1400 BACTERIAL POLLUTION OF WATER

The objective of this kit is to introduce the student to bacterial pollution found in water. General bacterial population and E. coli or coliform bacterial populations are examined. Students perform experiments on water samples they collect. Enough material is contained in the kit to allow 10 samples of water to be tested for total bacterial population and 10 samples of water to be tested for coliform bacteria. If the petri dishes are prepared with the agar medium before the class period and the samples collected ahead, then the placing of the samples on the culture medium can be done in a class period. The observation and counting of the bacterial colonies should be performed over a 2-4 day period. The teacher may want to perform a demonstration experiment before the class undertakes their own experiments. Because samples polluted with coliform and other harmful bacteria may be used, it is extremely important that the teacher and students follow careful safety procedures.

Additional Materials Required:
Ethanol (Denatured Alcohol)
Cellophane tape
Heat Source (Bunsen Burner or Hot Plate)
Light Source (60 Watt Bulb) for
Culture Incubation
Magnifying Glass
Distilled Water

For Your Information:
Please note that the Plate Count Agar is pale yellow and the EMB Levine Agar is reddish green/brown in color.

BACTERIAL POLLUTION #1400 Teacher Manual

Introduction

Bacterial pollution of lakes and rivers results from the release of materials that stimulate bacterial growth, or the release of bacteria in high concentrations. The number of bacteria in natural waters commonly varies between one thousand and one million bacteria per 100 milliliters of water. The introduction of nitrogen, phosphorus, or new organic matter sets off a chain of events that can increase bacterial populations by a factor of a thousand. Substances such as nitrogen and phosphorus stimulate algae and plant growth. With the introduction of these new nutrients, microscopic decomposers grow in number, depleting the oxygen level of the water. As the oxygen level decreases, anaerobic bacteria begin to multiply and thrive, often producing toxins as metabolic by-products.

Sewage bacteria (such as E. coli) are known as coliform bacteria, since they are found in the lower digestive tract (colon). Such bacteria are released from sewage treatment plants and from septic tank overflows. Sewage includes fecal bacteria (coliform) and other harmful pathogenic bacteria. Coliform bacteria are ubiquitous bacteria; they are normally present in the lower digestive tract and play an important role in digestion. If the bacteria are ingested, however, they can cause gastrointestinal disease. The overwhelming majority of sewage bacteria are coliform, with populations as high as 20 million per 100 ml of water. Since there are no routine methods for detecting the presence of sewage, and all varieties of pathogenic bacteria associated with sewage, coliform populations are used as indicators of sewage pollution. Waters with coliform populations above 2000 per 100 ml of water are considered polluted. Water with a coliform population of above 240 per 100 ml should not be used for swimming, and water with a coliform population of above 1 per 100 ml should not be drunk. It should be noted, however, that certain pathogenic bacteria bear no direct relation to coliforms, and so waters with low coliform populations but high total bacteria count should be viewed with suspicion.

In all state and federal laboratories, the procedure for testing for bacterial pollutants is the same. Samples of the water to be tested are placed on petri dishes which have been filled with material conducive to the growth of bacteria. After an incubation period, the

number of bacterial colonies are counted and then multiplied by a certain factor to ascertain the number of bacteria per 100 ml of water.

In the past fifty years, the science of culturing bacteria has become extremely precise, so that nutrient media can now be formulated to grow a specific kind of bacteria, and not support others. Usually, two different cultures are done. When doing water pollution tests, federal and state authorities generally use two kinds of nutrient media. One is a general nutrient which will support all bacteria. This is called "plate count" agar, since every colony on the agar plate is counted for complete total. The other nutrient is called EMB- Levine agar, after its discoverer, and is specially designed to grow coliform (sewage) bacteria.

The most common bacteria are the coliforms, and other bacteria associated with sewage pollution. However, some dangerous bacteria do not come from sewage, and are not indicated by the presence of coliform bacteria on a culture dish. The "plate count" shows the total abundance of bacteria in the water. If there are many bacteria in the water, then there is a very good chance that there are harmful bacteria in it which will not be indicated on an agar plate designed to grow coliform bacteria. When a sample shows many colonies of bacteria, even if there are no coliform colonies, it should be viewed with suspicion.

To give the teacher and the class the best opportunity to duplicate the same experiments carried out by state and local health authorities, this kit contains all the necessary apparatus to test ten different samples for both total bacteria and coliform bacteria by means of culturing the colonies in petri dishes containing nutrient medium. In order to be able to contrast the two kinds of medium, the kit was designed with "split" plates with a ridge down the middle. This allows one half to be filled with "plate count" agar, and the other with EMB-Levine agar so that comparisons can easily be made.

Preparation of the Agar Dishes

When doing experiments which involve the detection and culturing of bacteria of any kind, extreme care must be taken to avoid unwanted contamination from bacteria in the air, or on the person of the experimenter. The agar dishes are packed in a plastic sleeve to protect them from dust and contaminants. The collection tubes are similarly sealed in sterile packages, so that they are opened only when the sample is taken, and then closed again with a screw top. After performing the experiments, it is suggested that both teacher and student wash their hands thoroughly. All involved should exercise caution to avoid contaminating the samples and the plates during the few moments that they are open to the air. The pipets, which are not available in sanitary wrappers, should be allowed to soak for a few hours in a beaker of 95% alcohol before use to help sterilize them.

Despite all precautions, unwanted contamination may result. It is for this reason that enough materials are included for ten tests. Even if one or two plates should pick up an unwanted microorganism before the sample is added to it, there will still be enough plates left for a number of experiments.

There are two bottles of prepared medium included in the kit. The agar in these bottles is jelled, and need only to be warmed in hot water before being poured. In the interests of sterility, it is strongly suggested that all the plates be poured at one time. If the covers are replaced immediately and sealed with cellophane tape, and the plates kept in a cool location, there is no reason they will not remain ready for use for at least two weeks without drying out.

Each bottle contains 100 ml of agar, which should be just enough to pour ten half-plates of each. It is not necessary to fill the plates to the brim with the medium; overfilling the first few will only mean that there will not be enough medium left for the last few.

To prepare the plates, you should follow this simple procedure. Although assistance by the students is possible, and perhaps even helpful and instructive, it is important that the poured plates not be exposed to the air any longer than absolutely necessary.

Place the two bottles of prepared agar, with screw caps still on, in a beaker or other container filled with water. Do not put so much water in the container that the bottles are totally immersed, but allow the water to surround them. Place the container over a heat

source such as a Bunsen burner or a hot plate. If neither of these is convenient, an inexpensive immersion heater is slower, but will do a perfectly adequate job. Heat the water to boiling.

In a short while, the agar in the bottles will become liquid. When this occurs, lift the bottles from the container with a pot holder or other protective device and unscrew the top of one of the bottles. Then carefully, one at a time, lift the covers from the petri dishes, pour in a little medium (about 10 ml) and immediately cover the dish again. After pouring three or four, check the level of agar remaining in the bottle; it should be half gone by the fifth plate. When you have filled one side on each of the ten plates with one bottle, repeat the procedure, filling the other half of each plate with the contents of the other bottle. The two agars are of different colors, so there will be no mistaking which is the total bacteria side and which is the coliform. When all ten dishes have been poured, seal the top to the dish with cellophane tape, being careful not to tilt the dish, and put them aside to cool. DO NOT STACK THE WARM DISHES. If they are stacked when still hot, the agar will not set up properly.

If the dishes are placed in a cool location, they should be jelled within an hour. They are ready to be used at this point, but they may be kept for two weeks if necessary. They must be kept in a fairly cool place, however, or else the agar will re-liquefy and cause problems. It is all right to stack the dishes, to conserve space, after they are jelled. It is suggested that the teacher wait a couple of days before conducting the initial experiments to be sure that none of the culture dishes were inadvertently contaminated. If the plates are still clear after two days, they are sterile and ready for use.

Teacher's Demonstration Experiments

Before letting the students collect their water samples, the teacher should perform experiments for total bacteria and coliform bacteria as a demonstration. This teacher-run demonstration is suggested for two reasons. First, it gives the students a clear idea of how they are to run their own experiments. Second, it provides an example of the microbial growth which the students may find if the samples which they collect are sufficiently polluted. In many cases, water samples are surprisingly unpolluted, and despite the relief afforded by such a result, those students who have collected clean water may be understandably frustrated if their sample grows few colonies or none at all.

Finding bacteria to culture should not be too difficult. Obtain one of the prepared dishes. Good locations for high bacteria populations include storm sewers, sink drains, gutters, and even human saliva. Finding sewage (coliform) bacteria may be more difficult, but if there are no nearby sewers or cess pools, or even sewage treatment plants, a recently used toilet bowl will usually have a sizable coliform population. Use the sterile culture tubes for collection.

It is suggested that you collect the total count sample first, and then collect the coliform sample. After soaking the pipet in alcohol, rinse it thoroughly in distilled water. Using the pipet, remove a sufficient sample from the collection tube. Remove the top of the petri dish and carefully deposit two drops on the total count side of the dish. Since twenty drops are approximately one milliliter, two drops are approximately one tenth of one milliliter. Two drops may seem like a small sample, but they will contain quite enough of whatever bacteria may be present. After dropping the two drops onto the surface of the agar, discard out the remaining water, without touching the end of the pipet to anything, and using the tip of the pipet, smear the water around the half of the agar plate to cover as much of the agar as possible with a film of water. When this is done, re-cover the dish to protect it from airborne bacteria. Thoroughly flush the pipet with distilled water. Now collect the sample for the coliform test, and repeat the procedure with two drops on the coliform side of the dish. Remember that it is quite important to keep the cover off for as short a time as possible. When the second sample has been applied, re-cover and reseal the dish and set it aside.

Since the bacteria grow best at a temperature of about 98°F, the growing culture should be incubated by placing the sealed agar dish about two feet away from a 60 watt light bulb. The light will not harm the growing bacteria. The incubation period should last for about two days.

After two days, there should be gardens growing on the surface of the agar. The coliform bacteria may require an extra day or two, but if nothing at all shows up after 72 hours of incubation, the sample did not contain a detectable coliform population. On both sides of the agar plate, the bacterial colonies will appear as spots. They may be counted visually (Do not open the plates when counting

bacterial colonies!), or with the aid of a small hand magnifying glass. On the coliform side of the plate, you should only count those colonies with a distinct metallic green color. Bacteria come in all shapes and colors, but only coliform bacteria will have this green coloring, even though a few colonies of other varieties may have decided to grow on the coliform plate. When counting for total bacteria on that side of the plate, count every spot, but only count the ones with the metallic green sheen on the coliform side.

After counting the colonies on the surface of the two sides of the dish, simply multiply this number by 1000 to arrive at the approximate number of bacteria per 100 ml of water.

After you have made the count and allowed the class to verify your results, put the dish into boiling water for twenty minutes before disposing of it. This precaution is taken because there may be some rather unpleasant bugs growing on the agar, especially on the coliform side. This is why you should not unseal the plates to count the colonies. If one should open the plate and, say. touch a coliform colony. the bacteria could get onto one's skin and then into one's mouth, causing severe diarrhea and other gastric problems.

In performing this experiment, the teacher will have demonstrated every technique which the students will need to know when performing experiments with their own samples. It may be a good idea at this point to review the steps taken in performing the experiment so that the students completely understand the process of culturing bacteria on agar plates.

Student Experiments

The student experiments are simply a repeat of the teacher's demonstration, but using student collected samples. Divide the class into groups so that samples for all uncontaminated plates may be collected by equal numbers of students. Using the sterile-wrapped collection tubes, the samples may be collected from almost anywhere. As a "control test" it might be a good idea to collect one sample from the water faucet to demonstrate the purity of the drinking water after it has been purified by the water department. (Bacteria found in drinking-water samples were probably airborne). Other sources for water samples could include local rivers, storm sewers, swimming pools, water from near a sewage treatment plant, bays, and harbors. Bacteria live in both fresh and salt water, so beaches can also be counted on as possible sample sources. If an outfall is suspect, the sample should be taken directly from it or from waters within a few feet of the drain.

Of course, one ought not simply look for pollution in places where it can obviously be found. Samples should be taken from a variety of locations to get the larger picture of the water situation in a given locality. It is also a good idea to remind the students that the finding of no coliform bacteria is as scientifically valid as the detection of serious pollution, after all, most water is relatively unpolluted.

The work sheet included with the kit allows the students to describe where the sample was collected, and what was found, if anything. Each student sample is, of course, tested on each side of the split plate, using the same procedure as the teacher's demonstration.

Since the agar plates, once poured, will last up to several weeks, this series of experiments can easily be spread over a fairly long period of time if it is more convenient to do it that way. After the experiments are over all the culture dishes should be sterilized by boiling and then discarded without opening, Other glassware should be sterilized and put away for future use.

Analysis of Results and Follow Up Discussion

Student work sheets are provided in the kits for the students to record the results of their experiments. If by chance their findings are uniformly negative, the students will have little of an exciting nature to report. This is unlikely, although it is likely that some students will have located water with little or no detectable pollution. If there seems a scarcity of pollution in the students samples, there is little reason to terminate the learning program at that point. The kit is designed to provoke a wide variety of responses and interests in the scientific assessment of water quality, and the teacher can easily follow up the learning already achieved with research and discussion of the effects the unfound pollutants would have on the quality of the water, how these pollutants could have gotten into the water or might at some later date. Discussion could then follow concerning what techniques could be used to treat such pollution, and what personal and social policies could be adopted to forestall any future water pollution.

Should the results of any of the tests prove positive, the teacher should meet with no resistance in carrying the program into the discussion stage. Knowing that some local water sources are polluted, most students will be anxious to trace the polluting sources, examine their effects, and explore alternatives for treatment and future prevention.

It should be mentioned that although the experiments provide an unusually definitive set of tests, they are not as professional as those conducted by local and state laboratories. If the student samples show significant pollution, students may obtain new samples and present them to local health authorities for a complete, authorized test. Another possible procedure would be for students to write letters to industries or treatment plants near where the polluted samples were obtained, including the results of their tests. This will usually provoke concern on the part of the suspected polluter, and might result in a visit from a representative from that company or treatment authority to explain what efforts are being made to eliminate the pollutants which the students have detected.

Aside from the direct social action possibilities arising from the use of the kit, the teacher, may wish to steer discussions into the topic of water purification and control. There are many booklets and pamphlets currently available from both federal and state authorities dealing in detail with these problems and their solutions. Local water treatment plants are often open for tours, and most large industries which have made efforts to eliminate pollution from their waste water and exhausts are more than happy to demonstrate their contribution to the growing effort to halt pollution of our natural resources.

We would suggest that the learning program be concluded with a thoughtful examination of current legislation in the area of water ecology. Your class might write a letter to your state representatives and to the congressional representatives in Washington. Elected representatives are usually eager to make a good showing for their constituents. If this letter is sent out at the beginning of the program, you should have replies by the end of the experiment period pointing out what efforts are being made currently at both local and national levels to combat pollution and to preserve our natural environment.

BACTERIAL POLLUTION OF WATER #1400 Student Instructions

Introduction

The materials in this kit will allow you to test water samples for the presence of bacterial pollutants. The procedures you will be following are those followed by state and local health authorities. Follow the safety procedures as stated by your teacher.

The method of testing for bacterial pollutants is fairly simple. It is difficult to count single bacteria, so one simply prepares a "medium" which bacteria like to feed on, and then allows them to multiply. Each individual bacteria will form a colony of clones through repeated splitting (fission) that are large enough to see and count. This special medium is usually a jelly called agar, to which special nutrients have been added. Liquid agar is poured into a petri dish, and then allowed to harden. Some of your water sample (which may contain bacteria) is then spread on the surface of the agar. Any bacteria in the water begin to feed on the medium and multiply. After a couple of days of feeding and multiplying colonies of bacteria have grown from each original single bacterium in the water sample. The colonies resemble small dots, and if they keep growing, they will eventually cover the surface of the agar with a variety of mold-like growths. If one uses a standard amount of water (the usual amount is 1/10 of a milliliter) and waits a few days, one can then count the number of bacteria which were originally in the water sample. By multiplying this amount by 1000, health officials can determine how many bacteria would be present in a standard sample of 100 milliliters.

Using the materials in this kit, you will be able to collect a water sample and test it in two ways. One kind of agar is food for most kinds of bacteria, while the other is a special diet for the type of bacteria called E. coli. These bacteria are found in the lower digestive tract and in all human and animal waste. When present in the lower intestine, these bacteria are completely harmless, and are even helpful in the digestion process. If they get into the stomach through the mouth, though, they can cause diarrhea and other sicknesses. If any coliform (E. coli) bacteria is present it is a sure sign that the water has been polluted with waste materials.

It is important to remember that there are bacteria all around us. They are in the air, on your skin, and on this piece of paper. When testing for the presence of bacteria, then, it is very important not to let any bacteria get into the agar except from the **material or** water

you are testing. The teacher has prepared ten special agar plates which are split down the middle. One half (the clear side) will grow most kinds of bacteria, and the other side will grow the metallic-green coliform bacteria. Your teacher has been very careful when pouring the plates, to prevent airborne bacteria from contaminating the agar.

Collecting Samples

When you collect your water sample you will use a special, sterilized test tube. You should not unwrap the test tube until you are ready to dip it into the water. As soon as you have filled the test tube, put the cap on immediately. Do not touch the inside of the cap. Only in this way can you be sure that any bacteria which you locate came from the water, and not from the air or your hands. Wash your hands as soon as possible after collecting the sample.

Preparing Sample

After you have returned to the classroom with your sample, soak the plastic pipets in alcohol. This is to make certain that any bacteria you grow comes from your sample, and not from the dropper itself. When your teacher gives you your agar dish, handle it carefully. First wash your hands carefully and thoroughly. Then, using distilled water, wash the alcohol out of the dropper. Uncap your sample tube and fill the dropper about half full with the sample water. Now, carefully take the tape off the agar dish. Lift the cover and drop two drops of water on each side, on the surface of the agar medium. Close the cover. Now discard the rest of the water in the dropper. Lift the cover off the dish again, and using the tip of the dropper, smear the water around on the surface of the agar so as much of it as possible is covered with a film of water. Try not to pierce the agar surface. Do this quickly, and re-seal the dish with the tape. Your dish is now ready to grow the bacteria.

Examining Results

At the end of a few days, you may find bacterial colonies on both sides of the agar dish. Since one side will grow almost all bacteria, it will probably have more spots on it. E. coli bacteria, which may grow on the other side of the dish, have a distinct metallic green color. Only spots of this color should be counted on the colored side of the dish. It is possible, and even likely, that you will grow no E. coli colonies. This means that the water is not polluted by sewage. You can use a small magnifying glass to help you count the colonies, but do not take the cover off of the dish. If by chance you are growing a disease-causing bacteria. you might catch it if you open up the petri dish. Therefore. the dish should remain tightly sealed at all times.

When you have counted the bacterial colonies on the E. coli side, multiply the number of colonies by 1000, and you will then have the number of bacteria in 100 milliliters of water. Do the same for the general bacteria side.

In the case of general bacteria you should expect between one and a hundred little colonies; this is normal. If you have any more than that, the sample is probably polluted. Even if the bacteria you have grown are not harmful, a large number of them may mean that there is some other kind of pollutant in the water which is providing more bacteria food than normal (such as the phosphates in detergents). However, counts of 1000 to 1,000,000 bacteria per 100 ml are considered within the normal range. In the case of E. coli bacteria, water with a population of 2000 per 100 ml (2 colonies) is polluted. If you grow even one colony, your sample is dangerous for swimming and very dangerous for drinking.

BACTERIAL POLLUTION OF WATER #1400 DATA SHEET

NAME: _____

CLASS: _____

Date of sample collection: _____

Besides myself, I collected this sample with:

The sample was collected from:

Date the culture was prepared: _____

After culturing this sample, we counted _____ colonies of general bacteria and _____ colonies of coliform bacteria.

The total (general) bacteria count was therefore _____ per 100 ml.

The coliform count was _____ per 100 ml.

Additional remarks:

SAFETY INSTRUCTIONS: IMPORTANT
NOTICE TO TEACHERS REGARDING LABORATORY REAGENTS

Perhaps the best general rule regarding the safe handling of laboratory chemicals is to treat all of them as being potentially dangerous. This means that none of them should be taken internally, and that any external contact should be washed thoroughly. In fact, most of the chemicals provided in The Science Source kits are diluted enough that they are not hazardous. The following lists indicate appropriate antidotes for the hazardous chemicals. Check this list for the chemicals provided in the kit:

I. Concentrated Acids & Bases - Do not induce vomiting, dilute with water, then milk or egg white, call a physician immediately.

1. **25 % Acetic Acid**
2. **3M Hydrochloric Acid**
3. **Concentrated Sulfuric Acid**

II Dilute Acids & Bases - Dilute with water, then milk.

1. **1 M, 0.5M, 0.1 M Hydrochloric Acid**
2. **Oxalic Acid**
3. **Sodium Hydroxide**
4. **Ammonium Hydroxide**

III Miscellaneous Chemicals - Dilute immediately with water. Induce vomiting with warm salt water, or warm baking soda solution.

1. **Ammonium Chloride**
2. **Ammonium Oxalate**
3. **Barium Chloride**
4. **Biuret Reagent**
5. **Bromthymol Blue**
6. **Calcium Chloride**
7. **Ethanol (Denatured Alcohol)**
8. **Ferric Ammonium Sulfate**
9. **Hydrogen Peroxide**
10. **Janus Green B**
11. **Lead Nitrate**
12. **Lugol's Solution**
13. **Magnesium Reagent (Titan Yellow, Alcohol)**
14. **Mercuric Nitrate**
15. **Methylene Blue**
16. **Ninhydrin**
17. **Phosphorus Reagent (Ammonium Molybdate, Nitric Acid)**
18. **Potassium Ferricyanide**
19. **Potassium Permanganate**
20. **Silver Nitrate**
21. **Sodium Carbonate**
22. **Sodium Thiosulfate**
23. **Sudan IV**

IV. Organic Solvents - Do not induce vomiting. Dilute with water and milk. Call a physician immediately.

Isopropyl Alcohol