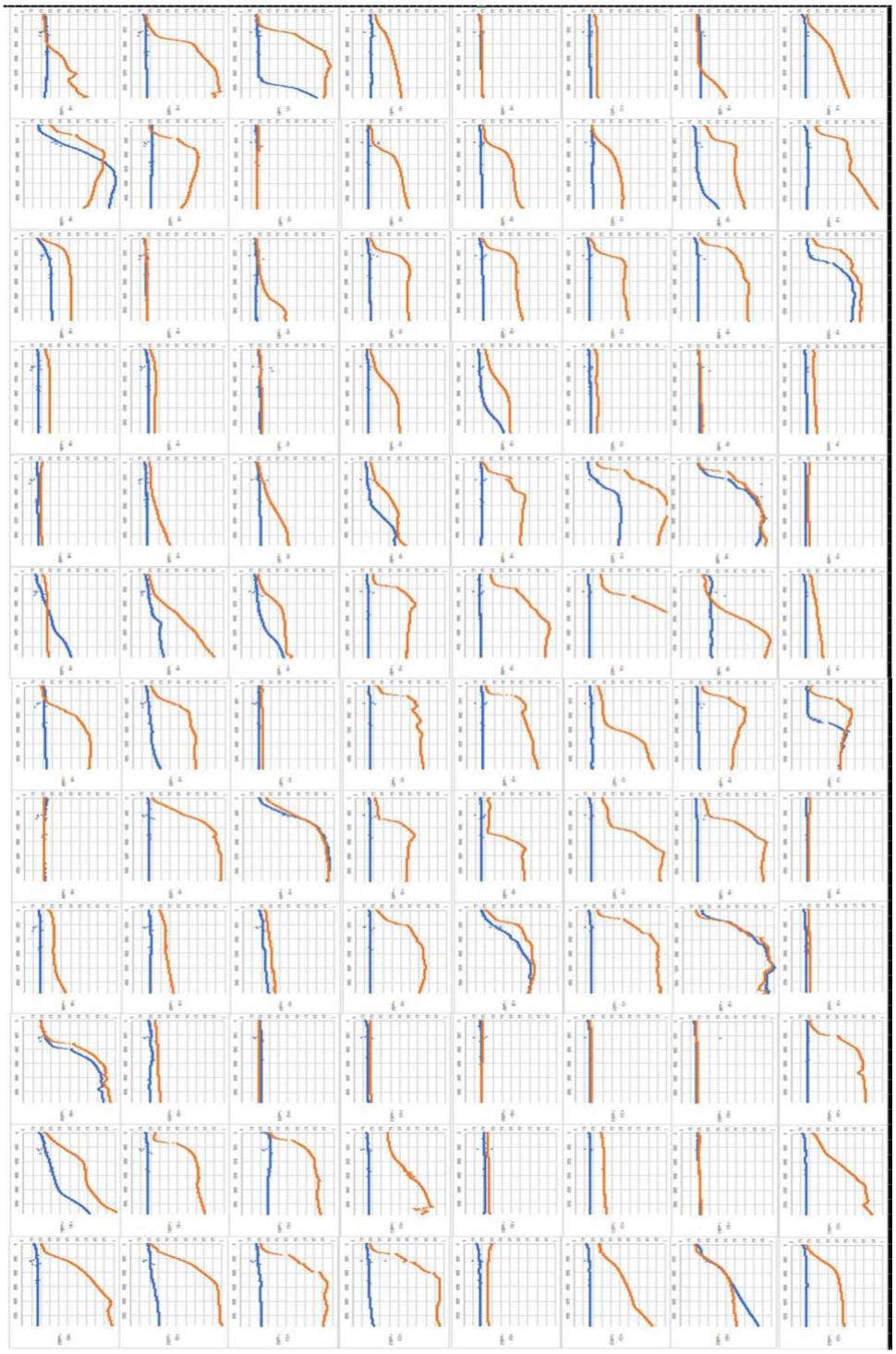
APPENDIX G

Paired growth curves for high nitrate condition. there is only one trial because there wasn't enough time to collect data for a second trial.



APPENDIX H

Paired strain growth (orange) vs individual strain growth (blue) in low pH condition.



APPENDIX I

Paired strain growth (orange) vs individual strain growth (blue) in high nitrate condition.