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Microbiological Skills
for Water and Wastewater Analysis

By Douglas W. Clark

Illustrated by Jan Carley

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The work upon which this publication is based was supported by funds provided through the New Mexico Water Resources Research Institute as a result of appropriations by the state of New Mexico.

NEW MEXICO WATER RESOURCES RESEARCH INSTITUTE

Report No. M16

CONTENTS

CAST OF CHARACTERS

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THE WIZARD OF BODS
"The Wizard of BODs" is a joint creation of Clark and Carley and appears in a regular cartoon strip in "The Bench Sheet," a newsletter for water and wastewater analysts published by Environmental Training Consultants, Inc., Corvallis, OR. "The Wizard of BODs" is a trademark of Laboratory Consultants; New Mexico Water Resources Research Institute authorized user.

ACKNOWLEDGEMENTS

Many individuals contributed to making this handbook possible. Among those whose efforts should be acknowledged are: Tom Bahr, Peter Herman, Darlene Reeves, Linda Harris and the rest of the staff of the New Mexico Water Resources Research Institute; Nanette Londeree of Miles Cutter Laboratories, Berkeley, CA; Cynthia Heirs-Robinson of the New Mexico State University Water Utilities Technology Program, Las Cruces, NM; April Owens of the April Owens Company, Rio Rancho, NM; and Bob Julyan and Neal Singer of the Adobe Writers Group, Albuquerque, NM.

Appreciation also goes to Sharon Clark for her willingness to endure yet another project in the hope that the author really had finally learned how to manage his time and energy.

This book is dedicated to Phil Weldon and James Talley, without whose support and encouragement during a critical time this and many other achievements would never have come about.

PREFACE

This handbook is concerned with the proper care and feeding of microorganisms recovered from water and wastewater samples. The microorganisms must be cultured and identified to determine not only what kinds of cells were present in the original sample, but also what concentrations they appeared in.

The skills required to do this are exacting. Like all living things, microorganisms can be damaged or killed by improper handling. In most tests, only certain microorganisms are to be recovered and grown, but each of the microorganisms selected for by the test must result in a colony if the results are to be accurate. Moreover, as they grow, the microorganisms must be confined to the culture vessel since they may be pathogenic or may contaminate other cultures.

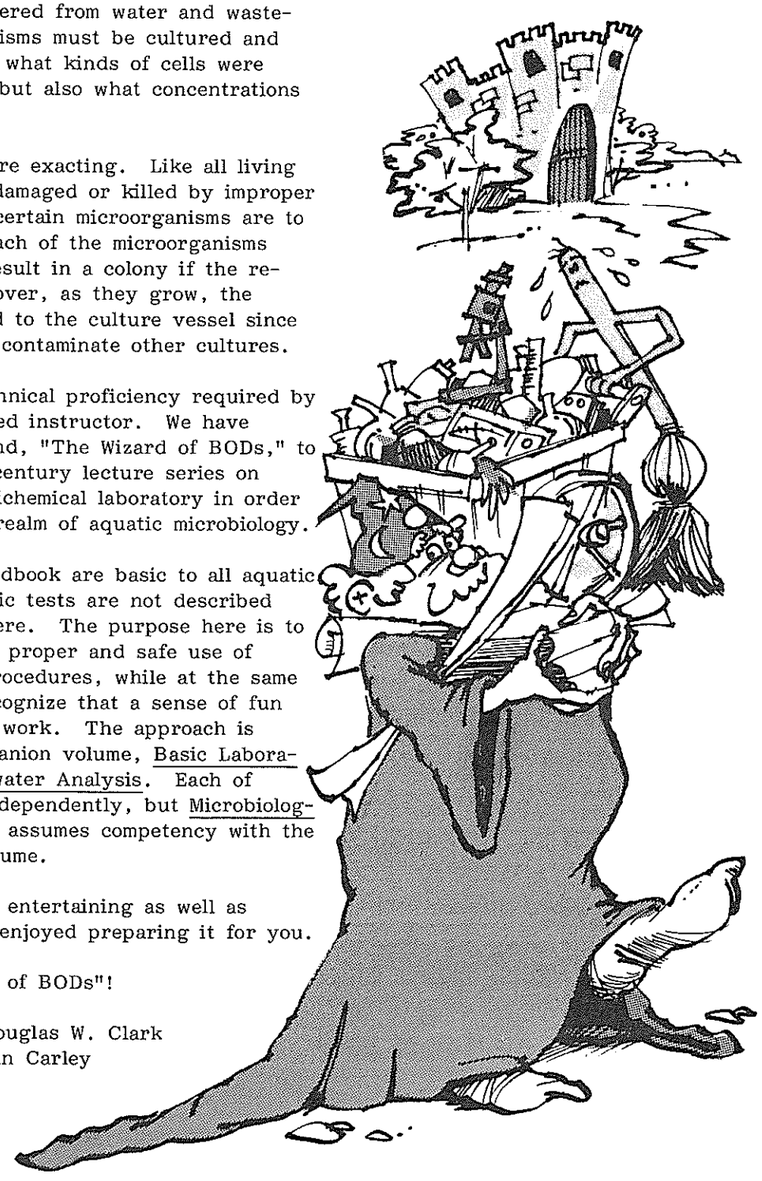
Conveying the high level of technical proficiency required by all this calls for an equally gifted instructor. We have therefore persuaded an old friend, "The Wizard of BODs," to postpone a scheduled fifteenth century lecture series on spontaneous generation in the alchemical laboratory in order to guide this tour through the realm of aquatic microbiology.

The skills he covers in this handbook are basic to all aquatic microbiological analyses. Specific tests are not described since these are available elsewhere. The purpose here is to provide a firm grounding in the proper and safe use of microbiological equipment and procedures, while at the same time encouraging analysts to recognize that a sense of fun can be an inherent part of this work. The approach is similar to that used in the companion volume, Basic Laboratory Skills for Water and Wastewater Analysis. Each of these handbooks can be used independently, but Microbiological Skills is more advanced and assumes competency with the topics covered in the earlier volume.

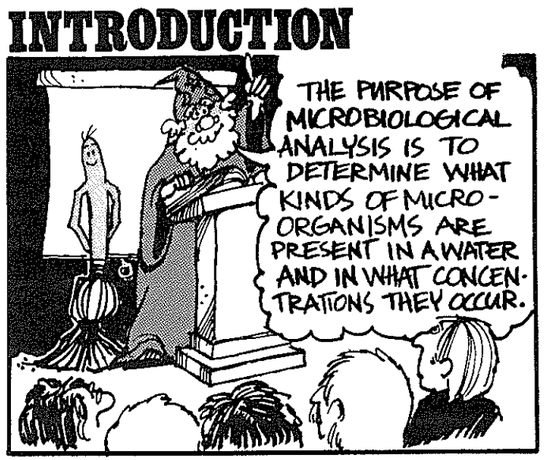
We hope you find this handbook entertaining as well as informative. We have certainly enjoyed preparing it for you.

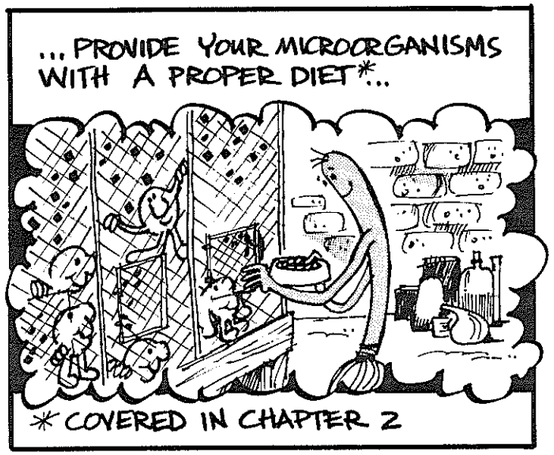
And now, bring on "The Wizard of BODs"!

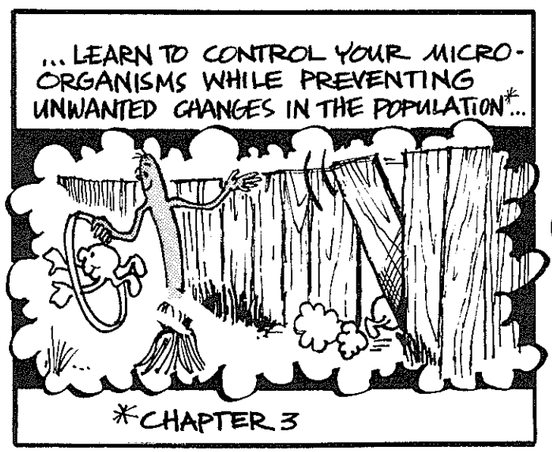
Douglas W. Clark
Jan Carley

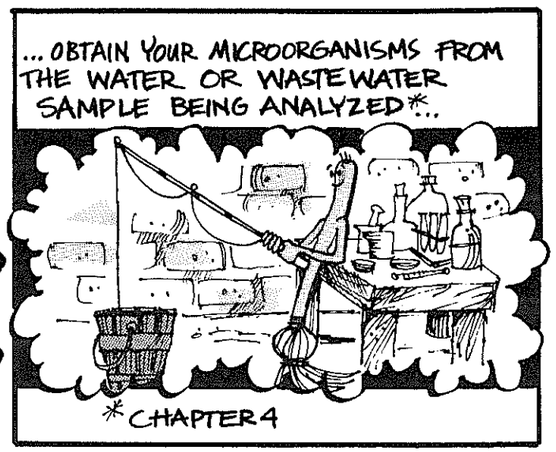


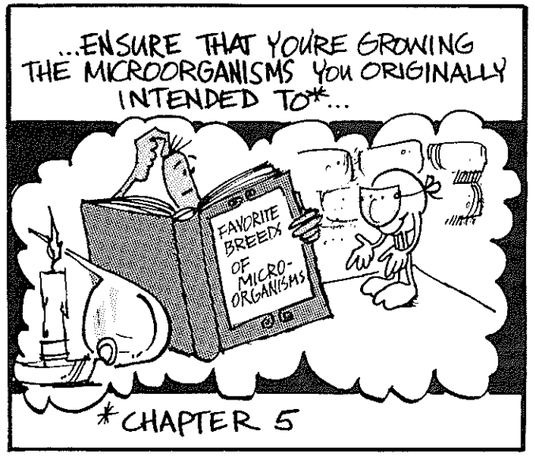
INTRODUCTION

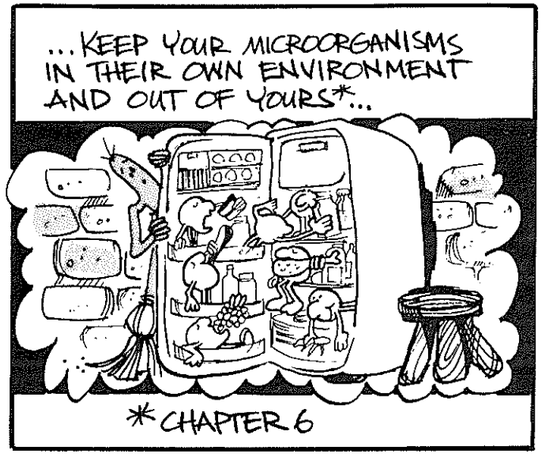


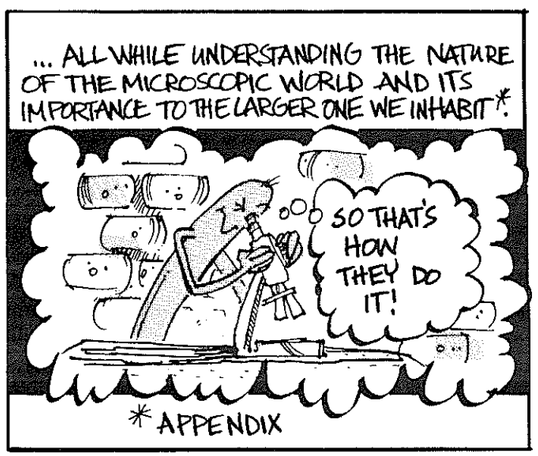


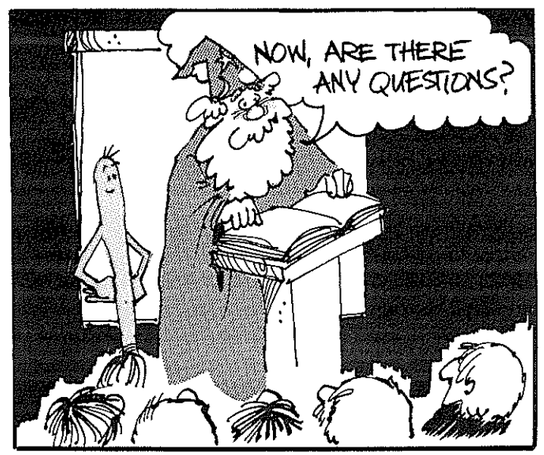












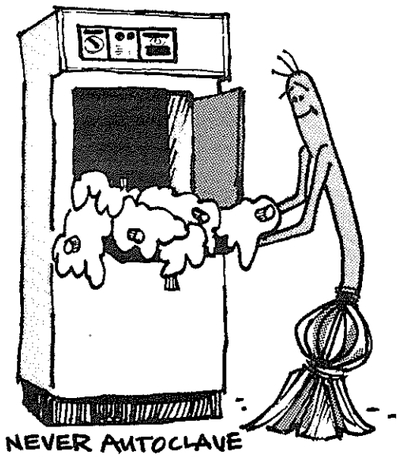
Equipment & Supplies

When you scoop up a water sample for microbiological analysis, you trap in the sample vessel a diverse and dynamic community of microorganisms. Unless stopped, the relationships between life forms in the bottle will change constantly. Some organisms will achieve dominance over other groups, only to be replaced in turn by still other varieties. At any given time, one kind of organism may be dying off while another multiplies prolifically.

Yet it is the analyst's job to determine what relationships existed among the organisms in the original water source. Therefore, from the time the sample is taken until the analysis is complete, the analyst must strive to keep the organisms in check, to restrain further growth (except when required by the analytical procedure, in which case the growth is usually selective) while at the same time preventing organisms from dying. And during all of the analytical procedures, the analyst must be sure not to introduce foreign microorganisms into the sample or release organisms from the sample into the laboratory environment.

Because these requirements are so demanding, great care must be taken during all steps in microbiological analysis, including the selection and handling of basic equipment and supplies. This chapter examines some of the criteria such equipment must meet to be suitable for microbiological analysis.

SAMPLE CONTAINERS



Sample bottles should be wide mouth, at least 125 mL in volume and must be resistant to both sterilization and the solvent action of water. Borosilicate glass bottles with screw-cap or ground-glass stoppers are recommended. Heat-resistant plastic bottles are also acceptable provided they can be sterilized without producing toxic materials. Screw-caps must have leakproof liners and must not produce bacteriostatic or nutritive compounds upon sterilization. Regardless of type, all caps and stoppers must be water-tight. Discard any bottles having chips, cracks or etched surfaces.

Thoroughly wash all bottles and caps or stoppers prior to use with a suitable detergent and hot water, followed by a hot water rinse to remove all traces of detergent. Rinse three times with laboratory-pure water. If necessary, test for bacteriostatic or inhibitory residues left by the detergent. Prior to sterilization, protect the tops and necks of glass-stoppered bottles from contamination by covering them with aluminum foil or heavy kraft paper.

Sterilize glass or heat-resistant sample bottles by autoclaving them at 121° C for 15 minutes. Dry glassware may also be sterilized in a hot air oven at 170° C for not less than two hours. For plastic bottles susceptible to heat distortion from autoclaving, sterilize with ethylene oxide gas (see Chapter 3). Store sample bottles sterilized by gas overnight before using to enable the last traces of gas to dissipate.

When the water or wastewater to be sampled is expected to contain residual chlorine, add sodium thiosulfate to the bottle prior to sterilization at a concentration of 0.1 mL of a 10 percent solution for each 125 mL sample volume. This is sufficient to neutralize approximately 15 mg/L of residual chlorine.

A chelating agent should be added to the sample bottles prior to sterilization when the water or wastewater to be sampled is expected to contain heavy metals such as copper, nickel or zinc in concentrations greater than 0.01 mg/L. Use 0.3 mL of a 15 percent solution of ethylenediaminetetra-acetic acid (EDTA) tetrasodium salt for each 125 mL of sample.

Commercially available sterile plastic bags (such as Whirl-Pak) may be used in place of plastic or glass sample bottles where no chlorine residual is anticipated and no chelating agent must be added.

PIPETS

Pipets are of two general categories: transfer (also called volumetric) and measuring. Transfer pipets have an enlarged bulb at the center and are calibrated to deliver a single fixed volume. The opening at the tip of the pipet is sized to provide optimum outflow rates when dispensing the contents. Transfer pipets are used for precise work and their tolerance limits are specified by federal standards.

Measuring pipets, also known as serological or Mohr pipets, are narrow straight tubes graduated to deliver variable amounts of liquids. They are not as accurate as transfer pipets and should not be used for precise work.

Pipets used for microbiological work must deliver the required volumes quickly and accurately within a 2.5 percent tolerance. Graduation markings should be distinct, tips unbroken and the sides free of frosting or etching. Bacteriological transfer pipets or pipets conforming to the APHA standards given in the latest edition of Standard Methods for the Examination of Dairy Products may be used.

When using a transfer pipet for precise measurements, first rinse the pipet with the solution to be measured and discard the rinsings. For very precise work, repeat this procedure at least twice. Then, whether using a transfer or a measuring pipet, fill to above the calibration mark, hold the pipet nearly vertical and gradually drain the excess solution until the meniscus is exactly on the mark, keeping the tip in contact with the wall of a dry beaker to remove any drops of solution which would otherwise adhere to the tip. The solution is then ready to be dispensed. Dispensing should also be done with the pipet in a nearly vertical position and with the tip in contact with the wall of the receiving container. The liquid should be allowed to flow out freely and the pipet should be drained for five seconds or more (up to 20 seconds for precise work) after the liquid is out. For pipets calibrated to deliver (marked TD), do not blow out or shake the pipet to remove the remaining drop inside the tip. The presence of this drop is accounted for in the calibration of the pipet. "To deliver" pipets are the only kind which should be used in microbiological analysis. "To contain" or "blow-out" pipets should not be used in microbiological work because of the danger of creating an aerosol containing pathogenic organisms.

All pipeting, especially in microbiological analysis, should be done with the aid of a pipeting bulb or similar device. This eliminates contact between the analyst's mouth and the pipet, thereby preventing accidental swallowing of dangerous reagents or contaminated samples.

Pipet containers, which maintain pipet sterility until use, should be of aluminum, stainless steel, pyrex glass or other noncorrosive heat-resistant material. Pipets may also be wrapped in kraft paper. Copper or copper-alloy containers must not be used.

GRADUATED CYLINDERS

Graduated cylinders may be used in the microbiological laboratory for measuring volumes greater than 10 mL. In general, however, they are only calibrated to a tolerance of one percent. They are not as accurate as most analytical measurements require and therefore should not be used for measuring critical volumes. When greater accuracy is required, use volumetric pipets or volumetric flasks. A graduated cylinder should be of such a size that the liquid being measured nearly fills it up.

DILUTION BOTTLES & TUBES

Dilution water blanks are prepared for the multiple tube test, membrane filtration and pour plate technique in either culture tubes containing 9 mL of diluent (for 1:10 dilutions) or in dilution bottles containing 99 mL of diluent (suitable for both 1:10 and 1:100 dilutions).

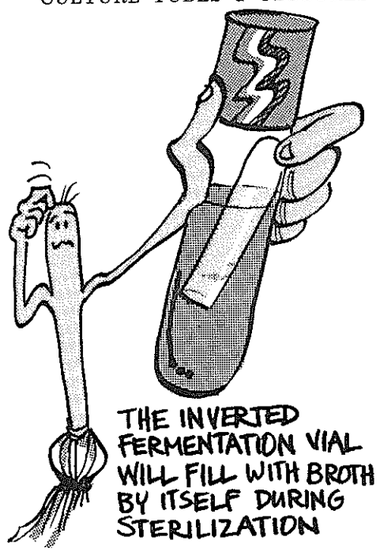
Bottles or tubes should be of borosilicate glass with glass stoppers or nontoxic screw caps having inert liners. Cotton plugs should not be used. Dilution bottles with ground glass stoppers must be covered with metal foil or kraft paper during sterilization, storage and use to prevent contamination around the bottle lip. Screw-cap closures eliminate the need for this extra protection. Indelibly mark 9 mL or 99 mL graduation levels on the sides of the dilution bottles or tubes with a diamond marking pencil or other glass marking tool. Plastic bottles may be used in place of glass provided they can be properly sterilized and do not contain toxic materials.

CULTURE TUBES & CLOSURES

Culture tubes are used for such purposes as multiple tube fermentation tests, biochemical tests for bacterial identification and preparation of stock cultures. The tubes must be large enough to contain both the culture medium and the sample portion without filling the tube more than about half way. Tubes should be of borosilicate or other corrosion resistant glass. Disposable tubes made of soda-lime glass (soft glass) are not recommended for bacteriological work because of interaction between glass and media during storage.

When gas production is to be observed, there should be sufficient media in the outer fermentation tube to fill the enclosed inverted vial after sterilization and partially submerge it. Culture tubes used for multiple tube fermentation tests should be closed with snug-fitting stainless steel or plastic caps or loose fitting aluminum caps.

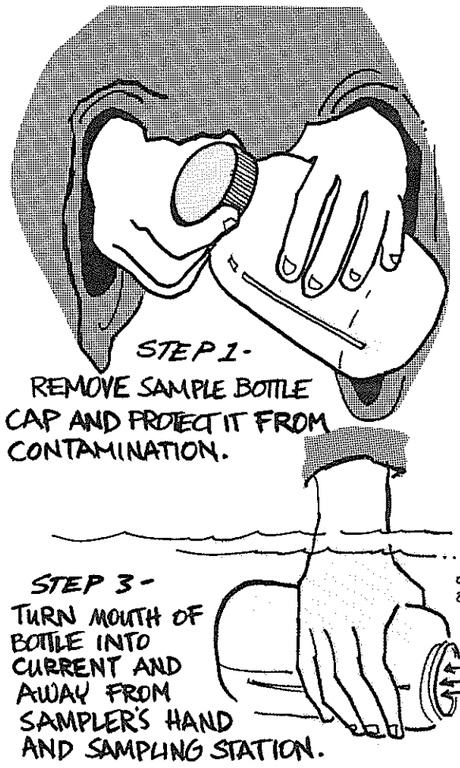
Tubes used in preparing media for biochemical tests and agar slants for stock culture collections should be closed with screw-caps to reduce excessive media loss through evaporation. Screw-cap tubes 16 x 150 mm are commonly used for biochemical tests and 16 x 125 mm for agar slants.

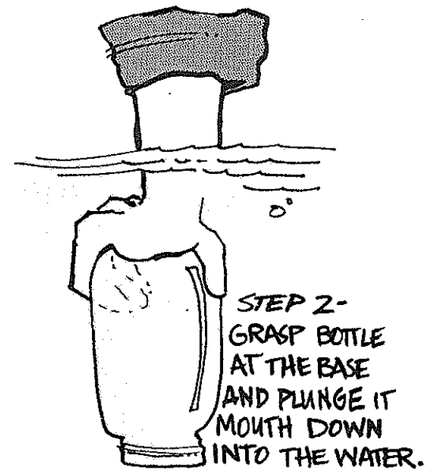


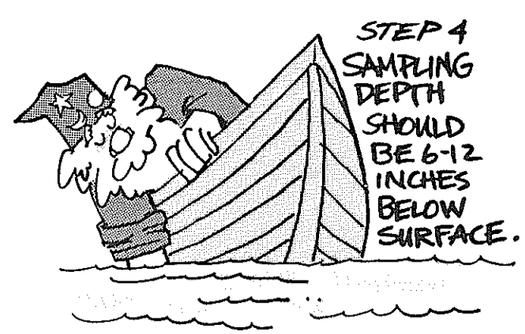
PETRI DISHES

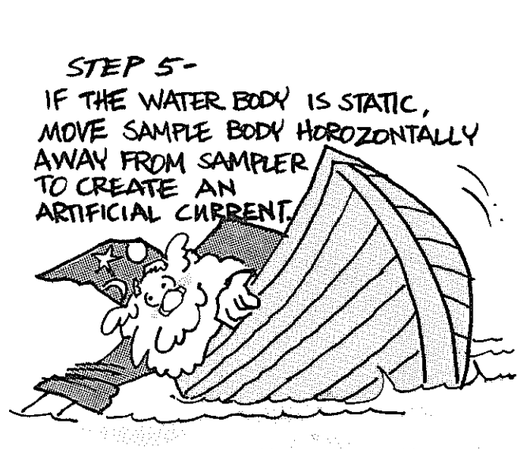
Petri dishes are used for membrane filtration cultures, standard plate count determinations and streak plate isolation of bacterial cultures. For membrane filtration (MF) work, use either tight-lid plastic dishes (50 x 12 mm) or loose-lid plastic or glass dishes (60 x 15 mm). The tight-lid dishes are preferred for membrane filter use since they retard evaporation loss from broth or agar media and help maintain a humid atmosphere in the culture dish. Loose-lid dishes should be used in conjunction with controlled humidity incubators or in tightly closed plastic boxes containing moist towels. For pour plates and pure culture isolations, dish size is usually 100 x 15 mm.

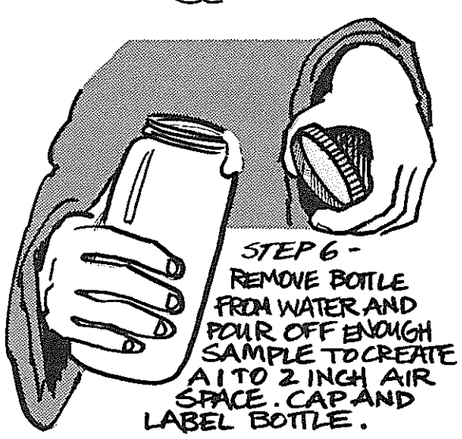
SAMPLING FROM SURFACE WATERS



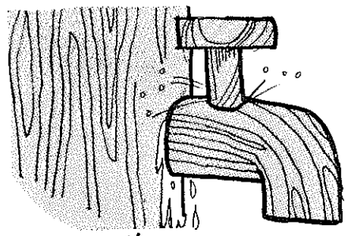




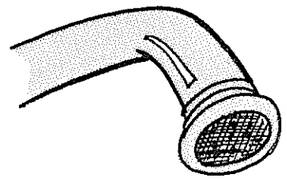




SAMPLING FROM WATER TAPS



STEP #1 -
DO NOT SAMPLE FROM SPIGOTS WITH LEAKING STEMS.



STEP #2 -
DO NOT SAMPLE FROM SPIGOTS CONTAINING AREATION DEVICES OR SCREENS.

STEP #3 -
FLUSH SPIGOT FOR 2 TO 3 MINUTES TO CLEAR SERVICE LINE.

STEP #4 -
ASEPTICALLY REMOVE CAP FROM BOTTLE.

STEP #5 -
HOLD SAMPLE BOTTLE UPRIGHT BY THE BASE WHILE IT FILLS TO WITHIN 1 INCH OF TOP. CAP AND LABEL BOTTLE.

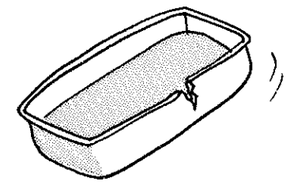
NOTE: IT IS NO LONGER CONSIDERED NECESSARY TO FLAME THE SPIGOT PRIOR TO TAKING A MICROBIOLOGICAL SAMPLE.

Regardless of whether the dishes are plastic or glass, they must be completely transparent for maximum visibility of colonies, have flat bottoms to ensure uniform medium thickness throughout, and be free from bubbles and scratches that might impair observation.

Glass petri dishes with loose tops must be enclosed in metal containers, aluminum foil or kraft paper during sterilization and storage. Copper and copper-alloy containers must not be used because of the possibility of introducing toxic contaminants from the container into the dishes. Presterilized plastic disposable petri dishes should be stored in the original protective packaging to ensure sterility is maintained.

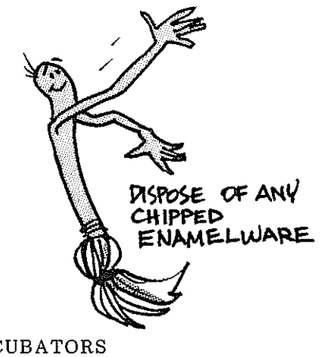
INOCULATING NEEDLES & LOOPS

Needles and loops are used to transfer microbes aseptically from one growth medium to another. Loops should be made of 22- or 24-gauge nickel alloy (chromel, nichrome or equivalent) or platinum or platinum-iridium wire and should be at least 3 mm in diameter. Needles and loops are usually sterilized by heating in a gas flame or electric incinerator until the wire glows red. Alternatively, they may be sterilized by dry heat or steam in the same manner as other equipment and supplies. Replicate transfers of the same bacteria or bacteria-containing materials to a sterile medium do not require resterilization of the needle or loop after the initial transfer. Sterile, disposable hardwood applicator sticks can be used to inoculate fermentation tubes. These should be 0.2 to 0.3 cm in diameter and at least 2.5 cm longer than the fermentation tube. Sterilize hardwood applicators by dry heat and store in a glass or other nontoxic container.



MEDIA PREPARATION UTENSILS

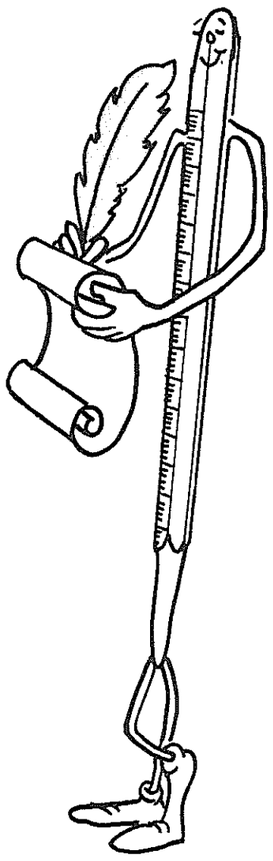
Media preparation utensils should be made of borosilicate glass or other suitable noncorrosive equipment such as stainless steel, plastic or non-chipped enamel. Chips in the porcelain glaze of enamelware expose the base metal to corrosion and interaction with media preparations. Aluminum, copper or zinc alloy utensils are not suitable for microbiological use because of possible reactions between the metals and media solutions and because toxic metal ions may be introduced into the media. Glassware should be clean and free of residues, dried agar or other foreign materials that could contaminate media. Utensils must be chemically clean before use to prevent contamination of media with chemicals such as chlorine, zinc, chromium and detergents. Any magnetic stirrers used for media solutions must also be thoroughly cleaned.



DISPOSE OF ANY CHIPPED ENAMELWARE

INCUBATORS

Constant, known temperatures are critical for bacteriological work. Microorganisms can be extremely sensitive to temperature and will not grow properly unless exacting conditions are met. If the temperature varies from the intended



A RECORDING THERMOMETER IS RECOMMENDED

setting, or if the wrong temperature is selected, the results of a test may appear normal while in fact being completely invalid. Incubators therefore perform a crucial role in water quality microbiological analysis and careful attention must be paid to their selection and operation.

Most incubators perform similarly to ovens, except that the temperature control is usually more precise. Water-jacketed or anhydric-type incubators are preferred over those with other types of insulation. Incubators should be equipped with low-temperature heating elements, rather than high-temperature ones, to prevent the formation of local hot spots during operation. Temperature control in incubators with mechanical air circulation is superior to that in incubators using only convection. If ordinary room temperatures in the laboratory vary widely, keep incubators in a special room maintained at a few degrees below incubator temperature. Avoid overcrowding incubators as this will interfere with the constancy of the desired temperature.

Incubators equipped with recording thermometers are recommended. Where these are not available, temperature readings inside the incubator should be taken at least once (and preferably twice) daily during use. Thermometer bulbs should be immersed in a liquid (glycerine, water or mineral oil) to minimize the effects of sudden temperature changes when the incubator door is opened. Place thermometers on each shelf used in an incubator. In addition, it is also recommended that a maximum and minimum registering thermometer be placed on the middle shelf of each incubator to record the gross temperature range over a 24-hour period. Avoid placing incubators where direct sunlight, drafts and other disturbances might affect temperature maintenance.

Humidity in air incubators should be kept high to prevent culture media from drying. This can be achieved by partially submerging a towel in a beaker of water and placing them both in the incubator. Some incubators have built-in water reservoirs which can be used instead.

Incubators for total coliform analysis must be capable of maintaining a temperature of 35 ± 0.5° C, which can be achieved with an air incubator. Fecal coliform analysis, however, requires a temperature of 44 ± 0.2° C, which necessitates using either a water bath or a heat sink incubator (for example, one using an aluminum block). Although mechanical circulation of the water in a water bath incubator may not be essential to maintain the temperature within the required range, it does greatly improve temperature control. Water baths must be provided with gabled covers to reduce water and heat losses. Maintain a water depth sufficient to immerse tubes to the upper level of the media.

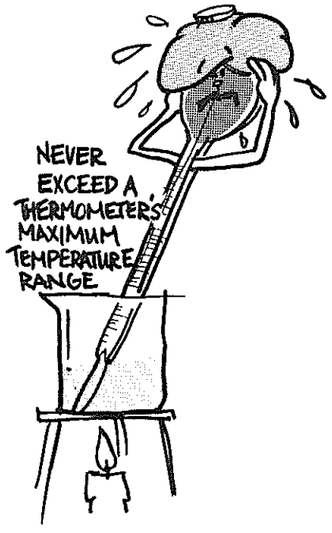
THERMOMETERS

With a few exceptions, temperature is measured in water quality microbiological laboratories by means of mercury-filled glass thermometers. These thermometers should be graduated in intervals at least as small as 0.1° C. Accuracy should be checked against a thermometer certified to meet

the requirements of the National Bureau of Standards (NBS), and readings taken from general laboratory thermometers should be corrected accordingly. A certified thermometer is provided with a plot verifying its accuracy; this plot is then used when establishing precise temperature measurements or when calibrating other laboratory thermometers. General thermometers should be checked for accuracy over the entire range of intended use because accuracy is not uniform over the full scale of a thermometer. Recheck thermometer calibrations periodically to detect inaccuracies caused by hairline breaks in the mercury columns. Certified thermometers are available either individually or in sets covering wide temperature ranges. Every laboratory should have at least one certified thermometer, and a full set is recommended.

Care should be taken to prevent thermometer breakage or separation of the mercury column. When long-stemmed thermometers are used in ovens and incubators so temperatures inside the instrument can be read without opening the door, the shelf around the thermometer bulb should be left vacant to reduce the chance of breakage. Placing the thermometer bulb in a beaker of fine sand in an oven or in a beaker of liquid in an incubator not only helps provide a more accurate indication of temperature readings inside thick-walled glass containers in the units, but also helps keep the mercury column from separating by buffering extreme temperature changes experienced when the door of the oven or incubator is opened and closed. Avoid placing a thermometer into solutions or environments with temperatures radically different from the initial temperature of the thermometer. The thermal shock may fracture the thermometer or cause the column to separate. Instead, allow the thermometer to gradually come within the temperature range to be measured prior to use. Never exceed the maximum temperature range of a thermometer. Thermometers which are to be used in the field should be protected by placing them in specially designed metal cases to reduce breakage. Discard thermometers if the graduations become illegible.

Recording thermometers may be used for processes which are highly temperature dependent, as in hot-air sterilizers, autoclaves and particularly incubators. Where recording thermometers are not available for incubators, a maximum and minimum registering thermometer should be used (in addition to a regular thermometer) so gross temperature variations over a 24-hour period can be determined. This additional thermometer should be placed on the middle shelf of the incubator.



pH METERS

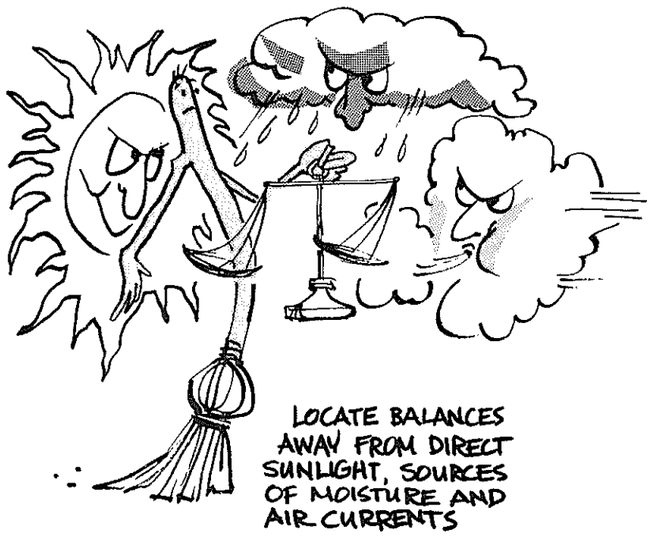
For determining pH values of media, use electrometric pH meters accurate to at least 0.1 pH unit. A sturdy, full-range combination electrode with either a plastic or glass body should be used. An electrode which contains a solid gel-type filling material has the added convenience of not requiring the normal maintenance which a liquid-filled electrode does.

Glass electrodes respond very rapidly in highly buffered solutions, but respond slowly in poorly buffered ones. This effect is compounded when changing the electrodes from buffered solutions to relatively unbuffered solutions. Therefore, thoroughly rinse electrodes with distilled water after each reading, then rinse with or dip the electrodes several times into the next solution to be measured before taking another reading. Stir weakly buffered solutions during a measurement. Keep the electrodes immersed in water between readings to prevent them from drying out.

BALANCES

For routine weighing of media and reagents, a single pan top loader balance having a sensitivity of 0.1 g at a load of 150 g should be used. For weighings of less than 2 g, use a four place analytical balance having a sensitivity of 1 mg at a load of 10 g.

Balances should be located away from direct sunlight, unusual heat, sources of moisture, air currents and heavy laboratory traffic. Analytical balances should be placed on sturdy tables, preferably ones designed specifically for this purpose. Before use, balances should be leveled and zeroed. Beam and pan arrests should be in the locked position when the balance is not being used or when changing weights on the balance pan. Calibrated reference weights should be manipulated only with forceps or a similar device, never with fingers. Moisture and oils from the skin can change the value of the weights. Objects to be weighed should also be handled with forceps or tongs for precise weighings. Never overload a balance; check the maximum rated capacity prior to use. Place heavy weights and objects in the center of the balance pans to keep the pans from swinging. Chemicals and corrosive materials should



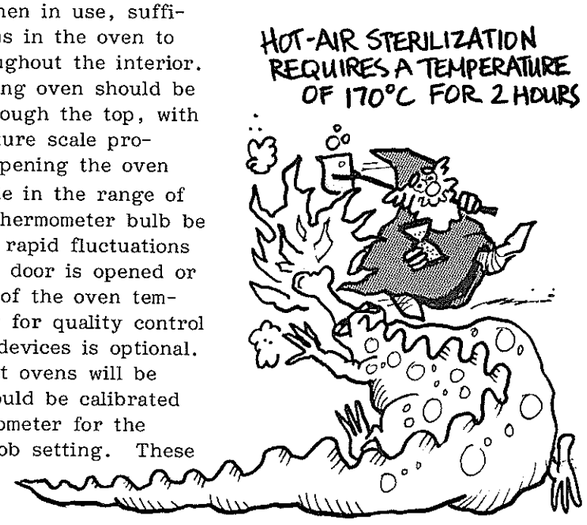
always be weighed in appropriate containers, never directly on the balance pans. Spilled chemicals should be removed and the balance cleaned immediately. Many dehydrated bacteriological media are very hygroscopic (water absorbing) and can cause erratic dampening of the balance point during zero balance and weighing operations. Balance pans should also be cleaned daily with a camel's hair brush to remove dirt and dust. Balances, particularly analytical ones, should be calibrated and serviced annually. Calibrate analytical balances with National Bureau of Standards (NBS) Class S-1 weights.

HOT-AIR STERILIZING OVENS

Before using an oven for hot-air sterilization, it should be checked to ensure that it will hold the required temperature of 170 ± 10° C for a two hour period. When in use, sufficient space should be left around the items in the oven to permit heat to be distributed evenly throughout the interior.

The temperature in a hot-air sterilizing oven should be monitored by a thermometer extending through the top, with the bulb inside the oven and the temperature scale protruding above so it can be read without opening the oven door. The thermometer should be accurate in the range of 160-180° C. It is recommended that the thermometer bulb be placed in a beaker of fine sand to reduce rapid fluctuations in the temperature reading when the oven door is opened or closed. A routine record should be kept of the oven temperature prior to its first use for the day for quality control purposes. Use of temperature-recording devices is optional.

The temperature control knob on most ovens will be graduated with arbitrary numbers and should be calibrated against the readings of an accurate thermometer for the actual temperatures obtained with each knob setting. These calibration values should be recorded.



AUTOCLAVES

Autoclaves sterilize equipment and culture media by exposing them to steam pressurized to 15 psi and having a temperature of 121° C for 15 minutes. Autoclaves should be large enough to allow plenty of room for the steam to circulate when the autoclave is loaded and should be provided with an accurate thermometer with its bulb located in the exhaust line to measure the minimum temperature in the sterilizing chamber. A recording thermometer is a desirable option. A pressure gauge and properly adjusted safety valve are required. Autoclaves should be capable of reaching sterilization temperature within 30 minutes. They should be cleaned and inspected regularly to prevent damage or deterioration. Pressure cookers and vertical autoclaves are not recommended.

Labeling tapes having heat-sensitive inks which undergo color changes at sterilization temperature, or similar temperature indicators, should be used during sterilization to monitor heat penetration throughout the contents of the autoclave.

COLONY COUNTERS

Colonies should be counted on a standard device equivalent to the Quebec colony counter (particularly the dark-field model). The device should provide good visibility, uniform intensity back lighting and magnification of at least 1.5 diameters on a non-glare ruled guide plate.

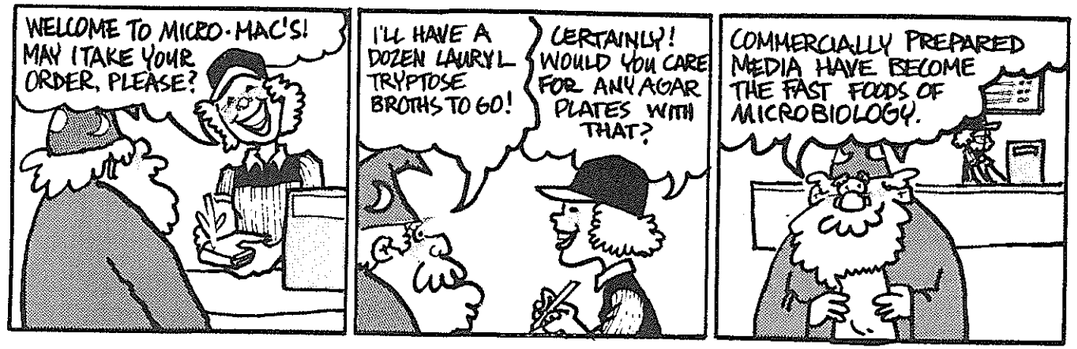
MICROSCOPES

Once the hallmark of the microbiologist, the microscope has now slipped to a position of secondary importance, at least insofar as bacteriological analysis of water is concerned. However, there are times when the use of a proper microscope is still desirable.

A stereoscopic, wide-field dissecting microscope with 10-15x magnification is recommended for counting membrane filtration colonies. A reading lens is generally ineffective for this purpose because of the low magnification such a lens provides and should not be used. Examination of MF total coliform cultures with the unaided eye is also not recommended.

A 4-watt daylight fluorescent tube mounted on a flexible arm is recommended for MF colony illumination. Colors of differentiated colonies should be observed with the lamp adjusted to an angle of 60-80° above the MF culture. Low-angle lighting must be used when detecting undifferentiated, nonpigmented colonies on MF cultures in order to provide adequate shadow contrast between the colonies and the background. The high-intensity incandescent lamps used with oil immersion microscopes or other incandescent light sources are not adequate for detecting colony sheens on Endo-type media.

For specialized bacterial observations, such as identifying cellular structure and detection of specific stains, a compound microscope with a 1000x oil-immersion lens should be used.



Media & Reagents

Since it is impractical to directly observe a water or wastewater sample for bacterial life -- tallying up each and every occurrence of a particular type of organism -- the kinds and numbers of microorganisms present in a sample must be estimated in other ways. This consists primarily of culturing water samples under conditions which will favor the growth of certain organisms over others that may be present. The media and reagents used must therefore meet rigid requirements to ensure that the appropriate cell types are selectively grown and identified while other organisms remain suppressed.

SOURCES OF MEDIA

Many of the standard media used in water quality analysis are now available from commercial outlets in dehydrated form, requiring only weighing and dissolving of a single powder in laboratory pure water. Where such commercially prepared dehydrated media are available, these should be used rather than preparing media from basic ingredients in the laboratory. Smaller laboratories which conduct few microbiological analyses in a given time should even consider going a step further by purchasing already prepared sterile tubes, culture plates, media ampules or preweighed dehydrated media vials. Use of these items, although more expensive on a per test basis, may be justified by the savings in time, labor and facilities, as well as by providing more uniformly high quality media.

All chemicals used in preparing culture media must be ACS (American Chemical Society) or AR grade. Chemical impurities found in commercial and other lower grades of chemicals may be present in sufficient concentrations to restrict bacterial growth. Similarly, all dyes used must be purchased from lots certified for bacteriological use by the Biological Stain Commission because of variations in biological activity of dyes from lot to lot and from manufacturer to manufacturer.

DEHYDRATED MEDIA SUPPLIES

DEHYDRATED MEDIA ARE OFTEN HYGROSCOPIC... DISCARD BOTTLES WHICH SHOW SIGNS OF MOISTURE CONTAMINATION

Commercial dehydrated media will slowly deteriorate when it is stored, producing by-products that may adversely affect the sensitivity and selectivity of the media. Many kinds of dehydrated media are also highly hygroscopic and will absorb moisture from the air if the bottles are improperly sealed. Store bottles of dehydrated media upside down to help seal the bottle screw caps and retard media decomposition. Periodically inspect media stocks and discard media which have been contaminated by moisture. Contaminated media will normally appear caked or viscous. Record in the quality control log the lot numbers of all commercial media used, together with the date of receipt and the date of opening for each bottle. Use older bottles of media first. Order media stocks in quantities which will be used within one year, preferably within six months. If a particular medium is used infrequently or is unusually hygroscopic, purchase it in quarter-pound bottles rather than in 1- or 5-pound sizes to minimize exposure of the media to the atmosphere. When a new lot number of a medium is used, test the contents for its performance characteristics. Store dehydrated media stocks in a cool, dry place away from sunlight.

MEDIA COMPOSITION

Whenever possible, use commercially prepared media instead of preparing media from basic ingredients in the laboratory. The procedures listed in this manual for preparing media normally do not include the basic ingredients required to formulate the media. Instead, catalog numbers from two U.S. manufacturers of microbiological media are given where possible. These numbers identify quarter-pound sizes of the appropriate media. Media from other manufacturers can also be used if they conform to the formulas cited and produce comparable results.

The media concentrations listed for broths yield normal- or single-strength broths. These are adequate where water sample volumes of 1 mL or less are added to 10 mL volumes of broth. However, when water sample volumes of 10 mL must be used, the broth should be double-strength so that the final concentration will be single-strength when diluted by the water sample.

Use pure grain alcohol (ethanol) to prepare such media as M-Endo Broth. The alcohol is essential for colony development with a maximum sheen and less tendency for confluent growth. Ethanol also helps suppress the growth of noncoliform organisms. Commonly available denatured ethanol must not be used since the denaturants used, commonly methanol or propanol, are toxic to coliforms. If state laws or other restrictions make the use of pure ethanol impractical, a stock of ethanol can be technically denatured for use in the MF procedure by adding a few grains of M-Endo powder. This satisfies the legal constraints on keeping pure ethanol in the laboratory yet leaves the ethanol suitable for formulating M-Endo medium.

MAINTAIN A STOCK OF PURE GRAIN ALCOHOL FOR MEDIA SUCH AS M-ENDO BROTH

When preparing media from the procedures shown in this manual, the dry ingredients listed should be added to one liter volumes of laboratory pure water. For liquids such as ethanol, add the liquid to a graduate and then bring the contents up to volume by adding laboratory pure water.

When using dry ingredients, the term "percent solution" is used to mean "grams of solute per 100 mL of solution."

REHYDRATING MEDIA

Reconstitute dehydrated media by slowly adding the appropriate weighed quantities to approximately one-half the total volume of distilled or deionized water required. (From here on, the term "laboratory pure water" will be used exclusively, except for sections dealing specifically with the preparation of distilled and deionized water. However, it should be understood that either distillation or deionization can, if properly used, produce water of suitable quality for media preparation.) The water should be freshly prepared before reconstituting a dehydrated medium, or else boiled prior to use to dispel absorbed gases which may alter the pH of the final medium. Use chemically clean glassware and stainless steel utensils for preparing and dispensing media. Gently agitate the mixture of medium and water either by hand or by magnetic stirrers to ensure rapid dissolution. Dissolution may also be aided by preheating the laboratory pure water to approximately 45 - 50° C. After mixing the solution thoroughly, rotate the container while slowly adding the remaining volume of laboratory pure water to wash any residual powder from the sides of the container.

Water-medium mixtures containing agar, gelatin or cystine should be allowed to soak for about five minutes to ensure a more uniform suspension. After that time, apply heat to complete dissolution and to enable the medium to be dispensed in culture tubes or bottles. Then sterilize. Dissolve gelatin-containing media by heating in a boiling water bath. Always be sure agar and gelatin are completely dissolved before dispensing and sterilizing a medium or the final medium may not be uniform and may even fail to properly solidify.

If heat is needed to prepare media, apply it carefully and for the shortest length of time possible. Direct heat, boiling water bath and flowing steam must be used selectively according to the type and volume of medium being pre-

AVOID EXCESSIVE HEAT WHEN RECONSTITUTING AND STERILIZING MEDIA

FAVORITE MICROBIOGICAL RECIPES

Tryptic Soy Broth (Difco 0370-02)
Trypticase Soy Broth (BBL 11767)
17.0 g Tryptone or Trypticase Peptone
3.0 g Soytone or Phytone Peptone
5.0 g Dextrose
2.5 g Sodium Chloride
2.5 g Dipotassium Phosphate

Add 30 grams of TS broth per liter of laboratory pure water. Warm the broth and mix gently to completely dissolve the medium. Dispense and sterilize for 15 minutes at 121° C (15 psi). Final pH: 7.3 ± .2

Use for general purpose cultivation of fastidious microorganisms.

Tryptic Soy Agar (Difco 036902)
Trypticase Soy Agar (BBL 11042)

15.0 g Tryptone or Trypticase Peptone
5.0 g Soytone or Phytone Peptone
5.0 g Sodium Chloride
15.0 g Agar

Add 40 grams of TS agar per liter of laboratory pure water and mix well. Heat in boiling water bath to completely dissolve agar. Dispense into tubes, bottles or flasks and sterilize for 15 minutes at 121° C (15 psi). Final pH: 7.3 ± .2

TS Agar (cont'd)

A general purpose medium for cultivating fastidious microorganisms. An excellent blood agar can be prepared by adding sheep blood.

For blood agar, cool the sterile, melted agar to 45-46° C and aseptically add 5 mL of sterile defibrinated sheep blood for each 100 mL of agar. Mix flask of agar by swirling and dispense into petri dishes. Blood from other species may be used for particular purposes.

Nutrient Agar (Difco 0001-02; BBL 11471)
5.0 g Peptone
3.0 g Beef Extract
15.0 g Agar

Add 23 grams of nutrient agar per liter of laboratory pure water and mix well. Heat in boiling water bath to dissolve agar completely. Dispense in screw-cap tubes, bottles or flasks and sterilize for 15 minutes at 121° C (15 psi). Remove tubes and slant. Final pH: 6.8 ± .1

Use to cultivate pure culture isolates for subsequent Gram stain and other examinations. For general cultivation of non-fastidious microorganisms.

Nutrient Broth (Difco 0003-02; BBL 11478)

5.0 g Peptone
3.0 g Beef Extract

Add 8 grams of nutrient broth per liter of laboratory pure water and warm to dissolve the medium completely. Dispense in containers and sterilize for 15 minutes at 121° C (15 psi). Final pH: 6.8 ± .1

Use for general cultivation of non-fastidious microorganisms.

Standard Methods Agar (BBL 11637)
Plate Count Agar (Difco 0479-02)
(Also called Tryptone Glucose Yeast Agar)

5.0 g Tryptone or Trypticase Peptone
2.5 g Yeast Extract
1.0 g Dextrose
15.0 g Agar

Add 23.5 grams of tryptone glucose yeast agar per liter of laboratory pure water. Mix well and heat in boiling water bath to completely dissolve agar. Dispense into screw-cap tubes, flasks or bottles and sterilize for 15 minutes at 121° C (15 psi). Final pH: 7.0 ± .2

Standard Methods or Plate Count Agar (cont'd)

Use for Standard Plate Counts with potable water samples and general purpose pour plate procedures.

Phenol Red Broth Base (Difco 0092-02; BBL 11505)

Difco:
1.0 g Beef Extract
10.0 g Proteose Peptone No. 3
5.0 g Sodium Chloride
0.018 g Phenol Red

BBL:
10.0 g Trypticase Peptone
5.0 g Sodium Chloride
0.018 g Phenol Red

Phenol Red Broth Base (card 2)

Add 15-16 grams (depending upon manufacturer) of phenol red broth base per liter of laboratory pure water. Mix well to dissolve. Add 10 grams of test carbohydrate, if heat-stable. Mix to complete solution. Distribute medium in fermentation tubes and sterilize not more than 15 minutes at 118° C (12 psi). Filter-sterilize heat-sensitive carbohydrates and alcohols and add to the sterile medium tubes. Check pH and adjust if necessary with 0.1 N NaOH after addition and solution of the carbohydrate. Final pH: 7.4 ± .2

Phenol Red Broth Base (card 3)

With the addition of carbohydrates, phenol red broth base is used in fermentation studies of microorganisms because its pH range of 6.8-8.5 indicates slight changes toward acidity. Although 0.5-1.0% carbohydrates have been used, the 1.0% level is recommended to prevent reversal of the reactions.

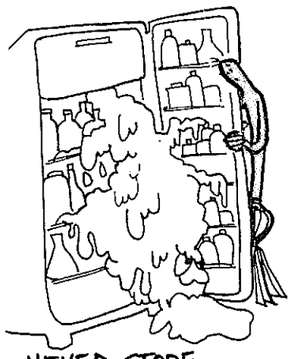
pared. For small volumes of MF broth, rehydrate in a boiling water bath for five minutes. MF agars and larger volumes of MF broth require direct heating to the first bubble of boil. When applying direct heat, stir and pay constant attention until the agar is dissolved. Other broths may require some heat from flame or waterbath to dissolve the ingredients prior to autoclaving. For other agars, use direct heat on large volumes to ensure rapid dissolution, but provide stirring and constant attention to prevent scorching.

ADJUSTING MEDIA pH

After being reconstituted, a medium will rarely require pH adjustment. However, if the medium has been improperly weighed or overheated, the final pH may be unacceptable. The pH of reconstituted media should therefore be checked with a pH meter and adjusted if necessary prior to sterilization.

The pH of a medium normally decreases about 0.1 to 0.2 pH units during sterilization, although the decrease may be as much as 0.4 in some cases. An exception to this is a medium containing buffering salts such as phosphates, which will exhibit a negligible decrease in pH during sterilization. To adjust the pH of an unacceptable medium, titrate a known volume of medium with a solution of NaOH to the desired pH. From this, determine the amount of NaOH solution needed to bring the bulk medium to this pH. Add this amount of NaOH solution to the bulk medium, mix thoroughly, then recheck the pH and adjust again if necessary. The required final pH is indicated in the directions for preparing each medium. If a final pH is not specified, no adjustment is necessary.

DISPENSING MEDIA



Once a medium is rehydrated, it should immediately be dispensed into appropriate culture containers and then sterilized. Delays may result in bacterial growth which can alter the pH of a medium or introduce toxic metabolic by-products. Therefore, the total time between media preparation and sterilization should not exceed two hours. Unsterilized prepared media should not be stored overnight prior to sterilization because even refrigeration will not completely suppress bacterial growth.

Broth and melted agar media are generally dispensed into culture containers by means of automatic pipettors. Rinse these pipettors immediately after use to remove any traces of medium which might contaminate the next medium dispensed with the pipettor. Keep agar media at a temperature above 60° C while dispensing to ensure that the media remain fluid enough to pass through the system and be flushed from the pipettor after use.

Immediately cap or close culture containers after dispensing media into them. Caps should be kept loose until after autoclaving so pressures inside the containers can

equalize with pressures in the autoclave during sterilization. Tightly capped glass containers may crack or even implode when removed from an autoclave.

STERILIZING MEDIA

Media should be sterilized within two hours after being rehydrated or prepared. Never refrigerate or attempt to store unsterilized, rehydrated media.

Different sources sometimes conflict in the sterilization procedures given for a particular medium. Whenever such a conflict arises, the procedure given in Standard Methods should be followed. In general, however, the following rules apply for sterilizing media:

-- MF and Salmonella media: heat to boiling only.

-- Media containing blood: autoclave and cool before adding blood.

-- Litmus milk and other milk-containing media: autoclave at 115° C for 20 minutes (this temperature yields a pressure of 10 psi).

-- Most media for fermentation reactions that contain carbohydrates (such as phenol red broth and triple sugar iron agar): autoclave at 118° C for 15 minutes (a pressure of 12 psi), or at 121° C for 12 minutes.

Sugar broths should not be exposed to any heat for more than 45 minutes, including the entire span from the time the loaded autoclave is closed until it is unloaded. In order to remain within this limit, preheat the autoclave before you load it. This will minimize the time required to reach full temperature once the sterilization cycle begins.

STORING STERILIZED MEDIA

Remove sterilized media from the autoclave as soon as the pressure returns to zero. If a medium is to be used right away, cool it first. Otherwise, store the media immediately. Allow refrigerated media to reach room temperature before use.

Tubes of fermentation media may dissolve enough air when refrigerated to create bubbles in the fermentation vials upon incubation. Therefore, these tubes should be incubated overnight immediately prior to use. If bubbles develop, discard those tubes. Fermentation media can be stored at about 25° C, but this may create excessive evaporation. For this reason, fermentation media should not be stored at this temperature for more than one week. Discard any tubes which lose more than 1 mL due to evaporation.

Media poured into petri dishes should be used on the same day they are poured or else should be refrigerated. Water loss from the media due to evaporation can be prevented by storing petri dishes in plastic bags. Invert the plates prior to storing so condensation will collect on the lid rather than on the media surface.

Culture media should be prepared in quantities which will be used within one week. An exception to this is media

contained in screw-capped tubes, which may be stored for up to three months. All media should be stored out of direct sunlight, away from sources of contamination and should be protected from excessive evaporation.

LABORATORY PURE WATER

To be suitable for preparing microbiological media and dilution or rinse water, laboratory water must meet several criteria. Primarily, these criteria require that the water be free of the following:

-- Substances which might either inhibit or encourage the growth of microorganisms;

-- Microorganisms themselves, which could introduce nutritive or toxic substances into the water in the form of metabolic by-products;

-- Pyrogens that are deleterious to virus tissue cultures; and

-- Substances which will interfere with sensitive chemical measurements.

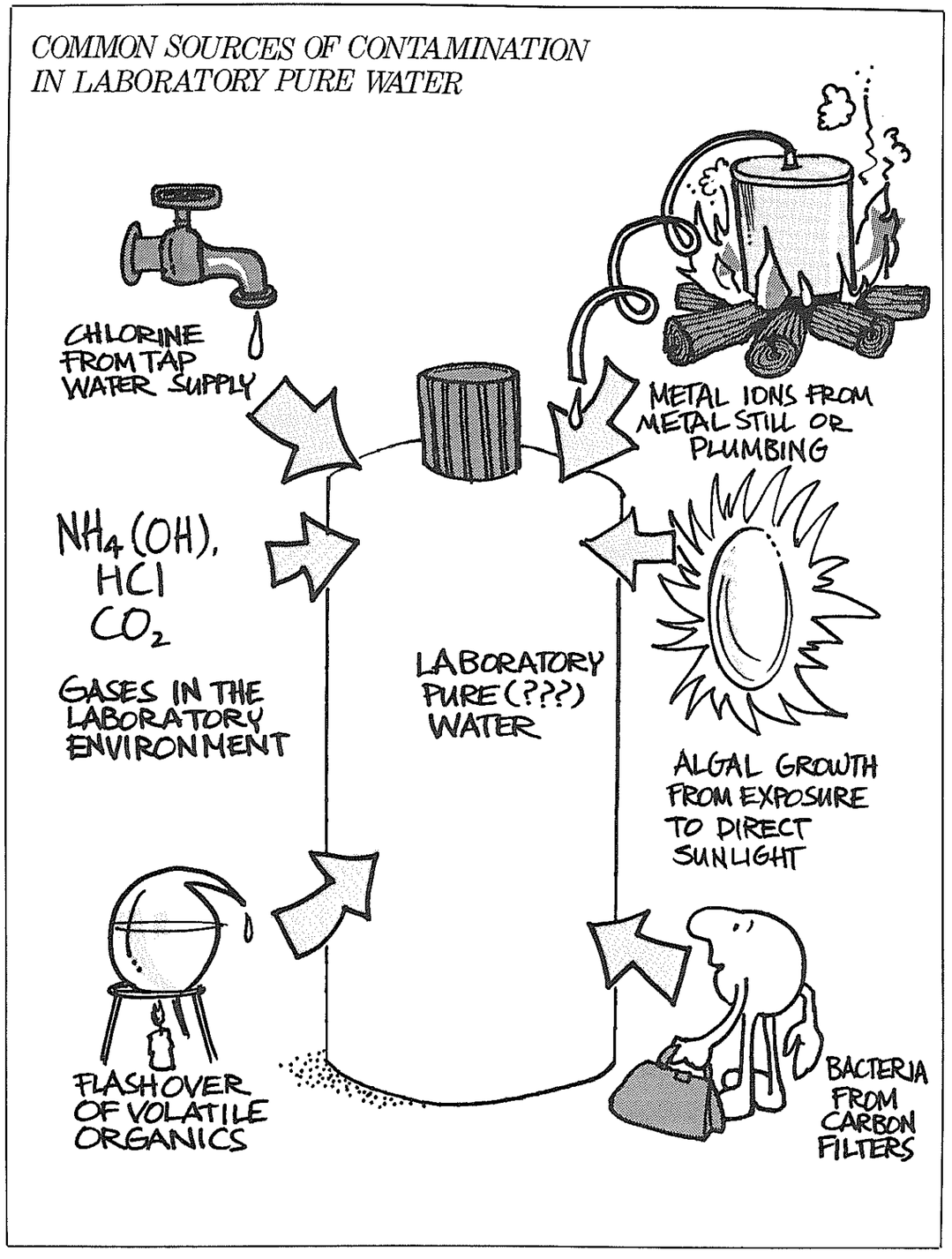
Either distilled or deionized water may be used if it meets the above requirements.

Many factors may affect the quality of the water produced by distillation or deionization. These factors include the design of the purification system, the raw water source, the condition and maintenance of system components, the chamber used for storing excess water produced, the temperature of the stored water, and the length of time the water remains in storage prior to use. The kinds of contaminants these factors can introduce into the final water include metal ions from stills and distribution systems; bacteria from carbon filters; ammonium hydroxide, hydrochloric acid and other fumes from the laboratory; chlorine from the tap water supply; and carbon dioxide from the atmosphere. As a result, the pH of laboratory water may vary.

Connecting plumbing for the laboratory pure water system should be constructed of stainless steel, Pyrex glass, or special pipes made of polytetrafluoroethylene (PTFE). Polyvinyl chloride (PVC) must not be used for laboratory pure water because it can cause serious contamination. Storage tanks for purified water should be constructed of stainless steel, fiberglass or suitable plastic (PTFE), and should be protected from contamination by dust. Avoid storing purified water in direct sunlight because this could cause algal growth and the production of organic nutrients or antibacterial metabolites.

QUALITY REQUIREMENTS

An essential measurement of the quality of laboratory pure water is conductivity. This value will reflect the presence of inorganic metals, salts and bases. It will not distinguish between toxic and nontoxic metallic ions, nor will it reveal the presence of organic contamination. Conductivity should be measured continuously as the water is produced, or at

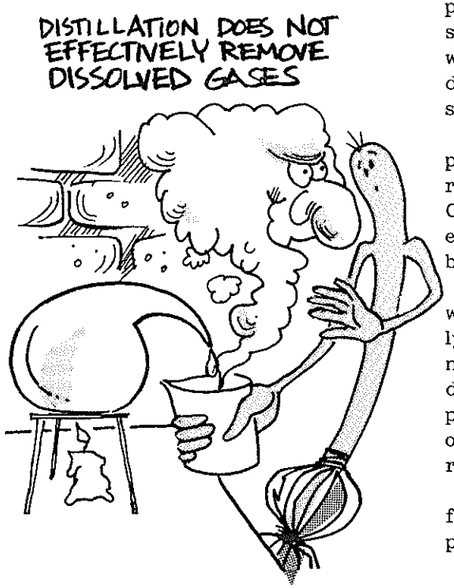


least with each use of the water. The limit for conductivity is 1-2 umhos/cm at 25° C.

Using municipal water supplies for input water in distillation systems may cause increased concentrations of ammonia and chloramines in the final water. Free chlorine is difficult to remove by distillation because it forms an azeotrope with water at pH values above 5.5. For this reason, flashover of chloramines is a particularly serious problem in water plant laboratories using freshly chlorinated water as a raw water supply. The limit for both ammonia/amines and free chlorine in laboratory pure water is less than 0.1 mg/L.

The pH of laboratory pure water should be between 5.5 and 7.5. Total organic carbon should be less than 1.0 mg/L. Individual trace metals should be less than 0.05 mg/L, and the total value for Cd, Cr, Cu, Ni, Pb and Zn combined should be less than 1.0 mg/L. The standard plate count for freshly produced laboratory pure water should be less than 1,000 bacteria/mL, and the count for stored water should be less than 10,000 bacteria/mL.

DISTILLED WATER



Properly maintained and cleaned stills can produce good grades of water over a long life span. The quality of water produced by a still normally deteriorates gradually as corrosion, leaching and fouling become apparent. Sudden loss of water quality rarely occurs unless caused by a structural defect. Using softened water as the raw water source for a still increases the interval between cleanings.

It is recommended that source water for a still first be passed through a deionizing column and a carbon filter to remove many of the inorganic and organic contaminants. Careful maintenance of these units must be followed, however, or the quality of the water delivered to the still could be lowered rather than improved.

Stills efficiently remove dissolved chemicals from the water, but they fail to remove dissolved gases. Even freshly prepared distilled water may contain chlorine and ammonia. During storage, the ammonia increases and carbon dioxide enters from the atmosphere. In addition, the water produced by stills may contain organics as a result of flashover during distillation. Toxicity in distilled water may also result from fluoridated water high in silica.

Distilled water systems should be monitored continuously for conductance, monthly for chlorine, ammonia and standard plate count, and at least annually for trace metals.

DEIONIZED WATER

Deionization systems alone can also produce good grades of pure water and, when used in conjunction with filtration and activated carbon in a recirculating system, are capable of producing ultrapure water. A reverse osmosis system can also be series-connected with one or more deionizing columns to improve the quality of the water produced.

Deionization systems do not deteriorate gradually the way stills do; rather they produce water of relatively uniform purity over a long period of time. Then, once the resins or carbon beds are exhausted, the water quality deteriorates rapidly. Sudden loss of good quality water can be avoided by continuously monitoring the system's performance and by anticipating the remaining life of cartridges so they can be replaced before failure occurs.

Types of potential contamination include amines eluted from the resin and organic carbon from organics in the source water or from bacterial growth in the columns. Bacteria may grow on charcoal filters, reaching extremely high levels and raising the possibility of bacterial or nutrient contamination of the water produced. Use of a 0.22 um final filter will remove any bacterial contamination from the water produced. However, dissolved metabolic by-products will still be carried through into the final water. A monthly check on the general bacterial population level will detect any excessive growth.

Monitoring for deionization systems should include conductance (continuously); ammonia/amines, total organic carbon, specific organics and standard plate count (monthly); and metals (at least annually).

DILUTION WATER

Frequently, it is necessary to dilute a sample prior to conducting such analyses as pour plates, spread plates, MF or MPN (most probable number). Otherwise the number of microbial cells per unit volume in the sample would far surpass the upper level of detection for the test. Dilution of the sample brings the microbial density into a range which can be accommodated. It also reduces particulates which might interfere with test results.

The water used for dilutions must be neutral in its effect on the bacterial populations present. It must promote cell stability, but at the same time it must avoid:

-- Stimulating cell growth and reproduction;
-- Damaging cells; or
-- Reducing the ability of the cells to survive, grow and reproduce.

Microbial survival in a particular dilution water can be quite variable, depending upon such factors as suspension time, temperature, pH, osmotic gradient, buffering, chelating capacity, and trace concentrations of magnesium, calcium and iron ions. The ideal dilution water must provide uniform results between laboratories. It must also contain the chemical elements and compounds which occur in natural conditions and which are necessary to insure a balance of cell solutes and to maintain cell turgidity. Various attempts have been made to develop an appropriate, ideal dilution water. Some researchers have copied the natural environment by using sterile fresh or marine waters, but these are non-standard. Others have used tap water, which also lacks uniformity and adds the potential for toxicity. Distilled or deionized water is inappropriate because it lacks essential trace metal ions as well as buffering and chelating capacity.

Synthetic dilution waters use combinations of inorganic constituents (such as sodium, potassium, magnesium, phosphate, chloride and sulfate), and soluble organics (such as peptone) to provide the required conditions. The pH of these dilution waters is normally kept near neutral. Two standards are phosphate buffered dilution water and peptone dilution water.

PHOSPHATE BUFFERED DILUTION WATER

Some degree of mineralization, similar to that in natural water, is necessary if bacterial growth with minimal lag is to be achieved when conducting microbiological analyses on high quality natural waters. Phosphate buffered dilution water comes close to meeting the optimum desired qualities.

Prepare a stock solution of phosphate buffered dilution water by adding 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) to 500 mL of laboratory pure water. Adjust the pH to 7.2 with 1 N NaOH and bring the final volume to 1000 mL with laboratory pure water. Sterilize by filtration or autoclave for 15 minutes at 121° C (15 psi).

Store the stock solution in the refrigerator after sterilization. The stock solution should be handled aseptically. Discard if evidence of turbidity, mold or other contamination appears and prepare a fresh stock solution.

To prepare a working solution, add 1.25 mL of the stock solution to 5 mL of magnesium chloride solution (81.1 g $MgCl_2 \cdot 6H_2O$/L) and 1000 mL of laboratory pure water. The pH of the working solution should be 7.2 ± 0.1.

PEPTONE DILUTION WATER

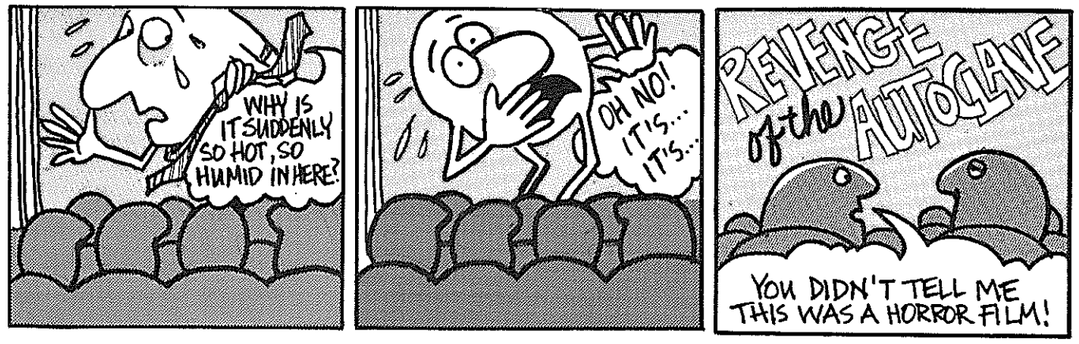
The use of a 0.1 percent peptone dilution water may be superior to phosphate buffered dilution water in recovering attenuated organisms from industrial wastes or from stream samples having high concentrations of heavy metal ions.

Prepare peptone dilution water by first mixing a 10 percent solution (10 g of peptone per 100 mL of solution) using laboratory pure water. Dilute a measured volume of this to provide a final 0.1 percent solution. The final pH of peptone dilution water should be 6.8.

PREPARING DILUTION & RINSE WATER

Dispense the working solution of either phosphate buffered or peptone dilution water in amounts that will provide 99 ± 2.0 mL or 9 ± 0.2 mL after autoclaving at 121° C (15 psi) for 15 minutes. Generally, 102 mL of solution will result in 99 mL after autoclaving. Loosen screw-caps during sterilization. Cool and separate bottles or tubes whose volumes fall outside the acceptable limits. Tighten screw-caps and store in a cool place.

Dilution water is also used for rinsing apparatus during microbiological manipulations. Prepare dilution water to be used for rinsing in 500 mL or larger volumes and autoclave for 30 minutes or more. Bottles and flasks must be well separated during autoclaving to provide sufficient circulation of steam.



Working with Microorganisms

There is a common thread that runs through all microbiological work, binding it together into a single, rigorous discipline. That unifying thread is the concept of aseptic -- or sterile -- technique. In its broadest sense, aseptic technique is the doctrine or code a microbiologist lives by. In a world teeming with microorganisms -- where every surface, every drop of moisture and even the air itself carries unseen flecks of life -- aseptic technique is the primary defense against contamination. It requires constant attention to details. The slightest lapse can introduce foreign organisms into a sample culture, or release cells from that culture into the laboratory environment. The consequences of contamination are far greater in microbiology, where a contaminant may grow and multiply, than in analytical chemistry, where at least contaminants can be counted on to remain static.

Aseptic technique begins with the cleaning and sterilization of equipment and supplies to be used in analysis. It also includes preparation and disinfection of the laboratory environment where the work is to be performed. And it culminates in the procedures the analyst uses while handling cultures of microorganisms during transfers, inoculations, dilutions, plating, culturing and other activities, right through final disposal of microbiological wastes.

At this point, all the necessary equipment and supplies have been gathered, all the proper media and reagents carefully prepared. Now it is time to bring the pieces together and begin actually working with microorganisms.

CLEANING GLASSWARE

The first step in working with microorganisms is to be certain all apparatus to be used is scrupulously clean. The large volume of glassware which must be cleaned on a regular basis in most laboratories justifies using mechanical glassware washers. These should be equipped with high pressure, directional jet streams which can break up and remove such difficult deposits as microbiological growth films, autoclaved proteins, agar, sediments, sludges, chemicals and wax markings. Washers should be easy to load and provided with accessory racks to handle the variety of glassware used in the laboratory. Wash, drain and rinse cycles must each be capable of operator adjustment, as well as providing automatic sequences of 71.1° C (160° F) detergent wash water, a clean water rinse at 82.2° C (180° F), and a final rinse in distilled water or equivalent. Plumbing should be of stainless steel or other nontoxic material. The washer must produce sparkling glassware which is free from acidity, alkalinity and toxic residues.

Where hand washing is necessary, laboratory detergents must be used rather than the milder detergents formulated for household use. Glassware must be vigorously scrubbed with appropriately sized laboratory brushes in hot (160-170° F) water. Washing should be followed by multiple hot water rinses, then at least three final rinses with laboratory pure water to ensure complete removal of any contaminating substances. Fogs or films which remain on washed glassware indicate inadequate cleaning. Permanently stained or etched glassware should be discarded.

DETERGENT TOXICITY

The detergent used must not leave any toxic or inhibitory residues. Test for the suitability of a detergent using glass petri dishes as follows (this procedure can be modified for culture tubes or dilution bottles in laboratories which use only plastic, disposable petri dishes):

-- Using the normal procedure, wash and rinse a few glass petri dishes.

-- Rinse a separate group of petri dishes six times with laboratory pure water.

-- Wash another batch of dishes with detergent and drain dry without rinsing.

-- Sterilize all three batches of dishes.

-- Use standard plate count agar to prepare duplicate or triplicate plates for each batch of petri dishes using a water sample which will yield 20 to 60 colonies per plate.

-- Incubate each group of plates at 35° C for 48 hours.

-- If the unrinsed plates have a bacterial count 15 percent or more lower than the well-rinsed plates, there is a bacterio-static substance in the detergent and a different detergent should be used.

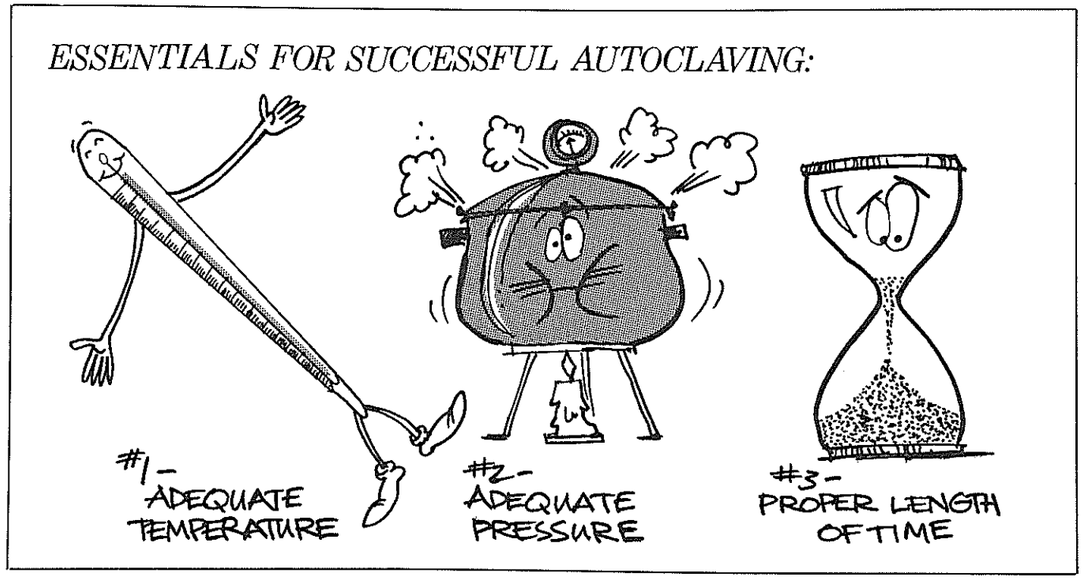
-- If the plates which underwent normal washing and rinsing have a bacterial count 15 percent or more lower than than the well-rinsed plates, a toxic residue from the detergent is remaining on the glassware and a different detergent should be used.

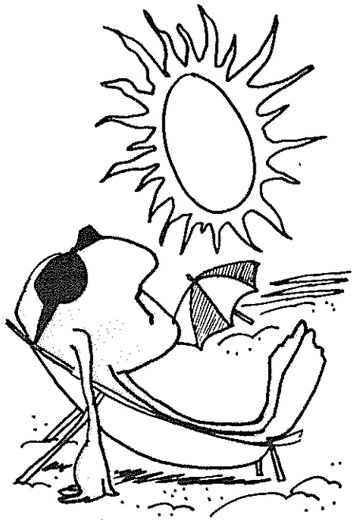
STERILIZATION

Sterilization ensures that no living organisms remain on equipment and materials to be used in microbiological analysis. Chemical or physical methods may be used for sterilization, depending upon the kinds of materials to be sterilized. Chemical agents, such as iodophor or quaternary ammonium, are used as disinfectants. Another form of disinfection is pasteurization, which is used with materials that might be damaged or altered by excessive heat. In pasteurization, low heat is applied one or more times to sensitive liquids to destroy vegetative cells. Other methods of sterilization include moist heat, dry heat, incineration, filtration, radiation, and ethylene oxide gas.

Moist heat sterilization is accomplished by means of the autoclave. Normal operation of an autoclave calls for steam held at 15 psi (yielding a temperature of 121° C, or 250° F) F) for 15 minutes at sea level. When held under pressure, steam has good penetrating power and coagulates microbial protoplasm, thereby affording effective sterilization. The three variables which must be closely monitored for effective autoclaving are temperature, pressure and time. Autoclaving is effective for sterilizing such materials as solid and liquid media, contaminated materials, discarded cultures, glassware and filtering units. Pressure cookers and vertical autoclaves should not be used for sterilization.

The dry heat provided by a hot-air oven can be used to sterilize heat resistant materials such as glassware, stainless steel utensils and hardwood applicators. It should not be used on liquids and other materials which might evaporate or deteriorate under heat. Since there is no moisture to provide steam in the hot-air oven, a temperature of 165-170° C must be maintained for two hours.





Burning destroys combustible contaminated materials and is effective for sterilizing inoculating loops and needles, and for flaming the lips of culture tubes and flasks during aseptic transfers and inoculations.

Heat-sensitive liquids can be sterilized by passing them through filters composed of one of the following: asbestos-cellulose (Seitz filter), cellulose esters (0.22 um MF or molecular filter), unglazed porcelain (Chamberland-Pasteur filter), or diatomaceous earth (Berkfeld filter). Normally, filtration is used to remove bacteria. However, some filters have sufficiently small pore sizes to also remove viruses.

Ultraviolet light consists of all radiation between 150 and 4000 Angstrom units (A.U.). However, radiations less than 1800 A.U. are absorbed by atmospheric oxygen. Radiation at 2600 A.U. has the greatest killing effect on microorganisms. Commercially available ultraviolet sterilization lamps emit light primarily around 2537 A.U., which has about 85 percent of the sterilization efficiency of 2600 A.U. ultraviolet light. Ultraviolet radiation can be used to sterilize such apparatus as membrane filter units, inoculating rooms, bacteriological hoods and glove boxes.

Another low temperature sterilization method involves the use of ethylene oxide gas. This is effective for heat sensitive plastics, rubber goods, delicate instruments and similar items which might be damaged by autoclaving. Be sure to allow sufficient time and ventilation to dissipate all traces of the gas prior to using items which have been sterilized by ethylene oxide.

PREPARING FOR MICROBIOLOGICAL ANALYSIS

Prior to beginning an analysis, be sure the sample identification, sampling conditions, chlorine residual, pH, temperature, sampling site, date and time of sampling, and other critical information have been identified and recorded on the bench sheet. There is no point in analyzing an improperly identified sample, for the results will be meaningless and the time wasted.

Once proper identification has been determined, the bench surface or work area can be disinfected and the analysis performed. Wipe the surface with a laboratory strength disinfectant and allow it to dry before use. During the procedure, keep a covered container of iodophor or quaternary ammonium disinfectant nearby in case of an emergency or spill. Phenolics can also be used as disinfectants as long as the laboratory does not perform analyses for these compounds. Be sure to disinfect the work surface again after completing the analysis. This reduces the chances of contamination being introduced to the next analysis performed in that area.

When the work area is ready, check the sample for any pretreatment that might be necessary. This involves testing for free chlorine residual and uneven distribution of microorganisms.

Microorganisms must be evenly distributed throughout the sample in order to achieve meaningful results when the sample is subdivided for analysis. When a sample contains high solids, as with primary effluents, sludge and highly turbid waters, the sample must be blended prior to removing any subsamples. Blend a sample with particulates in a Waring-type blender for no more than 30 seconds at about 5000 RPM to avoid overheating or shearing damage to the cells. Use only blender containers made of autoclavable Pyrex glass, stainless steel or plastic. The container should have a safety screw cover to prevent the release of aerosols containing microorganisms into the laboratory environment.

Sediments or soils which contain limited amounts of water should be diluted first at a 1:1, 1:2 or other ratio with dilution water. This will ensure good blending action and reduce heat generation during blending. Heat buildup can also be reduced by using a large blender container rather than a small one.

When a solid sample is to be analyzed for microorganisms, the results will be calculated as the number of organisms per gram of dry solids. It is therefore necessary to first mix the sample thoroughly and weigh a 50 g aliquot in a tared weighing pan. Dry the aliquot at 105-110° C until a constant weight is reached. Record the final weight. This provides the ratio of dry solids to initial sample weight. Then prepare an initial dilution of the sample by weighing out a second aliquot of 11 g from the original sample. Add this to 99 mL of buffered dilution water for a 1:10 dilution and blend aseptically, following the guidelines listed above. Transfer 11 mL of the diluted, blended sample to a second dilution bottle containing 99 mL of buffered dilution water and shake vigorously 25 times. Repeat this last process until the desired dilution is reached.

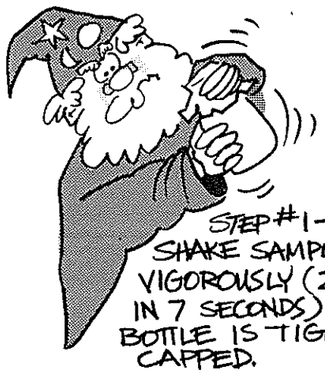
Where samples have high solids contents and low microbial populations, MPN or pour plate procedures may have to be used for the microbiological analysis. However, other high solids samples such as polluted soils and sludges will be appropriate for MF analysis after dilution of the initial sample.

HOMOGENOUS SAMPLES

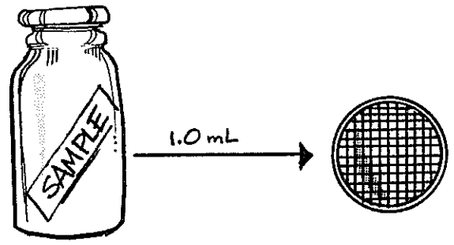
SAMPLE DILUTION

Many water and wastewater samples must be diluted prior to analysis in order to reach a microbial concentration which can be handled by the test procedure. The usual method for serial dilutions is to take a specified quantity of the sample (normally 1.0 mL, although other volumes can be used instead) and add it to either a 9.0 mL volume of dilution water (for a 1:10 dilution), or 99 mL of dilution water (for a 1:100 dilution). Then the same volume of sample is removed from this first dilution and added to another bottle of dilution water. Continue this procedure until the desired bacterial density is reached. The diluted sample can then be analyzed by the appropriate technique.

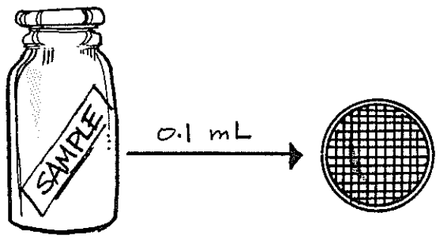
PREPARING SERIAL DILUTIONS



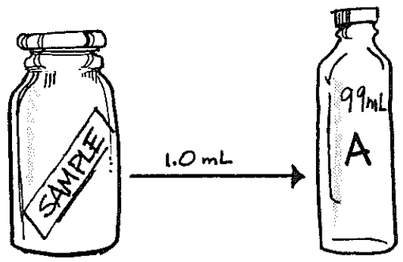
STEP #1 -
SHAKE SAMPLE BOTTLE VIGOROUSLY (25 TIMES IN 7 SECONDS). BE SURE BOTTLE IS TIGHTLY CAPPED.



STEP #2 -
1.0 mL OF ORIGINAL SAMPLE CAN BE USED DIRECTLY FOR 1 x 10° mL SAMPLE VOLUME.

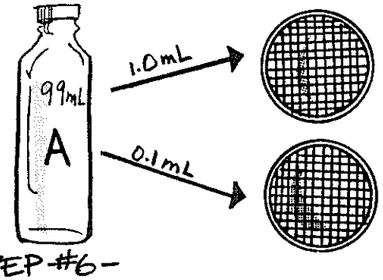


STEP #3 -
0.1 mL OF ORIGINAL SAMPLE PROVIDES 1×10^{-1} mL SAMPLE VOLUME.



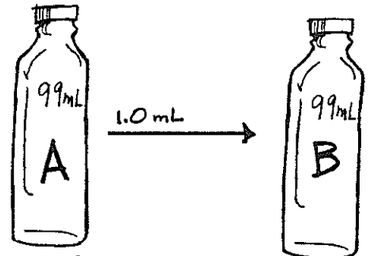
STEP #4 -
TRANSFER 1.0 mL OF SAMPLE TO 99 mL OF DILUTION WATER. THIS IS DILUTION "A".

STEP #5 -
SHAKE DILUTION "A" VIGOROUSLY.



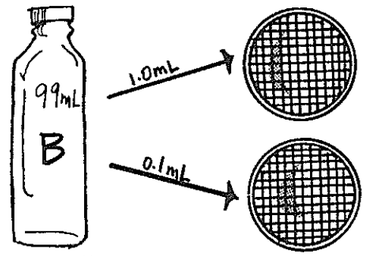
STEP #6 -
1.0 mL OF DILUTION "A" PROVIDES 1.0×10^{-2} mL OF SAMPLE.

STEP #7 -
0.1 mL OF DILUTION "A" PROVIDES 1.0×10^{-3} mL OF SAMPLE.



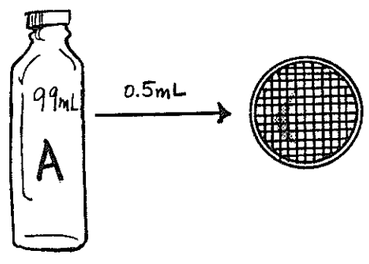
STEP #8 -
TRANSFER 1.0 mL FROM DILUTION "A" TO ANOTHER 99 mL DILUTION BOTTLE. THIS SECOND BOTTLE IS DILUTION "B".

STEP #9 -
SHAKE DILUTION "B" VIGOROUSLY.



STEP #10 -
1.0 mL OF DILUTION "B" PROVIDES 1.0×10^{-4} mL OF SAMPLE.

STEP #11 -
0.1 mL OF DILUTION "B" PROVIDES 1.0×10^{-5} mL OF SAMPLE.



ALTERNATE SAMPLE VOLUMES - USE DIFFERENT AMOUNTS FROM THE DILUTIONS TO OBTAIN INTERMEDIATE VOLUMES OF SAMPLE. FOR EXAMPLE, 0.5 mL FROM DILUTION "A" PROVIDES 5.0×10^{-3} mL OF SAMPLE.

STEP #12 -
CONTINUE THIS PROCESS UNTIL A DILUTION IS REACHED WHICH WILL PROVIDE A SATISFACTORY MICROBIAL DENSITY.

NOTE -
BE SURE TO FINISH USING ALL DILUTIONS WITHIN 30 MINUTES OF PREPARATION TO AVOID UNWANTED POPULATION CHANGES.

For simplicity, serial dilutions are usually prepared in succeeding ten-fold volumes known as "decimal dilution." However, the decimal dilution series must be modified when using the membrane filter technique in order to produce colonies within the accepted limits for MF analysis. The normal limits for MF plates are 20 to 60, 20 to 80, or 20 to 100 colonies per plate, depending upon the particular procedure being used. To obtain counts within these ranges, filter dilution volumes of the decimal series which have a factor of 3, 4 or 5 among them.

Phosphate buffered dilution water is potentially toxic to some microorganisms and peptone dilution water may stimulate microbial growth. Both actions increase rapidly with time. Dilutions should therefore be used promptly after preparation with no more than 30 minutes elapsing prior to use.

RECOMMENDED SAMPLE VOLUMES FOR MEMBRANE FILTRATION

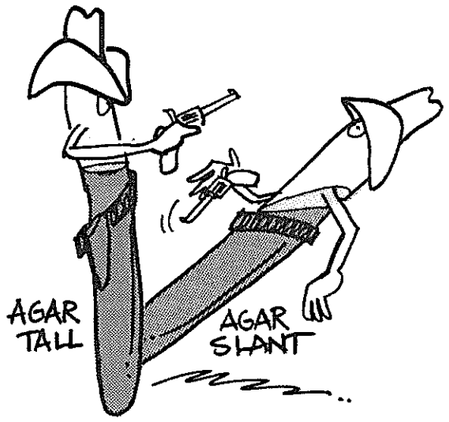
20 - 60 colonies/plate		20 - 80 colonies/plate		20 - 100 colonies/plate	
Sample (mL)	added as	Sample (mL)	added as	Sample (mL)	added as
.01	1 mL of 10^{-2}	.04	4 mL of 10^{-2}	.01	1 mL of 10^{-2}
.03	3 mL of 10^{-2}	.15	1.5 mL of 10^{-1}	.05	5 mL of 10^{-2}
.1	1 mL of 10^{-1}				
.3	3 mL of 10^{-1}	.5	5 mL of 10^{-1}	.25	2.5 mL of 10^{-1}
1.0	1 mL of sample				
3.0	3 mL of sample	2.0	2 mL of sample	1.25	1.25 mL of sample
10	10 mL of sample	8.0	8 mL of sample	6.0	6 mL of sample
30	30 mL of sample	30	30 mL of sample	30	30 mL of sample

CULTURING MICROORGANISMS

Often it is necessary to take a small population of microorganisms and encourage them to grow so a larger population can be obtained. This is the reverse of dilution and is used whenever the original population is too small to count, identify, select or otherwise examine directly. Culturing is, of course, the key step in most of the qualitative and quantitative analytical techniques involving microorganisms, but it may also be an intermediary step in other techniques, such as staining or the propagation of a pure culture.

Culturing is frequently done in glass test tubes called culture tubes. Culture tubes may be plain and have no lip, in which case the closures will be sleeve-like caps of stainless steel or plastic, or the tubes may be threaded and use screw-caps. If the screw-cap variety is used, the neck of the tube must be flamed prior to withdrawing microorganisms from or introducing them into the tube during aseptic laboratory procedures. When using sleeve-like caps and plain tubes, this step is unnecessary.

The media used in a culture tube may be either liquid broth or solid agar. When agar is used, it may be allowed to harden while the tube is in either an upright position (forming an "agar tall" or "agar deep"), or while the tube is in a slanted position (forming an "agar slant"). The advantage of the latter is that it provides a greater surface area for culturing aerobic microorganisms. Anaerobic organisms can be cultured in an agar deep or agar tall by stabbing an inoculating needle with the organisms into the agar and then withdrawing the needle along the line of entry. The microorganisms then grow within the interior of the agar, away from the presence of oxygen.

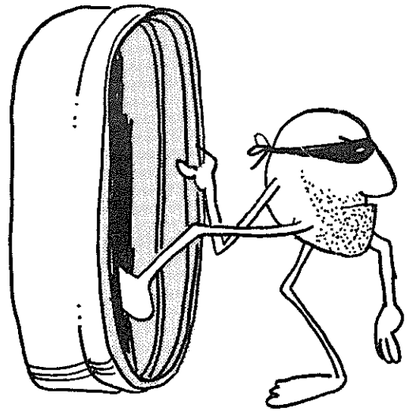


INOCULATIONS & TRANSFERS

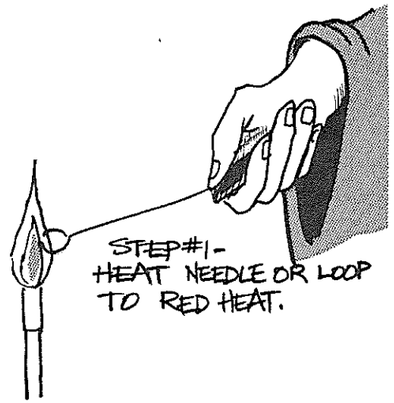
An inoculating needle or loop is used to transfer microbial samples from one culture medium or vessel to another. A needle may be preferable over a loop when it is necessary to obtain cells from a single colony on an agar or membrane filter surface, while a loop should be used when a culture is being withdrawn from a broth.

A culture transfer begins by first flaming the loop (or needle) to red heat. When using a Bunsen burner for this, adjust the flame so there is a distinct blue cone. Pass the wire to be sterilized through the tip of this cone. Then allow the loop to cool for 10-15 seconds. Pick up the culture tube from which the microbial culture is to be obtained in the opposite hand (this will be the left hand for right-handed analysts). Remove the cap from the culture tube using the crook of the little finger on the hand holding the loop. Keep the cap in your hand -- do not set the cap down. If the culture tube has threads and a screw-cap, pass the exposed neck of the culture tube through the burner flame once or twice to destroy any microorganisms which might be adhering to the top of the tube. Then slowly insert the loop into the tube and retrieve a small amount of inoculum. If the medium is a liquid, the amount adhering to the loop will normally be sufficient. If the medium is solid, such as agar, and the colonies are growing on the surface (as they will be with most types of cultures), do not gouge the agar or damage the surface when obtaining the inoculum. Only a very small amount of microbial material is necessary to transfer several million organisms.

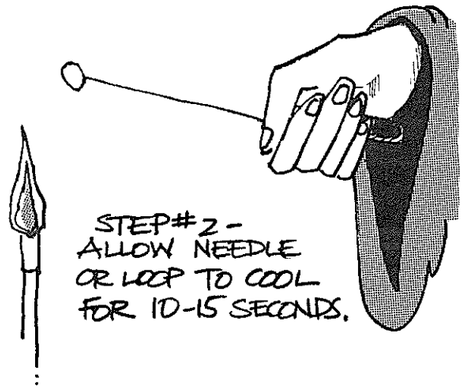
When the inoculum has been obtained, withdraw the loop and (for screw-cap tubes) again flame the neck of the culture tube. Then replace the cap and set the tube down. Pick up the fresh culture tube which is to be inoculated and



PERFORMING ASEPTIC INOCULATIONS

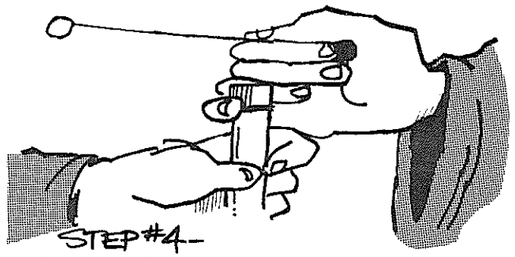


STEP#1-
HEAT NEEDLE OR LOOP TO RED HEAT.

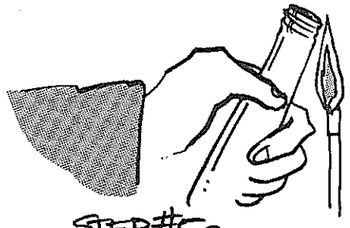


STEP#2-
ALLOW NEEDLE OR LOOP TO COOL FOR 10-15 SECONDS.

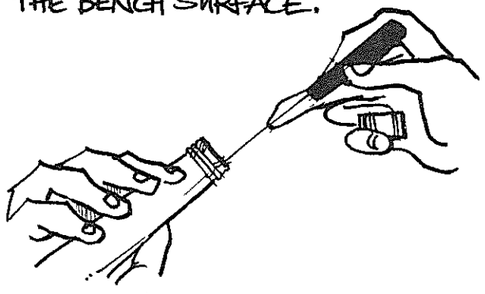
STEP#3-
PICK UP CULTURE TUBE FROM WHICH MICROBIAL SAMPLE IS TO BE OBTAINED WITH FREE HAND.



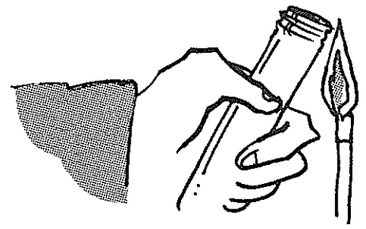
STEP#4-
REMOVE CLOSURE TO CULTURE TUBE ASEPTICALLY WITH FIRST HAND, USING THE CROOK OF THE LITTLE FINGER. NEVER SET THE CAP ON THE BENCH SURFACE.



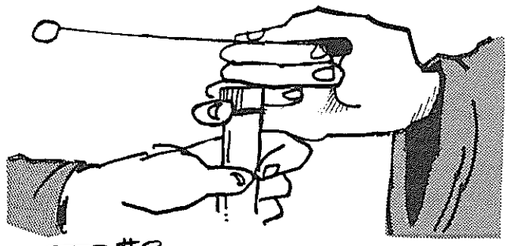
STEP#5-
FLAME NECK AND LIP OF CULTURE TUBE (SCREW-CAP TUBES ONLY).



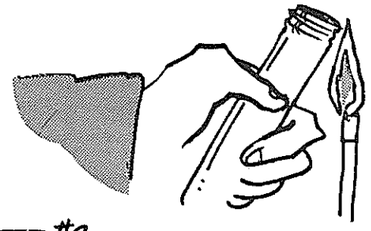
STEP#6-
INSERT NEEDLE OR LOOP INTO CULTURE TUBE AND OBTAIN A SMALL AMOUNT OF SAMPLE.



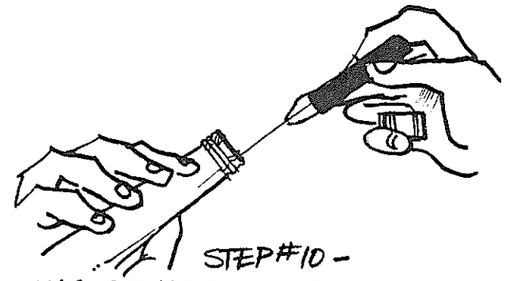
STEP#7-
FLAME NECK OF CULTURE TUBE ONCE AGAIN (SCREW-CAP TUBES ONLY) AND REPLACE CAP.



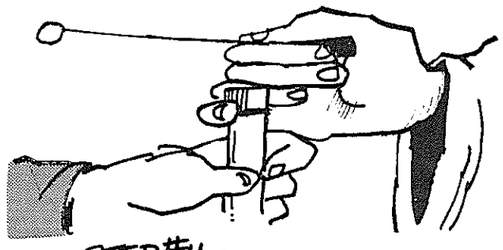
STEP#8-
PICK UP CULTURE TUBE TO BE INOCULATED AND REMOVE CAP AS BEFORE.



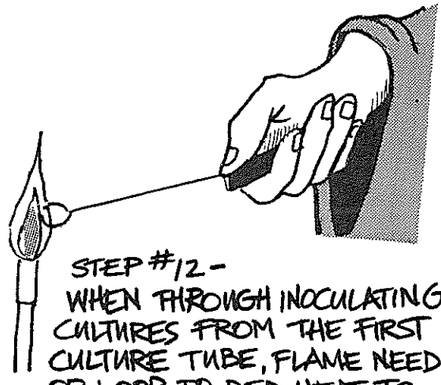
STEP#9-
FLAME NECK OF SECOND CULTURE TUBE (SCREW-CAP TUBES ONLY).



STEP#10-
INSERT NEEDLE OR LOOP TO INOCULATE STERILE MEDIUM IN SECOND CULTURE TUBE.



STEP#11-
FLAME SECOND CULTURE TUBE NECK AGAIN (SCREW-CAP TUBES ONLY) AND REPLACE CAP.



STEP#12-
WHEN THROUGH INOCULATING CULTURES FROM THE FIRST CULTURE TUBE, FLAME NEEDLE OR LOOP TO RED HEAT TO DESTROY ANY ADHERING ORGANISMS.

remove the cap as before. Flame the top of the tube, if necessary. Insert the inoculating loop into the tube. If the the medium is a broth or other liquid, simply plunge the loop into the liquid. If the medium is solid, streak the inoculum gently over the surface. Remove the loop, flame the neck of the culture tube if necessary and replace the cap. If another inoculum of the same culture is to be obtained from the first tube, this can be done without sterilizing the loop again. When all transfers involving that culture are completed, heat the loop to red heat once more to destroy any remaining organisms.

This same technique can be used with only minor modifications when transferring organisms to and from other kinds of culture containers, such as petri dishes, and onto slides for microbial smears.

PLATING TECHNIQUES

DURING THE FINAL STREAKING ON A STREAK PLATE, THE BACTERIA SHOULD BE FALLING OFF THE NEEDLE OR LOOP ONE CELL AT A TIME.

The three plate dilution methods commonly used for isolating or enumerating individual bacterial colonies are streak plate, spread plate and pour plate. These techniques use agar media to dilute the microorganisms, either on the surface or within the agar itself, so colonies resulting from isolated, individual cells can be distinguished. (However, because in actual practice colonies can result from more than one cell, the results should technically be reported as colony forming units, or CFUs.)

The streak plate method is used to isolate individual bacteria from mixed cultures. The analyst uses a loop or needle to take a small amount of microbial material from the surface of a solid medium and draws it lightly across an agar plate in a regular pattern. As more and more of the bacteria from the loop or needle drop off the wire and onto the agar, the bacterial population remaining on the wire thins out. Eventually, if performed properly, cells begin falling off one at a time. Retrieval of one of the resulting colonies enables a pure culture to be developed.

The spread plate method also isolates bacterial cells over the surface of an agar plate. In this method, however, the bacterial cells originate in a liquid medium which is spread over the surface by means of a bent glass rod.

The pour plate method, which is best known through the standard plate count technique, involves mixing a volume of liquid sample with melted agar and then plating the resulting suspension. Colonies develop both on the surface and within the interior of the agar plate, where they can be counted directly or retrieved for further analysis.

STREAK PLATE

Melt a nonselective agar, such as nutrient agar or Trypticase soy agar, and temper it in a water bath set at 44-46° C. Add about 15 mL of agar to each sterile petri dish and allow it to harden and dry before use.

To prevent damaging the surface of the agar during streaking, bend an inoculating loop (or needle) at an angle about 1 cm from the tip. Heat the loop to red heat to

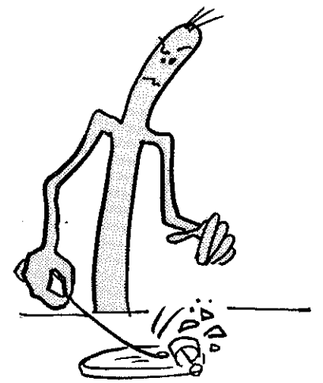
sterilize it and air cool for 10-15 seconds. Using the free hand, pick up the culture tube containing the bacterial culture to be streak plated. Remove the cap and hold it in the crook of the little finger on the hand with the loop. If the culture tube is the screw-cap variety, flame the neck once or twice.

With the loop, obtain a small amount of the bacterial growth from the culture tube. Flame the neck of the tube again (for screw-cap tubes) and cap it, then set it aside. Lift the petri dish cover with the free hand and draw the tip of the loop gently across 1/3 - 1/2 the surface of the agar, following a regular, non-overlapping pattern. Flame and cool the loop and rotate the plate a quarter turn. Draw the loop tip gently over another portion of the agar surface, passing it initially through the edge of the first area to re-inoculate the tip with a small portion of the bacterial culture. Flame and cool the loop again and rotate the plate another quarter turn. Draw the loop tip gently through the last area of the plate as before, passing it through the edge of the second area to be streaked to again re-inoculate it with bacterial cells.

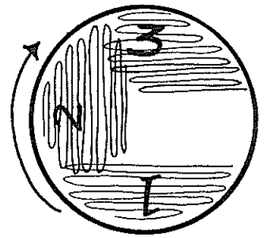
Replace the petri dish cover and flame the loop one final time to destroy any remaining cells. Invert the dish to prevent condensation from forming on the agar, as this would cause the bacteria to spread over the surface and eliminate the separation achieved by streaking. Incubate (normally at 35° C for 24 hours).

The cells deposited by the loop (or needle) should develop into pure bacterial colonies, each arising from an individual bacterium. The colonies in the portion of the plate which was streaked last should show the highest degree of separation as there were fewer cells in each successive area streaked. For further purification, choose a single, well-isolated colony and obtain a portion of it on a sterilized inoculating loop or needle. Suspend these cells in dilution water and restreak on another agar plate. If the colonies which form on the second plate are all alike, the culture can be assumed to be pure.

Selective or differential agars, such as Endo or EMB agars, can also be used for streaking in order to develop pure cultures of particular bacterial strains.



DRAW THE NEEDLE OR LOOP GENTLY OVER THE PLATE TO AVOID DAMAGING THE AGAR SURFACE.



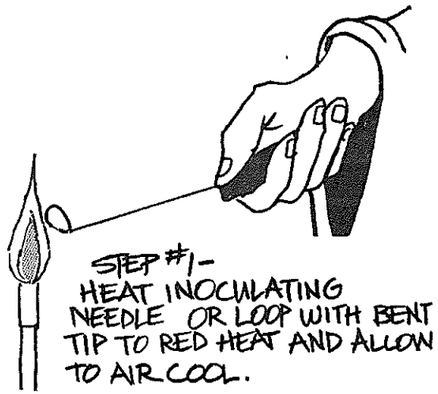
SUGGESTED PATTERN OF STREAKS FOR A STREAK PLATE.

SPREAD PLATE

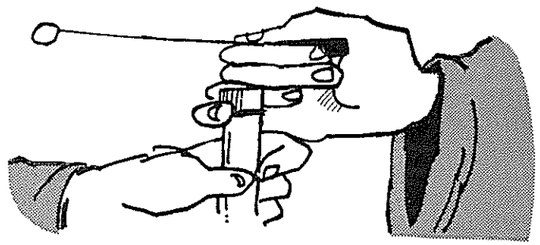
Pour melted agar of a type appropriate for the organisms to be cultured into petri dishes to harden. Use about 15 mL of agar for each 100 mm dish. Keep the covers to the plates slightly opened until the agar hardens and any excess moisture or condensation dries. Then close the dishes and store them, if necessary, in a refrigerator. Allow refrigerated plates to warm to room temperature prior to use.

Estimate the concentration of bacteria in the sample to be plated and prepare a series of dilutions that will result in 40-2000 cells/mL. When plated, a 0.1-0.5 mL quantity of such a dilution will then produce the 20-200 colonies needed to fall within the range for a successful plate. To be sure

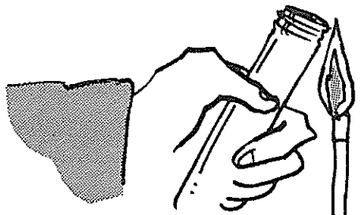
PREPARING STREAK PLATES



STEP #1 -
HEAT INOCULATING NEEDLE OR LOOP WITH BENT TIP TO RED HEAT AND ALLOW TO AIR COOL.

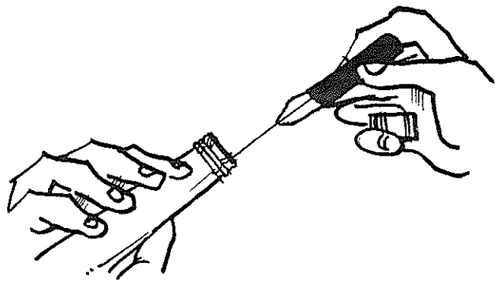


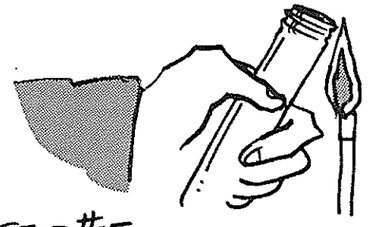
STEP #2 -
PICK UP CULTURE TUBE AND REMOVE CAP.



STEP #3 -
FLAME NECK AND LIP OF CULTURE TUBE (SCREW-CAP TUBES ONLY).

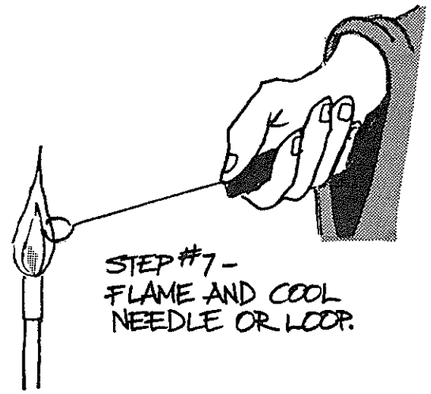
STEP #4 -
INSERT NEEDLE OR LOOP INTO CULTURE TUBE AND OBTAIN A SMALL AMOUNT OF SAMPLE.





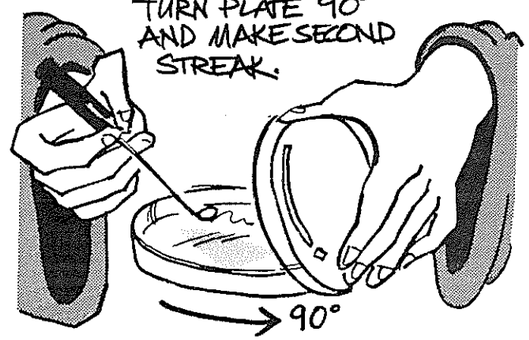
STEP #5 -
FLAME NECK OF CULTURE TUBE AGAIN (SCREW-CAP TUBES ONLY) AND REPLACE CAP.

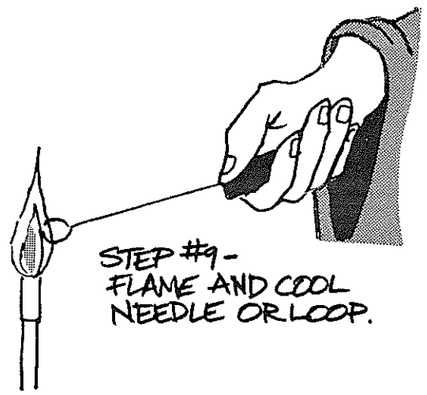
STEP #6 -
RAISE PETRI DISH LID UP SLIGHTLY AND MAKE FIRST STREAK.



STEP #7 -
FLAME AND COOL NEEDLE OR LOOP.

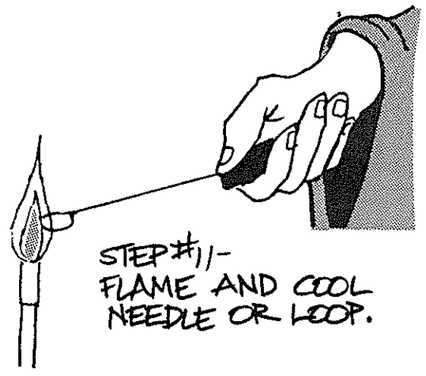
STEP #8 -
TURN PLATE 90° AND MAKE SECOND STREAK.





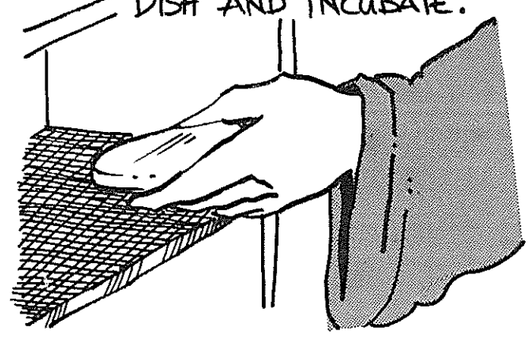
STEP #9 -
FLAME AND COOL NEEDLE OR LOOP.

STEP #10 -
TURN PLATE 90° AND MAKE THIRD STREAK.



STEP #11 -
FLAME AND COOL NEEDLE OR LOOP.

STEP #12 -
REPLACE LID. INVERT DISH AND INCUBATE.



BE SURE THE GLASS ROD SPREADER IS SUFFICIENTLY COOL TO PREVENT DAMAGE TO THE MEDIUM AND BACTERIAL CELLS.

of obtaining a plate within this range, bracket the estimated dilution by using one or two other dilutions to either side as well.

When plating, remember that using only 0.1 mL of a 10^{-1} dilution actually represents a final dilution of 10^{-2}.

After inoculating a plate, the inoculum is evenly distributed over the plate by means of a glass spreader -- a glass rod bent at an angle of about 120°. If only a single spreader is available, sterilize it between plates by soaking it in a beaker of alcohol. Flame it to remove excess alcohol and allow it to cool prior to use. To be sure the spreader is sufficiently cool, test it against the edge of the agar before placing it in contact with the inoculum. If several spreaders are available, they can be hot-air sterilized with the other glassware, thus eliminating the alcohol and flaming steps.

Place the spreader next to the inoculum so the bent portion extends radially across the plate with the bend at the center and the tip at the edge. Then either rotate the plate beneath the spreader, or rotate the spreader across the surface of the plate, for several revolutions. Lift the spreader from the plate and return it to the alcohol solution. Leave the lid of the petri dish slightly ajar for about 15-30 minutes while the excess moisture evaporates. Then fully close the dish, invert it and incubate.

After incubation, find the dilution which has produced isolated surface colonies within the acceptable counting range of 20-200 colonies.

POUR PLATE

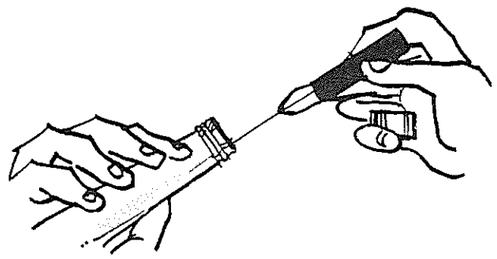
Place 0.1-1.0 mL of appropriately diluted sample directly into a series of sterile petri dishes. Then pour 12-15 mL of melted agar medium which has been cooled to 44-46° C into each dish. Mix the agar and inoculum by rotating the dishes carefully. Be sure not to spill the contents. To ensure complete mixing, use a sequence of rotation similar to the following: five rotations clockwise, five counterclockwise, and five from front to back.

Set the plates on a level surface to harden. Then invert and incubate. After inoculation, choose the plates with well-isolated surface and subsurface colonies for counting. These should have 30-300 colonies each.

STAINING

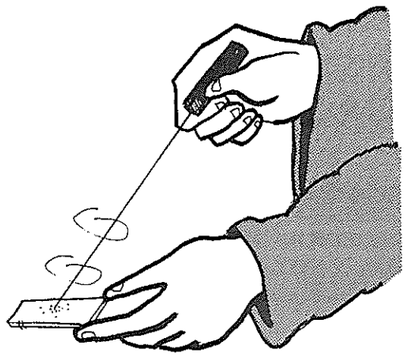
An important tool to be used when examining and identifying organisms under the microscope is staining. Sometimes a simple stain is used to highlight the organisms and set them off from the background medium. A simple stain affects all organisms in the same way. More often, however, a differential stain is used which will react one way with one type of organism or cell structure, and react another way with a different cell type or structure. A differential stain not only makes the organisms easier to observe under the microscope, but also separates the potential population into various categories for the purpose of identification.

PREPARING BACTERIAL SMEARS

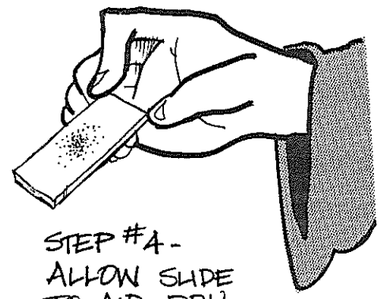


STEP #1 - OBTAIN SMALL AMOUNT OF BACTERIAL MATERIAL. (FOR BROTHS, 2-3 LOOPFULS ARE SUFFICIENT.)

STEP #2 - IF BACTERIA ARE FROM A SOLID MEDIUM, MIX WITH A DROP OF LABORATORY PURE WATER.



STEP #3 - EVENLY SPREAD BACTERIAL SUSPENSION OVER AN AREA THE SIZE OF A QUARTER.



STEP #4 - ALLOW SLIDE TO AIR DRY

STEP #5 - FIX SMEAR BY PASSING SLIDE THROUGH A FLAME SEVERAL TIMES. **DO NOT OVERHEAT.**

ONCE A SMEAR IS PREPARED AND STAINED, IT CAN BE KEPT IF NECESSARY FOR FUTURE REFERENCE.

Thoroughly clean the glass slides to be used in staining before use. First wash them in detergent and water, then rinse them with 95 percent alcohol. Allow the slides to dry, then flame the "up" sides of the slides for a moment in a Bunsen burner flame and allow to cool.

Once the slides are clean, a bacterial smear can be prepared. For a broth culture, simply transfer two or three loopfuls of the culture medium from an inoculating loop to the surface of the slide and distribute this over a small area. If the culture medium is solid, transfer a very small portion of bacterial growth and mix it on the surface of the slide with a drop of laboratory pure water. When the smear dries, it should look faintly white, like a hard water stain.

When the smear has been prepared, it must be fixed to prevent it from washing off during the staining process. Fixation also stops enzymatic action which might destroy cells prior to observation. To fix the smear, take the slide in a slide holder and pass it through a Bunsen burner flame several times. The glass should not be allowed to become too hot to touch.

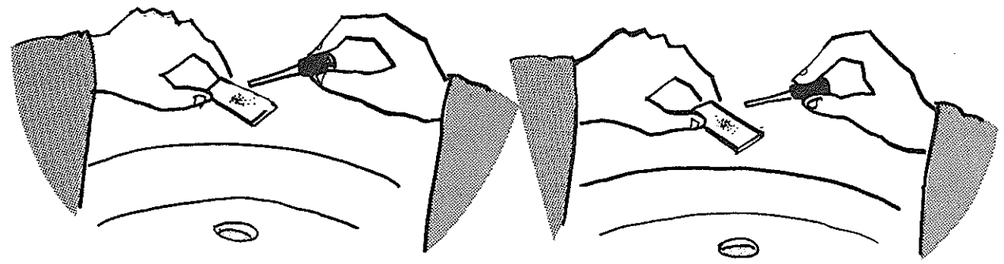
To clean a slide which has been used for a bacterial smear or stain, place several drops of xylene on the slide over a sink. Then rub off the smear using detergent and water. Repeat this process at least once to ensure complete removal of all organisms. Finally, clean the slide as usual for reuse.

GRAM STAIN

IN THE GRAM STAIN, GRAM POSITIVE ORGANISMS WILL APPEAR BLUE, WHILE GRAM NEGATIVE ORGANISMS WILL APPEAR RED.

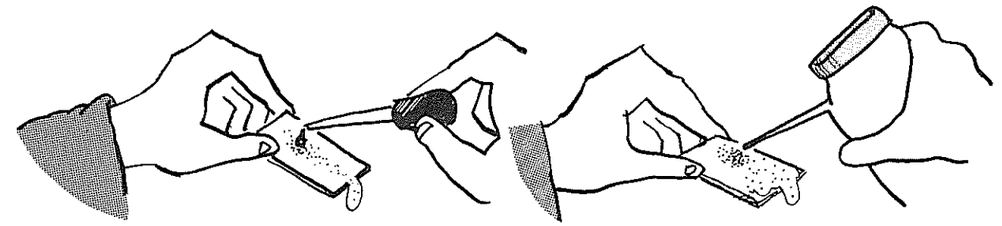
The most widely used differential bacteriological stain is the Gram stain, named after its originator, Dr. Christian Gram. The Gram stain is an extremely useful classification tool, particularly when combined with information on the shape of the cell. Four different reagents are employed in the Gram stain, and correct application of the procedure requires a great deal of skill from the analyst. Crystal violet (also called gentian violet) is applied as the first or primary dye. This imparts color to all organisms in the smear. The second reagent is a diluted iodine solution (usually called Gram's iodine or sometimes Lugol's iodine). This acts as a mordant -- strengthening the bond between the dye and its substrate. The resulting complex, which occurs only in Gram positive organisms, is relatively insoluble in alcohol. The third reagent is a decolorizer which dissolves and removes the primary stain from cells which do not bind firmly with the primary dye. The decolorizer is a mixture of equal volumes of acetone and 95 percent ethyl alcohol. The cells which cling to the primary stain despite the action of the decolorizer are Gram positive, while those which yield up the primary stain are Gram negative. The fourth reagent is a red dye called safranin. This is a counterstain which will be taken up by Gram negative organisms which lose their primary stain during decolorization. Without the counterstain, these organisms would be difficult to observe. Gram positive organisms will therefore appear blue and Gram negative organisms will appear red.

PREPARING GRAM STAINS



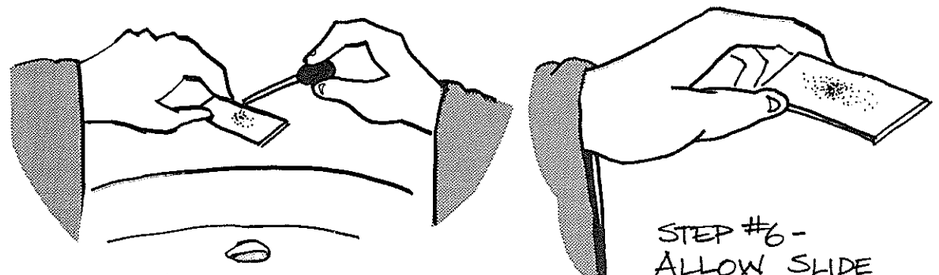
STEP #1-
FLOOD SMEAR WITH GRAM'S CRYSTAL VIOLET FOR 1 MINUTE. POUR OFF EXCESS AND RINSE WITH LABORATORY PURE WATER.

STEP #2-
FLOOD SMEAR WITH GRAM'S IODINE FOR 1 MINUTE. POUR OFF EXCESS AND RINSE WITH LABORATORY PURE WATER.



STEP #3-
ADD DECOLORIZING SOLUTION DROP BY DROP UNTIL COLOR JUST STOPS COMING OFF SLIDE.

STEP #4-
IMMEDIATELY RINSE SLIDE WITH LABORATORY PURE WATER TO STOP DECOLORIZING SOLUTION.



STEP #5-
FLOOD SMEAR WITH GRAM'S SAFRANIN SOLUTION FOR 10-20 SECONDS. RINSE WITH LABORATORY PURE WATER.

STEP #6-
ALLOW SLIDE TO AIR DRY.

limitations include samples containing large quantities of colloidal materials or suspended solids (iron, manganese or alum flocs or clay); the presence of algae; large non-specific bacterial populations; industrial wastes containing zinc, copper or other heavy metals; toxics such as chlorine or phenols; and indicator organisms which have been stressed in the environment.

SETTING UP

DO NOT OVERHEAT FORCEPS AS THIS WILL DESTROY THE TEMPER OF THE METAL

Open a sterile plastic petri dish, using the butt-end of the forceps to lever the halves of the dish apart. Dip the forceps in a beaker of alcohol and burn off the excess, being careful not to overheat the forceps and destroy the temper of the metal. Use the forceps to pick out one sterile absorbent pad from the packet. Touch the pad only at the edge. Place the pad in the bottom half of the petri dish and replace the forceps in the beaker of alcohol. Aseptically pipet 2 mL of broth medium from the flask containing the medium to be used. Add 1.8-2.0 mL of this broth to the absorbent pad. Saturate the pad but do not flood it. Tip the dish to drain off any excess broth. Loosely reassemble the petri dish halves, label them and arrange the dishes in order of use. (If an agar medium is to be used, the procedure is similar except that 5-6 mL of melted agar is added to each dish, to a depth of 2-3 mm.)

If a vacuum pump is to be used, turn it on and let it run for about 5 minutes to lubricate the motor. Then turn the pump off, attach a 1 L water trap flask and place the bottom portion of the sterilized membrane filtration apparatus in the flask. Turn the pump on and run for another 5 minutes, then turn it back off. Take the forceps from the alcohol and again burn off the excess. Pick one membrane filter from the package, handling it only along the outermost edge to avoid damaging the filter, and place it grid-side up on the support screen of the filter base. Be sure the filter is centered on the screen. Return the forceps to the beaker of alcohol. Set the funnel portion of the filtration apparatus onto the base, being careful not to damage or dislodge the membrane filter. Lock or tighten the funnel into place.

ADDING THE SAMPLE

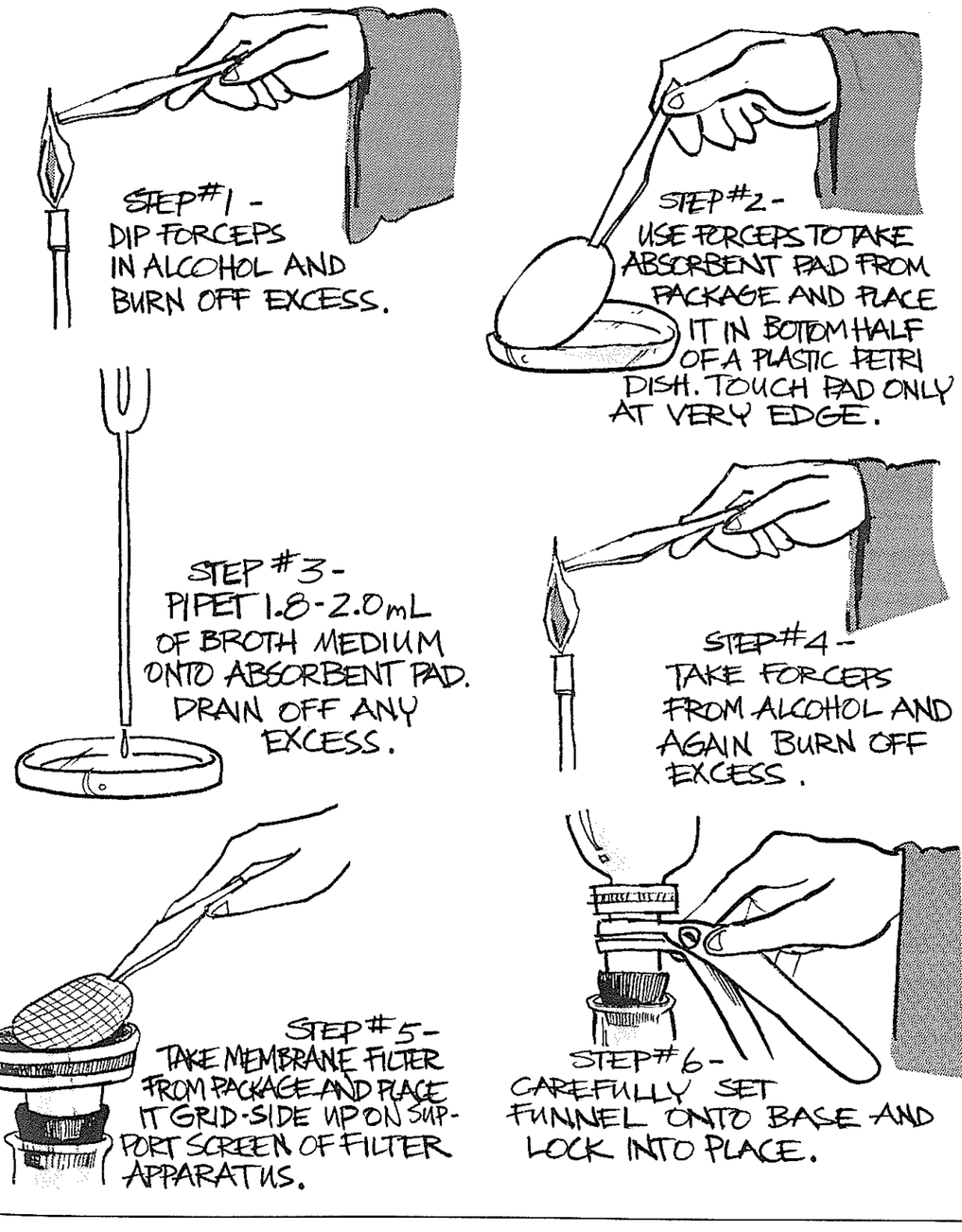
Shake the sample bottle (or diluted sample) vigorously about 25 times to ensure complete dispersion of the microorganisms. Make sure the vacuum pump is turned off before adding sample to the filtration funnel. How the sample is to be measured into the funnel depends upon the volume required:

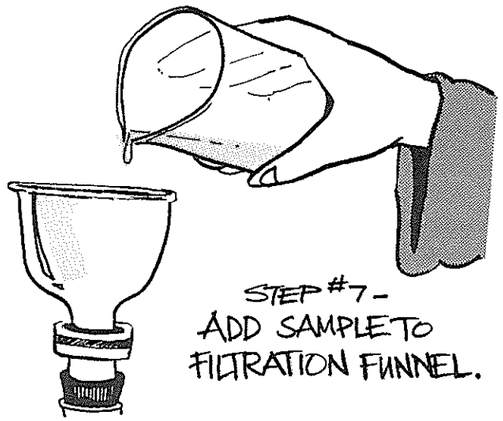
-- For 100 mL volumes of potable water: Measure directly in a precalibrated filtration funnel.

-- For volumes of 20 mL or more: Measure the volume in a sterile graduated cylinder and pour the contents into the filtration funnel. Rinse the cylinder at least twice with sterile dilution water and add the rinse water to the funnel.

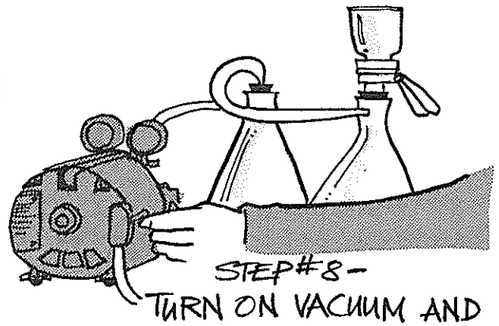
-- For volumes of 10-20 mL: Measure the volume with a sterile 10 or 20 mL pipet and release it into the filtration funnel.

PERFORMING MEMBRANE FILTRATIONS

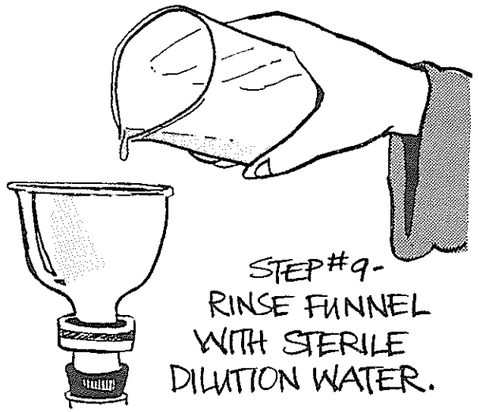




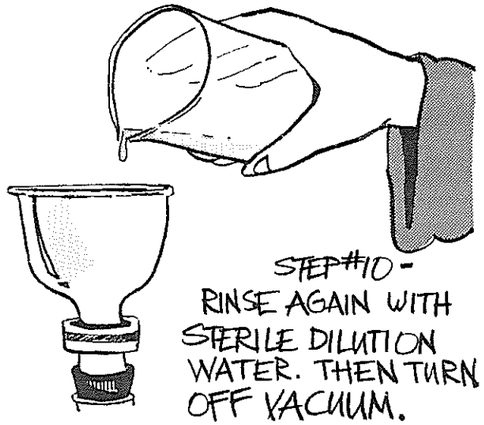
STEP #7 -
ADD SAMPLE TO FILTRATION FUNNEL.



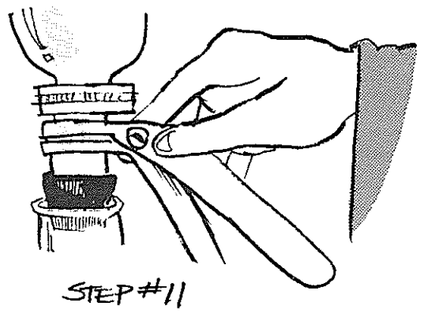
STEP #8 -
TURN ON VACUUM AND DRAW SAMPLE THROUGH FILTER.



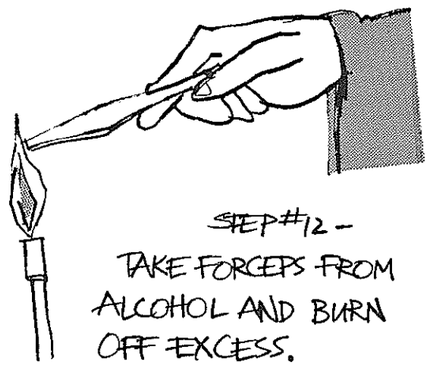
STEP #9 -
RINSE FUNNEL WITH STERILE DILUTION WATER.



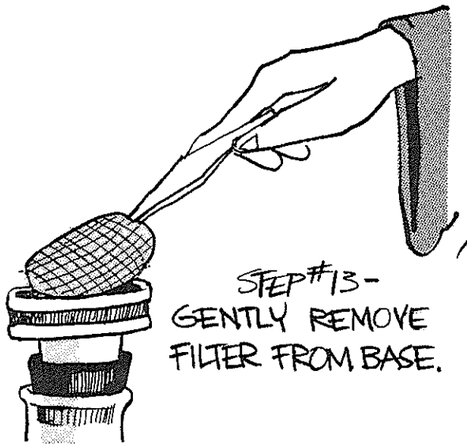
STEP #10 -
RINSE AGAIN WITH STERILE DILUTION WATER. THEN TURN OFF VACUUM.



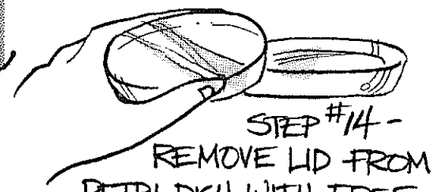
STEP #11
UNLOCK FILTRATION FUNNEL AND REMOVE IT.



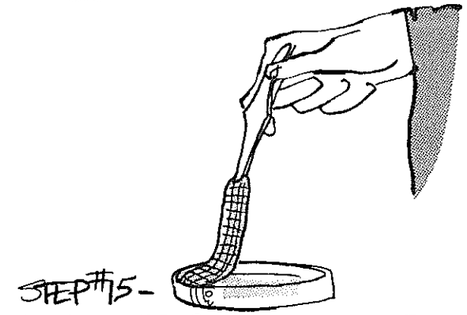
STEP #12 -
TAKE FORCEPS FROM ALCOHOL AND BURN OFF EXCESS.



STEP #13 -
GENTLY REMOVE FILTER FROM BASE.

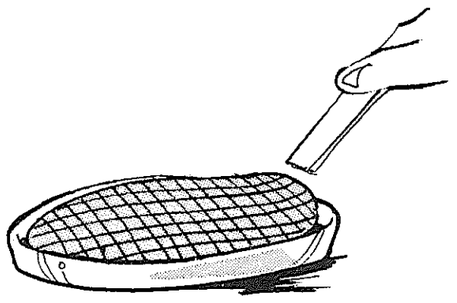


STEP #14 -
REMOVE LID FROM PETRI DISH WITH FREE HAND.

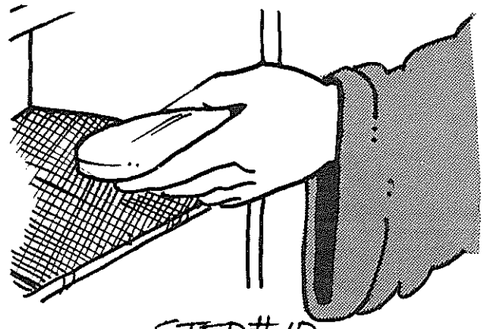


STEP #15 -
PLACE EDGE OF FILTER ON EDGE OF ABSORBENT PAD.

STEP #16 -
ROLL FILTER ONTO PAD WITHOUT TRAPPING AIR BUBBLES.



STEP #17 -
GENTLY TAMP LOOSE FILTER EDGE ONTO PAD.



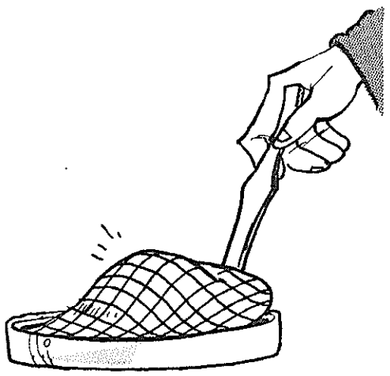
STEP #18 -
SEAL PETRI DISH LID, INVERT AND INCUBATE.

-- For volumes of less than 10 mL: Pour about 20 mL of sterile dilution water into the filtration funnel. Then measure the sample volume with an appropriately sized sterile pipet and add this to the dilution water in the funnel. The extra dilution water helps ensure even distribution of the sample over the membrane surface.

Do not attempt to directly measure sample volumes of less than 0.1 mL. Where such a volume is needed, first prepare one or more dilutions with sterile dilution water. Then proceed with an appropriate larger volume of the diluted sample as indicated above.

FILTERING THE SAMPLE

USE THE BUTT-END OF THE FORCEPS TO SEPARATE PLASTIC PETRI DISH HALVES



IF AN AIR BUBBLE BECOMES TRAPPED UNDER A MEMBRANE FILTER, PEEL THE FILTER OFF AND TRY AGAIN

Once the correct sample volume is in the funnel, turn on the vacuum and allow the liquid to be completely drawn through the filter. Leave the vacuum on while rinsing the sides of the filtration funnel with a volume of sterile dilution water at least equal to the volume of sample just filtered. Repeat the rinse, then turn off the vacuum. Loosen the halves of the correct petri dish and place the dish near the filtration apparatus. Unlock or unclamp the filtration funnel and remove it from the base, being careful not to dislodge the membrane filter. (If an ultraviolet sterilizer is used to sterilize the funnel between filtrations, place the funnel in the sterilizer at this time to ensure at least 2 minutes of exposure for complete sterilization before reuse. Use goggles, glasses or an enclosed UV chamber to protect your eyes from UV radiation.)

Pick up the forceps and burn off the excess alcohol. Carefully slip the tip of one of the forceps blades under the edge of the membrane filter. Gently grip the filter and peel it off the base unit. Do not excessively bend the edge of the filter with the forceps. Remove the lid of the petri dish and place the edge of the filter on the edge of the absorbent pad (or agar). Roll the filter onto the pad to avoid trapping air bubbles between them. If air bubbles do occur, peel the membrane filter off and reseat it. Only when the filter is correctly placed on the pad should you release it with the forceps. Gently tamp the loose filter edge down onto the pad and return the forceps to the alcohol beaker. Seal the lid on the petri dish, invert the dish and incubate.

For subsequent filtrations, the same filtration funnel can be used without resterilization only if the same sample (or sample dilution) is to be used. Otherwise, the funnel must either be resterilized or another sterilized funnel obtained. If more than 30 minutes elapse between filtrations, a sterilized funnel must be used regardless of the sample involved. Similarly, the same pipets or graduated cylinders can be used for subsequent measurements of the same sample (or sample dilution), provided that the volume being measured increases from one filtration to the next. If the volume decreases, sterile pipets or cylinders must be used each time. Again, if more than 30 minutes elapse between measurements, sterile pipets and cylinders must be used regardless of the relative volumes involved.

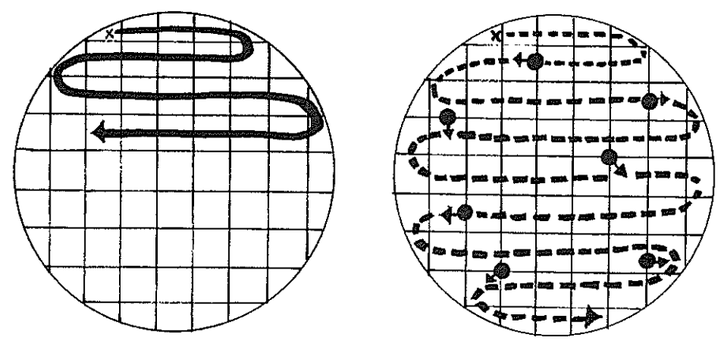
COUNTING COLONIES

At least 20 colonies must be present on a filter for the count to be statistically significant. The maximum number depends upon the particular test: 80 colonies for total coliform; 60 for fecal coliform; and 100 for fecal streptococci. If possible, choose sample volumes for counting which produce colonies within these ranges and discard sample volumes outside the appropriate range. In addition to these limits, the total coliform test also has a maximum limit of 200 colonies of all types on any given filter. This includes colonies identified as total coliforms (and therefore falling with the 20-80 colony range) and background, non-coliform colonies. Background colonies are rarely a problem in the other two tests since the growth media are much more selective.

To count colonies, take the plates from the incubator and remove the lids while the plates are still inverted. Then turn the plates upright and place the first one on the stage of the stereomicroscope. Adjust the magnification to 10x and position the fluorescent light source to the proper angle. Examine the entire surface for typical indicator colonies. As you do this, take into consideration the approximate number of colonies; the shapes, sizes and sheen of the colonies; and the presence of any background colonies. After the entire series of plates has been examined in this manner, select the plates which are actually to be counted. Count only those colonies which are typical of the indicator organism specific to the particular medium used -- do not include atypical colonies. Count these typical indicator organism colonies following the membrane grid in a regular, non-overlapping pattern. Where a colony is in contact with the lines of the grid, adopt a consistent policy of assigning it to the last square counted of those squares which the colony touches. This procedure will help reduce counting errors where a colony might otherwise be counted twice.

SOME PLATES SHOULD SIMPLY BE RECORDED AS "TNTC" (TOO NUMEROUS TO COUNT)

PATTERN FOR COUNTING MEMBRANE FILTERS



COUNT THE GRIDS THIS WAY... AND...ASSIGN BORDERLINE COLONIES THIS WAY.

When counting colonies on a membrane filter, the assumption is that each organism on the membrane surface results in one bacterial colony. From this count, the number of indicator organisms per 100 mL of water can be calculated using the following formula:

$$\text{Number of indicator organisms/100 mL} = \frac{\text{number of indicator organisms counted}}{\text{volume of sample filtered (mL)}} \times 100$$

If more than one plate contains a suitable number of organisms for an acceptable count (that is, within the colony limits for the particular indicator organism involved), determine the number of indicator organisms per 100 mL for each plate using the formula above, then average the results arithmetically for the final, reported value. This procedure works whether the plates are replicates of the same dilution, or whether different dilutions are involved.

If all the plates fall below the minimum colony level, count the plate having the largest number of colonies. Calculate the number of indicator organisms per 100 mL from this and report as an estimated count.

If no colonies are present on any of the plates, choose the plate representing the largest volume of sample. Determine the number of organisms per 100 mL for this filtration volume as if one colony had appeared on the plate. Report the results as being less than the value obtained.

If all the plates fall above the maximum colony level, count the number of colonies on the plate representing the smallest sample volume. Calculate the number of indicator organisms per 100 mL from this and report as an estimated count.

If the colonies are too numerous to count (TNTC) on every plate, choose the plate representing the smallest sample volume. Calculate what the number of indicator organisms per 100 mL would have been had there been only the maximum acceptable number of organisms on that plate. Report the final value as being greater than this number.

Quality Assurance

Quality assurance helps a laboratory ensure that its results adequately reflect the real world, that the numbers it reports reasonably approximate fact. Of course, no analytical result is ever entirely correct. Precision and accuracy are always limited. An element of chance inevitably enters the picture. But the values obtained must lie within accepted limits of error.

Quality assurance is more than a collection of steps or procedures to be carried out blindly. It is a state of mind, a basic approach to the whole issue of laboratory analysis. Its purpose is to reduce the level of uncertainty associated with reported analytical results. In the bacteriological laboratory, this uncertainty tends to be amplified by the nature of the living organisms involved, which continually change and adapt in response to their environment. A bacteriological quality assurance program must, therefore, emphasize control of laboratory operations and analytical procedures.

The bacteriological laboratory is under a further limitation, however. Because true test values cannot be provided for microbial parameters, microbiologists do not yet have the advantages available to their counterparts in chemical laboratories. There are no analytical standards, quality control charts or spiked samples in the microbiological laboratory. Because known values cannot be applied, the analyst must pay rigorous attention to those variables which are under his control, including sampling, personnel, analytical technique, materials, supplies and equipment.

DOCUMENTATION

WITHOUT ADEQUATE RECORDS, LABORATORY ANALYSIS BECOMES A GAME OF CHANCE

Laboratory records form the essential link between an analyst's two key functions: performing analyses and interpreting results. Without adequate records, a laboratory's results are worthless. More specifically, unless proper records are kept of the quality assurance checks and procedures employed, there is no proof of their performance, no value in future reference, and for all practical purposes, no quality assurance program in operation. It is, therefore, necessary to understand what records should be kept, the purpose for keeping them and how they should be used.

A laboratory operating manual should be prepared describing the operation, maintenance and quality control of all the laboratory procedures and analyses used. The manual should include review mechanisms and frequencies as a part of the quality assurance program.

A sample log is used to record information on samples received in the laboratory. This information should include details of sample identification and origin, necessary chain-of-custody information, analyses performed and final results.

A quality control record should be maintained on media preparation; instrument calibration; purchase of supplies; and quality control checks on materials, supplies, equipment, instrumentation, facilities, and analyses.

A record of analytical quality control checks should also be maintained on positive and negative controls, sterility checks, single-analyst precision, precision between analysts, and use-test results from comparison lots of media, membrane filters and other supplies.

A general rule to follow in documenting quality assurance measures is to record everything. When a thermometer is calibrated against a certified standard, tag the thermometer with that information. Include the analyst's name or initials and the date. Record the process in a laboratory notebook. Record each pH meter calibration, each reading of an incubator or hot-air sterilizer temperature, each cleaning of a still or deionizing system. Tape tables or charts to each piece of equipment which must be calibrated, next to a test tube for holding a pen. Make record keeping as simple and easy as possible, but see that records are maintained. Without thorough records, even a bench wizard is playing a numbers game of chance with his results.

ANALYTICAL QUALITY CONTROL

Quality control procedures should be established for each of the analyses routinely performed in a laboratory. Such quality control procedures, as a whole, should account for 15 percent of laboratory personnel time. The following are among the procedures which should be instituted:

-- Duplicate Analyses: Run duplicate analyses on 10 percent of the known positive samples analyzed, with a minimum of one duplicate per month. The duplicates may be run as split samples by more than one analyst.

-- Positive Control Samples: Test a minimum of one pure culture of known positive reaction per month for each parameter tested.

-- Negative (Sterile) Control: Include one negative control with each series of samples using buffered water and the medium batch at the start of the test series and following every tenth sample. When sterile controls indicate contamination, data on samples affected should be rejected and a request made for immediate resampling of those waters involved.

-- Colony Counting by More than One Analyst: At least once per month, two or more analysts should count the colonies on the same membrane from a polluted water source. Colonies on the membrane should be verified and the analysts' counts compared to the verified count.

-- Check Analyses on Water Supply Program by State Laboratories: In a local laboratory, a minimum number of the water supply samples should be analyzed by the State laboratory. For example, laboratories that are required to test less than 100 samples per month should submit an additional 10 percent of the number to the State laboratory for analysis. Water systems with sample requirements above 100 per month should submit an additional 2 percent to the State Laboratory for analysis.

-- Reference Samples: Laboratories should analyze reference samples quarterly when such samples are available for the parameters being measured in the particular laboratory.

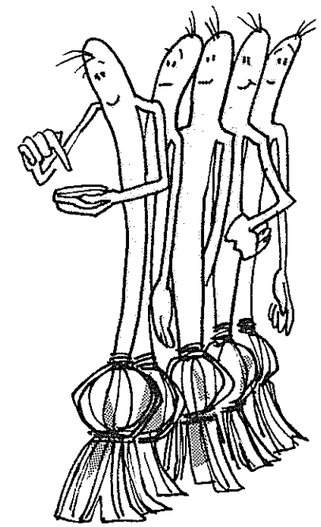
-- Performance Samples: Laboratories should analyze at least one unknown performance sample per year when such samples are available for the parameters being measured in the particular laboratory.

-- MF Verification: Five percent of the membrane filtration analyses performed should be verified, using the following procedures:

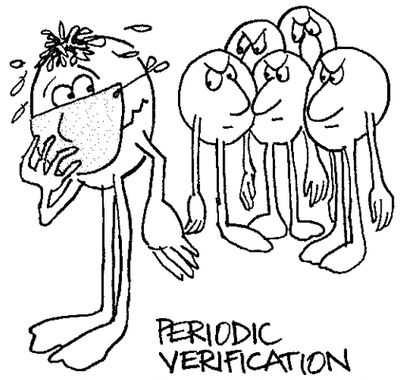
-- Total Coliforms: Pick at least 10 isolated sheen colonies from each sample. Transfer into lauryl tryptose broth. Incubate and read. Transfer positive tubes into brilliant green bile broth for verification of coliforms. Since samples from public water supplies with five or more sheen colonies must be verified, at least five colonies are picked from each positive potable water sample. The laboratory should make every effort to detect coliforms from samples with excessive non-coliforms on the membrane filter. Any sheen colonies appearing in mixed confluent growth must be verified.

-- Fecal Coliforms: Pick at least 10 isolated colonies containing blue to blue-green pigment and transfer to lauryl tryptose broth. Incubate and read. Transfer positive tubes to EC broth where gas production verifies fecal coliform organisms.

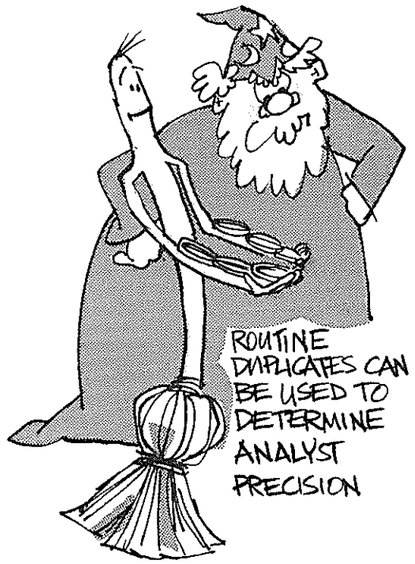
-- Fecal Streptococci: Pick at least 10 isolated pink to red colonies from MF or pour plates. Transfer to BHI agar or broth. After growth, perform catalase test. If negative (possible fecal streptococci) transfer growth to BHI and 40 percent bile broth tubes and incubate at 45° and 35° C, respectively. Growth at both temperatures verifies fecal streptococci.



NO TWO ANALYSTS COUNT COLONIES EXACTLY THE SAME... PERIODICALLY COMPARE AND VERIFY RESULTS OBTAINED BY DIFFERENT ANALYSTS FOR A SINGLE MEMBRANE FILTER.



PERIODIC VERIFICATION OF MEMBRANE FILTRATION RESULTS HELP PREVENT REPORTING OF FALSE POSITIVES



-- Measurement of Analyst Precision: If the routine work of the laboratory includes samples from different wastewaters, surface waters, water supplies or finished waters, the following steps should be accomplished for each type:

-- Step 1: Perform duplicate analyses on the first 15 typical samples with positive responses. Although each set of duplicates must be run by the same analyst, all analysts performing routine analyses should contribute a share of this initial data.

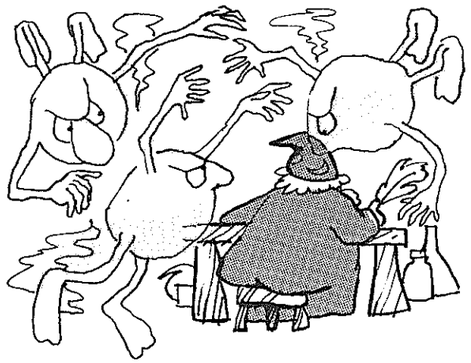
-- Step 2: Calculate the logarithms of results. If either of a set of duplicate results is zero, add 1 to both values before calculating the logarithms.

-- Step 3: Calculate the range (R) for each pair of transformed duplicates and the mean ($\bar{R}$) of these ranges.

-- Step 4: Thereafter, run 10 percent of routine samples in duplicate. Transform the duplicates as in Step 2 and calculate their range. If this range is greater than 3.27 $\bar{R}$, analyst precision is out of control and all analytical results since the last precision check must be discarded. The analytical problem must be identified and resolved before doing further analyses.

-- Step 5: In order that the criterion used in Step 4 be kept up-to-date, periodically repeat Steps 2 and 3 using the most recent sets of 15 duplicate results.

MONITORING LABORATORY CLEANLINESS

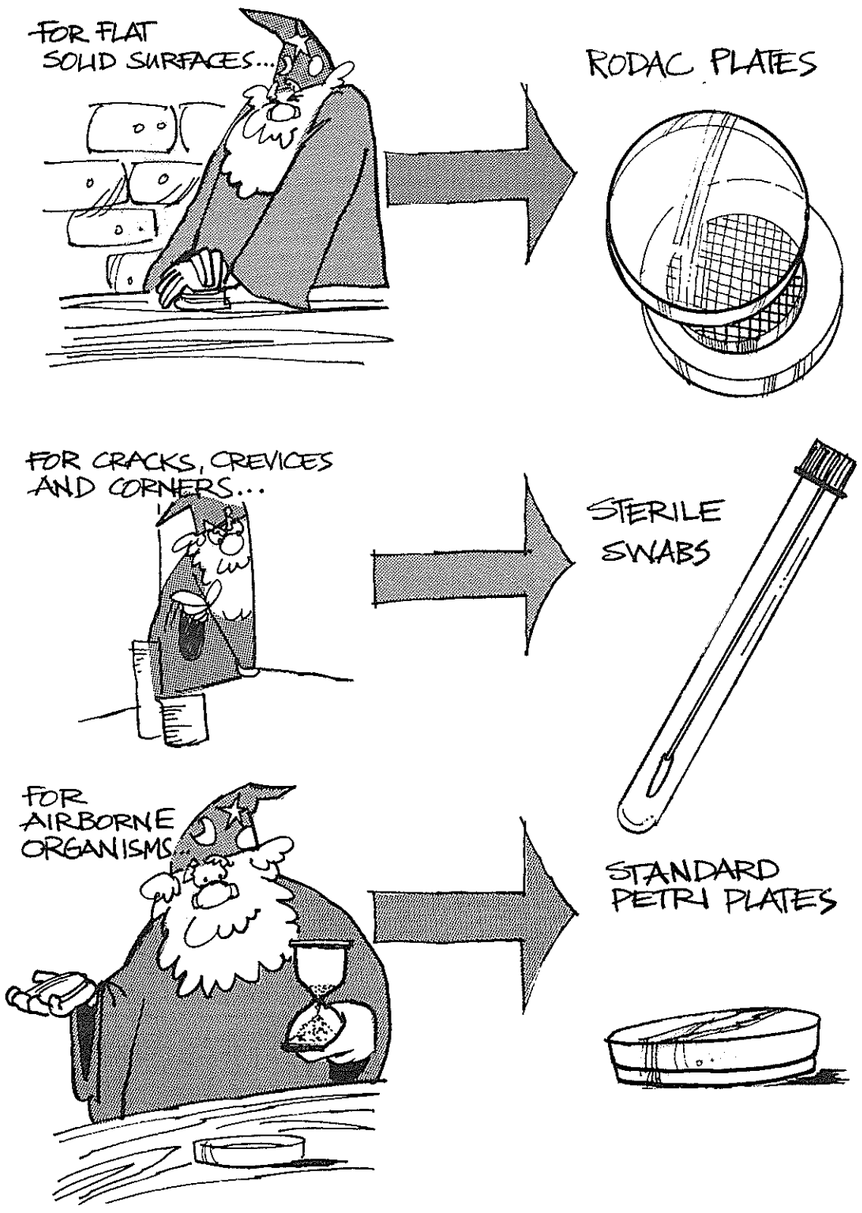


Rigid standards of cleanliness should be observed in the microbiological laboratory to reduce microbial populations in the work environment which might contaminate samples and cultures. It is impossible, however, to eliminate all such potential contaminants. The following tests are, therefore, designed to check the levels and types of bacterial populations which exist in the laboratory. Although absolute limits cannot be established for these bacteria due to incomplete recovery by the test procedures, the tests are useful as a relative measure of population changes over time.

RODAC Agar Plates: Microbial populations can be recovered from flat solid surfaces by RODAC (Reproducible Organism Detection and Counting) plates. The plates, which may be prepared in the laboratory or purchased prefilled with an appropriate agar, are embossed with Quebec-style grids for easy counting. The agar medium extends approximately 1 mm higher than the rim of the dish so it can be placed in direct contact with the surface to be monitored. After pressing the agar against the surface, the cover is replaced and the dish incubated. The medium used may be either general or selective. The resulting colonies are an indication of the bacteria present on the laboratory surface at the time of the test.

Swab Method: Bacterial popluations present in cracks, crevices, corners and rough surfaces can be monitored by the swab contact method. Moisten a sterile swab with a neutralizing buffer and rub it slowly over the surface area

MONITORING LABORATORY CONTAMINATION



to be monitored. Rinse the swab head in the neutralizing buffer between repeated contacts. After the final contact, cut off the swab head with sterile scissors and leave it in the rinse vial. Shake the vial vigorously to dislodge the bacteria on the swab head, then prepare pour plates with 1.0 and 0.1 mL aliquots of the rinse solution. Incubate the plates and count the colonies which result.

Air Density Plates: The numbers and types of airborne microorganisms can be determined by exposing petri plates for 15 minutes at points where inoculating, filtering, plating and transfers are being performed. The organisms recovered are those which might contaminate sterile media and equipment during routine microbiological analyses, thereby affecting test results. The colonies which result on the plates after incubation are approximately equivalent to the number of organisms settling from the air over a square foot of surface area each minute (a standard petri dish is roughly 1/15 sq. ft.). The microbial density should not normally exceed 15 colonies per square foot per minute. For routine monitoring, air density plates should be taken weekly during peak work periods.

LABORATORY GLASSWARE

Traces of cleaning solutions remaining on glassware may have a variety of effects on microbial populations. The general procedure for determining detergent suitability has already been described (Chapter 2). This procedure checks for toxic or inhibitory effects. Detergents may also leave acid or alkaline residues. A few drops of bromthymol blue or other suitable pH indicator solution can be used to spot-check glassware for such residues.

Sterilization procedures should also be periodically checked by adding an aerobic or anaerobic broth medium (such as lauryl tryptose or fluid thioglycollate broth) to a load of sterilized bottles, flasks or tubes. Check for the presence of microbial growth following incubation.

WATER SUITABILITY TEST

The water suitability test detects toxic or stimulatory effects of deionized or distilled water on bacteria. Reduction of 20 percent or more of an Enterobacter aerogenes culture in a chemically defined medium indicates water toxicity. An increase greater than 300 percent indicates growth stimulation.

The water suitability test should be performed at least annually on laboratory water supplies. However, the test is difficult and exacting to perform. It requires a four-day analysis period, ultrapure control water, very pure reagents and absolute cleanliness of equipment and supplies. Considerable analyst skill is also required. For these reasons, this test should not be performed in most microbiological laboratories. Rather, samples should be submitted to an appropriate outside laboratory which conducts the test on a more routine basis.

USE TESTS

Use tests are a pragmatic approach to evaluating the suitability of new batches or lots of media, membrane filters and laboratory pure water. Simultaneous pour plates or membrane filters are prepared on five water samples using both the new and the old shipment or lot of the one item to be checked. All other materials used in the test must be from single batches or lots to eliminate additional variables. When evaluating batches of laboratory pure water, prepare two sets of all reagents and media used, one with the old pure water batch, the other with the new batch.

Following incubation, compare the resulting bacterial colonies from the two lots for size and appearance. Record any observable differences between the two lots, regardless whether or not the difference in appearance is reflected in the colony count.

Count the colonies on each plate or filter and calculate the microbial density. Transform these results to logarithms. Calculate the mean and standard deviation of the transformed results for both lots. Compare the lots using the Student's t test. Use the critical value 2.78 representing the .05 significance level for five samples (four degrees of freedom). If the calculated t value for the two lots does not exceed 2.78, the lots produce significantly different results and the new lot may be unacceptable for use.

CULTURE MEDIA

As a general rule, unopened bottles of culture media should not be kept longer than two years. Opened bottles should be discarded six months after initial use. Limits for storing prepared media at 4° C are as follows:

-- MF broths in screw-cap flasks: 96 hours.

-- MF agars in plates with tight-fitting covers: two weeks.

-- Agar or broths in loose-capped tubes: one week.

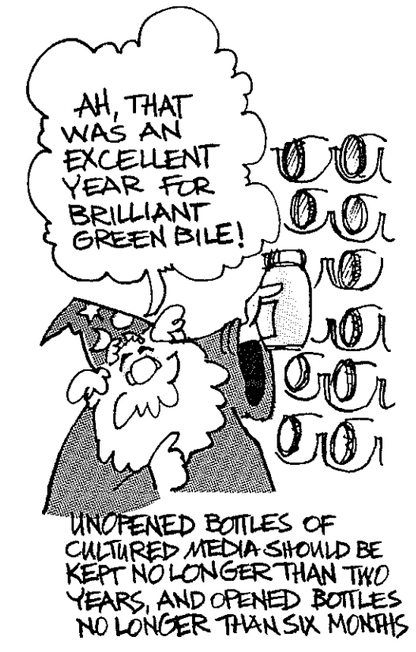
-- Agar or broths in tightly closed screw-cap tubes: three months.

-- Agar plates (non-MF) with loose-fitting covers, in sealed plastic bags: two weeks.

-- Large volumes of agar in tightly closed screw-cap flasks or bottles: three months.

When preparing culture media, note in the quality control record any unusual color development, darkening or precipitation of the media. Check the sterilization time and temperature for possible error. If the change in appearance is drastic, discard the medium and remake it. If the problem persists, remake the batch using a different lot of medium.

Always prepare broths or agars in containers twice the volume of the medium being prepared. Media should be stirred continuously during heating to avoid burning. Agar media are particularly susceptible to scorching and boilover. To prevent this, use a boiling water bath for small batches on a hot plate or burner. Use a combination hot plate and magnetic stirrer whenever possible for preparing media.



AGAR PLATES SHOULD BE FREE OF SURFACE IRREGULARITIES WHICH MIGHT INTERFERE WITH UNIFORM MICROBIAL DISTRIBUTION AND GROWTH

Check the effectiveness of autoclaving weekly using strips or ampules of Bacillus stearothermophilus spores. These spores are available commercially in ampules of growth-indicator media. Sterilization at 121° C for 12-15 minutes is sufficient to kill the spores. If growth of the spores occurs during incubation following autoclaving, the autoclave is not providing effective sterilization.

Agar plates which are to be used for streaking or spread plates should be kept slightly open for 15 minutes after pouring or after removal from refrigeration to evaporate excess moisture which could cause confluent growth of colonies. Agar plates used for membrane filtration and spread plates must be free of lumps, pock marks, bubbles, foam or other surface irregularities which would prevent good contact between the agar and the membrane filter or interfere with even distribution of microbial cells on spread plates.

Melted agars should be tempered in a water bath held at 44-46° C before contact with live cells. However, do not temper agar for longer than three hours.

Reduced media such as thioglycollate broth oxidize during storage. Heat these broths in boiling water before use for 20-30 minutes to reduce the medium.

For enrichment media, bring the base medium to 44-46° C before adding a labile constituent. Warm enrichments (such as blood or serum) to room temperature before adding them to a base medium. Once a labile material is added to a medium, prepare plates or tubes as soon as possible. Do not hold the batch medium in a water bath for more than 10 minutes.

When using commercially prepared media, incubate five percent of each batch of medium for two days at 35° C and examine for growth. Check each batch of medium when used by inoculating two tubes or plates with pure cultures of species producing positive and negative reactions for that medium. Test new batches of differential media by inoculating with organisms of known fermentative or other biochemical ability. Enrichment and selective media should also be tested for productivity of the desired microorganisms and inhibition of other microorganisms.

Laboratory Safety

Microbiologists face many of the same hazards as do their counterparts in analytical chemistry laboratories: fire, explosion, electrical shock, chemical burns and poisoning. But on top of all these, microbiologists face the added danger of much greater exposure to potentially pathogenic microorganisms.

Many infections of microbiological laboratory personnel appear to be caused by airborne pathogens. Among the common microbiological procedures which have been shown to produce aerosols (and are therefore capable of introducing pathogens into the laboratory air) are pipetting into petri dishes and flasks, opening lyophilized culture ampules, opening culture containers, inserting a hot loop or needle into a culture container and removing the cover from a blender after mixing a sample.

In addition to infection from airborne pathogens, microbiological laboratory personnel also face such sources of infection as accidental inoculation with syringes and needles, accidental oral aspiration of infectious material through a pipet, cuts and scratches from contaminated glassware, and spilling or splattering of pathogenic cultures on floors, table tops and other surfaces.

Unfortunately, these infections cannot normally be blamed on ignorance of proper procedure. The vast majority of reported cases of microbiological laboratory infection occur among highly trained personnel, according to a study by the Environmental Protection Agency. The inference is that carelessness, not ignorance, is at fault.

DEVELOPING A SAFETY PROGRAM

There are many rationalizations for failing to implement safety procedures, including excessive work loads and inflexible time schedules. However, it is the laboratory management's responsibility to ensure that an adequate safety policy is developed and enforced.

The first step in developing a laboratory safety program is to prepare a safety policy. This is a written statement from the head of the laboratory establishing safety as a concern which must not be ignored. Such a policy, if prepared in good faith and sincerity, commits laboratory management to pursue standards of safety with the same rigor as overall laboratory production, quality and profit.

THE SAFETY COMMITTEE SHOULD CONDUCT PERIODIC INSPECTIONS TO ENSURE COMPLIANCE WITH SAFETY REGULATIONS

The task of implementing the safety policy falls to a safety committee. Ideally, this committee should represent a broad cross section of laboratory personnel. The members of the committee should jointly possess a wide knowledge of the functions and activities of the laboratory. This knowledge, when combined with appropriate instructional tools and resources, will enable the committee to carry out the following duties:

-- Issue rules and regulations. The committee is responsible for developing and issuing specific written rules and regulations for the safe operation of the laboratory.

-- Conduct safety inspections. A critical function of the safety committee is to find and correct conditions or activities in the laboratory which could result in accident or injury.

-- Investigate and record accidents. The safety committee should keep a permanent record of all accidents within the laboratory and investigate accidents of unusual severity. The causes of serious accidents should be determined, preventative measures formulated and improved safety practices implemented to prevent such accidents from happening again.

-- Promote safety awareness. The safety committee should promote safety awareness among laboratory personnel and encourage active participation in carrying out the safety program. An essential element of this is to conduct safety training programs to ensure that all employees are familiar with the safety procedures to be incorporated into their jobs.

-- Act as liaison between employees and management. Just as employees must be informed of management's commitment to a safety program, so management must be informed of particular hazards or problems encountered by the laboratory staff. Communication must flow both ways for a laboratory safety committee to be effective.

RULES FOR PERSONAL SAFETY

Among the rules which should be adopted by the safety committee are several involving personal conduct and clothing. These include the following:

-- Each laboratory worker should familiarize himself with the safety procedures relevant to the operations he performs, as well as the locations of all safety equipment.

These procedures should then be followed strictly and the necessary equipment used.

-- Laboratory work must be recognized as a serious and potentially dangerous endeavor and each analyst should conduct himself accordingly at all times.

-- Smoking, eating and drinking should never be permitted in the laboratory, nor should laboratory vessels ever be used to hold food or beverages. Do not keep food or drinks in the laboratory refrigerator or cold room and do not brew tea or coffee in the laboratory area.

-- Coats, hats, jackets and other items of personal clothing should be stored outside the microbiological laboratory. Never mix laboratory clothes and street clothes in the same locker.

-- Wear a non-flammable laboratory coat or gown in the laboratory at all times. If clothing becomes contaminated, autoclave it before laundering. Do not wear laboratory clothing in clean areas or outside the building. Avoid wearing open-toed or other extreme shoe styles which inadequately protect your feet.

-- Wear goggles or safety glasses to protect the eyes from flying pieces of broken glass, splattered chemicals or cultures, ultraviolet irradiation and other hazards.

-- Wash your hands carefully after laboratory and field activities using a germicidal soap.

-- Use forceps or rubber gloves when there is a significant danger of contamination, such as during the clean-up of pathogenic material.

-- Never touch your face, lick labels, or put pencils or other materials in your mouth while in the laboratory.

-- Keep conversation to a minimum when performing bench work to reduce the chances of self-infection or loss of analytical data.

-- Keep reading matter, surplus materials and unnecessary equipment out of the laboratory area.

-- Laboratory and field personnel who handle polluted samples should be vaccinated against typhoid, tetanus and polio.

AVOID CLOWNING AROUND IN THE LABORATORY

LABORATORY FACILITIES & EQUIPMENT

In addition to the above rules for personal conduct and clothing, a number of other restrictions should be observed with regard to laboratory facilities and equipment:

-- Limit traffic through the work areas. Sample receiving, office and reception areas should have external access so visitors and other incoming traffic do not have to enter the laboratory work areas.

-- Treat all reagents, cultures and samples as if they are potentially toxic or pathogenic. In a microbiological laboratory, the risks are particularly great because microorganisms are produced in very large numbers.

-- Never pipet polluted water, wastewater or other potentially infectious or toxic fliuds by mouth. Some laboratories allow mouth-pipetting of potable water, provided pipets are plugged with non-absorbent cotton. Other labo-

Commandments of the Microbiological Laboratory

1. Thou shalt not smoke, eat or drink in the laboratory.
2. Thou shalt not behave in the manner of the court jester while in the laboratory.
3. Thou shalt wear a laboratory coat or gown at all times.
4. Thou shalt wear goggles, safety glasses or other eye protection.
5. Thou shalt treat all reagents, cultures and samples as potential toxins or pathogens.
6. Thou shalt keep all materials away from thy face while in the laboratory.
7. Thou shalt not pipet by mouth.
8. Thou shalt disinfect work surfaces before and after microbiological operations.
9. Thou shalt wash thy hands with germicidal soap after all microbiological operations.
10. Thou shalt limit thy conversation and thy coming and going while in the laboratory.

ratories forbid any mouth-pipetting at all, requiring a bulb or other mechanical device to be used at all times.

-- During culture work, use a hooded Bunsen burner or shielded electric incinerator to protect against splattering.

-- Allow sufficient bench space for each analyst and keep it clear and uncluttered for maximum efficiency and safety.

-- All culture work should be performed in a biohazard hood to protect cultures and workers.

-- Kitchen-type blenders should not be used for mixing materials containing infectious agents. Instead, use safety blenders in which such materials may be mixed without disseminating infectious aerosols.

-- Interpose suitable traps or filters when using vacuum lines to ensure that infectious agents do not enter the system.

-- When opening lyophilized ampules, wrap the ampule in disinfectant-soaked cotton before breaking to avoid releasing potentially infectious aerosols.

-- Freezers, ice chests and refrigerators should be cleaned periodically to remove any broken ampules, tubes or other containers of infectious agents. If pathogenic cultures are involved, wear rubber gloves during cleaning. Respiratory protection should also be used if there are easily disseminated agents such as actinomycetes and fungi involved.

ALLOW SUFFICIENT BENCH SPACE FOR EACH ANALYST

Another area which should be addressed when establishing rules for the microbiological laboratory is that of disinfection and sterilization. Among these rules should be the following:

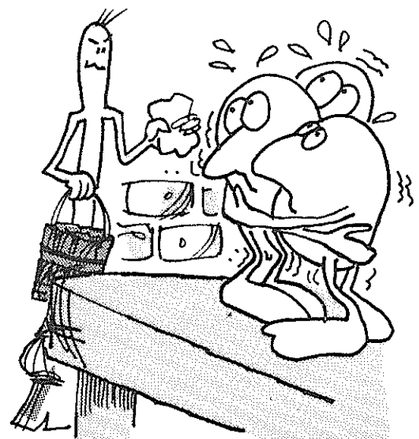
-- Table tops, carts and other work surfaces should be disinfected before and after performing microbiological operations. Keep a bottle of disinfectant and gauze squares or towelling available in the laboratory for routine or emergency use.

-- A disinfectant which specifies germicidal activity against the organisms most often encountered in the laboratory should be used. For general use, organo-iodine complexes, quanternary ammonium compounds, phenolics and alcohols which are effective against vegetative bacteria and viruses are recommended. These disinfectants are not sporocidal, however. If spore-forming bacteria are encountered, use formaldehyde or formaldehyde/alcohol solution. Do not use mercury salts, compounds containing chlorine, or products intended for home use in the laboratory.

-- Notify the laboratory supervisor immediately if a culture or infectious material is spilled, then disinfect and clean up the area.

-- Viable cultures and contaminated materials should never be disposed of down the sink. Never leave infectious materials or equipment unattended during use.

-- Pipets should be completely immersed in a container of disinfectant immediately after use. Cultures and contaminated materials should be placed in color-coded biohazard



DISINFECT THE BENCH AREA BEFORE AND AFTER EACH MICRO-BIOLOGICAL OPERATION

bags and sealed immediately after use. Autoclave the disinfectant containers of pipets and sealed biohazard bags together prior to cleaning or disposal.

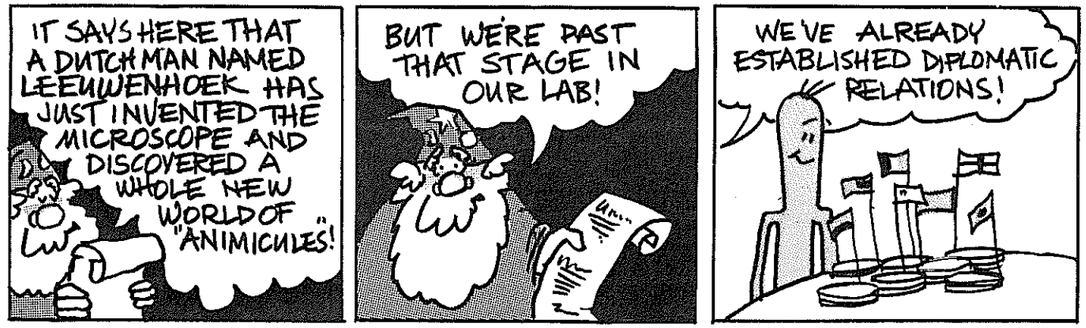
-- When autoclaving, place plastic items in cans separate from glassware to prevent fusing of plastic around glass items.

-- Contaminated items should be marked as such before removal from the laboratory for autoclaving. Use temperature-sensitive tape which will indicate when such items have been decontaminated by autoclaving.

-- Wet-mop laboratory floors with a disinfectant weekly. If vacuum cleaners are used in the microbiological laboratory, they should be equipped with high-efficiency exhaust air filters. Use bacteriostatic floor waxes if available.

DISINFECTANT CONCENTRATIONS FOR NORMAL USE

Disinfectant	Use Concentration
Organo-Iodine Complexes	100-150 mg/L
Quaternary Ammonium Compounds	700-800 mg/L
Phenolics	½-1 %
Alcohol	70 %
Formaldehyde	8 %
Formaldehyde in 70% Alcohol Solution	8 %



The Microbial Bestiary

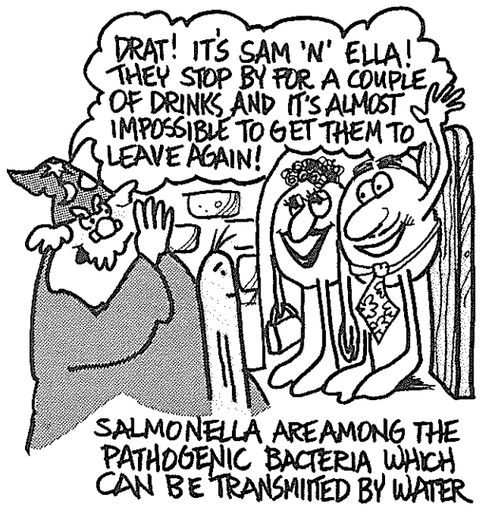
The effects of microorganisms form a critical aspect of water and wastewater management. Microorganisms contribute to the cycling of materials used by all living things. These cycles make up the natural purification processes which have cleansed the waters of our planet since life began, and which now form the basis of modern biological wastewater treatment.

However, some microorganisms also create nuisances in water and wastewater operations. In water treatment, they may be responsible for taste and odor problems, discoloration of finished waters and even blockage of pumps and raw water intakes. Wastewater treatment problems may include "bulking" sludge, sour digestors and turbid effluents.

Also, a few microorganisms cause diseases in man and domesticated animals. Primary diseases which may be caused by waterborne microorganisms include typhoid fever, cholera, dysentary, polio and infectious hepatitis. Other less specific problems may not be caused by any one microorganism in particular, but rather by a change or increase in the total population of organisms inhabiting a drinking water. Travelers often notice this when exposed to different water supplies. Normally nonpathogenic organisms may also sometimes cause severe infections in persons whose systems are susceptible or already out of balance, such as small children, the elderly and the ill. The organisms involved in these kinds of diseases are called secondary pathogens.

Now let's take a brief look at the amazing bestiary which makes up the world of waterborne microorganisms.

BACTERIA



The group of organisms of primary interest in aquatic biology are bacteria. Bacteria are extremely small, ranging from approximately 0.5 to 5 micrometers. At least 500 billion bacteria can be packed into a volume of one cubic centimeter. Under normal conditions, an adult human excretes between 100 billion and 100 trillion bacteria in feces each day.

Bacteria occur in a variety of shapes, including rods, spheres, commas and spirals. They may occur singly or aggregate in pairs, packets or filamentous chains. Filaments may be either simple or branched. Other aggregates may take the form of an egg, a star, a ribbon, a net or a sheet. The cell shape and form of aggregation are characteristic for particular organisms.

Most aquatic bacteria are motile -- capable of directed movement. Most of these have some form of flagella, although some move by creeping along solid surfaces. The majority of aquatic bacteria are also saprophytic, living on organic material from dead plants and animals. A few are capable of either carrying out photosynthesis or oxidizing inorganic compounds to obtain energy and reducing carbon dioxide to synthesize organic material.

True aquatic bacteria are able to use the extremely small concentrations of nutrients typically found in natural waters to grow and multiply. Other bacteria may also find their way into water sources from soils and the intestinal tracts of animals. Some of these organisms are also capable of living in water, while others only survive in water for short periods of time.

Bacteria of particular significance to water resources include those of the genera Nitrosomonas, which oxidize ammonia nitrogen to nitrite, and Nitrobactor, which oxidize nitrite to nitrate. Desulphovibrio desulphuricans reduces sulfate to sulfide, which in turn is released as hydrogen sulfide gas. Sphaerotilus natans is a filamentous bacteria inhabiting water heavily laden with organic material and often accounts for "bulking" in activated sludge.

Filamentous bacteria of the genera Leptothrix and Crenothrix frequently inhabit water pipes where dissolved iron is available. They oxidize the iron as an energy source using bicarbonates as a carbon source and deposit the iron as $Fe(OH)_3$ in slimes around the cells. The oxidized iron imparts a yellow or reddish color to the water. Eventually the bacteria die and decompose, causing foul tastes and odors.

Fecal bacteria typically found in domestic sewage include Escherichia coli, Aerobacter aerogenes and Streptococcus faecalis. Saprophytic bacteria also associated with sewage wastes include Pseudomonas fluorescens, Ps. aeruginosa, Proteus vulgaris, Bacillus subtilis, B. cereus, Aerobacter cloacae and Zoogloea ramigera, among others.

Pathogenic bacteria which can be transmitted by water include Salmonella typhi, which causes typhoid fever; Salmonella typhimurium, S. schottmulleri and S. choleraesius, responsible for the enteric fevers known as "paratyphoid fevers"; Vibrio cholerae, responsible for cholera; as well as Shigella dysenteriae, which causes bacterial dysentery.

FUNGI

Fungi, which include yeasts and molds, have much larger cells than bacteria and show greater morphological variation. They often produce complicated fruiting bodies. There are both single and multicelled fungi. As with bacteria, some fungi found in waters are true aquatic fungi while others also grow in other habitats as well, particularly soils.

All fungi are consumers, depending upon an external source of organic material. Aquatic fungi may be saprophytic, parasitic or sometimes both depending upon the circumstances. Other aquatic fungi are predacious, catching protozoa, rotifers and nematodes.

Together, bacteria and fungi remineralize the organic material present in water on a large scale, breaking down almost any naturally formed organic compound into its inorganic components -- carbon dioxide, water and various inorganic salts. They play a significant role in the cycling of biological nutrients. In doing this, they also frequently affect the dissolved oxygen content of water resources. Decomposition activities result in oxygen consumption and may lead to the complete disappearance of dissolved oxygen. When this occurs, anaerobic respiration and fermentation take place, resulting in fish kills and various undesirable metabolic end products such as hydrogen sulfide and methane gases.

Fungi may also form filamentous growths in acitivated sludge that interfere with proper settling characteristics.

ALGAE

Algae are relatively simple aquatic organisms which may be responsible for as much as 90 percent of all the photosynthetic production on earth. They produce both oxygen and organic materials which can be used by other organisms. There are approximately 30,000 species of algae, ranging from single-celled organisms to such seaweeds as giant kelp which grows in strands up to 200 feet long.

Excessive growths of algae in water bodies and reservoirs can result in offensive tastes and odors. In addition, algae require oxygen to support metabolism during periods of dark when photosynthesis ceases. Large quantities of algae in a body of water may, therefore, seriously deplete the dissolved oxygen level at night. This can result in the killing of aerobic organisms (including algae itself) and the initiation of anaerobic decomposition in the same manner as can an overabundance of bacteria and fungi.

OTHER ORGANISMS

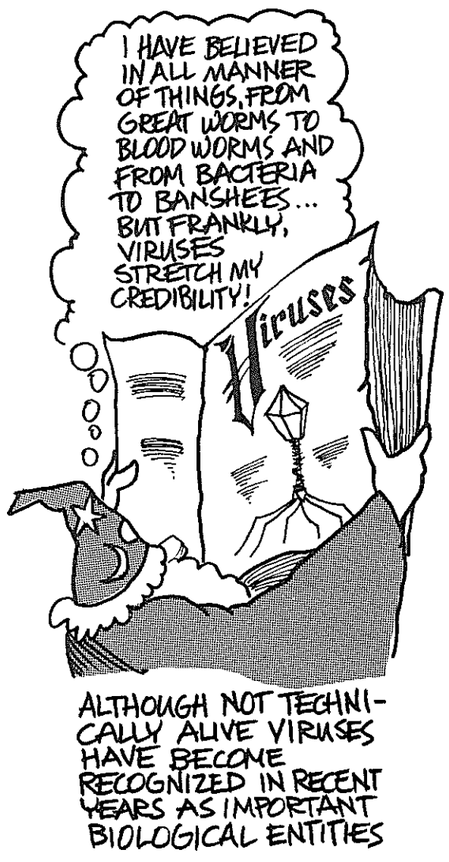
Protozoa are single-celled organisms ranging in size from 10 to 100 micrometers. They are motile, generally nonphotosynthetic, strict aerobes and feed on bacteria. A protozoan of particular interest in water quality management is Entamoeba histolytica, which causes amoebic dysentery.

Rotifers are simple multicellular animals. They are strict aerobes, requiring relatively high levels of dissolved oxygen, and feed primarily on bacteria. They are found exclusively in waters with low organic content and are thus good indicators of low pollution levels.

Crustaceans are multicellular animals with rigid shell structures. They are strict aerobes, feed on bacteria and algae and are limited primarily to relatively stable streams and lakes.

Worms and larvae normally inhabit organic muds and biological slimes. Nematodes occur in activated sludge and trickling filter slimes, although their function is not entirely understood. Worms of the genus Tubifix, also known as blood worms because of their red color, are indicative of highly polluted waters. The midge fly larvae, Chironomidae, are also red and are occasionally mistaken for Tubifix, although they occur at points in a stream where recovery from active decomposition begins.

VIRUSES



A virus is little more than a molecule of nucleic acid enclosed in a protein coat. The nucleic acid can be either DNA or its close relative, ribonucleic acid (RNA), depending upon the particular virus. A virus by itself is not a living organism -- it has no cytoplasm, no energy production or consumption, no synthesis.

However, viruses are biological entities which do reproduce, even though they are unable to accomplish this by themselves. Instead, the nucleic acid is injected into a suitable host cell, whereupon the viral nucleic acid takes over command of the cell's synthetic machinery. The resources of the cell are diverted from their normal functions to the production of more viruses. The cell fills with viruses until it bursts, releasing the new viruses to infect other cells. In the process, the host cell is destroyed.

Concern has developed in recent years over the presence of viruses in water because of their extremely small size (0.01 - 0.2 um), which enables them to pass through filtering systems which would retain bacteria. Other concerns include their greater resistance to traditional disinfection techniques such as chlorination and the ability of a single virus to produce an infection in a host organism (as compared to the hundreds of bacteria which must be present to cause infection in multicellular hosts such as man). Particular attention has been directed toward determining what viral diseases can be transmitted by water and how various treatment processes affect them. Better detection methods are also being developed.

Viral diseases in man which can be transmitted by water include those caused by poliovirus (responsible for poliomyelitis), coxsackieviruses (herpangina, aseptic meningitis and myocarditis), reovirus (respiratory and gastro-intestinal disease) and adenovirus (atypical pneumonia, conjunctive and acute respiratory disease).