

Limiting Nutrient and Algal Growth: Designing an Individualized Student Project

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In this original research project, students chose one of the design options to determine the limiting nutrient in an aquatic system. A 'plant pot' method involves filling porous plant pots with agar and a nutrient mixture. The plant pots were then placed in clear cups containing pond or stream water. Spectrophotometry was used to estimate chlorophyll a concentration to estimate algal growth. In another design, students grew *Chlamydomonas reinhardtii* in defined media or natural water enriched with specific nutrients in test tubes. They estimated cell density using a hemocytometer or spectrophotometry and conducted statistical tests. Finally students produced a research paper from this study.

Keywords: nutrient limitation, phytoplankton, nutrient enrichment, phosphorus, nitrogen, fertilizer

Introduction

The purpose of this lab is to allow multiple options for students to design their own research project at every level of the study to determine the limiting nutrient in an aquatic system. Students may bring their own water or use the provided water. They may use the method to simulate the natural process of nutrient leaching in soil and connect this study to algal bloom or they may test the limiting nutrient in a single, provide algal species. The design may be a two-sample study or a correlation study for statistical test. Ultimately, this lab is a good candidate for students to produce a scientific paper. Although the introduction of limiting nutrient in this paper refers to a virtual lab designed by Simbio®, the concept of limiting nutrient may be included in a lab handout without the subscription of the software. This lab requires a minimum of two weeks, one week for set up and one week for assay of algal growth. To allow time for students to decide on a project and have enough time to prepare the materials requested by students, three weeks of lab, each three-hours, would be ideal.

Table 1. Synopsis of activities in the multi-week module.

Week	Exercise	Approximate Time (min.)
Week 1	Conduct Simbio® simulation	45-60
	Students form research groups	15
Week 2	Design experiment, submit study proposal and request materials	45
	Instructor approves of experimental design	30
Week 3	Set up experiment	30-90
	Estimate algal growth using either spectrophotometry or hemocytometer	45- 150
	Conduct statistical tests	30

Student Outline

Laboratory Synopsis

In this laboratory you will be engaged in a multi-week project of scientific investigation that allows you to design your own experiment on nutrient enrichment. The first week you will conduct a computer simulation in lab using SimBio® and complete the questions in the worksheet. Your group will design its own experiment based on interest and available resources. You will request the materials you will need to set up the experiment in the second week. In the third week you will use one of the three analytical techniques to estimate the mass of the algae. Each of your team members will write a lab report following a format similar to a primary scientific paper.

Laboratory Objectives

Conceptual

At the end of this laboratory you should be able to...

1. identify the limiting nutrient and secondarily limiting nutrient and predict their impact to algal blooms.
2. apply some commonly used estimator such as chlorophyll a concentration for algal mass.
3. evaluate data by choosing appropriate statistical tests mentioned in this lab.

Procedural

At the end of this laboratory you should become proficient in using...

1. a spectrophotometer to measure percentage of transmittance and absorbance for a specific pigment and convert the absorbance to chlorophyll a concentration.
2. a microscope to count cells with a hemocytometer and estimate cell density.

Introduction

Week one – limiting nutrient

In the first week of the three-week module you will conduct a computer simulation using a virtual lab produced by SimBio®. You will download the software on your computer and read the following information for the concept of limiting nutrient in aquatic system. At the end of the simulation you are expected to understand how the knowledge of a limiting nutrient can help researchers control the growth of algae to maintain good water quality. In addition, scientists may apply the knowledge to help growing algae as a source for biofuel.

The composition of elements in phytoplankton and the supply of these elements are not proportional (Table 2). Because the elemental composition is relatively fixed in phytoplankton, the elements in low proportion in water become limiting nutrients for the growth of phytoplankton. This shortage is especially prominent in nitrogen and phosphorus where the demand over supply is more than ten thousand fold. If these limiting nutrients are added to the water, phytoplankton will grow rapidly and produce algal blooms. In the 1970's a few prominent large-scale field experiments supported the fact that phosphorus was limiting the growth of phytoplankton and addition of the element would produce algal blooms (Schindler, 1974).

As described in the SimBio® simulation exercise, algae are able to utilize their limiting nutrient(s) when they become available and reproduce rapidly to create algal blooms. In addition to toxins produced by some species in algal blooms, when algae die they become food for bacteria in the benthic environment. Decomposition of dead algae by bacteria consumes a large amount of oxygen needed by many benthic organisms, which causes die-offs of these organisms. The subsequent underwater barrens resulting from these die-offs in oceans and lakes are commonly known as **dead zones**. Dead zones are often found at the mouth of major river systems. These river systems pass through agricultural regions with heavy use of fertilizers containing limiting nutrients such as phosphorus and nitrogen for algal growth. Garden fertilizers and untreated household sewage can also contribute to algal bloom in lakeside communities and deteriorate water quality.

Algae are a vital part of the ecosystem as they produce the majority of oxygen on Earth and are the primary producers in the aquatic food chain. Besides monitoring algal blooms, in recent years, researchers are studying the use of algae to clean up wastewater, harvest biofuel, and feed fish in farms. Therefore learning how to control algal growth is the first step to potentially utilize this versatile group of organisms.

The purpose of this lab is to identify possible limiting nutrients in a freshwater system by enriching the system with nutrients. If the nutrients are indeed limiting the growth of algae, we will see an increase of algal mass compared to the same system without the added nutrients.

There exist a few design choices depending on which one interests you most. Consequently the materials required for setup and the method require for analyzing algal biomass may vary. Table 3 is a synopsis of the possible questions you may ask and

Table 2. Essential elements in freshwater phytoplankton and in the water of average world river systems. Adapted from Vallentyne (1974).

Element	Average phytoplankton content or requirement (%)	Average supply in water (%)	Ratio of phytoplankton content to average supply
Oxygen	80.5	89	1
Hydrogen	9.7	11	1
Carbon	6.5	0.0012	5,000
Silicon	1.3	0.00065	2,000
Nitrogen	0.7	0.000023	30,000
Calcium	0.4	0.0015	<1,000
Potassium	0.3	0.00023	1,300
Phosphorus	0.08	0.000001	80,000
Magnesium	0.07	0.0004	<1,000
Sulfur	0.06	0.0004	<1,000
Chlorine	0.06	0.0008	<1,000
Sodium	0.04	0.0006	<1,000
Iron	0.02	0.00007	<1,000
Boron	0.001	0.00001	<1,000

Table 3. Possible choice of research questions and analytical methods for nutrient enrichment.

	If you are interested in finding out the limiting nutrient(s) for...	You will grow algae in...	Analytical equipment/method
Types of Experiment	single algal species in defined growth media	test tubes and defined growth media of unknown nutrient deficiency and enrich with nutrients (Figure 1)	1. Cell counting using hemacytometer, or 2. Spec20* on cell density, or 3. Spec20 on chl-a** concentration
	single algal species in natural freshwater	test tubes and filtered creek water of unknown nutrient deficiency (Figure 1)	1. Spec20 for chl-a concentration, or 2. Spec20 on cell density
	algal assembly from field (concentrated on filter first)	test tubes and defined growth media of unknown nutrient deficiency (Figure 1)	1. Spec20 for chl-a concentration, or 2. Spec20 on cell density
	algal assembly from field	cup of creek water containing plant pot filled with nutrients (Figure 2); nutrients may be compounds, fertilizers, or others.	1. Spec20 for chl-a concentration
	nutrient enrichment in the field	Plantations pond: submerged plant pots contain nutrients held in solidified agar (Figure 3); nutrients may be compounds, fertilizers, or others.	1. Spec20 for chl-a concentration

*Spec20: Spectronic 20®, the spectrophotometer used in this lab.

**Chl-a: chlorophyll a.

the materials and method suitable for the experimental design. Keep in mind that variation exists in biological systems, so you should include enough replicates for each treatment. The number of replicates (sample size) often is dictated by the statistical test you plan to use. You should read the relevant Statistical Reference chapter to choose a suitable test for this research project. The next section of this chapter also includes space for recording data for three possible statistical tests.

You may also come up with your own design not listed in Table 3. If so, bring materials that you are interested in testing to see whether they contain the limiting nutrient in the aquatic environment you are investigating. Between the end of this lab and the beginning of the next lab, your TA will approve or suggest modifications to your research design.

The experimental setups listed in Table 3 are divided into two types: one which includes growing algae in test tubes where nutrients are added directly to the water (Fig. 1) and the second method requires adding nutrients in terra cotta plant pots and submerging them in either cups of water (Fig. 2) or in the field (Fig. 3). The 'test-tube design' allows quick determination of possible limiting nutrients while the 'plant-pot design' simulates a natural leaching process where nutrients are released from the soil into freshwater systems or oceans.

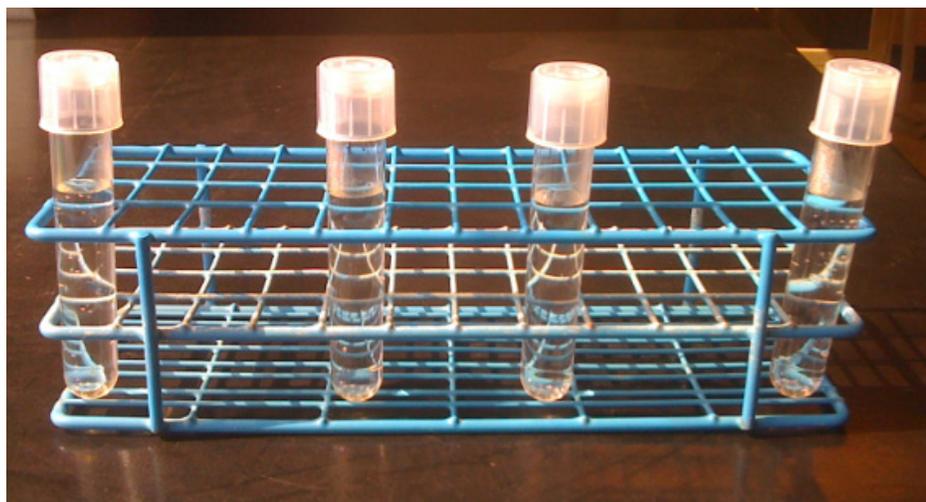


Figure 1. Experimental set up using test tubes. Algae are grown in nutrient-enriched growth media or natural water enriched with nutrients. Four replicates are shown.



Figure 2. Experimental set up using plant pots in plastic cups. Each inverted plant pot contains nutrient-blended agar and is sealed with Parafilm. Only one replicate is shown. The set-up is kept in the lab during the growth period.



Figure 3. Plant pot method in the field. Nutrient containing plant pots are lowered into a pond to test the nutrient deficiency in pond water. Adapted from Bushong & Bachmann (1989).

Procedures

Setting Up the Experiment

The set up is for each group of three students.

A. Experiments using test tubes

- 20 polystyrene test tubes with tops
- Defined growth medium or filtered sterile creek water
- Nutrient of choice: phosphorus compound, iron compound, nitrogen compound.
- (If you bring your own nutrient, this nutrient needs to be fully dissolved in water)
- Single algal species (*Chlamydomonas reinhardtii*), or natural algal assembly
- 10 mL graduated cylinder
- Micropipet
- If you choose to bring your own water, you will need $\frac{1}{2}$ gallon freshwater from a natural source, e.g., stream, pond, or lake, boil it and let it cool.

B. Experiments using plant pots

- 12 two inch terra cotta pots (30 mL capacity)
- Twelve 500 mL wide-mouth jars with lids
- 2% agar melted in a water bath
- Masking tape
- Parafilm®
- Stir sticks
- Small petri dishes
- Rubber bands
- Creek water for group using plastic cup, or board for group conducting a field study
- Nutrients: fertilizers, nitrogen compound, iron compound, phosphorus compound, or bring your own nutrient
- If you choose to bring your own water or potential nutrients, you will need $\frac{3}{4}$ to 1 gallon of freshwater from a natural source, e.g., stream, pond, or lake

Experimental Considerations and Procedure

A. For the 'test tube' groups

1. Each of the test tubes can take up to 10 mL of total volume.
2. You may test whether the increase of algal biomass follows the increase of nutrient concentration. The sample size should be at least 6 test tubes.
3. If your group is interested in testing the difference between presence and absence of nutrients, you will need at least 6 replicates for the control and 6 for the experimental group.
4. When adding the algal sample it is important that a consistent amount is added to each replicate.
5. The completed replicates should be labeled with your group name, section number, and TAs name and date. Test tubes will be kept in the test tube rack and left in lab during the experimental period.
6. The position of each replicate in the test tube rack may produce an unintended effect on the growth of algae. When your group has completed all the replicates, you should consider randomizing the sequence of the replicates in the test tube rack to minimize the effect of positional effect to the results.

Use the following procedures to set up your experiment:

1. Retrieve test tubes, test tube caps, glass pipettes, pipette dispenser, algae, and nutrient.
2. Write the information on each of the test tubes about 2 cm from the top with your sample number/name. Record the content of each test tube in your notebook.
3. Using a glass pipette, transfer 10 mL of the growth medium to each of the replicate test tubes.
4. Using a 20-200 μ L micropipet, transfer the desired volume of nutrient to each test tube. If this nutrient is provided for you, write down its name and concentration here: _____.

Depending on your experimental design, if you add different volumes of the nutrient in the test tubes and the total volume of growth medium becomes quite different between replicates, you should add de-ionized water to make up the difference so the concentrations of all other substances are equal.

5. Using a glass pipette, transfer 0.5 mL of algal culture of your choice to each of the test tubes.
6. Place a cap on each of the test tubes. The cap has two stops: the first is a loose setting which allows air exchange and the second is a tight setting which makes it leak-proof. Make sure caps are on the loose setting.
7. With your TAs assistance, use the random number generator from the website random.org to randomize the order of your samples. Follow the order from its output and arrange your test tubes on the test tube rack.
8. Use a small tape to label your group name on one end of the row. Leave the test tubes on the rack until next lab.

B. For the 'plant pot' groups

The groups using the plant pots require some assembly as described in the following procedure.

1. Apply a small piece of masking tape to cover the drain hole of each plant pot on the inside. Press the tape with the eraser end of a pencil or a similar item to seal. Label the side of each pot with masking tape and indicate either control or experimental group, the replicate number, and your group name, section number, TA, and date.
2. Pour 2% liquid agar to each plant pot up to the half full mark, add the desired amount of nutrient, and gently stir to mix well. Immediately add more agar until the pot is almost full. Let the agar solidify without disturbance.
3. After the agar is solidified, seal the large opening of the pot by stretching a piece of Parafilm® to cover the opening and run your finger around the rim. Attach a petri dish to the Parafilm seal and tie the petri dish and plant pot together with a rubber band.

Sealing the large opening of the plant pots with Parafilm minimizes rapid release of nutrients through this opening.

4. Remove the label from the assembled pot and place it on a clear plastic cup (or label the treatments on the board if setting up in the field). Invert the pot and gently place it into the jar receiving the label. Add 250 mL of freshwater to allow complete submersion. Place the lid on the jar without screwing it tight to allow for gas exchange.

5. Repeat this procedure to all replicates in the experimental group.
6. Repeat this procedure to all replicates in the control group but omit adding nutrients.
7. Leave the experimental setups undisturbed at a designated location until the next lab.

For the plant pot group to conduct the experiment in the field (in a pond or other natural water), the procedure has the following modifications:

1. Follow the procedure in section B steps 1 through 3 EXCEPT that you will label each replicate **on the inside of the small petri dish** using a permanent marker. This way the identity of each replicate will not be washed off by the pond water.
2. When each of the replicate plant pots is completely assembled, assign to each replicate a number from 1 to 12. Use the random number generator from the website random.org with your TA's assistance to randomize the placement of each replicate in the provided cage, which has 3 plant pots x 4 plant pots arrangement.
3. Secure the lid of the cage with the twist ties provided and bring the cage to the plantation pond using the provided transportation. Course staff will assist your set up at the pond.

Introduction to Week of Results

A. Estimating algal biomass using chlorophyll *a*

Photosynthesis is a process for organisms to transform light energy to chemical energy using specific proteins that absorb light at specific wavelengths. Because these proteins also reflect or transmit light in the visible spectrum between 400 nm and 700 nm, we see them as pigments of various colors from the wavelengths of light that they **do not** absorb. Although the composition of pigments varies depending on the taxonomy of the organisms and their ecological conditions, a great majority of photosynthetic organisms require *chlorophyll a* to convert light energy to chemical energy, so the concentration of chlorophyll *a* is a good indication of the amount of photosynthesis, thus an accurate measurement of algal biomass (Gregor & Marsálek, 2004). Chlorophyll *a* has two absorption peaks, one at the blue color range around 430 nm and the other at the red range of 665 nm. Because the wavelengths within the green color range are reflected, we see chlorophyll *a* as a green pigment. In its pure form, chlorophyll *a* concentration can be measured at either one of its two absorption peaks. However, because many accessory pigments such as *chlorophyll b* and carotenoids also absorb light at the blue color range in natural extracts from algae or other photosynthetic organisms, we should use 665nm as the wavelength in spectrophotometry to determine chlorophyll *a* concentration.

The chemical structure of chlorophyll *a* has a ring of carbon and nitrogen with magnesium at the center and a long chain composed mostly by hydrocarbon. When zooplankton consume algae, the acidity in their digestive tracts converts chlorophyll *a* to various types of **pheopigments**, for example, pheophytin *a* by losing the magnesium from the center of its ring structure, or pheophorbide *a* by losing both its magnesium and its hydrocarbon chain. These pheopigments have similar absorption peaks, but at a weaker absorption level. Because these pheopigments are not photosynthetically active, the reading from these pigments should be subtracted from the total reading of chlorophyll *a* so as not to over estimate the algal biomass (Lorenzen, 1967).

B. Estimating cell density using Spectronic® 20

The density of algal cells is a direct measurement of algal mass and direct evidence on the influence of nutrients. Rather than extracting photosynthetic pigments and reading the chlorophyll *a* concentration, this method reads turbidity of cell suspension. The level of turbidity is correlated to cell density with higher turbidity, less transmittance of light indicating higher concentration of cells. This method, however, is not as sensitive as the chlorophyll *a* method in detecting slight differences. Also, the correlation between the cell density and turbidity reading holds only within a certain range. Whether your results are suitable for this analysis, depends on the appropriate range of concentration in your samples at the end of the growth period.

C. Estimating cell density using hemocytometer

Originally designed to count blood cells, hemocytometers have been widely used for counting the number of yeast and algal cells in a solution. A hemocytometer is similar in shape to a microscope slide but is much thicker. It has a fixed volume when the coverslip is applied allowing the cell count to be readily converted to cell density.

Procedures

Depending on your group design and choice, **you will need to use only one of the three methods** below to analyze your algal mass unless you have time and are interested in learning the correlation between the methods. The chlorophyll *a* method may take the longest time to complete but is also one of the more reliable methods. The cell counting using the hemocytometer is also accurate but can be tedious if cells are dense and you should properly dilute the culture if necessary. The optical density reading using spectrophotometer is the fastest method. It, however, has the limitation of being less sensitive and is reliable only with a certain range.

A. Chlorophyll *a* concentration

To measure chlorophyll *a* concentration, the first step is to concentrate cells on a filter membrane. You will first use a water pump to concentrate algae from a known volume of water and collect algae on a cellulose filter. You will then extract chlorophyll *a* and other photosynthetic pigments from the filter with 95% ethanol placed in a hot water bath. The ethanol extract will be transferred to a cuvette and the percent transmittance of light at 665 nm is measured using a spectrophotometer. As described in the introduction for this week's activity, the presence of pheopigments can cause overestimation of chlorophyll *a* concentration. After completing the initial spectrophotometric reading, you will acidify the pigment extract to turn all chlorophyll *a* to pheopigments and then conduct the reading for percent transmittance again. You will then convert the percentage transmittance to absorbance using a handout. Using the provided formula, you will estimate the concentration of chlorophyll *a* as a measure of algal biomass.

Material list per group

- 1 test tube brush or hard bristle toothbrush
- 1 test tube holder
- 1 forceps
- 1 small scissors
- 1 10 mL graduated cylinder
- 12 test tubes
- 1 bottle of 95% ethanol
- 1 5 mL glass pipette with dispenser
- 12 cuvettes
- 1 strip of aluminum foil
- 1 strip of Parafilm®
- 1 20-200 µL micropipettor
- 1 box of micropipettor tips
- 1 bottle of 0.1 N HCl

Filtration Stations

There are 4 filtration stations in each lab room, and each station has:

- 1 filtration device (made up of 2-L vacuum flask, funnel with support plate, clamp, reservoir, supporting stand and aspirator tubing connected to faucet)
- 1 box of cellulose filters
- 1 100 mL graduated cylinder

Collection of algae

For all sample cups:

1. While the plant pot is in the cup, use a test tube brush to thoroughly brush off algae attached on the surfaces of the plant pot and glass jar into the water.
2. Use the test tube holder to lift up the plant pot (or use a blunt probe to lift the rubber band). Discard agar and Parafilm.
3. With the test tube brush, do a final removal of algae from the inside of the jar; clean and blot dry the brush between samples; repeat procedure to all sample cups.
4. Label each test tube. Label one extra test tube "filter blank". Bring jars, lids, scissors and labeled test tubes to filtration station.



Figure 4. Assembled filtration flask, funnel, filter, and clamp attached to water pump.

Algal Filtration

See the filtration station set up in Fig. 4.

1. Use forceps (not fingers) to pick up one cellulose filter and place it at the center of the funnel support plate. Place the reservoir small side down to cover the filter and align with the funnel. Hold parts together with a clamp.
2. For each sample, mix the algae with water thoroughly using the test tube brush or swirl the sample jar.
3. Immediately measure 100 mL of water sample with the graduated cylinder then transfer it into the reservoir.

Note: Larger volume is more ideal as there is less sampling bias. However, if sample water appears dense with algae, use 50 mL or 25 mL instead so as not to clog the filters and delay filtration. Record your volume.

4. Turn on aspirator faucet.

When water travels from a pipe of wider diameter to another with a narrower diameter, it produces a lower pressure at the pipe with the narrower diameter. Since the narrower tube is connected to the side arm of the flask, the low pressure in the flask pulls water through the filter to facilitate filtration.

5. Turn off the faucet after sample water has completely passed through filter and the filter appears dry.
6. Unclamp funnel and glass base; remove funnel. Fold filter in half with forceps, algae side in, and blot dry on paper towel.
7. Cut filter into four wedges and place them in appropriate test tube.
8. To prepare a filter blank, measure 100 mL (or another volume you have used) of deionized water and filter it through filtration devise following the procedure above.

Note: Light causes extracted pigments to break down, so all filtered algae samples should be handled under dim light for the subsequent procedures.

Water Bath Incubation (adapted from Pápista & Böddi, 2002)

1. To each test tube add 10 mL of 95% ethanol. Cover each test tube with aluminum foil and incubate in 75°C water bath for 5 min.
2. While algal filters are in the hot water bath, label cuvettes near their top with masking tape.
3. Retrieve test tubes from the water bath.

CAUTION: the test tubes are hot; hold them by their top and bring them to your bench to cool for 1-2 min.

4. With a finger pressing the aluminum foil to seal, invert the test tube 1-2 times to mix. Transfer 5 mL of pigment extract to each corresponding cuvette and seal with Parafilm.

Spectrophotometry Before And After Acidification

You should have read the Appendix on Spectrophotometry using Spectronic 20®. Your TA will show you a Spec20 video to ensure proper handling of this instrument. For measuring chlorophyll a concentration, the wavelength should be set for 665 nm.

1. While no cuvette is in the sample holder, turn the zero control knob of Spec 20 to 0%T (transmittance).
2. Insert cuvette of the filter blank completely in the sample holder with its white, vertical line aligning with the raised line on the edge of sample holder. Turn 100%T knob to 100%. Remove blank.
3. Insert sample in the sample holder in the same manner as you did with filter blank. Read %T value to the nearest 0.25%. Record data in Table 4 under *Before Acidification*.
4. Repeat procedure to all prepared samples and record your %T data in Table 4.
5. Add 150 μ L of 0.1 N HCl to cuvette, cover with Parafilm, invert twice to mix well, and let stand for 1.5 min. Make sure you do not allow acidification to proceed longer than 1.5 min.
6. Measure %T after 1.5 min. Record data in Table 4 under *After Acidification*.
7. Repeat acidification procedure to all samples and record %T.
8. Repeat Algal Filtration procedure on samples in the experimental group if necessary.
9. Use the provided handout to convert %T to Absorbance and record in Table 4.
10. Subtract absorbance value after acidification from absorbance before acidification. Record data.
11. Follow Clean Up Procedures and dispose of ethanol-containing pigment extracts in the designated container.

Table 4. Percentage transmittance and absorbance before and after acidification of each algal sample.

Replicates	Volume filtered (mL)	Before Acidification		After Acidification		Difference in Absorbance
		%T	Absorbance (A_o)	%T	Absorbance (A_a)	$A_o - A_a$
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						

Calculation of Chlorophyll a Concentration

As described above, among all photosynthetic pigments chlorophyll *a* is the only pigment at the center of the reaction for photosynthesis in most organisms, and as such its concentration can provide direct estimation of the level of algal biomass. There is a direct relationship between the absorbance and the amount of photosynthetic pigments. By plugging in the value of $A_o - A_a$ from Table 4 to the equation below, you can convert absorbance into chlorophyll a concentration using the following equation (Nusch, 1980).

$$Chl - a(\mu g/L) = \frac{29.6(A_0 - A_a)}{V_f \cdot l}$$

where

29.6 is the combination of chlorophyll a absorption coefficient and the factor to equate the reduction in absorbance to initial chlorophyll concentration,

A_0 = Original absorbance at 665 nm,

A_a = Absorbance after acidification at 665 nm,

V = Ethanol extract volume in mL,

V_f = Volume of water filtered in liter,

l = length of light path in cuvette in cm, ours is 1.2 cm.

Calculate the concentration of chlorophyll *a* for each of the replicate and record values in Table 5.

Table 5. Concentration of chlorophyll *a* ($\mu\text{g/L}$) in control and experimental groups.

Sample	1	2	3	4	5	6	7	8	9	10	11	12
[Chl-a]												

B. Cell density using Spectronic® 20

The measurement of cell density will be conducted at the wavelength of 665 nm, which measures the absorbance or optical density (OD) of the cells. The term optical density is perhaps more appropriately used in suspension where light scatters when it hits the particles in the suspension. Within a certain range of OD, there is a linear relationship between the OD and cell concentration, so the cell density as the indicator for algal biomass is indirectly measured through optical density.

You should have read the Appendix on Spectrophotometry using Spectronic 20®. As mentioned in the Spectrophotometry section above, your TA will show you a Spec20 video to ensure proper handling of this instrument. Make sure your wavelength is set for 675 nm for cell density reading.

Notice that normally a blank should contain all constituents of the sample measured except for the amount of the substance that gives the optical density. Since the media of various nutrient compositions and concentrations are fully dissolved and do not carry any optical property additional to pure water, we will use *deionized water* instead as the blank for each of the replicates.

However, you should verify the optical density value of the nutrient solution, use either one of the provided ammonium sulfate (100 mM) or potassium phosphate (10 mM) you used when you set up the experiment.

1. Prepare a blank by filling a cuvette at least halfway with the deionized water supplied from the spring-controlled faucet.
2. While no cuvette is in the sample holder, turn zero control knob of 'Spec 20' to 0%T (transmittance).
3. Insert cuvette of blank completely in sample holder with its white, vertical line aligning with the raised line on edge of sample holder. Turn 100%T knob to 100%. Remove blank.
4. Insert the provided nutrient in the cuvette in the sample holder in the same manner as you did blank. Read %T value to the nearest 0.25%.
 - What is the reading of the nutrient solution 100% T or nearly 100% T?
 - The 100% in transmittance value indicates that the various concentration of nutrients added does not influence the optical property of the sample.
5. Return the nutrient solution to your lab instructor.
6. Transfer about 4 mL of a sample to a new cuvette. Use the procedures described from step 2 through step 4 for each sample and record your %T data in Table 6. *Note, if your cells are clumped at the bottom of the test tube, you should first press down the cap to seal the tube and then invert the sample to homogenize the suspension.*

Within the range of about 10%T to 90%T (absorbance 1.000 to 0.0458) at 665 nm, there is a linear relationship between cell density and optical density. If the **Transmittance** reading from any of your samples is lower than 10%, the cell density may be too high to be accurately read by the spectrophotometer. (Recall that the higher the concentration of the substance being read, the lower the reading of %T.)

7. If needed, you should make a 1:1 dilution to reduce the concentration then repeat the procedure for %T on the 1:1 dilution. Use the deionized water to make the dilution. If the transmittance value is *higher* than 10%, no dilution is necessary. Record this information in the Notes column of Table 6 as needed.

8. Convert %T to absorbance (OD) using the provided handout.

Again, although absorbance is the term listed, it is interchangeable with optical density, which is more commonly used in checking density of cells and molecules such as DNA, RNA, protein, etc.

The following procedure is conducted to produce your own conversion factor between O.D. and cell density.

9. Retrieve a hemocytometer and follow the procedure described below in section C.

C. Cell Counting Using Hemocytometer for one sample.

Count the number of cells in 5 squares as described and record in the first blank 4 by 4 grid below.

10. Convert the cell count to cell density (cells/ μ L).

11. Refer back to the OD of this sample and use the following formula to produce cell concentration of 1 OD.

$$\frac{\text{Sample cell concentration}}{\text{Sample OD reading}} = \frac{\text{General cell concentration}}{1 \text{ OD}}$$

Write your own conversion (if applicable): 1 OD = _____ cells/ μ L

Table 6. Cell density measured by spectrophotometry.

Replicate	%T	Absorbance (Optical Density, OD)	Cell density (cells/ μ L)	Notes
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

D. Cell counting using a hemocytometer

Groups will be provided with hemocytometers and hand counters. The hemocytometers are pricy and breakable so handle them carefully. Figure 5 describes the structure of a hemocytometer and indicates where to locate the two grid areas for cell counting. Notice that a counting chamber is created when a coverslip is placed on top of the coverslip mounting supports. The moat around the grid areas allows excess liquid to drain away from the counting chamber to produce a chamber of 0.1 mm in depth.

Sample preparation

1. Start from the samples that appear to have the densest growth. Close the cap of the sample and invert the test tube a few times to homogenize the algal cells.
2. Use a provided dropper to transfer one or two drops of this sample onto one of the grid areas on a hemocytometer. Gently lower one edge of the coverslip to cover the grid area to allow the coverslip to rest on the two supports. If there are large bubbles, repeat the procedure.
3. Follow the microscopy procedure on p. A3 of Appendix 1 to observe this sample.

Warning: the hemocytometer is much thicker than normal microscope slide so you need to be extra cautious when lowering objectives to avoid damage to both the objective and the hemocytometer.

4. Using 4X objective, focus on the edge of the counting area to locate the grid. Close the iris diaphragm and reduce the light by turning down the power wheel can help you find the etched lines of the grid. The entire grid should fit within the viewing field from a total of 40X magnification (4X of objective multiplied by 10X of ocular). Observe the algal cells. If you are studying *Chlamydomonas*, you should see many moving spherical cells. If you are studying the lab provided natural samples, you may see the needle-shaped *Ankistrodesmus* cells.
5. While the cells are in focus, turn the 10X objective into position. Now the viewing field allows you to see the entire 1 mm x 1 mm area. Focus on one of the four corners. This 4 x 4 square area will be the main area for your cell counting.

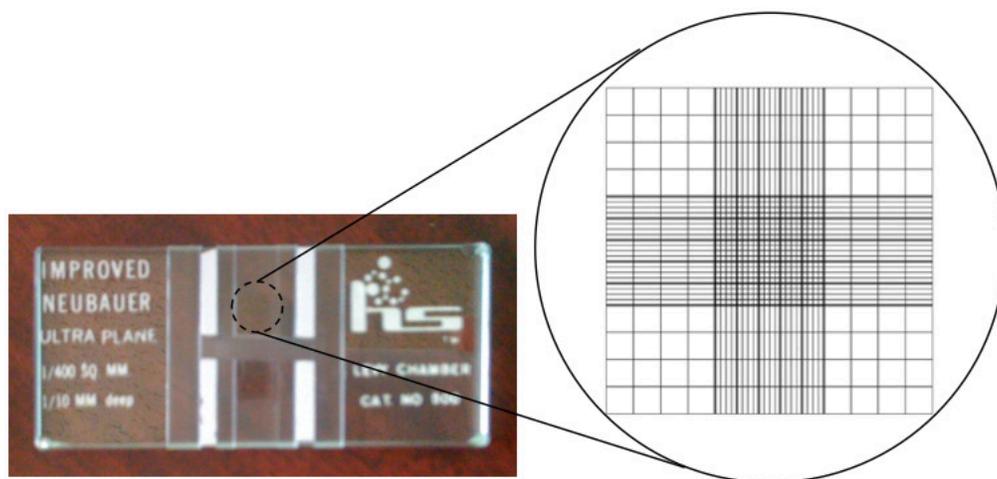


Figure 5. The view of one of the two complete grid areas on a hemocytometer under 40X total magnification and the profile of the counting chamber. Top figure, an identical grid is located at the opposite side of the moat facing the moat. The bottom figure is the profile showing the coverslip is supported and a depth of 0.1 mm is created for the sample.

Counting cells in the 1 mm x 1 mm area

As described above, you will use one of the four 1 mm x 1 mm areas located at the four corners of the grid. This area does not have the fine lines found at the center area of the counting chamber that forms a cross (Fig. 6). Each 1 mm x 1 mm area has 4 x 4 squares and each square is 0.25 mm x 0.25 mm. You will only count the squares at the four corners plus one at the center.

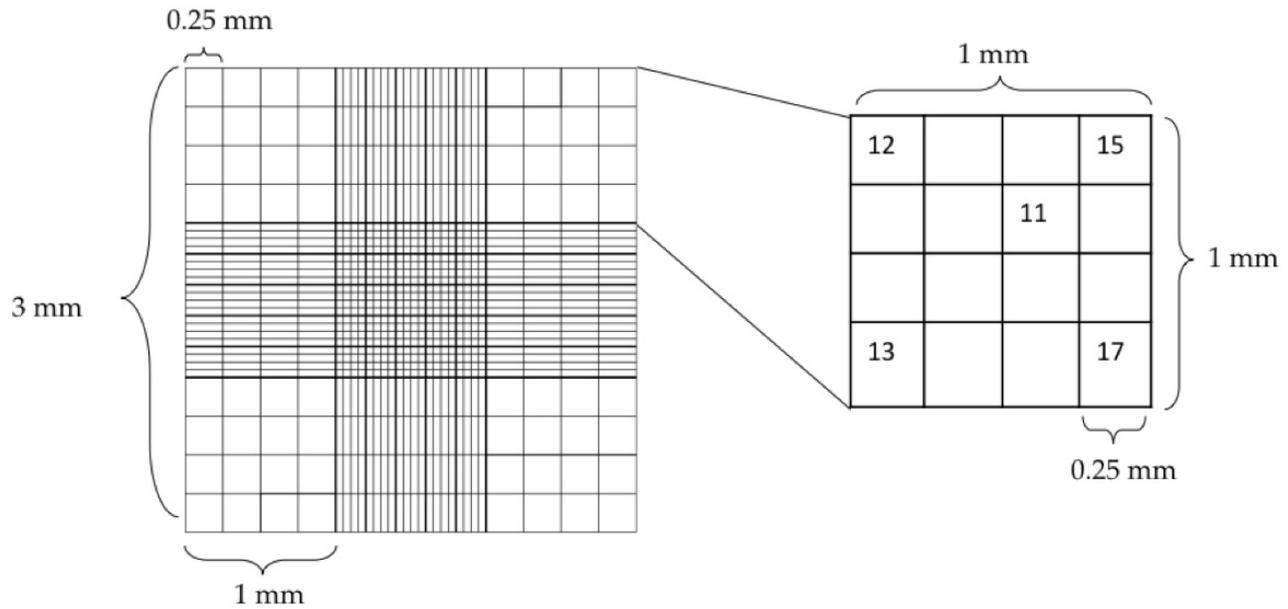


Figure 6. Dimension of an entire grid area and the area for cell counting. Under a total magnification of 100X one of the 1 mm x 1 mm area can fill the entire viewing field. The 4 x 4 squares at right are shown as an example result of cell counts in the corresponding squares.

While holding the tally counter in your non-writing hand, scan the 0.25 mm x 0.25 mm squares in a systematic manner to count all cells within the square. It is common to find cells sitting on the lines. To avoid over- or under estimating the cell numbers, count the cells when they are on the upper and left boundaries of the square and do not count them when they are at the bottom and right boundaries.

1. Record the number in the corresponding square using the blank grid provided on the next page. Repeat counting to the three other corner squares in this 1 mm x 1 mm area. Make sure you zero the tally counter before you count the next square. Count one more square from one of squares in the middle circle.
2. Repeat the next sample and record data on the next blank grid on the next page. Label each 4 x 4 squares as needed for later analysis. (The cell density of each sample is calculated in the next section.)

Calculation of cell density

For each 0.25 mm x 0.25 mm square, the volume is equal to 0.25 mm x 0.25 mm x 0.1 mm (depth) = $\frac{1}{4} \times \frac{1}{4} \times \frac{1}{10} \text{ mm}^3 = 1/160 \mu\text{L}$, since $1 \text{ mm}^3 = 1 \mu\text{L}$. To calculate the concentration of cells using the example in Fig. 5, then

$$\frac{(12+15+11+13+17)}{5} \div \frac{1}{160} \mu\text{L} = \frac{68}{5} \times \frac{160}{\mu\text{L}} = 2176 \text{ cells}/\mu\text{L}$$

So the generic equation for cell concentration will be:

$$\frac{\text{Total number of cells counted}}{\text{Total number of (0.25 mm x 0.25 mm) squares counted}} \times \frac{160}{\mu\text{L}} = \text{Number of cells } /\mu\text{L}$$

Calculate the cell density of each sample and record it under the corresponding data squares.

Blank Grid for Recording Counts

1	2	3	4	5																																																																																
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Cell density: _____
 (#/ μ L)

6	7	8	9	10																																																																																
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Cell density: _____
 (#/ μ L)

11	12	13	14	15																																																																																
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Cell density: _____
 (#/ μ L)

16	17	18	19	20																																																																																
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Cell density: _____
 (#/ μ L)

After you have completed calculating the concentration of chlorophyll a or obtain cell density for all replicates, you should read the appropriate section in the statistics appendix to decide on an appropriate statistical test. As you may quickly realize, most of you are conducting the experiment on two groups of data, one with and one without the treatment, in hope to see if any difference exists between them. It becomes obvious from the chart that you are making a choice between **t test** and **rank sum test**. If none of your replicates have failed due to contamination or other factors, and you have a large enough combined sample size, such as 12 or larger, you may choose to use t test because it is more powerful in detecting differences. If, however, some of your replicates turned out to be unusable, you may still conduct a statistical test using the rank sum test, which can accommodate as low as a total of 8 of total number of replicates.

Statistical Tests

t test

As a convention on statistical tests, you always start by stating your null hypothesis (H₀) and alternative hypothesis (H_A). For our purposes, the two competing hypotheses maybe be stated as follows:

H₀ = There is no difference in algal mass between control and experimental groups.

H_A = There is a difference in algal mass between control and experimental groups.

Consult the symbols and equations in the statistical reference handout. Continue from Table 5, Table 6 or from the cell density you have calculated above and organize your flow of calculation as follows:

Control Group	Experimental Group
n ₁ =	n ₂ =
\bar{x}_1 =	\bar{x}_2 =
SS ₁ =	SS ₂ =
SS _p ² =	
SS $\bar{x}_1 - \bar{x}_2$	
t=	

where

$$S_p^2 = \frac{SS_1 + SS_2}{(n_1 - 1) + (n_2 - 1)}$$

$$S_{\bar{x}_1 - \bar{x}_2} = \sqrt{\frac{s_p^2}{n_1} + \frac{s_p^2}{n_2}}$$

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{\bar{x}_1 - \bar{x}_2}}$$

You should use the sum of (n₁-1) and (n₂-1) as your total sample size and look up the tabular statistic of that sample size at the 0.05 level. If your t statistic is greater than the tabular statistic, you should reject your null hypothesis stating that the two groups have no difference. You should then conclude that the two groups are significantly different in biomass. If your t statistic is greater than the tabular statistic at the 0.01 level, you should further conclude that the difference of biomass between the two groups of data is **highly** significant.

Spearman Rank Correlation Test

If your study is on the correlation between chlorophyll *a* concentration or cell density and the amount of nutrient, you should use Spearman Rank Correlation as your statistical test. You should have at least four concentrations to be able to use the statistical table. This is a nonparametric test so no mean or standard deviation is measured. It also does not assume the distribution of samples. As always in a statistical test, you should state your null hypothesis and alternative hypothesis.

H_0 = No significant correlation exists between the quantity of nutrient and algal mass.

H_A = A significant correlation exists between the quantity of nutrient and algal mass.

Variable pair	Nutrient amount	Rank	[Chl-a] or cell density	Chl-a rank	d	d ²
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						

$n =$

$\sum d^2 =$

$$r_s = 1 - \frac{6 \sum d^2}{n(n^2 - 1)}$$

Test statistic (r_s) =

Tabular statistic =

Rank Sum (Mann-Whitney) Test

You can use the non-parametric method of rank sum test if your sample size is smaller. As described in the Statistical Reference Appendix, rank sum test convert data into ranks and generate statistic. Again, as a non-parametric method, rank sum test does not assume the distribution of samples and does not measure mean or standard deviation of samples.

H_0 = There is no difference in the algal mass between control and experimental groups.

H_A = There is a difference in the algal mass between control and experimental groups.

Follow the procedure in the Statistical Reference Appendix to complete the following table.

Chlorophyll <i>a</i> concentration ($\mu\text{g/L}$) or Cell concentration (number/mL)			
Rank of control	Control	Experiment	Rank of experiment
$T_1 =$			$T_2 =$
$n_1 =$			$n_2 =$
Test statistics (the smaller of T_1 and T_2) =			
Tabular statistic =			

If your test statistic is **smaller** than the tabular statistic at the alpha level of 0.05, you should **reject** the null hypothesis and conclude that there is a difference between the two groups of data. Again, if you can reject the null hypothesis at the alpha level of 0.01, you should conclude that the difference is highly significant.

Materials

Equipment List (per 18 students, or 6 groups)

Qty.	Unit	Equipment	Notes
6	Piece	Hemocytometer	Improved Neubauer or disposable
6	Unit	Compound microscope	
6	Box	Microscope cover slip	
4	Unit	Spectrophotometer	
4	Set	Filtration device for algae	Including reservoir, funnel, rubber stopper, 2-L flask
4	Set	Clamp and stand	For supporting filtration device
4	Set	Aspirator	
72	Piece	Millipore filter	0.8 micrometer pore size
4	Piece	10 mL Graduated cylinder	
30	Piece	Cuvettes	
30	Piece	Test tube	
4	Piece	Test tube brush	
1	Unit	Water bath of 75 C	
4	Piece	20-200 μ L micro-pipettor	
4	Bottle	95% ethanol	Each 50 mL or more
4	Vial	0.1 N HCl	Each 12 mL or more
1	Roll	Aluminum foil	
4	Pair	Small scissors	
4	Piece	5 mL glass pipette	
24	Piece	Flower pot	
24	Piece	Small petri dish	
24	Piece	Plastic cups with lid	
10	Piece	Coffee stirrer	
1	Jug	Creek water	
		Fertilizer	
4	Vial	<i>Chlamydomonas reinhardtii</i>	
4	Beaker	2% agar, each 25 mL	

Notes for the Instructor

1. To allow students the flexibility to design their experiment, it will be very helpful to prepare a material request list (example in Fig. 7) for each student group during the design phase. Students submit their proposal and material list to the instructor and receive approval before the week of the experimental setup.
2. Students may be allowed to bring their own nutrients, such as fertilizers, nutrient supplements, or detergent (detergent may make it difficult to filter algae). Some of the nutrients supplied by students in the past included vitamins, coffee grounds, rock salt, motor oil, cow manure, etc. These 'nutrients' are more suitable to be used in the plant pot method because particulate matter may interfere with cell count on optical density.
3. The groups may finish the estimation of algal growth at different time; checking chlorophyll a concentration will take the longest time while using spectrophotometer to check the cell density will take the shortest time to complete the procedure.
4. To help students to make a decision on choosing the design, the instructor may describe the pros and cons of each of the three analytical methods. In general the chlorophyll a assay is most widely used in research that collects samples from the field. Both cell counting using hemocytometer and cell density measurement using optical density are direct measurement of algal mass but is more meaningful if used to compare single species and tested in controlled condition in lab.

Acknowledgments

The author would like to thank Nelson Hairston and Martha Lyon for the development phase of this lab and Martha Lyon and Mark Sarvary for the presentation of this workshop.

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TA Name: _____ Day: _____ Room: _____ Time: _____

Nutrient Enrichment Materials Request Sheet

- Maximum 6 groups/lab, each group with 2 or 3 students.
- Maximum 4 groups using test tubes and staff-provided algae; 20 test tubes/group max; leave unused ones in lab.
- Maximum 2 groups using plant pots; 12 plant pots/group max; return unused ones.
- Staff will provide both liquid and dry nutrients with sufficient quantity; return remaining items.

Supply or Activity	Student groups using test tube and staff-provided algae [Complete with Option A, B, or E below and indicate with a ✓ mark in the blanks. Only one group can choose Option E.]				Total
	1. Option <u>A</u>	2. Option <u>A</u>	3. Option <u>B</u>	4. Option <u>E</u>	
Chlamydomonas (12 mL)	✓	✓	✓		3
Mixed algae (12 mL)				✓	1
Sterile pond water			✓		1
TAP medium, deficient for A	✓				1
TAP medium, deficient for B		✓		✓	2
Chlorophyll-a analysis by Spec 20. (Option E only)				✓	1
Supply or Activity	Student groups using plant pots [Option C or D; indicate with a ✓ mark in the blanks]				Total
	1. Option <u>C</u>	2. Option <u>D</u>			
Plant pots with agar					2

Figure 7. Example of a material request sheet for each lab section to determine the specific materials they need.

Appendix A

Student Study Proposal

Project Title:

Group Members:

What is your research hypothesis?

What prediction(s) of your research hypothesis will you test?

Describe your test of hypothesis (study) in terms of:

Set up of equipment (draw a diagram?):

Measurements you will make:

Chen

Sources of random variation and how you might control for them:

Number of Replicates Planned:

What are your expected results?

How will you analyze the data you have collected?

Possible Sources of Error:

Approved by (instructor)_____

Appendix B

Percentage Transmission-Absorbance Conversion Table

T%	Absorbance				T%	Absorbance			
	(0.0)	(.25)	(.50)	(.75)		(0.0)	(.25)	(.50)	(.75)
1	2.000	1.903	1.824	1.757	51	.2924	.2903	.2882	.2861
2	1.699	1.648	1.602	1.561	52	.2840	.2819	.2798	.2777
3	1.523	1.488	1.456	1.426	53	.2756	.2736	.2716	.2696
4	1.398	1.372	1.347	1.323	54	.2676	.2656	.2636	.2616
5	1.301	1.280	1.260	1.240	55	.2596	.2577	.2557	.2537
6	1.222	1.204	1.187	1.171	56	.2518	.2499	.2480	.2460
7	1.155	1.140	1.126	1.112	57	.2441	.2422	.2403	.2384
8	1.097	1.083	1.071	1.059	58	.2366	.2347	.2328	.2310
9	1.046	1.034	1.022	1.011	59	.2291	.2273	.2255	.2236
10	1.000	.989	.979	.969	60	.2218	.2200	.2182	.2164
11	.959	.949	.939	.930	61	.2147	.2129	.2111	.2093
12	.921	.912	.903	.894	62	.2076	.2059	.2041	.2024
13	.886	.878	.870	.862	63	.2007	.1990	.1973	.1956
14	.854	.846	.838	.831	64	.1939	.1922	.1905	.1888
15	.824	.817	.810	.803	65	.1871	.1855	.1838	.1821
16	.796	.789	.782	.776	66	.1805	.1788	.1772	.1756
17	.770	.763	.757	.751	67	.1739	.1723	.1707	.1691
18	.745	.739	.733	.727	68	.1675	.1659	.1643	.1627
19	.721	.716	.710	.704	69	.1612	.1596	.1580	.1565
20	.699	.694	.688	.683	70	.1549	.1534	.1518	.1503
21	.678	.673	.668	.663	71	.1487	.1472	.1457	.1442
22	.658	.653	.648	.643	72	.1427	.1412	.1397	.1382
23	.638	.634	.629	.624	73	.1367	.1352	.1337	.1322
24	.620	.615	.611	.606	74	.1308	.1293	.1278	.1264
25	.602	.598	.594	.589	75	.1249	.1235	.1221	.1206
26	.585	.581	.577	.573	76	.1192	.1177	.1163	.1149
27	.569	.565	.561	.557	77	.1135	.1121	.1107	.1093
28	.553	.549	.545	.542	78	.1079	.1065	.1051	.1037
29	.538	.534	.530	.527	79	.1024	.1010	.0996	.0982
30	.523	.520	.516	.512	80	.0969	.0955	.0942	.0928
31	.509	.505	.502	.498	81	.0915	.0901	.0888	.0875
32	.495	.491	.488	.485	82	.0862	.0848	.0835	.0822
33	.482	.478	.475	.472	83	.0809	.0796	.0783	.0770
34	.469	.465	.462	.459	84	.0757	.0744	.0731	.0718
35	.456	.453	.450	.447	85	.0706	.0693	.0680	.0667
36	.444	.441	.438	.435	86	.0655	.0642	.0630	.0617
37	.432	.429	.426	.423	87	.0605	.0593	.0580	.0568
38	.420	.417	.414	.412	88	.0555	.0543	.0531	.0518
39	.409	.406	.403	.401	89	.0505	.0494	.0482	.0470
40	.398	.395	.392	.390	90	.0458	.0446	.0434	.0422

41	.387	.385	.382	.380	91	.0410	.0398	.0386	.0374
42	.377	.374	.372	.369	92	.0362	.0351	.0339	.0327
43	.367	.364	.362	.359	93	.0315	.0304	.0292	.0281
44	.357	.354	.352	.349	94	.0269	.0257	.0246	.0235
45	.347	.344	.342	.340	95	.0223	.0212	.0200	.0188
46	.337	.335	.332	.330	96	.0177	.0166	.0155	.0144
47	.328	.325	.323	.321	97	.0132	.0121	.0110	.0099
48	.319	.317	.314	.312	98	.0088	.0077	.0066	.0055
49	.310	.308	.305	.303	99	.0044	.0033	.0022	.0011
50	.301	.299	.297	.295	100	.0000	.0000	.0000	.0000

Appendix C

Statistical Tests Handout

t Test

t test is one of the most commonly used statistical tests involves comparing the differences between two samples of data. Because we directly process the measurement we require the two populations where the samples were obtained to follow a normal distribution and have the same variance. So the purpose of the test is to compare the means. To derive at the test statistic you will divide the difference of means of the two samples by the **standard error** of the difference between the sample means. It will become clear with the following description of the procedure.

1. Calculate the mean (\bar{x}) of each of the two samples.
2. Calculate the sum of squares (SS) of each of the two samples from Equation II in the Descriptive Statistics section.
3. Add the two sums of squares from the two samples and divide this by the sum of (n_1-1) and (n_2-1) , n_1 and n_2 being the sizes of the two samples. The quotient will be the **pooled variance** for the two samples.
4. Divide the pooled variance by n_1 , and then divide the pooled variance by n_2 to yield two quotients. Take the square root of the sum of the two quotients. This will be the standard error of the difference between the sample means.
5. The *t* statistic is the difference of the two means divided by the number you derived at in 4 above.
6. Look up tabular statistic for the total of $(n_1-1) + (n_2-1)$ at the 0.05 level in Table 4 below. If the test statistic is higher than the tabular statistic, reject H_0 . If the test statistic is higher than the tabular statistic at the 0.01 level, reject the H_0 and conclude the difference is **highly** significant.

Example: In order to determine if a size difference exists between male and female black-capped chickadees, an investigator obtains wing length measurements for both sexes from birds of known sex. The following data on 10 adult male and 10 adult female chickadees illustrate the use of *t* test to determine if the two groups are significantly different. Although the samples have the same size (number of replicates), *t* test can work with samples of different sizes.

Wing Length(mm)	
Male	Female
63.8	62.5
65.8	62.0
62.8	61.5
65.8	62.0
63.0	62.0
65.0	63.0
66.0	61.0
64.0	62.8
62.8	61.5
66.3	59.8

H_0 : there is no difference in wing lengths of males and females.

H_A : there is a difference in wing lengths of males and females.

$$n_1 = 10$$

$$n_2 = 10$$

$$n_1 - 1 = 9$$

$$n_2 - 1 = 9$$

$$\bar{X}_1 = \frac{\sum_{i=1}^{n_1} X_i}{n_1} = 64.53$$

$$\bar{X}_2 = \frac{\sum_{i=1}^{n_2} X_i}{n_2} = 61.81$$

$$\begin{aligned} SS_1 &= \sum_{i=1}^{n_1} (X_i - \bar{X})^2 \\ &= (63.8 - 64.53)^2 + \dots + (66.3 - 64.53)^2 \\ &= 17.881 \end{aligned}$$

$$\begin{aligned} SS_2 &= \sum_{i=1}^{n_2} (X_i - \bar{X})^2 \\ &= (62.5 - 61.81)^2 + \dots + (59.8 - 61.81)^2 \\ &= 7.869 \end{aligned}$$

$$s_p^2 = \frac{SS_1 + SS_2}{(n_1 - 1) + (n_2 - 1)} = \frac{17.881 + 7.869}{9 + 9} = 1.430$$

$$s_{\bar{X}_1 - \bar{X}_2}^2 = s_{\bar{X}_1 - \bar{X}_2} = \sqrt{\frac{s_p^2}{n_1} + \frac{s_p^2}{n_2}} = \sqrt{\frac{1.430}{10} + \frac{1.430}{10}}$$

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_{\bar{X}_1 - \bar{X}_2}} = \frac{64.53 - 61.81}{0.5349} = \frac{2.72}{0.5349} = 5.085$$

Tabular statistic $[(n_1 - 1) + (n_2 - 1) = 18, \alpha = 0.05] = 2.101$

Tabular statistic $[(n_1 - 1) + (n_2 - 1) = 18, \alpha = 0.01] = 2.878$

Test statistic $5.085 >$ Tabular statistic 2.878 ; therefore reject H_0 and assume a highly significant difference between male and female wing lengths.

Table C1. t test tabular values at $\alpha = 0.05$ and $\alpha = 0.01$. These values or larger for the test statistic cause rejection of H_0 (d.f., degree of freedom).

<i>d.f.</i>	α	
	0.05	0.01
1	12.70	63.65
2	4.303	9.925
3	3.182	5.841
4	2.776	4.604
5	2.571	4.032
6	2.447	3.707
7	2.365	3.499
8	2.306	3.355
9	2.262	3.250
10	2.228	3.169
11	2.201	3.106
12	2.179	3.055
13	2.160	3.012
14	2.145	2.977
15	2.131	2.947
16	2.120	2.921
17	2.110	2.898
18	2.101	2.878
19	2.093	2.861
20	2.086	2.845

Rank Sum (-Whitney) Test

The rank sum test converts primary data into ranks in order to obtain the test statistic. It is designed to determine if two samples are significantly different from each other. The two samples could be experimental and control groups in an experimental test of hypothesis or, as in the following example, two randomly selected groups in a nonexperimental test of hypothesis. The sample size need not be the same for both groups. The following procedure is used in calculating the test statistic:

1. Rank the combined data values from both groups sequentially. Note: if the two sample sizes are not equal ($n_1 \neq n_2$) rank the data values (either smallest to largest or largest to smallest) so that the group with the smaller sample size has the smallest rank sum (see following example).
2. Sum the ranks of each sample separately (T_1 and T_2).
3. The test statistic is the smaller of the two rank sums.
4. Compare the test statistic with the value of the tabular statistic (Table 5) at the appropriate sample sizes, and alpha levels (.05 or .01). The tabular statistic is the largest value for the smaller rank sum allowable in order to reject H_0 . In using the table when sample sizes are unequal, let the smaller sample size equal n_1 and the larger, n_2 .
5. If the test statistic is equal to or less than the tabular statistic you can reject H_0 . If the test statistic is greater than the tabular statistic, you cannot reject H_0 .
6. Data which tie for two or more ranks are given average ranks.

Example: We will use the same data in the t test example to demonstrate the rank sum test.

Wing Length (mm)			
Rank	Male	Female	Rank
14	63.8	62.5	8
17.5	65.8	62.0	6
10	62.8	61.5	3.5
17.5	65.8	62.0	6
12.5	63.0	62.0	6
16	65.0	63.0	12.5
19	66.0	61.0	2
15	64.0	62.8	10
10	62.8	61.5	3.5
20	66.3	59.8	1

$$T_1 = 151.5$$

$$n_1 = 10$$

$$T_2 = 58.5$$

$$n_2 = 10$$

H_0 : T_1 does not differ significantly from T_2 ; there is no difference in the wing lengths of males and females

H_A : T_1 does differ significantly from T_2 ; there is a difference in the wing lengths of males and females

Test Statistic = $T_2 = 58.5$

Tabular Statistic ($n_1 = 10, n_2 = 10; \alpha = .01$) = 71

Test Statistic (58.5) < Tabular Statistic (71); therefore, reject H_0 and assume a highly significant difference between groups

If the two sample sizes are larger than the table's values, calculate an approximation to the normal curve as follows:

$$Z = \frac{|\mu - T| - 0.5}{\sigma}$$

where: T = the smaller rank sum (T_1 or T_2)

$$\mu = \frac{n_1(n_1 + n_2 + 1)}{2} \quad n_1 = \text{smaller sample size}$$

$$n_2 = \text{larger sample size}$$

$$\sigma = \sqrt{\frac{n_2 \cdot \mu}{6}}$$

Then, if the value of $Z \geq 1.96$, reject H_0 at $\alpha = 0.05$;
if the value of $Z \geq 2.58$, reject H_0 at $\alpha = 0.01$.

Table C2. Rank sum tabular values at alpha = 0.05 (5) and alpha = 0.01 (1). These values or smaller for the test statistic cause rejection of H_0 .

→ n_1	2		3		4		5		6		7		8		9		10		11		12		
↓ n_2	5	1	5	1	5	1	5	1	5	1	5	1	5	1	5	1	5	1	5	1	5	1	
4					10																		
5			6		11		17	15															
6			7		12	10	18	16	26	23													
7			7		13	10	20	17	27	24	36	32											
8	3		8		14	11	21	17	29	25	38	34	49	43									
9	3		8	6	15	11	22	18	31	26	40	35	51	45	63	56							
10	3		9	6	15	12	23	19	32	27	42	37	53	47	65	58	78	71					
11	4		9	6	16	12	24	20	34	28	44	38	55	49	68	61	81	74	96	87			
12	4		10	7	17	13	26	21	35	30	46	40	58	51	71	63	85	76	99	90	115	106	
13	4		10	7	18	14	27	22	37	31	48	41	60	53	73	65	88	79	103	93	119	109	
14	4		11	7	19	14	28	22	38	32	50	43	63	54	76	67	91	81	106	96	123	112	
15	4		11	8	20	15	29	23	40	33	52	44	65	56	79	70	94	84	110	99	127	115	

Spearman Rank Correlation Test

This test determines if two variables are related and specifies the degree of relationship. The test statistic is the Spearman rank correlation coefficient, r_s . In perfect positive correlations between two variables, $r_s = +1.0$. In a perfect negative correlation, $r_s = -1.0$. If no relationship exists between two variables, $r_s = 0$. The following procedure is used to obtain the Spearman rank correlation coefficient.

1. Separately rank each group of variables.
2. Compute the differences (d) of the ranks for each pair of variables, square these differences (d^2), and sum them (Σd^2).
3. Use the following formula to obtain r_s .

$$r_s = 1 - \frac{6 \sum d^2}{n(n^2 - 1)}$$

where: d^2 = square of the difference of the ranks for each pair of variables and
 n = number of pairs of variables

4. The **test statistic** is the calculated r_s for a set of data. Compare r_s with the tabular statistic in Table C3. The tabular statistic equals the smallest allowable r_s in order to assume that a significant correlation exists between two variables (at a specified n and alpha level). Therefore, if the absolute value of the test statistic is equal to or greater than the tabular statistic, reject H_0 . If the absolute value of r_s is less than the tabular statistic, you cannot reject H_0 .

Example: An investigator wants to determine if a correlation exists between the numbers of cucumber seeds that germinate and the concentration of a specific herbicide to which the seeds are exposed. In the study, cucumber seeds in four dishes (15 per dish) are each wetted with a different concentration of the herbicide. The seeds in a fifth dish, serving as the control, are wetted with distilled water. Therefore, in this study the two variables being tested for a correlation are herbicide concentration and cucumber seed germination. The investigator predicts that a negative correlation should exist between these two variables.

Variable Pair	Herbicide Conc. (mg/l)	Rank	Seeds Germinated	Rank	d	d ²
1	0.000	1	14	4.5	3.5	12.25
2	0.001	2	14	4.5	2.5	6.25
3	0.010	3	12	3	0	0
4	0.100	4	6	2	-2	4
5	1.000	5	2	1	-4	16

n = 5

Σd² = 38.5

$$r_s = 1 - \frac{6(38.5)}{5(5^2 - 1)}$$

$$r_s = 1 - \frac{231}{120} = 1 - 1.925$$

$$r_s = -0.925$$

H₀: no significant correlation exists between the variables

H_A: a significant correlation exists between the variables

Test Statistic = r_s = -0.925

Tabular Statistic (n=5; α = 0.05) = 0.900

Absolute value of Test Statistic (0.925) > Tabular Statistic (0.900); therefore, reject H₀ and assume that a significant correlation (α = 0.05) exists between variables. Negative sign of r_s indicates that it is a negative correlation

Table C3. Absolute (+ or -) tabular values for the Spearman rank correlation test (n = number of pairs of variables).

n	alpha level (α)	
	0.05	0.01
4	1.000	---
5	.900	1.000
6	.829	.943
7	.714	.893
8	.643	.833
9	.600	.783
10	.564	.746
12	.506	.712
14	.456	.645
16	.425	.601
18	.399	.564
20	.377	.534
22	.359	.508
24	.343	.485
26	.329	.465
28	.317	.448
30	.306	.432

*Note: if n > 30 consult Siegel (1956) p. 212.

About the Author

Kuei-Chiu Chen received her Ph.D. in biology from New York University. She has worked in New York City's Forensic Biology Department of the Office of Chief Medical Examiner. She was the lab coordinator for three introductory biology courses at UCLA. In 2005 she joined Cornell University as Director of Introductory Biology Laboratories, which was later changed to Investigative Biology Laboratory after a curricular reform. Currently she is Director of Instructional Laboratories, Premedical Program at Weill Cornell Medical College in Qatar.

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