October 3, 2023

Background Research

Azotobacter Characteristics:

- Role in Nitrogen Cycle: Azotobacter plays an important role in transforming atmospheric nitrogen (N_2) into ammonium ions (NH_4^+)

- Nitrogenase Types in Azotobacter: ~~Azotobacter~~ Azotobacter employs various types of nitrogenase for nitrogen fixation, with the molybdenum-iron nitrogenase being the most basic one used
  - ↳ This allows Azotobacter to easily adapt to various environmental conditions

- Symbiotic Relationship or Free-living?: While some species establish symbiotic relationships with plants, the majority function independently in nitrogen fixation, making them versatile contributors to the nitrogen cycle and excellent candidates as bio fertilizer for nonlegumes

- Physical attributes: Also known as diazotrophs, they exhibit motility and take on oval or spherical shapes. They form durable cysts, contributing to their resilience in various soil conditions,

- Habitat: Thrives as aerobic soil microbes Azotobacter is commonly found in alkaline soil, aquatic environments and occasionally on specific plant surfaces

- Applications: Azotobacter's oxygenation as a biofertilizer stems from its ability to enhance soil fertility. Additionally it serves as a key model organism for the study of diazotrophs and find applications in the production of food additives and specific bio polymers.

Page 3

October 5, 2023 Background Research

Azotobacter Nitrogen Fixation:

- process:

Nitrogen fixation process involves capturing atmospheric nitrogen directly without relying on symbiotic partnerships with plants

- Enzymes:

Azotobacter possesses a diverse array of enzymes that facilitate nitrogen fixation, including nitrogenase. The process of nitrogen fixation is also sensitive to oxygen, prompting the bacteria to develop mechanisms to mitigate oxygen exposure.

- Defense mechanisms

In response to Oxygen, Azotobacter employs defense mechanisms that involve intensifying metabolic activities while concurrently reducing cellular Oxygen levels ensuring efficient Nitrogen fixation.

October 17th, 2003

Pag 3: Characteristics of Nitrogenase

- Nitrogenases: Enzymes catalyzing conversions of atmospheric nitrogen to ammonia

- Various types:
  - Molybdenum (Mo)
  - Vanadium (V)
  - Iron-only (Fe) nitrogenase

- Adaptability: Different nitrogenases for efficiency in diverse environmental conditions

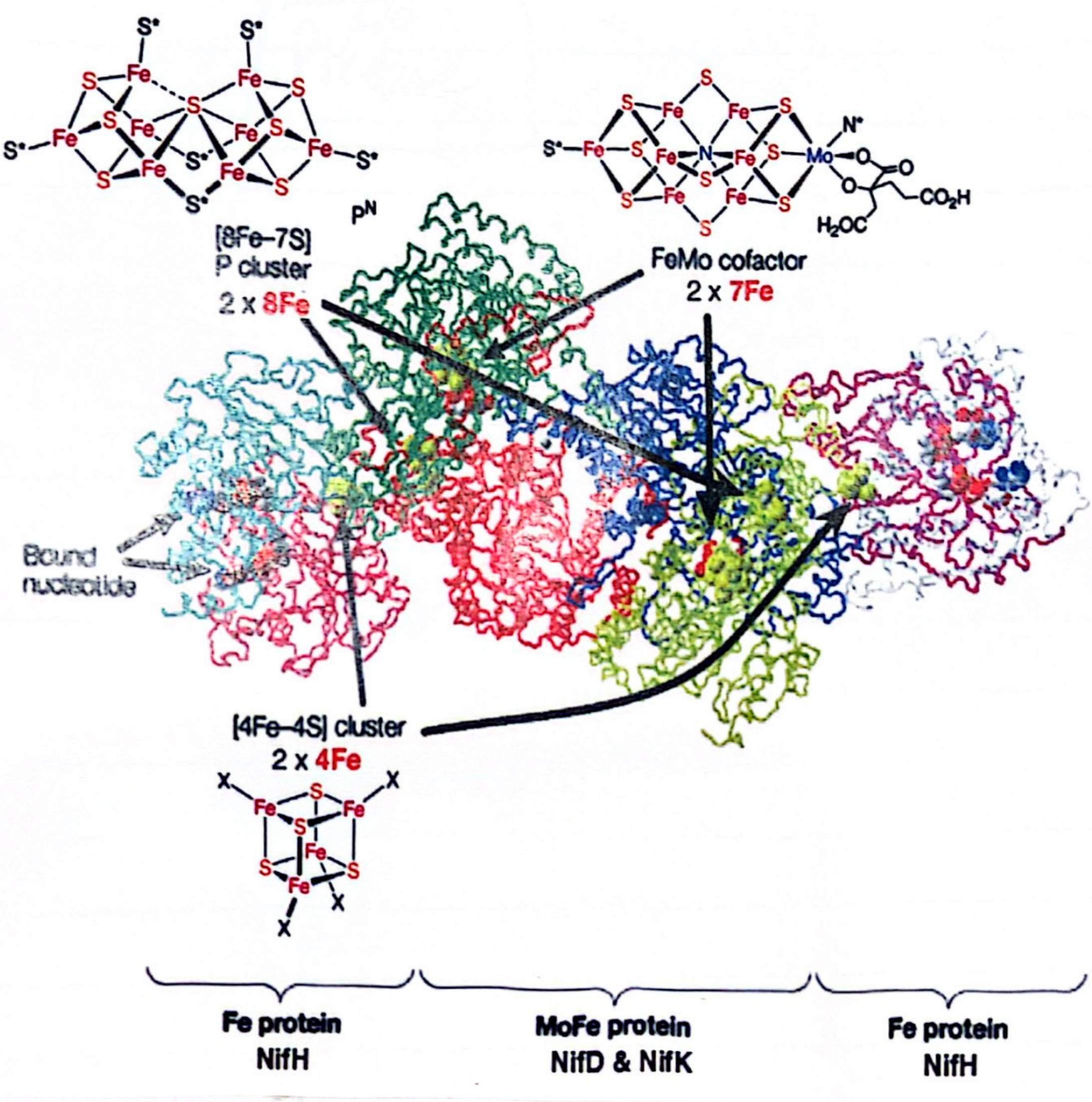
- V-Fe nitrogenase: More active at low temperatures and colder environments

- components of nitrogenase
  - Mo-Fe protein: Houses reaction machinery, requires constant energy supply

  - Fe protein: Transports electrons to Mo-Fe protein using ATP breakdown

Electron requirement: 6 e^- needed to split each ~~[illegible]~~ nitrogen molecule into two ammonia molecules

- ATP consumption: 2 ATP molecules expended for each electron transferred
- Hydrogen formation: Nitrogenase simultaneously converts H^+ to $H_2(g)$, potentially consuming additional ATP



October 22nd

Page: Nitrogen Cycle

- Earth's atmosphere: 78.1% nitrogen gas (N_2), 20.9% O_2, 1% CO_2 and trace gas

- Nitrogen not directly usable by most organisms

- Nitrogen fixation - certain bacteria convert N_2 into ammonium.

- Nodes on legume roots - specialized bacteria fix nitrogen, forming a symbiotic relationship with plants.

- Ammonium generation:
  - Ammonification: Decomposers break down organic matter to produce ammonium.
  - Soil bacteria convert ammonium to nitrite (NO^{2-}) and (NO^{3-})

- Nitrogen utilization: Plants use nitrate a a nitrogen source for growth.

- Denitrification: Denitrifying bacteria convert nit nitrite back to nitrogen gas in Oxygen limi environment.

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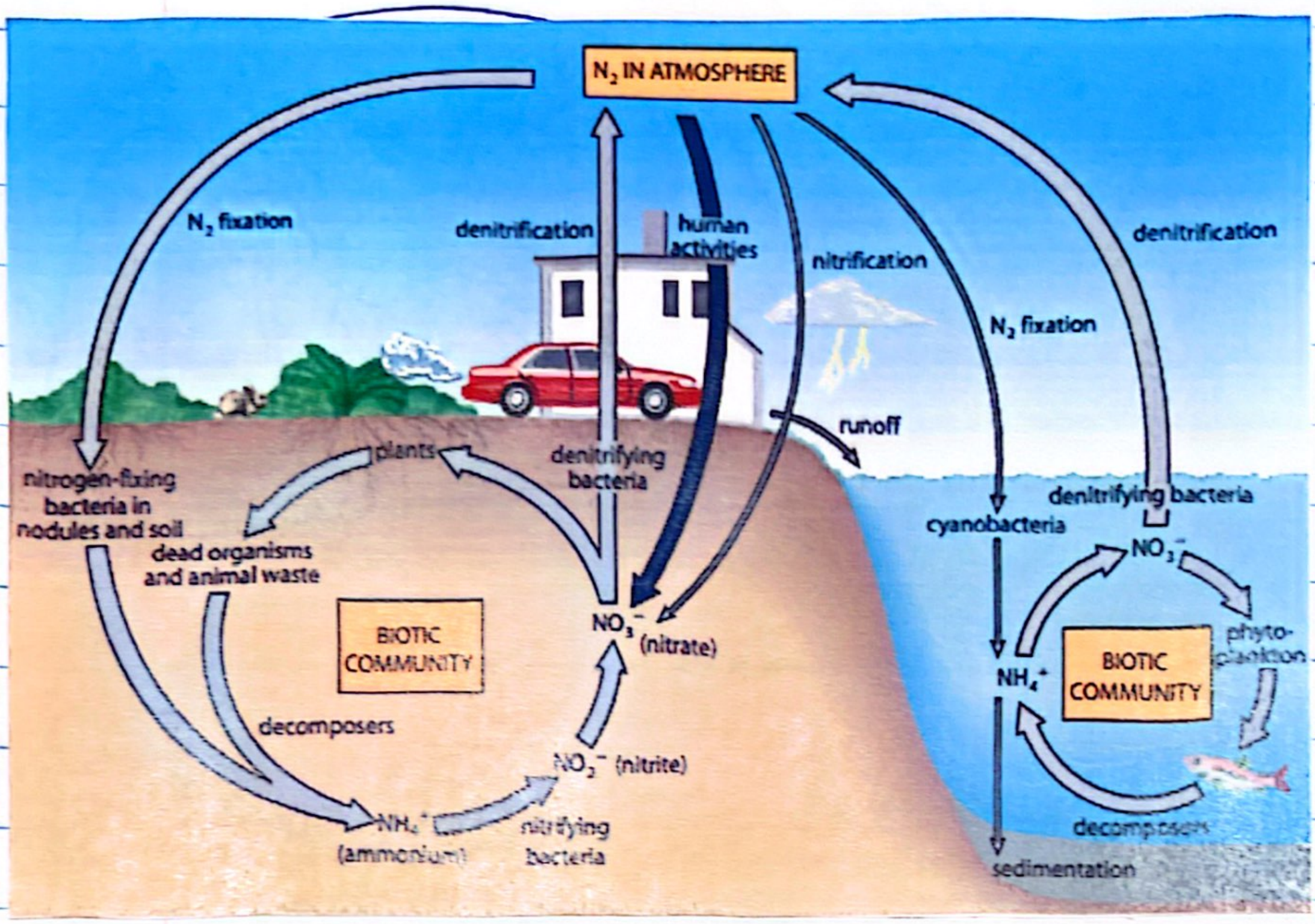

November 3rd, 2023

- Biological nitrogen fixation converts atmospheric nitrogen to ammonia using nitrogenase enzyme.

- Overall reaction: $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$

- Requires hydrolysis of 16 ATP

- Occurs at iron - molybdenum cofactor and involves protonation and reduction steps at the active site

- Free living diazotrophs integrate ammonia into glutamate via glutamic synthase.

- Nif genes essential for nitrogen fixation are found in different microbes across diverse environments.

- Nitrogenase sensitivity to Oxygen degradation:
  - Many bacteria cease enzyme production in oxygen rich environments
  - Some nitrogen-fixing organisms thrive in anaerobic conditions.
    - Reduces oxygen levels via respiration
    - Utilize oxygen biding proteins

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November 7th, 2025

Introduction Research

Haber Bosch Process

- Revolutionized agriculture by mass-producing ammonia from atmospheric nitrogen and hydrogen

- Enabled widespread use of synthetic fertilizers

- In addition to the significant carbon footprint, while depleting the nutrients in the soil

- Biological Nitrogen Fixation (BNF)
  - Offers sustainable alternative to synthetic fertilizers

- Involves Nitrogen-fixing microorganisms forming symbiotic relationships with plants or present freely in soil
  - Effective only in legumes
  - Majority of crops are non legumes

Does this through root nodules directly supply the nitrogen host to

Free-living Bacteria
→ Crucial for BNF for nolegumes

~~Hypothesis~~ 3

~~Our hypothesis suggests that the addition of ferrous sulphate to the growth~~

Hypothesis

~~To~~ Our Hypothesis suggests that the addition of ferrous sulphate to the growth medium will augment the efficiency of nitrogen fixation by Azotobacter strains, owing to ~~the~~ its provision of essential iron co-factors crucial for Nitrogenase enzyme activity. Conversely it also suggests that excessive concentrations of sodium chloride may impede Nitrogen fixation efficiency due to potential osmotic stress and disruption of cellular process, despite its necessity for bacterial growth.

Thus, the hypothesis anticipates that optimizing ferrous sulphate concentration while maintaining low, sodium chloride levels will yield optimal nitrogen fixation efficiency in the modified Azotobacter strains.

November 9th

Variables

Independant variables:

- Concentration of Iron (II) Sulphate: The different concentrations of iron (II) sulphate represent an independent variable as they are deliberately manipulated to assess their effects on nitrogen fixation.

- Concentration of sodium chloride: Similarly, the different concentrations of Sodium chloride represent another as that is also manipulated to observe its impact on nitrogen fixation.

Dependent Variables

- Ammonium ion Concentration: The Concentration of ammonium ions in the testing solution derived from the agar samples serve as a dependent variable. This variable is measured to assess the effectiveness of nitrogen fixation by the modified Azotobacter strains under different nutrient compositions.

Controlled Variables:

- Type of Azotobacter

- Temperature of the room: By maintaining a constant temperature in the experimental environment, we ensure that external fluctuations do not affect bacterial growth. Temperature variations can profoundly impact microbial metabolism and growth rates. Consistency in temperature provides a stable and controlled setting for observing the effects of the manipulated variables.

- pH of growth medium: The pH level of the growth medium plays a crucial role in bacterial metabolism and nutrient uptake. Fluctuations in pH can influence enzyme activity, microbial growth rates, and nutrient uptake. By keeping the pH of the growth medium constant, we eliminate potential pH-related variations that could confound the results. This ensures that any observed differences in nitrogen fixation efficiency is attributable to the manipulated variables rather than pH fluctuations.

Time allowed for bacterial growth:
providing a constant duration for
bacterial growth allows for uniformity
in observing nitrogen fixation efficiency
across all experimental trials.
Bacterial growth rates can vary
depending on various factors, including
nutrient availability, temperature, and
strain characteristics. By maintaining a
fixed incubation period, we ensure
that all bacterial cultures have
sufficient time to reach comparable
growth stages, facilitating accurate
comparisons of nitrogen fixation
efficacy.

Nutrient composition of the growth medium
Apart from the manipulated variable,
such as iron(II) sulfate or
sodium chloride concentrations, the
nutrient composition of the
growth medium should remain
constant. Nutrients other than
the ones under investigation
can also influence bacterial
growth and nitrogen fixation.
Therefore, keeping the composition
of other nutrients consistent
ensures that any observed

B

effects on nitrogen fixation, are solely attributable to the manipulated variables and not to variations in external variability

- Shaking/agitation of the growth medium: Uniform mixing or agitation of the growth is essential for ensuring equal distribution of nutrients, oxygen, and bacterial growth and nitrogen fixation within and across experimental samples. By maintaining consistent stirring or agitation techniques across experiments, one minimize the potential for localized gradients and ensure homogeneous growth for all bacterial cultures

November 17th

Procedure

Step 1: Preparation - Agar Plate (Repeat for modification)

~~100g of ferrous sulphate fertilizer was dissolved in~~

~~Addition~~

Addition of Substances:

- Dissolve 50 mg of the iron (II) sulfate crystals to 100 mL of water to make a 500 ppm solution
- ~~Add~~

- Dissolve 500 mg of iron (II) sulfate to 100 ml of water to make the 1000 ppm iron growth medium

- Dissolve 150 mg of iron (II) sulfate to 100 ml of water to make a 1500 ppm solution

- Dissolve 1g of sodium chloride to 100 mL of water to make a 1% solution

Add 2.5g of Nutrient Agar each

17

- Dissolve 2g of sodium chloride to 100 ml of water to make 2% solution
- Dissolve 3g of sodium chloride

Plate pouring:

- ~~Dissolve~~
- Heat each solution to 85°C
- Allow each solution to cool down to 60°C and pour each solution into 3 separate petri dishes
- Allow the poured agar to solidify in the petri dishes and cool down to room temperature forming a nutrient-rich medium for bacterial growth

- ~~Stir~~ The solution was stirred magnetically on a hot plate stirrer to ensure uniform agitation

Prelude to step 1: Ferrous Sulphate Purification:

- 20 g of ferrous sulphate was dissolved in 160 mL of distilled water.

- The solution was allowed to cool down to room temperature (around 20°C) overnight and was then left outside at 4°C for two days to allow crystallization.

- After two days, the crystals of pure ferrous Sulphate were harvested and dried.

Step 1: ~~Preparation~~ - Agar plate setup

Addition of substances:

- Dissolve 50 mg of the ferrous Sulphate crystals into 100 mL of water to make a 500 ppm solution.

- Dissolve 100 mg of the ferrous sulphate crystals into two mL of water to make a 1000 ppm solution.

- Dissolve 150 mg of the ferrous Sulphate crystals into 100 mL of water to make a 1500 ppm solution.

Step 2: Culture system setup - Initial phase:

Environmental conditions:
- Place the plated plates in a controlled environment set to a constant temperature of 18 degrees celsius.

- Properly label each plate to ensure accurate tracking and observation throughout the experiment.

Incubation period:
- Allow sufficient time, 6 days, for the bacterial colonies to grow and establish themselves on the nutrient-enriched agar.

Sample collection for testing:
- Following the growth period, collect samples of agar growth medium from a pippete tip, maintaing a constant size of 2.5 mm^3.

Preparation of solutions for testing
- Dissolve the collected samples each in 10 mL of distilled water. Ensure thorough mixing to create consistant solutions.

Ammonium Ion Concentration test:
- Utilize a specialized ammonia test kit to measure ammonium ion concentrations accurately.
- Adhere strictly to the provided instructions within the kit to execute the test protocol for each solution from the agar samples.

Step 3: Determining the Ideal Nutrient Composition.

- Analyze the results obtained from the initial phase to determine the optimal combination and quantities of iron (II) Sulphate and Sodium chloride that produced the maximum nitrogen fixation efficacy.

~~Chemical Absorption Period:~~
- ~~Allow the~~

→ Chemical Absorption Period:
- Allow the Azotobacter strains to grow and acclimate to the specific nutrient environment provided by the formulated plates for 6 days.

Step 4: Assessment of Ammonium Ion Concentration

Sample Collection for Testing

- collect agar samples from each of the three plates containing the modified Azotobacter strains following the 5-day period

Preparation of Testing Solutions

- Dissolve the collected agar portions from each plate in equal volumes of distilled water to create standardized testing solutions for ammonium ion concentration assessment

Ammonium Ion Concentration Test

- Use the strips from the ammonia kit to measure how much ammonium concentration is in each solution

Interpretation of Results:

- Record and compare the ammonium ion concentration levels obtained from the testing solutions derived from the modified ~~Acetobacter~~ Azotobacter strains.

November 30th

23

Part 1 of Experiment

followed procedure in step 1 - 2.

- Prepared and settled up Culture systems

- allowed bacterial growth for <u>five days</u>

November 3rd

Mathematical Logic 24

To calculate the actual concentration of Fe^{2+} in the trials, we first find the molar mass of ferrous sulphate heptahydrate.

Molar mass of $FeSO_4 \cdot 7H_2O$
$= 278.02$

Molar mass of iron $= 55.845$

$$\frac{55.845}{278.02} = 0.200876\ldots$$

This means that only around 20.087... % of the mass added actually consists of iron (II) ions.

Therefore, in the trials with 500 ppm ferrous sulphate heptahydrate, the actual concentration of Fe^{2+} is about 100 ppm.

Similarly, the trials with 1000 ppm $FeSO_4 \cdot 7H_2O$ only contains 200 ppm Fe^{2+}.

The trials with 1500 ppm $FeSO_4 \cdot 7H_2O$ contains 300 ppm Fe^{2+}.

Iron in soil
- Good levels should be between 50 and 100ppm for plants

December 3rd 20

Moderately Saline soil typically has a Salinity of 8 - 15 dS/m.

A 1% Concentration of NaCl is equal to 10000 ppm.

Assuming that 1 mS/m = 6.4 ppm (roughly)

Then; a Concentration of 10000 ppm is equal to 1562.5 mS/m.

Since 1 dS/m = 100 mS/m

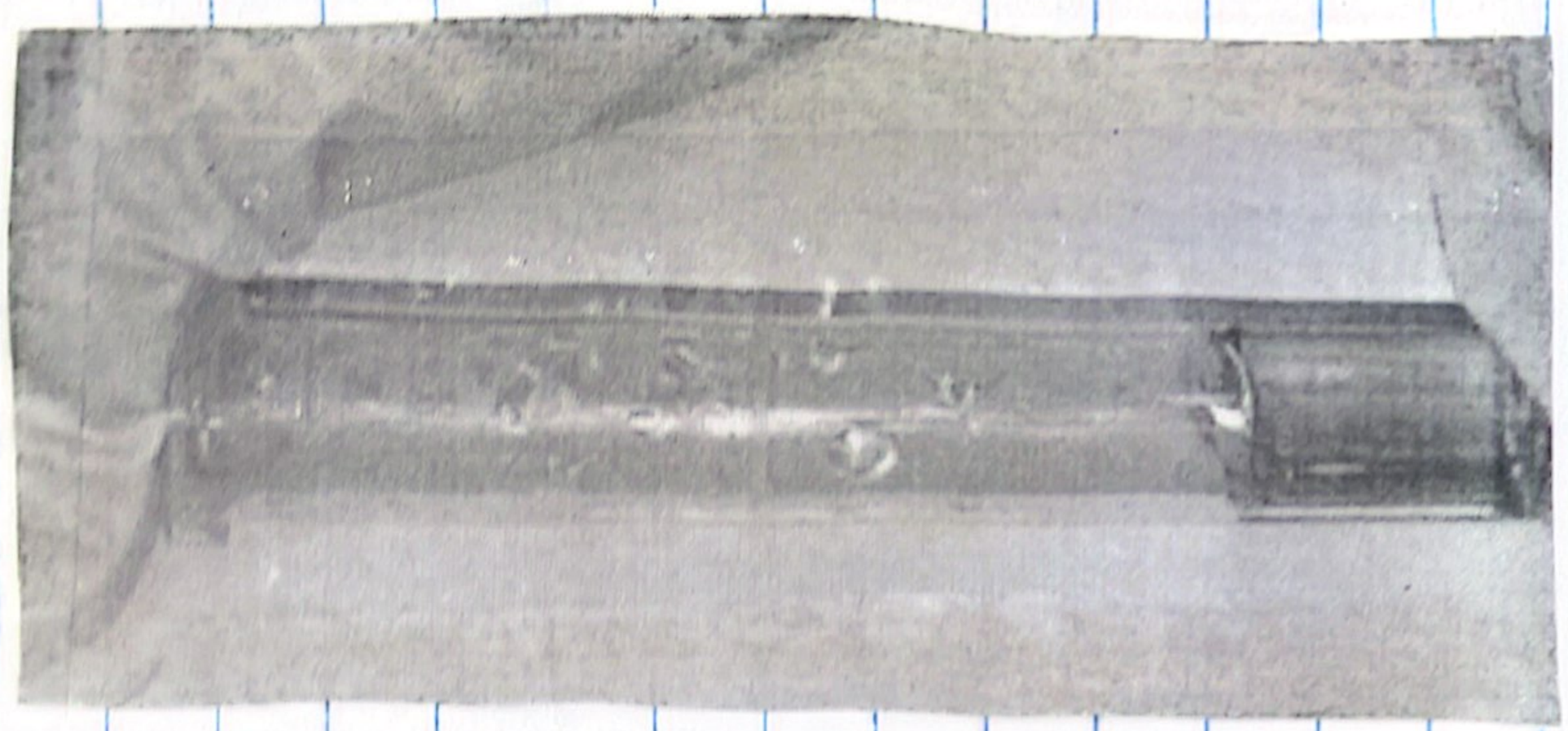
1562.5 mS/m = 15.625 dS/m

Therefore, a Concentration of 1% NaCl Correlates to just above moderately Saline Soil.

Similarly,

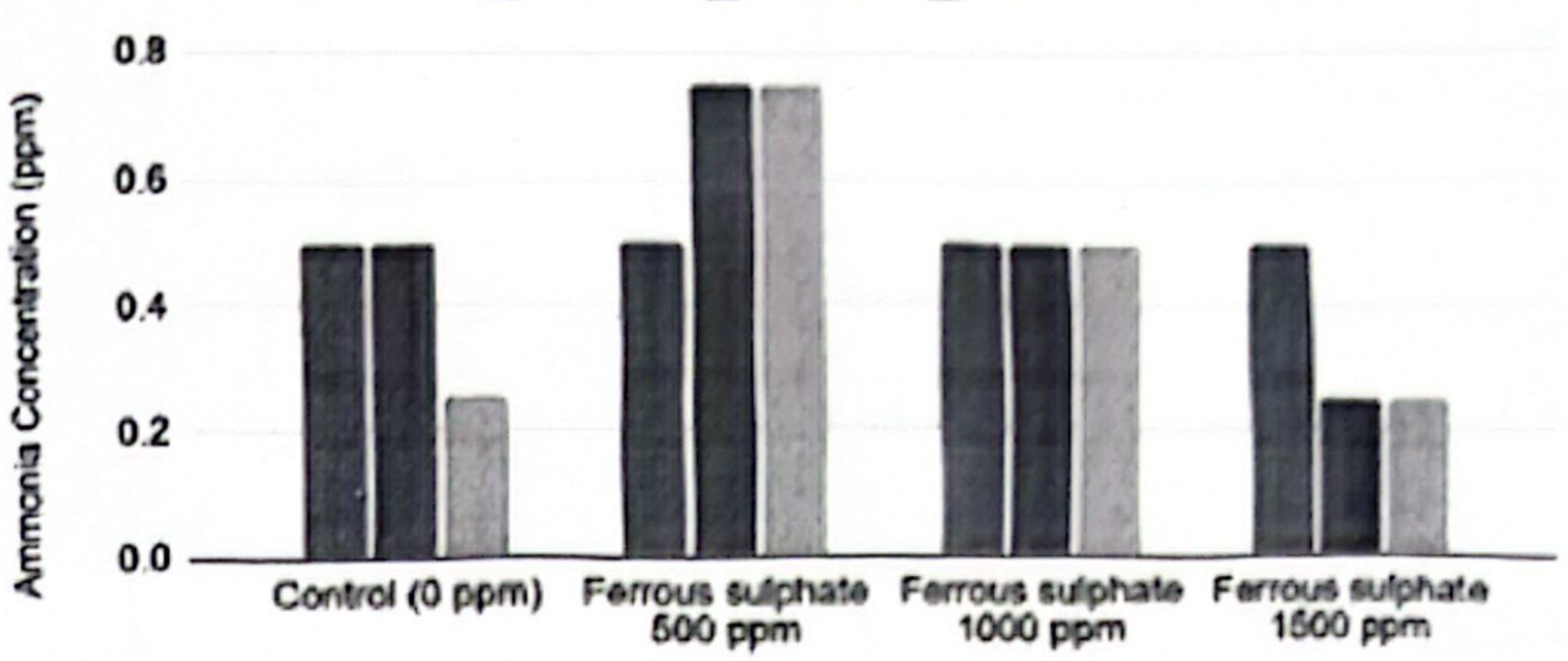
2 % = 31.25 dS/m
3 % = 46.875 dS/m

These all correspond to highly Saline Soil.



(5th day of experiment)

Effects of Various Ferrous Sulphate Concentrations on the Nitrogen Fixing Activity of Azotobacter



Table

Ammonia Concentration (ppm)			
Trial #1	Trial 2	Trial 3	
0.5	0.75	0.75	
0.5	0.5	0.5	
0.5	0.25	0.25	
0.5	0.5	0.5	
0.5	0.5	0.5	

Scanned with CamScanner

Table 2

~~Average~~

	Ammonia Concentration (ppm)		
	Trial 1	Trial 2	Trial 3
Control	0.5	0.5	0.25
1% NaCl	0.5	0.5	0.75
2% NaCl	0.5	0.25	0.5
3% NaCl	0.5	0.5	0.5

~~Table 2~~

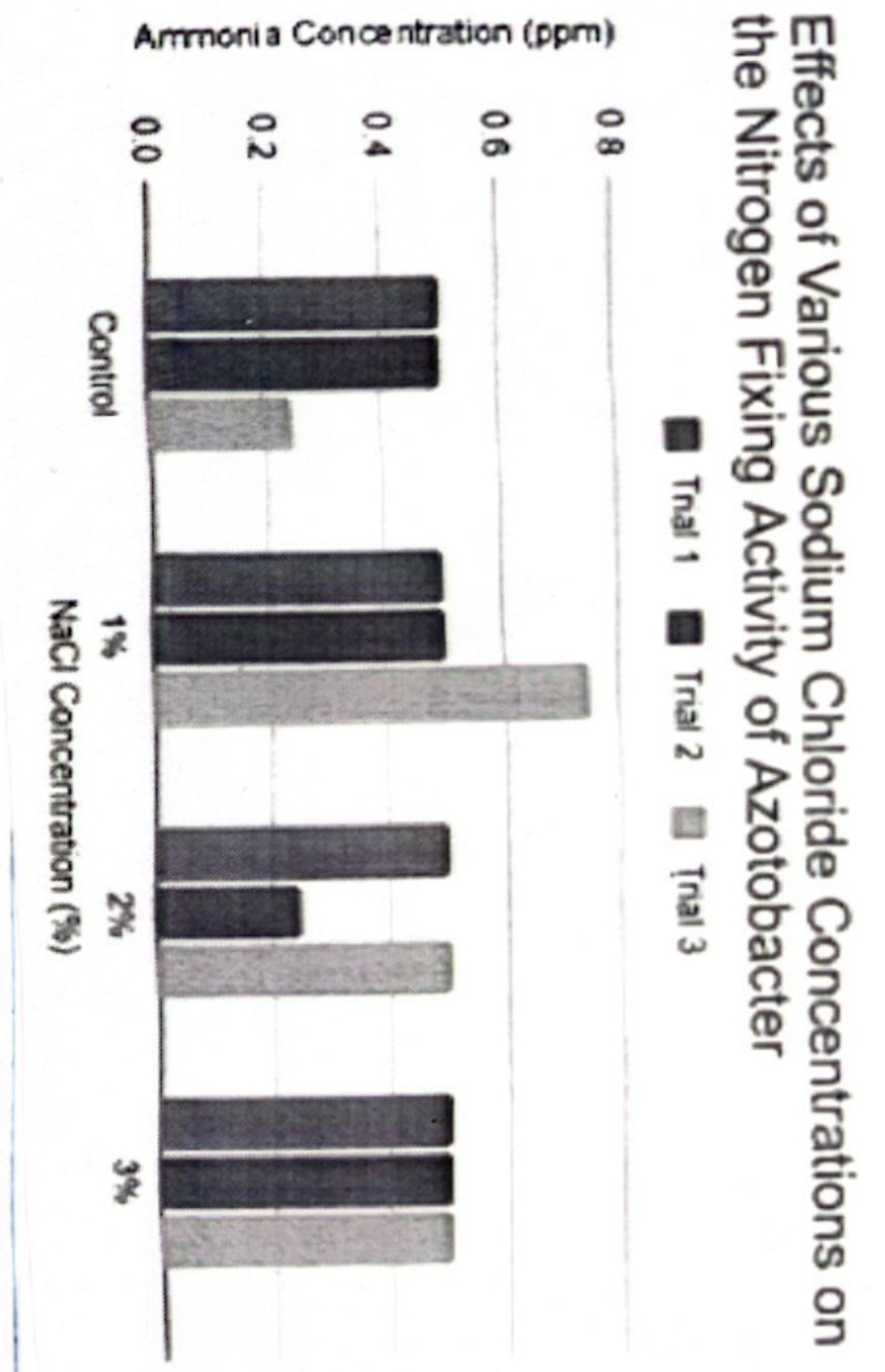


Table 3

	Average Ammonia Concentration (ppm)
Control	0.4166...
$FeSO_4 \cdot 7H_2O$ 500 ppm	0.6666...
$FeSO_4 \cdot 7H_2O$ 1000 ppm	0.5
$FeSO_4 \cdot 7H_2O$ 1500 ppm	0.3333...

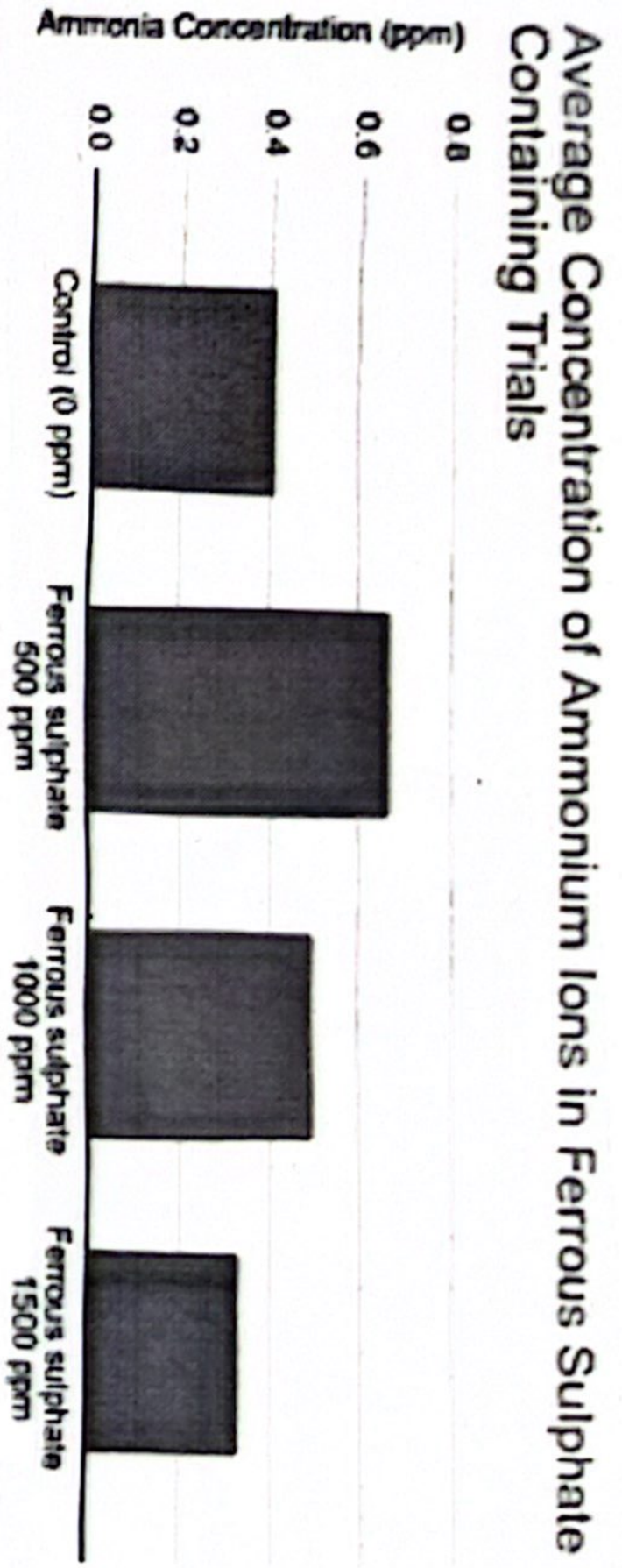
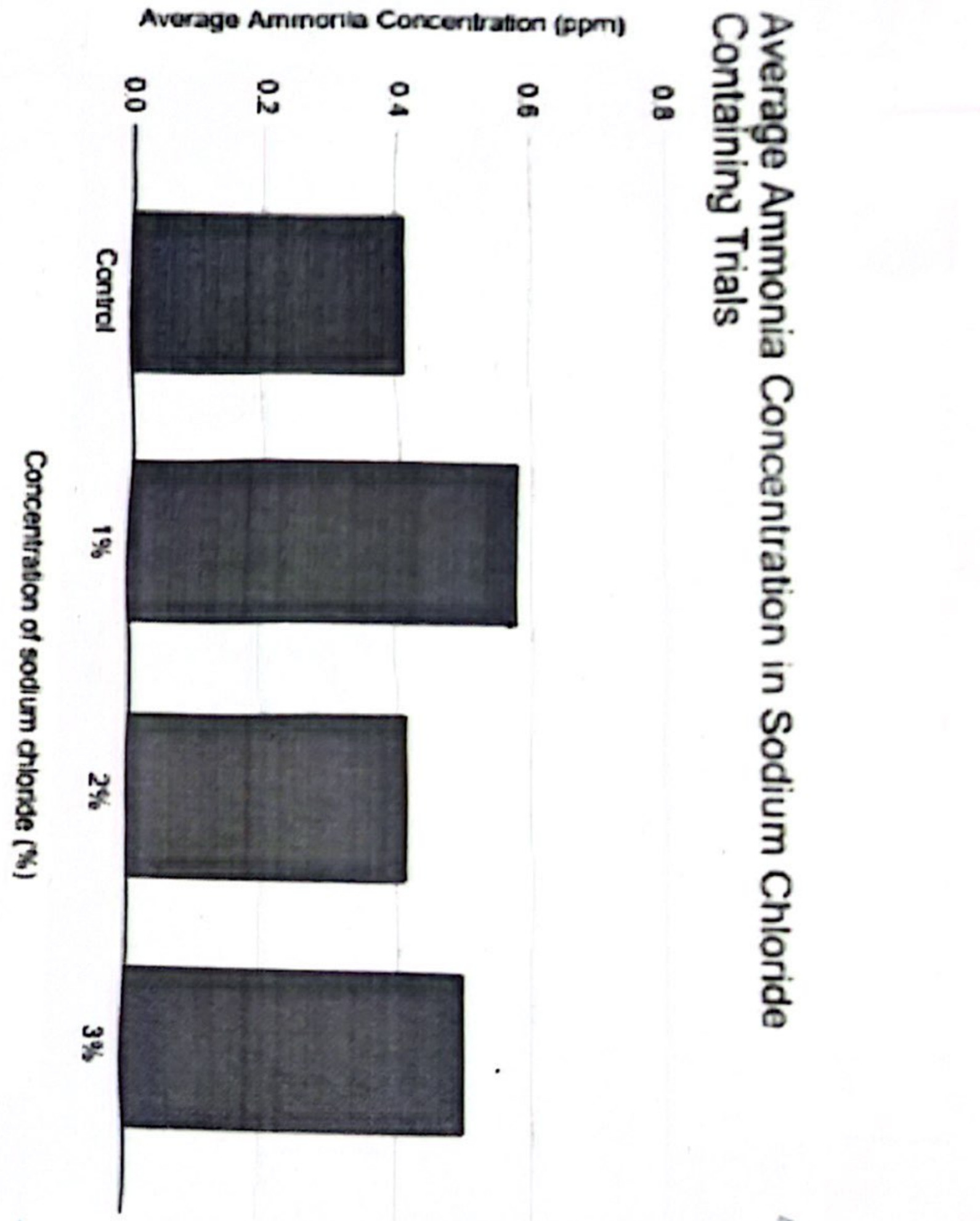
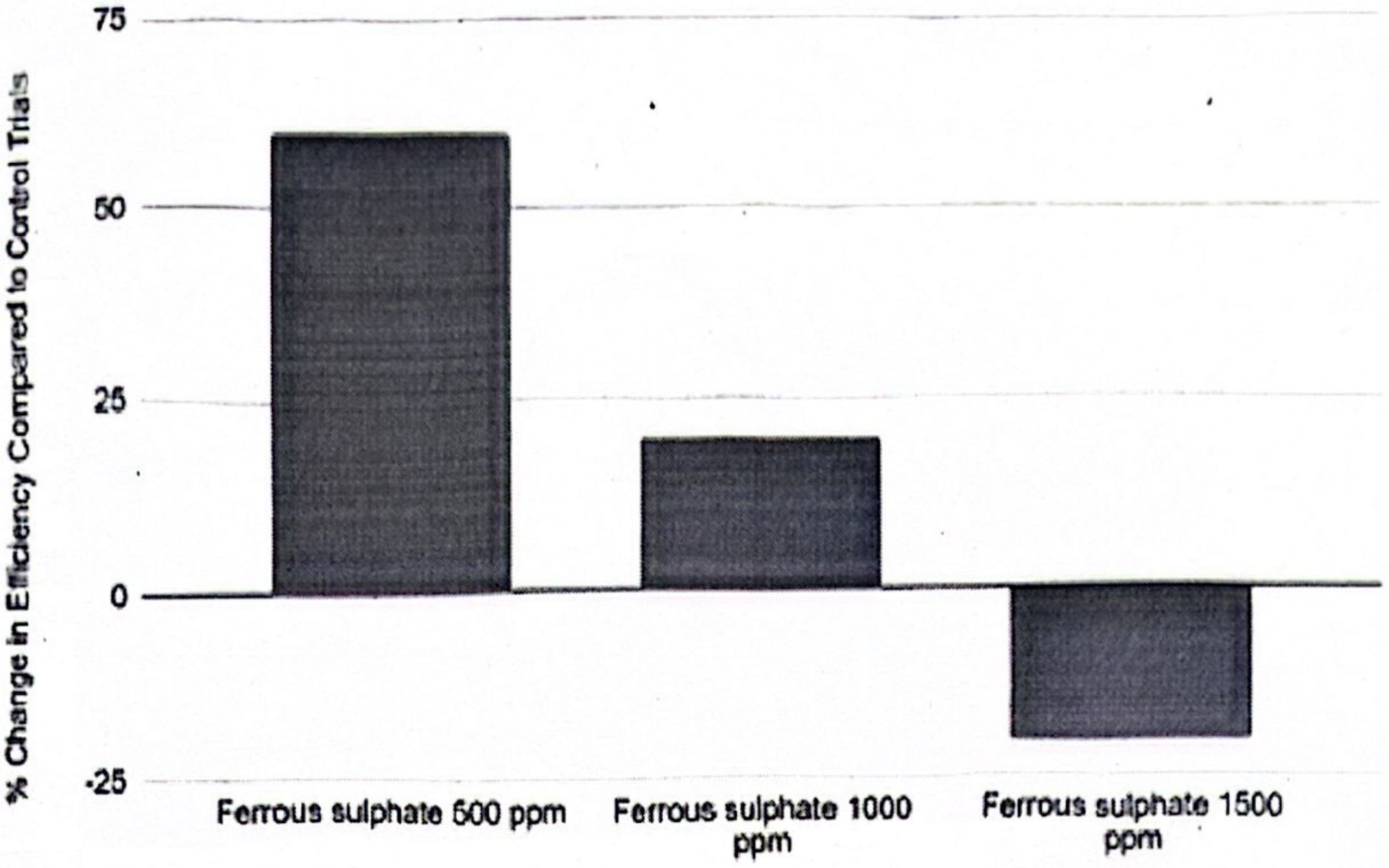


Table 4

	Average Ammonia Concentration (ppm)
Control	0.4166...
1% NaCl	0.5833...
2% NaCl	0.4166...
3% NaCl	0.5



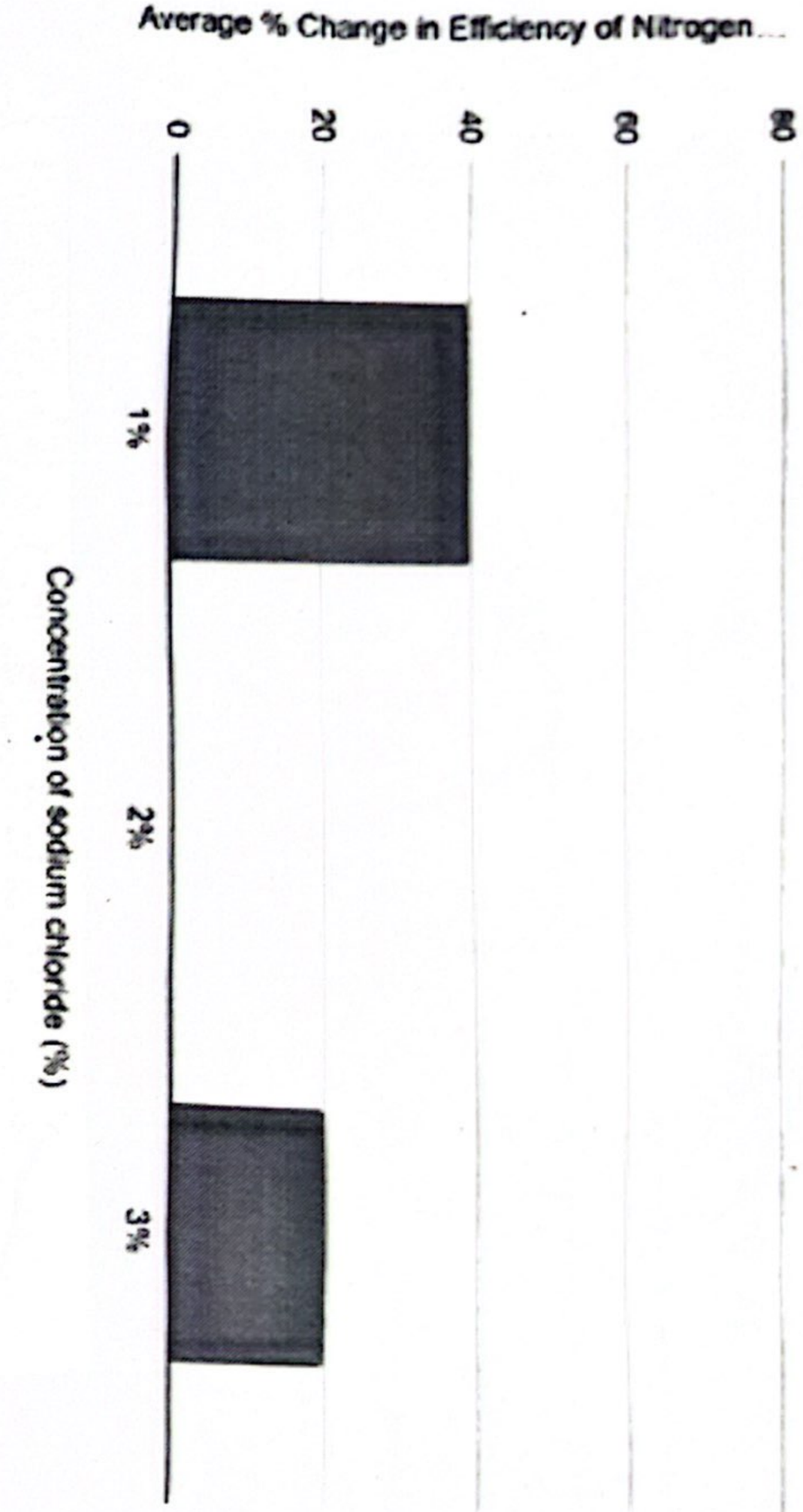
Average % Change in Efficiency in Nitrogen Fixation in Ferrous Sulphate Containing Trials Compared to Control Trials



	Average % change in efficiency compared to control
$FeSO_4 \cdot 7H_2O$ 500 ppm	60
$FeSO_4 \cdot 7H_2O$ 1000 ppm	20
$FeSO_4 \cdot 7H_2O$ 1500 ppm	- 20

Table 6

	Average % Change in efficiency Compated to Control trials
1% NaCl	40
2% NaCl	0
3% NaCl	20



Average % Change in Efficiency of Nitrogen Fixation Compared to Control Trials in Sodium Chloride Containing Tr...

December 14th

Table 1:

- Control group consistently displayed moderate nitrogen fixation (0.25 - 0.5)
- 500 ppm of $FeSO_4 \cdot 7H_2O$ showed some impact on nitrogen fixation
- 1000 ppm of $FeSO_4 \cdot 7H_2O$ showed no impacted
  - ↳ similar to control group
- 1500 ppm of $FeSO_4 \cdot 7H_2O$ resulted in a slight decrease in nitrogen fixation compared to control

Table 2:

- Control group and 1% sodium chloride showed consistent ammonia concentrations
- Increasing in NaCl concentration (2% - 3%) only led to slight decrease in ammonia concentration

Table 3:

- 500 ppm of $FeSO_4 \cdot H_2O$ resulted in the highest average ammonia concentration indicating enhanced nitrogen fixation

- 1000 ppm and 1500 ppm led to no effect and gradual decreasing in ammonia concentrations respectively
  - ↳ Suggest inhibitory effect

- Table 4.
  - Control group and 1% Sodium chloride had similar average ammonia Concentrations.
  - Increase in Sodium chloride Concentrations (2% and 3%) led to a noticible decrease in ammonia Concentrations

Table 5

- At 500 ppm of ferrous sulphate, average percent change in efficiency: 60 %, indicating optimal range for nitrogen fixation by azotobacter.

- At 1000 ppm, average percent change in efficiency drops to 10 %, suggesting reduced effectiveness.

- -20 % average percent change in efficiency at 1500 ppm suggests potential toxic effects of high iron concentrations.

- Emphasizes importance of optimizing nutrient concentrations.

- 10% NaCl trials - Average percent change in efficiency is 40 %

-2 % NaCl - 0 %

-3 % NaCl - 20 %

- Slight increase in Nitrogen fixing efficiency at 1%, while efficiency returns to more or less normal at 5 NaCl concentration reaches 2% and 3%

30 December 30th

Analysis

$FeSO_4 \cdot 7H_2O$

- The efficiency of Fe^{2+} ions in promoting nitrogen fixation follows established principles of microbial ecology.

- At 800 ppm of $FeSO_4 \cdot 7H_2O$ efficiency is ~~reduced~~ notably higher (60%), indicating an optimal range of nitrogen fixation

- Iron is essential role in enzymatic reactions involved in nitrogen metabolism vitally

- Efficiency decreases at higher concentrations (1000 ppm and 1500 ppm), suggesting toxicity at excess iron

- Iron

o Formation of iron (III) ions through Oxidation process may exert toxic effects on microbial cells, disrupting cellular functions vital for nitrogen fixation.

- Negative efficiency at 1500 ppm underscores severity of toxicity, overwhelming the benefits from increased iron.

Sodium Chloride:

- Relationship between NaCl Concentration and efficacy is evident.

- Control group establishes baseline efficiency

- At 1% Sodium chloride efficiency increases (40 %)

- Efficiency returns to control levels at 2% and 3 % Sodium chloride concentrations

- Increase in efficiency at 1% suggests essential roles of NaCl as macronutrients

- Decline at higher concentrations of NaCl indicate inhibitory effects due to osmotic stress.

- However, Azotobacter maintaining nitrogen fixation comparable to control trials, suggests strong evolutionary tolerance to salinity variations.

- Experimental data shows the relationship between Nutrient concentrations and Nitrogen fixation by Azotobacter strains.

- Fe^{2+} ions exhibit optimal efficacy at 500 ppm $FeSO_4$ with efficacy declining at higher concentrations

- Excessive iron levels beyond optimal range reduce toxicity, inhibiting bacterial activity and nitrogen fixation

- Negative effects observed at 1500 ppm $FeSO_4$ indicate ~~Stress~~ toxicity effects

- NaCl at low concentrations briefly increases nitrogen fixing but declines at higher concentrations

- Increase in NaCl results in lesser decline than $FeSO_4$

40

- Drill acts as important macronutrient but imposes stress at higher concentration due to osmotic stress

- Azotobacter exhibits relative tolerance to higher salinity levels
  to likely stems from Azotobacter adaptability to various soil environments

- Hydrolase was presently correct.

Jumong 10th

Application 4

- Growing interest in alternate nitrogen fertilization methods due to sustainability issues in agriculture

- BNF offers sustainable nitrogen enrichment through microorganisms like Azotobacter

- Optimal iron levels are crucial for plant physiological process

- Application of 500 ppm $FeSO_4$ presented in highest nitrogen fixing activity
  - ideal
  - ~~Overdosage~~ iron levels in soil are 100 ppm. More moderate levels are higher

- Low concentrations of and Cl% may reduce nitrogen fixing activity, but 2% and 3% may impose stress on bacteria

  - 500 ppm $FeSO_4$ ⇒ 100 ppm Fe^{2+}
    1000 ppm $FeSO_4$ ⇒ 200 ppm Fe^{2+}
    1500 ppm $FeSO_4$ ⇒ 300 ppm Fe^{2+}

- NaCl concentrations and salinity levels
  - EC => 15.6 dS/m
    - highly above moderate
  - 2% => 31.2 dS/m
  - 3% = 48.6 dS/m
    - highly saline

for.

goes to show that even under high salt stress, Azotobacter can still fix nitrogen

January 23rd

Sources of Error 43

- Dissolution of $FeSO_4$ and subsequent heating in presence of O_2 under likely caused some Fe^{2+} ions to oxidize ~~to~~ to Fe^{3+}.
  - Fe^{3+} is reduced biologically unavailable to Azotobacter leading to minimal conversion of Fe^{2+}
  - Controlling atmospheric O_2 was done by covering up the $FeSO_4$ crystals, but the little amount of O_2 that reacted was beyond our control
- Slight fluctuations in temperature
  - Inconsistent heating schedules & weather conditions
- Impurities in agar may interfere in testing

CH,

- Subjectivity in identifying colors of testing solution may reduce evaluation

Citations

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