Investigating Bacteria with the Winogradsky Column
http://www.woodrow.org/teachers/bi/2000/Winogradsky_Column/winogradsky_column_p8.html

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Abstract
As an investigation that was part of a larger module, a Winogradsky Column was developed to culture a mixed flora of bacteria. A column was created and other additional columns were started with differing conditions to determine the effects of bacterial growth. Extensions were developed to help expand the use of the Winogradsky Column as a teaching tool.

Objectives

- construct a Winogradsky column
- identify the various bacteria that grow
- chronicle the communities that develop
- determine the biogeochemical cycles that exist and the bacteria associated with each part thus associating microbial and functional diversity.

Background and Curriculum
The Winogradsky Column is an exceptionally elegant lesson in understanding microbial ecology. It can be grown by anyone from middle to high school but the real depth and breadth of its use would be most appreciated within the high school science curriculum; especially advanced classes.

WWNFF Theme
As a study for biodiversity in the microbial world, this investigation is by far essential for a class to devote time towards studying. In a small tube filled with soil and water, a complete ecosystem will develop, exhibit microbial succession over time and create numerous biogeochemical cycles that are essential to life on Earth. Students can create a unique environment with slight variations on the "recipe" and soon have an inquiry project that could easily occupy the entire year.

With the simple extensions given, teachers can isolate and grow a number of the species found in the column and extend this column to a number of examples in the real world showing the relevance of this complex yet deceptively simple lab.

Introduction
In many schools across the country in biology and life science classes, the topic of microbiology is often relegated to a short discussion of diseases and viewing bacteria under the microscope. While some instructors are able to grow cultures so that students see different species of bacteria, rarely is it show how they can interact with each other in the environment. Students often have the misconception of bacteria as static, immovable organisms. The Winogradsky Column investigation can be used as a tool to understand microbial life. It is an excellent way to understand the growth of microbial communities and to show succession as the column

develops. In addition, it helps many students see the difference between aerobic and anaerobic communities. However, the investigation often stops there once the column develops since very few teachers understand the complexity that lies within the depths of the ecosystem that has developed.

The Winogradsky Column was first used in the 1880's by Sergei Winogradsky (1856-1953), a Russian microbiologist, to study the complex interactions between environmental conditions and microbial activities and the role of soil enrichment in the isolation of pure bacterial cultures. Many microbiologists of the time, such as Louis Pasteur and Robert Koch isolated cultures for study, but Winogradsky's work was the first to study mixed environments of microorganisms.

The column is not a natural environment. All of the organisms are mixed during the preparation and it is merely to study environments that develop over time. When it is sealed and exposed to light, a succession of microbes will develop according to the concentrations of oxygen, nutrients and light available. Depending on the various concentrations of nutrients and the type of soils used, a variety of bacteria will appear over time. However, it is an excellent model of microbial ecology. Each organism is dependent on the other to set the conditions for development and the entire column is run on the energy of light. The Winogradsky Column is a classic demonstration of the metabolic diversity of prokaryotes.

Methodology

Building the Basic Column

- A glass or plastic container minimally15 cm in height and 5 cm in diameter is ideal but any size container will do. Plastic bottles are ideal because they can be manipulated easily to allow for extraction of species of culturing. If possible, the container's sides should also be smooth to make observing the bacterial growth easier. As well, very tall and wide containers take longer to grow and are more difficult from which to extract bacteria.
- Clear plastic film and a rubber band
- A long wooden dowel
- Cellulose source--Shredded paper, grass, leaves, lettuce, sawdust or wood chips
- Sulfur source--calcium sulfate, magnesium sulfate, egg yolk. This should be added to be about 1-2% approximately of the weight.
- Carbon dioxide source(optional)--calcium carbonate – 1-2%. Both of the previous sources can be approximate
- A selection of soils--pond mud, river mud, saturated soil
- Water from the source of the mud

Procedure

2. Check the assigned soil sample for clumps that will prevent packing. Break up the soil if necessary. It can be stirred up to gain a uniform consistency. (note: avoid rocks and large solid clumps when scooping up mud from the source.)

2. The amount of mud you add will depend on the size of the container. As well, it's important to keep the type of mud consistent with the water (fresh with fresh, marine with marine, etc.) Mix the sulfur source and carbon dioxide source (optional) in with the mud. They should be 1 to 2% of the final mass. (note: mud should be of milk-shake consistency before added sources below)
3. Then mix in an equal volume of cellulose. The cellulose should be near the bottom but it can be in the middle if a variation is desired. However, it can't be near the top since the bacteria involved are anaerobic.
4. Add the mud and pack this material into layers of 2-3 cm. Use the dowel or something equivalent to tamp down the mud to force out trapped air. Your last layer (water) should be about 5 cm from the top.
5. Cover the top with plastic film and secure with a rubber band. Place the column next to a low heat/high intensity light source probably less than 60 watts. It is important that it doesn't over heat.
6. Examine the columns weekly for at least a month, recording changes in color and depth as they occur.
7. Sampling can occur at weekly intervals to check succession or can be done at the end of the month to see the final flora of bacteria that develops.

Variations

If a freshwater model as described above is used, this is the standard Winogradsky Column. However, with just a few changes, some different columns can be created to compare for growth rates, etc.

a. sodium sulfide can be added in place of a sulfate. This may inhibit growth of the sulfur reducing bacteria bringing about different species of bacteria.
b. Changing the pH may effect which species grow. Many of the standard sulfur reducers are comfortable in a pH of 6-8 (Brock 2000) Creating a more acidic or alkalinic environment may change the species diversity as well.
c. Enriching for Extremophiles. There are three basic types that may be created:
    - Thermophiles – these species are very tolerant of high heat and if you put the column in front of a light source that creates more heat, thermophiles may be developed.
    - Acidophiles – will develop when the pH is significantly acidic.
    - Halophiles – If you opt to create a marine environment, Halophilic bacteria will predominate. The same can occur if your soil is inoculated with a 10% NaCl solution. Pickles or Saurkraut in the substrate would work as well (Levandowsky, oral communication)
d. **Classroom Notes**

    In order for this to be successful, enough time has to be given for the columns to develop. They will have growth in a week but they won't fully form and stabilize for about four weeks. This investigation is designed as an ongoing investigation in a unit on ecology, microbiology, biodiversity, evolution or any number of other biological themes. The ideal situation would be for students to grow and investigate them over the course of the year.

e. Isolating these bacteria can be tricky and, like the developing column, many of them, especially the anaerobes, take time to develop. The recipes and isolation techniques given below will give you the basic bacteria that are listed in the chart but these are only the "standard" species and there may be others that develop in the column. There are books listed in the references that will help you to identify other bacteria.
Table 1.

f. **Physical Properties of Bacteria**

Bacteria	**Shape**	**Gram stain**	**Classification**
Beggiatoa	Filamentous, long, gliding	-	Non-photosynthetic Chemolithotropic sulfur oxidizing
Thiobacillus	Rods	-	Colorless sulfur aerobic Chemolithotropic
Rhodospirillum	Spirals, polarly flagellated	-	Purple, non-sulfur Anoxygenic photosynthetic
Rhodopseudomonas	Rods, polarly flagellated; divide by budding	-	Purple, non-sulfur Anoxygenic photosynthetic
Chromatium	Ovals or rod, polarly flagellated Sulfur deposits internally	-	Purple sulfur anoxygenic photosynthetic
Chlorobium	Straight or curved rods; nonmotile	-	Green sulfur anoxygenic photosynthetic
Clostridium	Rod	+	Endospore forming anerobic
Desulfovibrio	Vibrio	-	Sulfur Reducing Anaerobic

g.

Isolation Techniques

Unlike many of the bacteria that are used in the classroom, the above eight bacteria, which represent the most common species found in the column, have isolation techniques that are a little more complex.

h. ***Rhodospirilium and Rhodopseudomonas*** – both of these organisms are non-sulfur purple bacteria and are cultured using Pfennig's medium (Brock, 1988). The concentration of the sulfide should be reduced to .01 to .02% $Na2S \cdot 9H2O$ or eliminated and an organic substance added to provide Carbon.

i. ***Chromatium and Chlorobium*** – both Chromatium (Kingdom I: Proteobacteria) and Chlorobium (Kingdom VII: Green Sulfur) are both photosynthetic anoxygenic bacteria. Though they are unrelated they have the same function. They each use a specialized medium to isolate them. The basic procedure is to add mud to a jar and enough of the respective medium covers the mud to a depth of .5 cm. It is then incubated for 7 days at room temperature while exposed to light. Then .1ml of the medium is then transferred to enriched agar shake deeps and incubated and additional 4-7 in the same conditions. Slides can then be made from the isolated colonies. The media recipes and more detailed

procedure can be found in a number of microbiology laboratory manuals some of which are listed in the reference section.

j. ***Beggiatoa***-(Bej je-ah to'ah) these are extremely interesting organisms. They were the initial bacteria that Winogradsky used to study the role of microorganisms in the cycling of sulfur. Beggiatoa, when isolated under the microscope, can be observed to have sulfur granules stored in its cells. Beggiatoa also produce filaments and can migrate. The organism is cultivated by placing them in a sulfide agar closed tube and overlayed with initially sulfide-free mineral agar. Beggiatoa grow at a well defined interface between O2 and the H2S which migrates upward.

k. ***Clostridium***-these are endospore forming bacteria. They don't reduce sulfates and are fermentative. They have strong industrial use as producers of ethanol, acetone and butanol. Their main habitat is soil and can have harmful effects on humans causing botulism and gangrene.

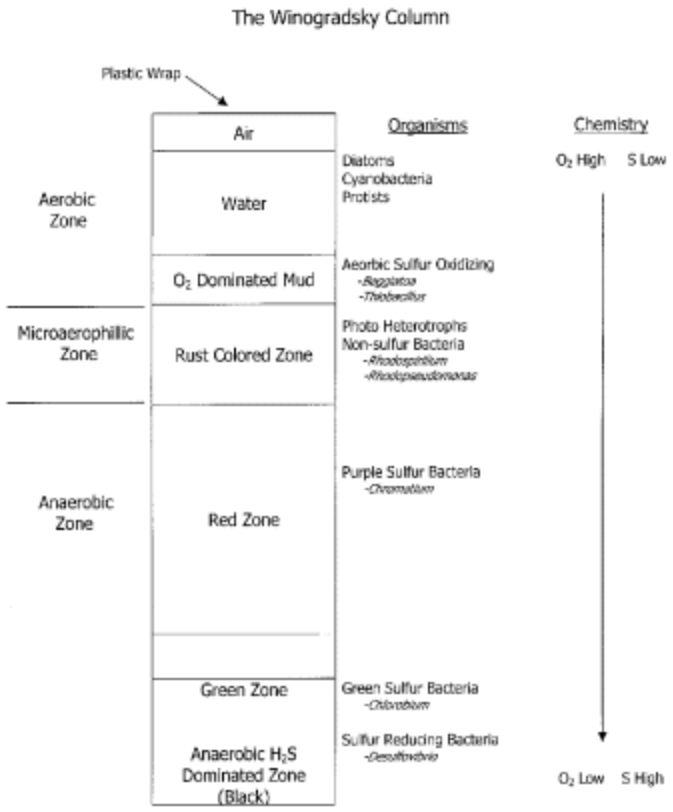
l. ***Thiobacillus***-Thiobacillus are chemoautotrophs and require an inorganic source of energy. They are found under aerobic conditions that contain sulfur or sulfides. The predominant method to isolate them is a mixture of Starkey's Medium, Thiosulfate Medium and a coal dust innoculant. This does take time but is effective. Another method is to take the soil sample and cook it to 80 C. Thiobacillus will sporolate and they can then be isolated and grown.

m. ***Desulfovibrio***-is a sulfate reducer obligate anaerobe. They may also reduce nitrates and just use sulfate in place of the nitrates. Desulfovibrio uses a specialized Desulfovibrio Medium to isolate them.

n. **EXTENSIONS**

General Biology of the Column

After a month to six weeks, the column should stabilize into three distinct environments and develop communities of bacteria specific to their environmental requirements and should resemble Figure 1.



o. **Aerobic Zone (Oxygen Rich)**
The top of the water column can contain large populations of diverse bacteria. These are aerobic organisms that are found in organic-rich freshwater habitats such as shallow ponds, polluted streams, etc. These are generally flagellated which allows the bacteria to migrate and establish themselves in new areas. In addition, there may be a diverse phototrophic fauna as well from the original water and mud source. At the very top of the zone the mud is characterized by a light brown color. This is the most oxygen rich part of the mud and the most sulfur poor.

p. Photosynthetic **cyanobacteriacan** grow in the upper zones. This area is characterized by a Grass green color These are the only bacteria that have photosynthesis like that of plants. In fact, there is very strong evidence that the chloroplasts of plants were originally ancestral cyanobacteria that established themselves as symbionts inside the cells of a primitive eukaryote. Similarly, there is equally strong evidence that the mitochondria of present-day eukaryotes were derived from purple bacteria.

q. From the mud source below, H2S will diffuse upward into the aerobic zone and can be oxidized to sulfate by the sulfur-oxidizing bacteria such as Beggiatoa and Thiobacillus. These bacteria gain energy from oxidation of H2S, to elemental sulfur and they synthesize their own organic matter from CO2. So they are termed **chemoautotrophs**

r. **Microaerophillic Zone (Oxygen Scarce)**
In this zone oxygen diffuses down from the surface but is limited in concentraction.

Sulfur from the lower part of the column has begun to move up in the form of H2S. This diffusion of H2S from the sediment into the water column enables anaerobic photosynthetic bacteria to grow. They are seen usually as two narrow, brightly colored bands immediately above the sediment - a zone of **green sulfur bacteria**, such as Chlorobium, characterized by a green/olive color indicative of growing anaerobic conditions, then a zone of **purple sulfur bacteria**, such as Rhodospirilum and Rhodopseudomonas, which takes on a red/ orange or rust color.

s. The green and purple sulfur bacteria gain energy from light reactions and produce their cellular materials from CO2 in much the same way as plants do. However, there is one essential difference: they do not generate oxygen during photosynthesis because they do not use water as the reducer; instead they use H2S. The following simplified equations show the parallel processes:

t. $6\ CO_2 + 6\ H_20 = C_6H_{12}O_6 + 6\ O_2$ (plant photosynthesis)

u. $6\ CO_2 + 6\ H_2S = C_6H_{12}O_6 + 6\ S$ (bacterial anoxygenic photosynthesis)

v. **Anaerobic Zone (Oxygen Depleted)**
The only organisms that can grow in anaerobic conditions are those that ferment organic matter and those that perform anaerobic respiration. **Fermentation** is a process in which organic compounds are degraded incompletely; for example, yeasts ferment sugars to alcohol. **Anaerobic respiration** is a process in which organic substrates are degraded completely to CO2, but using a substance other than oxygen as the terminal electron acceptor

w. There are three basic levels that form in the lower level of the column. At one level purple sulfur bacteria such as *Chromatium*, in a Red to Purple layer, are processing Sulfates into Sulfur. At another point *Gallionella*, a stalked bacteria, processes Iron to help create the black layer that forms just below. This level is marked by a strong rust/orange color.

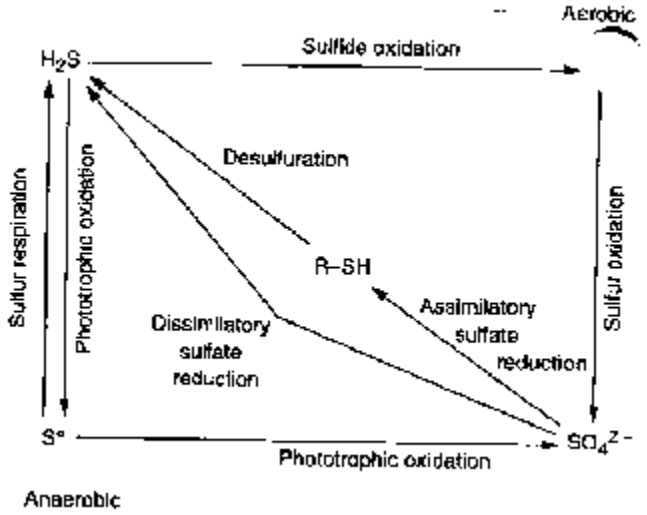
x. Some **cellulose-degrading *Clostridium*** species start to grow when the oxygen is depleted in the sediment. All Clostridium species are strictly anaerobic because their vegetative cells are killed by exposure to oxygen, but they can survive as spores in aerobic conditions. They degrade the cellulose to glucose and then ferment the glucose to gain energy, producing a range of simple organic compounds (ethanol, acetic acid, etc.) as the fermentation end products.

y. Deeper in the column, the **sulfur-reducing bacteria**, marked by a deep black layer and typified by Desulfovibrio,canutilize these fermentation products by **anaerobic respiration**, using either sulfate or other partly oxidized forms of sulfur (e.g. thiosulfate) generating large amounts of H2S by this process. The H2S will react with any iron in the sediment, producing black ferrous sulfide. This is why lake sediments (and our household drains) are frequently black. However, some of the H2S diffuses upwards into the water column, where other organisms utilize it.

z. Finally, at the bottom, depending on the source of the mud, a pink layer will develop due to purple sulfur bacteria with gas vesicles. A characteristic species is Amoebobacter. This environment is very high in H2S and is more tolerant of air and light

aa. **The Sulfur Cycle**
The sulfur cycle is a poorly understood process by high school students. In fact, beyond the carbon, nitrogen and oxygen cycle, many students are unaware that other essential

elements have a well-defined cycle thoroughout the biosphere. The Winogradsky Column is an excellent way to illustrate this process.



bb.

cc. Any diagram of the sulfur cycle will show that the entire cycle is represented by bacteria species that are present in the Winogradsky Column (Madigan et. al. 2000) Each can be isolated and grown to show that, indeed sulfur is cycled through nature just as nitrogen and carbon dioxide.

dd. However, the isolation of each species is difficult due to the fact that they are anaerobes and special media are needed as well as time to effectively culture them.

ee. Each bacterial species that can be found in the Winogradsky column has a role to play in the cycling of sulfur through the system.

ff. *Thiobacillus* and *Beggiatoa* are sulfur oxidzers breaking down H2SO4 and H2S

gg. $S^o + 3/2\ O + H_2O \rightarrow H_2SO_4$

hh. They use the energy to fix Carbon Dioxide. If there is a significant source of easily degradable carbon then they may be inhibited.

ii. *Chromatium* and *Chlorobium* are photoheterotrophs and can oxidize reduced sulfur compounds under anaerobic conditions

jj. $H_2S \rightarrow S^o \rightarrow SO_4^{2-}$

kk. *Desulfvibrio* is anaerobic but grows lithtrophically with H2 as the electron donor, sulfate as the acceptor and CO2 as the sole carbon source. Also nitrate can be substituted as the acceptor. It has a very complex biochemistry.

ll. There are far more reactions that occur and this is just a few of the pathways that exist in the column. There are also more species that inhabit the environment and each has its own unique contribution to the sulfur cycle.

mm. **Iron Chemistry**

Sulfur is not the only element being cycled through the column, iron is as well. As the column develops, the mud blackens this is due to the migration of H2S upward and being replaced by FeS .

The Winogradsky Column as a Window on the Past

As we view new wonders of the world, climb to new heights and plunge to new depths, we see not only some amazing sights but we also increase the range of biodiversity and see yet a new

angle on evolution. One particular example are hydrothermal vents, first observed when the submersible vehicle Alvin explored the Mid-Atlantic spreading ridge where the North American and European plates are inexorably moving apart.

When looked at closely, this highly volcanic environment is a mirror of what is occurring in the Winogradsky Column, albeit at lower temperatures. Sulfates are being reduced and elemental sulfur is being bound into sulfate salts. Ancient bacteria, Extremophiles, that are not dissimilar to the anaerobic bacteria accomplishing their task in the column at lower temperatures, are doing all of this at high temperatures deep in the ocean where it was once believed no life existed.

As well, it is a window on the past. In a recent article in Nature, Euan Nisbet paints a picture of an environment of early organisms in the Archean period 2.5 to 4 billion years ago that are analogs to those found in hydrothermal vents. They did the same work of the deep ocean vents of today cycling metals, CO2 and sulfur.

In a sense, by creating a Winogradsky Column we are reclaiming the ancient environments of the past.

Further Questions
There are a number of other areas that can be investigated and as an activity with students it is, in fact, limitless in scope of what might be accomplished.

- If a protist population is established in a series of columns, what effect will the development/succession of the column have on it? Is there a relationship between the protist population and the cycling of sulfur? What might happen if the growth of the column were suppressed?
- Do the columns generate energy? Would a small light bulb light? Why might this occur? Where does the energy come from for the bacteria?
- How barren a soil can there be before no growth will occur?
- If iron is increased how might that affect the population diversity?
- Are there methanogens in the column? How would they be detected? How might their growth be enhanced?

Conclusions
As can be seen the Winogradsky Column is a far more complex and fascinating system than it seems at first glance. It is an excellent example of an investigation that can span the level from guided inquiry all the way to very opened ended projects that can keep students investigating for months. As well, it is a window on the biodiversity of our world. Microbes are poorly documented and very misunderstood. The Winogradsky column is an excellent way to show students that in a sense as we investigate what is growing in the columns we are investigating our world and ourselves.

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