



Lesson #7

Colorimetric Assay

Objectives:

- The learner will demonstrate comprehension of basic lab techniques by following lab procedures.
- The learner will demonstrate analysis of data by analyzing the raw data gathered.
- The learner will demonstrate synthesis of the processes taking place in the bottle by using the analyzed data to write a conclusion about those processes.

National Science Education Standards:

UCP 3, PS 3, LS 3, ST 2, HNS 2

Benchmarks:

2B, 4B, 4D, 4E, 5E, 8C, 9D, 11A, 12D

Materials:

spectrophotometer (562 nm)
Winogradsky column aged 0, 7, 14, or 21 days
0.5 N HCl
ferrozine reagent (1 gram of ferrozine per liter of 50mM HEPES buffer)
iron standards
test tubes
pipettes (0.1 ml to 5.0 ml)
syringes and needles (1 ml syringe with 1/10 ml graduations and 21 gauge needle)

(Note - the modified activity only requires the Winogradsky column, syringes and needles, and a commercially prepared iron test kit.)

Material Source Information:

Many materials can be obtained through Fisher Scientific. It is advised that you request the general catalog (as opposed to the Education catalog) for ordering the ferrozine, HEPES buffer, FeCl₂ (for iron standards) and HCl. If you will be doing the modified activity, three test kits were tested while creating these activities. EM Quant Test Strips for iron worked well and measured within the ranges needed for this activity. They can be found in the general Fisher catalog. (Note – EM Quant Iron Test Strips must be refrigerated.) The LaMotte Iron in Water Kit and the Flinn Iron in Water Test Kit were both found in the Flinn Catalog. They are also very good test kits, but because their test range is much lower than the levels in the columns, diluted samples must be tested and then calculations must be made to return to the actual concentration. Syringes can be ordered through VWR International.

Fisher Scientific	1-800-766-7000	www.Fishersci.com
Flinn Scientific Inc.	1-800-452-1261	www.Flinnsci.com
VWR International	1-800-932-5000	www.VWR.com



Modifications:

This activity is designed to help students understand the processes occurring inside the column. Because of available materials and safety restrictions, there are two options for completing this activity. The teacher pages give all of the information necessary for the standard colorimetric assay. In the variations section there are many ideas for using this activity with commercially prepared iron test kits instead of the HCl and ferrozine reagents. The student activity is presented for both the standard colorimetric assay as well as a modified colorimetric assay. The modified activity does not give specific procedures as they will vary depending on the type and brand of test kit being used. It is recommended that you give each lab group a copy of the instructions and review them with the class.

Please read each of the student packets as well as all of the teacher information to make an informed decision about which activity (standard for modified) is best for your students. Remember, you know your students and their abilities better than anyone else.

Background:

As the iron in the system is reduced, it changes from Fe^{3+} to Fe^{2+} . Similarly, as the iron is oxidized, the Fe^{2+} is converted to Fe^{3+} . The bacteria which are helping this to take place have specific conditions in which they thrive. Certain bacteria need oxygen while others only live well in the absence of oxygen. Because of these requirements, the different bacteria will be found in different parts of the column. Aerobes will grow at the top where some oxygen gets in. Anaerobes will be concentrated at the bottom where all of the oxygen has been used up. The products of their activity will also be found in different places. Oxidized Fe^{3+} will be found at the top and more reduced Fe^{2+} will be at the bottom. We will be using specific chemicals to help determine the concentrations of the Fe^{2+} at all four port locations on the bottle. Students will use these facts to help them formulate their hypotheses.

Preparation:

Ferrozine Reagent:

To prepare the ferrozine reagent, Mix 1 gram of ferrozine per liter of 50 mM HEPES buffer. If you would like to prepare the buffer with the ferrozine at the same, time measure out the ferrozine and buffer powders. Place the buffer powder in an Erlenmeyer flask with distilled water and swirl gently. Next, add the ferrozine and swirl gently again. Both reagents should go into solution fairly easily.

You will need 5 ml of the ferrozine reagent for each test tube. This would mean 45 ml for each standard curve you set up. You will need 20 ml for each Winogradsky column which you test (5ml X 4 samples per bottle). Spend a few minutes deciding how much ferrozine you will need to prepare before you begin. Remember, you will be testing on days 0, 7, 14, and 21. Because of this you may want to calculate the amount needed for your classes to test once and then multiply by 4. The ferrozine reagent should keep well.



Use the following to help you.

	<u>For 1/2 liter</u>	<u>For 1 liter</u>
Distilled Water	500ml	1 liter (1000 ml)
Ferrozine	0.5 g	1.0 g
HEPES	5.96 g	11.92 g

Fe²⁺ Standard Curve:

Before the colorimetric assay can be run, you will need to create a standard curve for determining the actual concentration of Fe²⁺ in the samples. Start by making a 20 mM solution of FeCl₂ in 0.5 M HCl. To do this, weigh out 0.9g of FeCl₂ and dissolve it in 0.5 liters of 0.5 N HCl. Then make the standard curve by following these steps:

1. Label 8 test tubes (0.00, 0.15, 0.3, 0.6, 1.25, 2.5, 5.0, and 10.0 mM).
2. Add 0.1 ml of 0.5 N HCl to each of the tubes.
3. Take 0.1 ml of the 20mM FeCl₂ standard and add it to the test tube labeled 10.0 mM.
4. Swirl gently to ensure mixing.
5. Take 0.1 ml of the 10.0 mM solution and add it to the tube labeled 5.0 mM.
6. Swirl gently and continue the serial dilution until you reach the tube labeled 0.15 mM.
7. Take 0.1 ml of the solution in the 0.15 mM tube and place it in your waste container. There should be no FeCl₂ added to the tube labeled 0.00.
8. Add 3 ml of 0.5 N HCl to each of the tubes.

These standards will be treated the same as samples from the columns by adding 5 ml of ferrozine reagent and measuring the absorbance on a spectrophotometer set at a wavelength of 562 nm. (Note - If you do not have a spectrophotometer, you may want to set up the standards and have the students compare the colors of their samples with those standards. From this the students will be able to get a concentration range which their sample fits in. You can then use data provided to create the standard curve with your students so that they will have the same experience of calculating concentration from absorption as they would have had with a spectrophotometer.)

Warm-Up:

It is very important to begin this lesson with some safety procedures. Both the HCl and ferrozine reagent are irritants and should be considered hazardous. Eye protection, skin protection, spill and disposal procedures and general lab safety should be addressed. In addition, syringes with needles will be used for the extraction of the samples from the ports. It is imperative that sharps safety be discussed and demonstrated. If you have any diabetic students (who use insulin) in the class, this may be a wonderful time for them to share their expertise. It is advisable to monitor each student one at a time while they are using the syringe if you decide to allow them to take the samples. An excellent alternative would be to take the samples



yourself while students give you instructions as to how to proceed. Safety can not be emphasized enough in the science lab.

Begin reviewing the processes that are occurring in the bottle with your students. This is a wonderful place to review the oxidation-reduction information which students learned in the background of the Biogeochemical Cycles lesson. Once this is reviewed, ask students, “What is happening to the iron? How is it changing? Where do you think there will be more Fe^{2+} based on what you have learned so far?” Spend some time discussing the background and hypothesis with the students.

Procedures:

1. Place four test tubes in a rack and label them A, B, C, and D.
2. Using a syringe and needle, gently extract 0.1ml of water from the top port on the bottle. Transfer the sample to the test tube labeled A. Do the same for the other three ports, transferring the sample to tubes B, C, and D. (Some patience is required to get the samples.) If you encounter any leaking at the port sites, add a little more caulk.
3. To each of those four test tubes, add 3ml of 0.5 N HCl.
4. Add 5 ml of ferrozine reagent to each tube and to the standard curve tubes.
5. Gently cap and invert each test tube and allow them to stand for 10 minutes.
6. Measure the absorbance of the standard curve tubes using the spectrophotometer set at a wavelength of 562 nm.
7. Record the absorbance for each of the standard tubes to use later in creating a standard curve.
8. Measure the absorbance of each of the sample test tubes and record the results.
9. Dispose of your waste according to local guidelines.
10. If using this procedure, students will need to complete the graphing activity in Lesson #8 before completing their conclusion. Be sure to record the absorbances for the standard curve so students can use those numbers to create their graph.

Variations and Follow-Up Activities:

If you do not have the necessary materials or are concerned with using the materials required for the colorimetric assay you can still perform this activity with relatively good accuracy. There are several commercial test kits available which will allow you to test for the concentration of Fe^{2+} in your samples without using the procedures described above. See the “Materials Source Information” section of this lesson for ordering information.

Iron test strips allow for a sample to be taken from the port and simply dropped onto the test strip. The strip will turn a color within a few seconds. Students would be able to read the concentration of the Fe^{2+} in that sample using a color guide on the test strip container.

Another option is an iron in water test kit. Many of these kits are designed to test for the amount of iron found in drinking water. To make this test kit work for the activity, simply dilute your sample with distilled water. This can be a great math activity for students as they will need to calculate the concentration of Fe^{2+} in the sample by using the dilution factor.

As a follow up activity, it is suggested that the graphing activity be completed. Most teachers will not have access to a spectrophotometer, so many of the students will be measuring their Fe^{2+} concentrations using either the test kits or the comparison of the samples to the



standards set up by the teacher. With this in mind, a set of real results has been included in the graphing activity. After recording their own data, students can use this real data to complete the analysis of data from a spectrophotometer. Any teacher with a spectrophotometer can take this activity and substitute the students' results for the ones provided.

Assessments:

There are many opportunities for assessment during these activities. If students will be doing the measuring involved in this activity, assessments of measurement skills can be obtained.

In the follow up activity, students will be graphing the standard curve and finding the formula of the curve to calculate the concentration of Fe^{2+} from the absorbance measured by the spectrophotometer. This will provide for an excellent assessment of students graphing skills and analysis skills. Spend some time talking to the students as they proceed with the graphing activity and assess their understanding of not only graphing, but also the processes which they are analyzing. Ask why knowing the concentration of Fe^{2+} in the different layers of the bottle is important.

Further important assessments are provided during the activity while observing students' lab techniques. A clipboard with a set of notes can be a valuable assessment tool in recording students' practices and behaviors in the lab.

Finally, students' lab reports are an excellent means for assessing students' knowledge and understanding of the activity and its greater purpose in this study of the environment.

Observations:

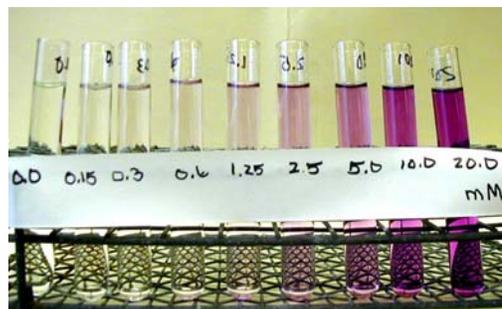
My students loved the color changes of the iron test strips. These results led us into some great discussions about what was going on inside the bottle and the activities of bacteria at the top and bottom of the bottle. Students discussed how both the presence and absence of O_2 in the bottle affected the bacteria.

Prepare the syringes ahead of time. My students did not like watching me put the needle on the syringe, yet they were fine while watching me extract the liquid samples with the needle and syringe. This is also a great time to review sharps safety. I NEVER allowed my students to handle the needles or syringes, and was always careful to both demonstrate and explain proper safety procedures for sharps.

Colorimetric Assay

A sample standard curve.

Fe^{2+} concentrations range from 0 - 20mM. Each tube contains: 3ml of 0.5N HCl, 5ml of ferrozine reagent and 0.1ml of the FeCl_2 standard.

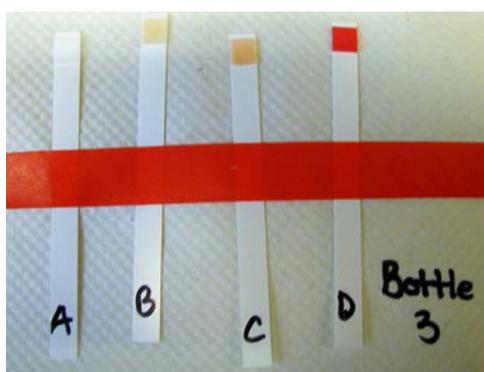
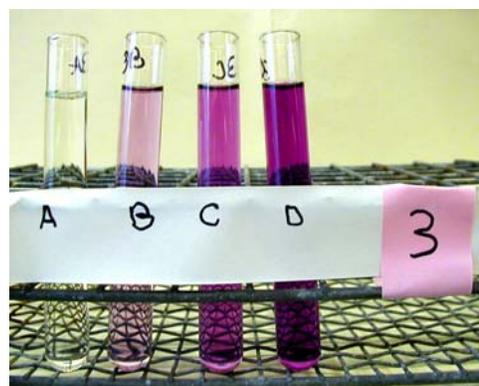


Using the spectrophotometer.

The wavelength is set to 562 nm and the absorbance is measured directly in the test tubes.

Data from one column.

Each tube represents one sample port from top (A) to bottom (D). The darkness of color indicates the amount of reduced iron.



Alternative method.

Reduced iron can be measured using EM Quant iron test strips. Conc. is determined by comparing the strip to a color scale.



Name _____

Date _____

Colorimetric Assay (standard)

Problem: There are many changes occurring inside of this system. To better understand these changes, it is helpful to think about how the top and bottom of the bottle are different. How can we measure the changes in the iron to give a "picture" of what is occurring inside the bottle?

Background:

As the iron in the system is reduced, it changes from Fe^{3+} to Fe^{2+} . Similarly, as the iron is oxidized, the Fe^{2+} is converted to Fe^{3+} . The bacteria which are helping this to take place have specific conditions in which they thrive. Certain bacteria need oxygen while others only live well in the absence of oxygen. Because of these requirements, the different bacteria will be found in different parts of the column. The products of their activity will also be found in different places. We will be using specific chemicals to help determine the concentrations of the Fe^{2+} at all four port locations on the bottle. Use these facts to help you formulate your hypothesis.

Hypothesis: If _____,

then _____,

because _____

Materials:

- spectrophotometer (562 nm)
- Winogradsky column aged 0, 7, 14, or 21 days
- 0.5 N HCl
- ferrozine reagent
- iron standards (your teacher will prepare these)
- test tubes
- pipettes
- syringes and needles



Procedures:

1. Place four test tubes in a rack and label them A, B, C, and D.
2. Using a syringe and needle, gently extract 0.1ml of water from the top port on the bottle. Transfer the sample to the test tube labeled A. Do the same for the other three ports; tubes B, C, and D.
3. To each of those four test tubes, add 3ml of 0.5 N HCl.
4. Add 5 ml of ferrozine reagent to each tube and the standard curve tubes.
5. Gently cap and invert each test tube and allow them to stand for 10 minutes.
6. Measure the absorbance of the standard curve tubes using the spectrophotometer. Your teacher will give you specific directions as to the proper use of your model spectrophotometer.
7. Record the absorbance for each of the standards to use later in creating a standard curve.
8. Measure the absorbance of each of the sample test tubes and record the results.
9. Dispose of your waste according to your teachers instructions.
10. Be sure your lab station is clean and your materials have been returned to their appropriate locations.

Results and Analysis

Date	Port A (top)	Port B	Port C	Port D (bottom)

Describe the colors that were generated for each of the four samples. How were they similar, how were they different? _____



Conclusions

Use the following to help you write a well developed conclusion. This means you must explain your findings and include details.

- Was your hypothesis supported? Why or why not?
- Explain your findings and relate these findings to what is happening in the bottle.
- How does this test give you a "picture" of the changes which you can not see occurring in your bottle?
- What can you learn about the environment around you from this activity?



Name _____

Date _____

Colorimetric Assay (modified)

Problem: There are many changes occurring inside of this system. To better understand these changes, it is helpful to think about how the top and bottom of the bottle are different. How can we measure the changes in the iron to give a "picture" of what is occurring inside the bottle?

Background:

As the iron in the system is reduced, it changes from Fe^{3+} to Fe^{2+} . Similarly, as the iron is oxidized, the Fe^{2+} is converted to Fe^{3+} . The bacteria which are helping this to take place have specific conditions in which they thrive. Certain bacteria need oxygen while others only live well in the absence of oxygen. Because of these requirements, the different bacteria will be found in different parts of the column. The products of their activity will also be found in different places. We will be using specific chemicals to help determine the concentrations of the Fe^{2+} at all four port locations on the bottle. Use these facts to help you formulate your hypothesis.

Hypothesis: If _____,

then _____,

because _____

Materials:

- Winogradsky column aged 0, 7, 14, or 21 days
- syringes and needles
- iron test kit



Procedures:

1. Obtain the materials your teacher has gathered for this activity.
2. You will need to follow the package instructions for the specific iron test kit you will be using. Your teacher will provide you with this information.
3. Begin your tests by extracting the designated amount of sample from the top port. This is sample A. Continue down the bottle ending with the bottom port (D).

Results and Analysis

Bottle Name	Port A (top)	Port B	Port C	Port D (bottom)

Describe the colors that were generated for each of the four samples. How were they similar, how were they different? _____

Conclusions

Use the following to help you write a well developed conclusion. This means you must explain your findings and include details.

- Was your hypothesis supported? Why or why not?
- Explain your findings and relate these findings to what is happening in the bottle.
- How does this test give you a "picture" of the changes which you can not see occurring in your bottle.
- What can you learn about the environment around you from this activity?