NASA CONTRACTOR
REPORT

NASA CR-184033

MICROBIAL ECOLOGY LABORATORY PROCEDURES MANUAL NASA/MSFC

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September 1990

Final Contractor Report

Prepared for
NASA, George C. Marshall Space Flight Center
Marshall Space Flight Center, Alabama 35812

(NASA-CR-184033) MICROBIAL ECOLOGY LABORATORY PROCEDURES MANUAL NASA/MSFC Final Report (Sverdrup Technology) 77 p CSCL 06C

N91-13856

Unclas
G3/51 0319621

Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
NASA CR-184033		

4. Title and Subtitle	5. Report Date
Microbial Ecology Laboratory Procedures Manual NASA/MSFC	October 1990
	6. Performing Organization Code
7. Author(s)	8. Performing Organization Report No.
Timothy L. Huff	
	10. Work Unit No.
9. Performing Organization Name and Address	11. Contract or Grant No.
Sverdrup Technology Materials and Processing Department Huntsville, Alabama 35806	NAS8-37814
12. Sponsoring Agency Name and Address	13. Type of Report and Period Covered
National Aeronautics and Space Administration Washington, D. C. 20546-0001	Final Contractor Report
	14. Sponsoring Agency Code

15. Supplementary Notes

Contract Technical Monitor: Elizabeth Rodgers, Science and Engineering, Materials and Processes Laboratory, MSFC, Alabama.

16. Abstract

An essential part of the efficient operation of any microbiology laboratory involved in sample analysis is a standard procedures manual. The purpose of this manual is to provide concise and well-defined instructions on routine technical procedures involving sample analysis and methods for monitoring and maintaining quality control within the laboratory. Of equal importance is the safe operation of the laboratory. This manual outlines detailed procedures to be followed in the microbial ecology laboratory to assure safety, analytical control, and validity of results.

17. Key Words (Suggested by Author(s))	18. Distribution Statement
microbiology, microbial ecology, media, bacteria, identification, safety, culture.	Unclassified-Unlimited

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of pages	22. Price
Unclassified	Unclassified	76	NTIS

NASA FORM 1626 OCT 86

For sale by the National Technical Information Service, Springfield, VA 22161-2171

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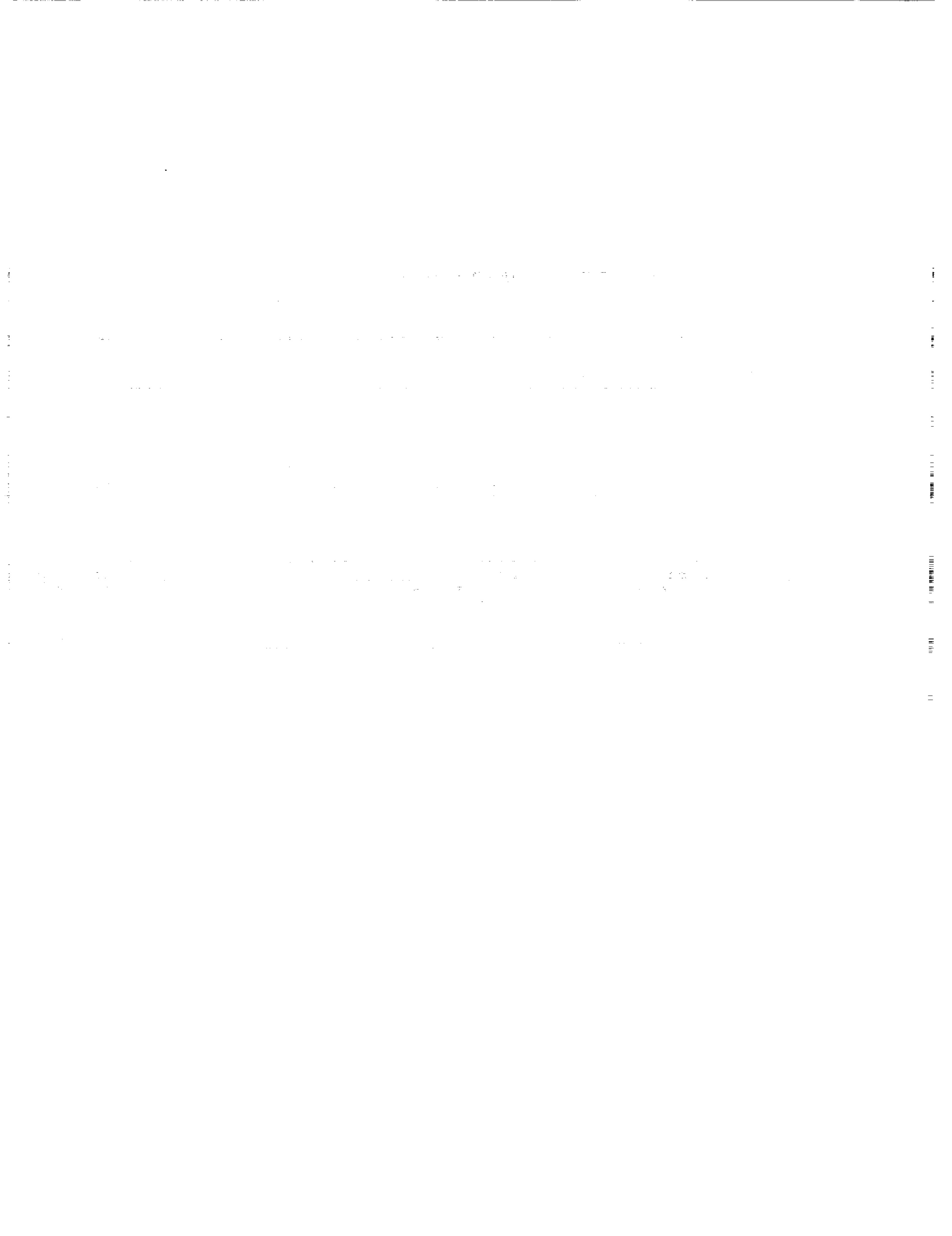

1.0 INTRODUCTION

An essential part to the efficient operation of any microbiology laboratory that may be involved in sample analysis is a standard procedures manual. The purpose of this manual is to provide concise and well-defined instructions on routine technical procedures involving sample analysis and methods for monitoring and maintaining quality control within the laboratory. Of equal importance is the safe operation of the laboratory.

In order for the laboratory to generate and report accurate sample data, personnel must be cognizant of the importance of their work during each step of an operation. Any data released from the laboratory are subject to the scrutiny of others and, as such, reflects directly on the competence and quality control measures employed by the technician. In order to meet such challenges, it is the responsibility of each analyst to maintain written records of performance and quality control measures.

In summary, the intent of this manual is to provide direction and to assure:

* That accepted staining and testing procedures are understood in principle and practice;
* That proper procedures for sample manipulations are provided. This includes the collection, tracking, reporting, analyzing and maintaining of samples;
* That enumeration methods are presented and defined;
* That media and reagents required for laboratory operations are provided and defined where required;
* That analytical controls to assure the validity of the whole spectrum of laboratory operations are maintained;
* That the laboratory is operated in a safe manner;

Incorporated into each procedure is a brief description of the principles and/or purpose of each operation. Specific quality control guidelines are included to insure accuracy in operation.

2.0 CLASSIFICATION AND IDENTIFICATION OF MICROORGANISMS (BACTERIA)

Bacteria can be classified and identified based on a variety of criteria including their morphology (structure) and biochemical responses to defined substrates. In general, the morphology of a bacterium provides important preliminary information on the organism which can then be applied to more specific testing by biochemical methods.

Known organisms are employed as controls in the performance of many of these tests. Available commercially, these organisms are usually received freeze-dryed (lyophilized) and may be reactivated by addition of a small volume of a suitable liquid medium and incubated at proper temperatures. Section 7 provides a more detailed description for storage and maintenance of these and all other organisms. Table 1 lists all control organisms which will be available for use in the laboratory. Included is a reference number for each organism assigned by The American Type Culture Collection (ATCC), an organization which maintains and distributes pure cultures of known organisms to researchers.

2.1 Morphology

The morphology of a bacterium is comprised of a colonial and cellular classification as described below.

2.1.1 Colonial

The colonial morphology of an organism describes the overall appearance of an isolated colony (large population of one bacterial species), usually on a solidified medium. Viewed under low magnification, criteria to be evaluated can be found in Table 1. Although usually visible through a petri plate, condensation within the plate may make examination difficult without removing the cover. If the plate is to be saved for further evaluation, the cover should not be placed on any surface as contaminants may be introduced when refitted. Analysis should be done quickly but carefully and the lid promptly replaced after observation. Plates may be stored at room temperature or refrigerated or discarded into an autoclave receptacle.

The following procedure may be used to observe colonial morphology.

1. Place the sample to be examined under a dissecting microscope and focus on an isolated colony using the coarse adjustment.

2. Slowly move the fine focus knob to bring colony into view.

3. Use Table 2 to record results on Culture Data Sheet (Form 1).

4. Disinfect any areas that may have been contaminated during the procedure by using a squeeze bottle containing disinfectant (Section 6.6) and wiping the area with a sponge or towel. Discard this into an autoclave receptacle.

2.1.2 Cellular

Cellular morphology refers to the structure of a single bacterium and is usually observed by staining of the cell in some manner on a glass slide. Staining procedures can provide general, and in some cases, specific information about the identity of a microorganism. Care should be taken in performing these procedures as the organisms are viable during sample preparation. Good laboratory practice includes applying a disinfectant to the working surface prior to and after use. If using a bunsen burner for sterilization, the inoculating loop or needle containing the excess organisms should be placed in the uppermost part of the flame first and slowly brought into the center of the flame. This will reduce the possibility of organisms "splattering" during the process. Electrical sterilization devices such as the "Bacti-Cinerator" should be turned on 10-15 minutes prior to use. An orange glow should be visible within the chamber before inserting the inoculating loop or needle. Heat the loop or needle to redness before removing. All slides should be cleaned and properly labeled so that they may be retrieved as needed. Slides are to be stored in a slide box. The date and reference number should be included on the slide. This number is included on the Culture Data Sheet (Form 1) and consists of the slide box letter, position within the slide box and position on the slide e.g. A-3-4 indicates slide box A-third position-fourth sample on the slide.

In most instances, control organisms can be run with the methods and are recorded on Form 1. If the expected results are not obtained, the procedure should be repeated. Results found in error again should be reported to senior laboratory personnel.

2.1.2.1 Sample Preparation

With the exception of the wet mount procedure (2.1.2.7) all methods presented here require the addition of chemicals (stains) to bacterial "smears" on glass slides. Commercial stains should be within expiration dates listed on the bottle. Stains prepared in the laboratory should not be used after their expiration dates as defined in Section 6. Unless noted in the specific procedure the following steps should be used in preparing the sample for staining. A 24 hour bacterial sample or control culture is used unless noted otherwise.

1. If using an organism from solid media, prepare the slide by placing a small drop of tap water on the slide. Using an inoculating loop or needle mix a small amount of the culture in the liquid, spread out the drop, and allow it to dry. If a broth culture is used, no liquid is needed.

2. After air drying, heat fix the prepared slide by passing it over a flame several times (warm to the touch).

3. Slides not intended for storage should be discarded in an autoclave receptacle.

2.1.2.2 Gram Stain

The Gram stain is used to distinguish two major subgroups of bacteria (gram positive and gram negative) based upon their ability to take up and retain different stains. The exact mechanism of the Gram reaction is not known but may be due in part to differences in the cell wall composition of the subgroups. Gram negative cell walls have a higher lipid content than gram positive cell walls, and although the crystal violet-iodine complex forms in both, the alcohol step removes the lipid from the gram negative cell wall, thereby increasing cell permeability and resulting in loss of the dye complex. The complex is retained in the gram positive cells. Gram negative bacteria then take up the counterstain.

1. Flood the slide with Gram's Crystal Violet for 1 minute. Rinse.

2. Flood the slide with Gram's Iodine for 1 minute. Rinse.

3. Apply the decolorizer until it flows colorless from the slide. Rinse.

4. Counter stain by flooding the slide with Gram's Safranin for 30 seconds. Rinse.

5. Observe stained preparation using ordinary light microscopy with an oil immersion objective (100X).

6. A Gram positive reaction is blue to purple and a Gram negative reaction is pink to red.

7. Record the reactions on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Use a Gram Stain Control slide (Fisher Scientific) to determine staining efficiency. The gram negative control should appear red upon microscopic examination while the gram positive control appears purple.

2.1.2.3 Wirtz-Conklin Spore Stain

This stain is used to determine the presence of spore-producing bacteria. Due to the resistance of spores to physical and chemical agents heat is required to promote penetration of the stain into the spores. Vegetative cells will not retain the stain and are counterstained to allow differentiation. In many instances, extended incubation periods (up to 7 days) on a sporulating agar (Section 5.1) are required before spores can be observed by this staining procedure.

1. Place a piece of bibulous paper over the prepared slide and flood with 5% aqueous malachite green.

2. Steam the slide, keeping it moist with stain, for 3 to 7 minutes and rinse with tap water.

3. Counterstain with Gram's Safranin for 30 sec.

4. Observe using ordinary light microscopy with an oil immersion objective.

5. Spores should stain green and the vegetative cell and debris should stain red.

6. Record the reactions of the unknown on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Use Bacillus subtilis as a positive control and Escherichia coli as a negative control.

2.1.2.4 Kinyoun Acid Fast Stain

This stain is used primarily to distinguish acid-fast bacteria of the genera Mycobacterium and Nocardia. Due to their high lipid content, once they are stained with a high affinity dye, decolorization with acid is slow. Other organisms decolorize rapidly and pick up the counterstain.

1. Flood the prepared slide with Kinyoun Carbolfuchsin and let it sit for two minutes. Wash the slide with tap water.

2. Decolorize by dropping acid alcohol over the smear until the alcohol flows colorless from the slide. Wash with tap water.

3. Flood the slide with Methylene blue and let stain for 20-30 seconds. Wash the slide and blot it dry.

4. Observe using ordinary light microscopy with oil immersion objective.

5. Acid fast organisms will stain red and everything else is blue.

6. Record the results on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Because of the hazards of working with acid-fast organisms, a commercially available acid fast control and test slide kit is used (Fisher Scientific). The positive control will stain red and the negative control will stain blue.

2.1.2.5 Poly-B-hydroxybutyrate (PHBA) Granule Stain

This procedure is used for the detection of bacterial cytoplasmic granules containing poly-B-hydroxybutyrate. The stain has a high degree of affinity for PHBA which makes the granules stand out against the counterstained cytoplasm.

1. Prepare a smear on a clean slide and allow it to air dry. Gently heat fix the specimen, but do not overheat it.

1. Flood the prepared slide with Sudan Black B (Section 6.9) and allow it to stand undisturbed for 15 minutes. Drain and gently blot dry.

2. Wash and clear the smear with xylol for approximately 10 seconds.

3. Counterstain with Safranin O for 10 seconds, rinse and blot dry.

4. Observe using the ordinary light microscope with oil immersion objective.

5. The cells stain red and the highly refractile poly-B-hydroxybutyrate granules are blue black.

6. Record the results on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Use Corynebacterium renale as a positive control and Escherichia coli as a negative control.

2.1.2.6 Capsule Stain

The capsule stain is used to detect the presence of a polymeric material (gel) produced by some bacteria. This gel surrounds the cell creating a capsular body. The dye is unable to penetrate the polymeric material, causing the capsule to stand out as a clear halo against a dark background.

1. Place two loopfuls of a bacterial suspension on a clean slide. If the sample is from solid media, put a few loopfuls of water on the slide prior to adding sample.

2. Add one small drop of Nigrosin stain (Section 6.10) to the slide and mix.

3. Using another glass slide, carefully spread the stain over the surface of the first slide to get a thin smear.

4. Allow the slide to air dry. Do not heat fix.

5. Observe slide using ordinary light microscopy with an oil immersion objective.

6. The area around the cells will appear dark blue and the cells will be colorless.

7. Record the results on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Use Acinetobacter calcoaceticus as a positive control and nigrosin alone as a negative control.

2.1.2.7 Wet Mount

The wet mount is used to determine the morphology of a microorganism. Because no chemical reagents are added, the bacteria retain their normal shape and size. This method is also useful for verifying the presence of spores.

1. Label a clean slide with the organism being tested.

2. Place a drop of water on the slide and mix a small amount of bacteria with it.

3. Carefully place a cover slip on the slide. To avoid air bubbles, touch the liquid with the edge of the cover slip and gently drop it.

4. Observe slide under the microscope with oil immersion using phase contrast. Microorganisms will appear as clear bodies with dark outlines. Any spores present will appear as highly refractile bodies.

5. Record the results on the Culture Data Sheet (Form 1).

QUALITY CONTROL

For spore controls use Bacillus alvei as a positive control and Escherichia coli as a negative control. Spores appear as highly refractile bodies and are best observed under a phase contrast microscope.

2.2 BIOCHEMICAL TESTS

The use of individual biochemical tests can provide both general and specific information about the identity of an organism. This information combined with morphological data provides the basis for selection of a battery of biochemical tests. These tests are available commercially and are designed to identify the organism at the species level. This laboratory employs the Minitek Identification System for the identification of unknown organisms.

The analyst should employ the following procedure when attempting to identify an aerobic or facultatively anaerobic organism. A more detailed discussion of each step in the procedure is found in the following section as listed below. It is important that in all cases a 24-hour pure culture or control grown on tryptic soy agar or other approved medium is used. All reactions are recorded on the Culture Data Sheet (Form 1).

Many of the procedures described in this section contain quality control guidelines which are recorded on the Culture Quality Control Data Sheet (Form 2). If unexpected results are obtained for any of these controls, senior laboratory personnel should be notified.

1. Perform a Gram Stain as per procedure 2.1.2.1.

2. For Gram positive rods, perform a wet mount (procedure 2.1.2.6) or a spore stain (procedure 2.1.2.2). If spores are not visible, place the organism on a sporulating agar slant and incubate at 35 degrees Celsius for 3-7 days. Repeat the wet mount or spore stain. If spores are found, the organism is considered to be a *Bacillus sp.*

3. For all other bacterial cultures, perform the following tests and all other tests that may be requested.

TEST	PROCEDURE
Catalase	2.2.1
Oxidase	2.2.2
O-F	2.2.3
MacConkey	5.10

4. All cultures, except Gram positive rods, are run on the Minitek Identification System (BBL, Division Beckton, Dickenson, and Co.). The following describes which Minitek to use.

ORGANISM	MINITEK	PROCEDURE
Gram positive cocci	Gram Positive Minitek	2.2.4.1
Gram negative rods (OXI-POS)	Nonfermentor Minitek	2.2.4.2
Gram negative rods (OXI-NEG)	Enterobacteriaceae Minitek	2.2.4.3

2.2.1 Catalase Test

Catalase is an enzyme found in most bacteria which catalyzes the breakdown of hydrogen peroxide, releasing free oxygen gas. This test is especially useful in the separation of _Staphylococci_ (positive) and _Streptococci_ (negative).

1. Using an inoculating loop, pick the center of an 18-24 hour pure colony and place on a clean, glass slide.

Note: Do not use a culture from blood agar as a false positive result may occur.

2. A nichrome wire, plastic loop or wooden stick must be used for the catalase test.

Note: A platinum loop will cause a false positive.

3. Add a drop of 3% hydrogen peroxide over the organism on the slide using a Pasteur pipette. Do not mix the culture with the hydrogen peroxide. The hydrogen peroxide must be stored in a dark bottle in the refrigerator at all times.

4. Do not reverse the order of the procedure as false positives may occur.

5. Observe for immediate bubbling (gas liberation:positive result)

6. Record the results on the Reference Culture Data Sheet (Form 1).

QUALITY CONTROL

Known positive and negative controls must be run with each set of organisms tested. For a positive result use _Staphylococcus aureus_. For a negative result use _Streptococcus faecalis_. Control results are recorded on Form 2.

2.2.2 Oxidase Test

The oxidase test is used to detect the presence of a bacterial enzyme (indophenol oxidase) in certain bacteria. If present, the enzyme will oxidize a redox dye, which results in color development of the bacterial colony. Performance of this test is considered to be of diagnostic importance in the identification of aerobic bacteria.

1. Hold the Oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) dispenser upright and with the tip pointing in an outward direction, squeeze gently to crush the glass ampule inside the dispenser.

2. Place 2-3 drops of the reagent onto the designated test area of a piece of filter paper.

3. Using a platinum loop or a wooden applicator stick, pick a portion of the colony to be tested and rub it into the reagent-saturated site of the filter paper. A nichrome loop may cause a false reaction. The organisms should be 18-24 hours old and cultured on TSA or other approved medium.

4. Examine for the appearance of a dark purple color within 10 (positive reaction). Formation of a purple color after this time should be recorded as negative.

5. Record the results on the Reference Culture Data Sheet (Form 1).

6. After testing, flood paper with disinfectant (Section 6.6) and discard in autoclave receptacle.

QUALITY CONTROL

Known positive and negative controls must be run with each set of organisms tested. For a positive control use Pseudomonas aeruginosa. For a negative control use Escherichia coli. Results are recorded on Form 2.

2.2.3 Oxidative/Fermentative (O-F) Test

The oxidative/fermentative test is used to distinguish bacteria based upon their ability to metabolize carbohydrates under aerobic (oxidative) and/or anaerobic (fermentative) conditions.

1. Prepare the appropriate O-F media using procedure 5-5 of this manual. This test procedure is used for either formula of O-F media.

2. An 18-24 hour pure culture should be taken from any suitable growth medium (except selective media).

3. Label two tubes for each organism being tested. In addition, label two tubes for the positive control and label two tubes for the negative control.

4. Using an inoculating needle, obtain a light inoculum and stab all tubes to approximately one-quarter inch from the bottom.

5. Cover one tube of each set with 1-2 ml of sterile mineral oil.

Note: To sterilize mineral oil, add 1 ml of water to 100 ml of mineral oil in a 200 ml flask. Sterilize at 121 degrees Celsius for 15 minutes.

6. Incubate all tubes at 35 degrees Celsius for 48 hours or longer. Some organisms may require up to 3-4 days of prolonged incubation. Read final results after 7 days.

7. A yellow color in the tube indicates acid production. An organism that produces acid only in the open tube is OXIDATIVE. An organism that produces acid in both tubes is FERMENTATIVE. When no acid is produced in either tube the organism is inert. A blue color indicates a basic reaction. Bubbles in the media indicates gas production.

8. Record gas, acid or base production in each tube on the Reference Culture Data Sheet (Form 2).

QUALITY CONTROL

Use <u>Escherichia coli</u> as a positive control and <u>Pseudomonas aeruginosa</u> as a negative control. An uninoculated O-F tube is compared to inoculated tubes when reading results. The positive control tubes should read oxidative and fermentative (both tubes are yellow) and may indicate gas production. The negative control should be inert (both tubes show no change). Record these results on (Form 2).

2.2.4 Minitek Identification System

The Minitek Identification System (MIS) employs a series of paper discs impregnated with specific substrates that are placed in wells and inoculated with a suspension of the organism to be tested. After incubation at the specified temperature the wells are scored as positive or negative for each substrate and assigned a numerical value. These values form a serial code number that is then checked in a data base of known organisms for matching codes. In this way unknown organisms may be identified. More specific information, including the complete substrate name and specific biochemical reaction involved may be found in the MIS instruction booklets located at the identification station. It is highly recommended that all personnel using the MIS for the first time read these booklets prior to performance of the tests described below. Control organisms that do not key out correctly should be reported to senior laboratory personnel.

The MIS may be used to identify members of the following groups: Gram negative nonfermenting bacteria, Gram positive coccoidal bacteria and the Enterobacteriaceae.

2.2.4.1 Minitek Test For Gram Positive Cocci

1. Set up the appropriate number of disc plates by adding the following discs in the order indicated using a 10 place disc dispenser. Be sure to make enough plates for the controls. These may be made ahead of time and stored for up to one week at 2-8 degrees Celsius.

1. Arginine(ARG)	6. Esculin(ES)	11. B-Glucosidase(BGS)	16. Voges-proska
2. Arabinose(AR)	7. Maltose(M)	12. Salicin(SA)	17. Phosphatase(
3. Galactose(GA)	8. Mannitol(MN)	13. Sorbitol(SO)	18. Hippurate(HI
4. Inulin(IN)	9. Mannose(MA)	14. Trehalose(TR)	19. Pyroglutamic
5. Lactose(L)	10. Raffinose(RA)	15. Dextrose(D)	20. Leucine(LEU)

2. Remove the lid and label the Minitek plate with the organism identification number in the appropriate space and on the Gram positive broth vial.

3. Using a sterile loop, wooden applicator stick or cotton-tipped swab, pick colonies from the agar plate and suspend them in the broth to give an inoculum concentration that is equivalent to a McFarland Standard 5 (1.5 x 10E9) bacteria/ml). Standards are available at the identification station. Vortex the vial 2-3 minutes.

4. Aseptically place a Minitek pipette tip on the Minitek pipetter by firmly inserting the end of the pipetter into the tip which is held upright in the tip tray. Withdraw the tip carefully to avoid contamination.

5. Place the tip into the inoculum and, using the finger-operated trigger of the pipetter, dispense 0.05 ml (1 shot) of inoculum into each well containing a disc. Use 2 shots for arginine. Do not use the last shot in pipette tip. When using more than 15 discs, obtain additional inoculum from the vial.

6. Eject tip from the pipetter into a biohazard receptacle.

7. Overlay the <u>Arg</u> disc with at least 0.1 ml of sterile mineral oil. Any mineral oil spilled on the plate surface should be thoroughly cleaned off to avoid sealing off wells which should not be overlaid.

8. Replace the lid on the plate and place the inoculated Minitek plate into the humidor (with moistened sponge) and cover with the humidor lid.

9. Incubate for 18-24 hours at 35 to 37 degrees Celsius.

10. Read all reactions that do not require the addition of reagents first. Next, add the reagents and read those results. The following chart describes all reactions.

DISC	NEGATIVE	POSITIVE
Carbo-hydrates	Orange to Red	Yellow to Light Orange
ARG	Yellow to Gold	Orange to Red
ES	White (colorless)	Light brown to dark brown
HIP	White (colorless) (after 3 min.)	Purple to Dark Blue (within 3 min.)
BGS	White (colorless)	Light Yellow to Yellow
PH	White (colorless)	Pink to Red (within 30 sec.)
LEU	White (in 1 min.)	Pink to Purple (in 1 min.)
PYR	White (in 1 min.)	Pink to Purple (in 1 min.)
NR *	White (colorless)	Pink to Red (in 1 min.)
VP	White (colorless)	Pink to Red (in 10 min.)

* Prior to addition of zinc dust. After addition, reactions are reverse

REAGENTS TO ADD

WELL	DISC	REAGENT
15	D	Nitrate Solution A, 1 drop Nitrate Solution B, 1 drop Zinc dust
16	VP	40% KOH, 2 drops 5% 1-napthol, 2 drops
17	PH	40% KOH, 1 drop
18	HIP	Ninhydrin reagent, 1 drop
19	PYR	Cinnamaldehyde, 1 drop
20	LEU	Cinnamaldehyde, 1 drop

11. Record reactions on the code pad. A seven digit profile number is derived by adding the positive values. Use the Minitek Gram-Positive Code Book to identify the organism with the corresponding profile number.

12. Record all pertinent information on the Culture Data Sheet (Form 1).

QUALITY CONTROL
Inoculate one plate with Staphylococcus aureus and record results on the Culture Q.C. Data Sheet (Form 2).

2.2.4.2 Minitek Test For Gram Negative Nonfermenters

1. Set up the number of appropriate disc plates by adding the following discs in the order indicated using a 10 place disc dispenser. Be sure to make enough plates for the controls. These may be made ahead of time and stored for up to one week at 2-8 degrees Celsius.

1. Dextrose (DEX;ANA)
2. Dextrose (DEX;AER)
3. Maltose (M)
4. Sucrose (SU)
5. Xylose (X)
6. Arginine (ARG)
7. Lysine (LY)
8. Ornithine (OR)
9. Urea (UR)
10. O-Nitrophenyl -galactopyr.(ONPG)
11. BLANK
12. Nitrate reductase (NR)
13. Starch (ST)
14. Phenylalanine (PA)
15. Citrate (CIT)

2. Remove the lid and label the Minitek plate with the organism identification in the appropriate space and on the Gram negative broth vial.

3. Using a sterile loop, wooden applicator stick or cotton-tipped swab, pick colonies from the agar plate and suspend them in the broth to give an inoculum concentration that is equivalent to a McFarland Standard 5 (1.5 x 10E9 bacteria/ml). Standards are available at the identification station. Vortex the vial 2-3 minutes.

4. Aseptically place a Minitek pipette tip on the Minitek pipetter by firmly inserting the end of the pipetter into the tip which is held upright in the tip tray. Withdraw the tip carefully to avoid contamination.

5. Place the tip into the inoculum and, using the finger-operated trigger of the pipetter, dispense 0.05 ml (1 shot) of inoculum into each well containing a disc. Use 2 shots for arginine. Place 3 shots of the inoculum into well 11 for the indole test. Do not use the last shot in pipette tip. When using more than 15 discs, obtain additional inoculum from the vial.

6. Eject tip from the pipetter into a biohazard receptacle.

7. Overlay the DEX (ANA), UR, LY, OR and ARG discs with at least 0.1 ml of sterile mineral oil. Failure to overlay the urea disc within 10 minutes may result in an erroneous urease reaction. Any mineral oil spilled on the plate surface should be thoroughly cleaned off to avoid sealing off other wells.

8. Replace the lid on the plate and place the inoculated Minitek plate into the humidor (with moistened sponge) and cover with the humidor lid.

9. Incubate for 24-48 hours at optimal temperature for growth (temperature yielding best growth from previous biochemical tests e.g. oxidase, catalase). Urea and ONPG reactions should be read after 18-24 hours.

10. Read all reactions that do not require addition of reagents first. Next, add the reagents and read those results. The following chart describes all reactions.

DISC	NEGATIVE	POSITIVE
DEX (ANA)	Orange to Red	Yellow
DEX (AER) and carbohydrates	Red	Orange to Yellow
ARG	Yellow to Orange	Red
LY	Yellow to Light Orange	Deep Orange to Red
OR	Yellow to Orange	Red
UR	Light Tan	Dark Purple
ONPG	White	Yellow
IND	Yellow	Pink to Red
NIT	Colorless	Pink to Red
N2	Pink to Red	Colorless
ST	ST obscured	ST visible
PA	Light Yellow	Green to Dark Green
CIT	Green	Dark Blue

REAGENTS TO ADD

WELL	DISC	REAGENT
11	BLANK	Kovac's Reagent, 1 drop
12	NR	Nitrate Solution A, 1 drop
		Nitrate Solution B, 1 drop
		Zinc Dust
13	ST	Grams Iodine
14	PA	10% Ferric Chloride, 1drop

11. Record reactions on the code pad. A seven digit profile number is derived by adding the positive values. Use the Minitek Gram-Negative Nonfermenter Code Book to identify the organism with the corresponding profile number.

12. Record all pertinent information on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Inoculate one plate with Pseudomonas aeruginosa and record results on the Culture Q.C. Data Sheet (Form 2).

2.2.4.3 Minitek Test For Gram Negative Enterobacteriaceae

1. Set up the number of appropriate disc plates by adding the following discs in the order indicated using a 10 place disc dispenser. Be sure to make enough plates for the controls. These may be made ahead of time and stored for up to one week at 2-8 degrees Celsius.

1. Nitrate Reductase(NR)
2. Phenylalanine (PA)
3. Hydrogen Sulfide (H2S)
4. Indole (IND)
5. Voges-Proskauer (VP)
6. O-Nitrophenyl-Galactopyr(ONPG)
7. Urea (UR)
8. Lysine (LY)
9. Arginine (ARG)
10. Ornithine (OR)
11. Dextrose (DEX)
12. Malonate (MAL)
13. Adonitol (AD)
14. Arabinose (AR)
15. Inositol (I)
16. Raffinose (RA)
17. Sorbitol (SO)
18. Lactose (L)
19. Rhamnose (R)
20. Sucrose (SU)

3. Remove the lid and label the Minitek plate with the organism identification in the appropriate space and on the Gram negative broth vial.

4. Using a sterile loop, wooden applicator stick or cotton-tipped swab, pick 1 colony from the agar plate and suspend them in the broth to give an inoculum concentration that is equivalent to a McFarland Standard 1 (3 x 10E8 bacteria/ml). Standards are available at the identification station. Vortex the vial for 2-3 minutes.

5. Aseptically place a Minitek pipette tip on the Minitek pipetter by firmly inserting the end of the pipetter into the tip which is held upright in the tip tray. Withdraw the tip carefully to avoid contamination.

6. Place the tip into the inoculum and, using the finger-operated trigger of the pipetter, dispense 0.05 ml (1 shot) of inoculum into each well containing a disc. Use 2 shots for arginine. Do not use the last shot in pipette tip. When using more than 15 discs, obtain additional inoculum from the vial.

7. Eject tip from the pipetter into a biohazard receptacle.

8. Overlay the ARG, H2S, LY, OR and UR discs with at least 0.1 ml of sterile mineral oil. Failure to overlay the urea disc within 10 minutes may result in an erroneous urease reaction. Any mineral oil spilled on the plate surface should be thoroughly cleaned off to avoid sealing off other wells.

9. Replace the lid on the plate and place the inoculated Minitek plate into the humidor (with moistened sponge) and cover with the humidor lid.

10. Incubate for 18-24 hours at 35 to 37 degrees Celsius.

11. Read all reactions that do not require addition of reagents first. Next, add the reagents and read those results. The following chart describes all reactions.

DISC	NEGATIVE	POSITIVE
NR	Colorless	Pink to Red
PA	Light Yellow	Green to Dark Green
H2S	White to Beige	Grey to Black
Indole	Colorless	Pink to Red
VP	Colorless	Pink to Red
CIT	Green	Blue
ONPG	White	Light Yellow to Deep Yellow
UR	Light Tan to Light Lavender	Deep Purple
LY	Yellow to Gold	Orange to Red
ARG	Yellow to Orange	Red
OR	Yellow to Orange	Red
DEX	Red-Orange	Yellow to Gold
MAL	Light Yellow	Blue
All carbohydrates	Red-Orange	Yellow to Gold

REAGENTS TO ADD

WELL	DISC	REAGENT
1	NR	Nitrate Solution A, 1 drop
		Nitrate Solution B, 1 drop
		Zinc Dust
2	PA	10% Ferric Chloride, 1 drop
3	H2S/Indole	Read H2S/Kovac's Reagent, 1 drop
4	VP	40% Potassium Hydroxide, 1 drop
		5% 1-Napthol

12. Record reactions on the code pad. A seven digit profile number is derived by adding the positive values. Use the Minitek Gram-Negative Enterobacteriaceae Code Book to identify the organism with the corresponding profile number.

13. Record all pertinent information on the Culture Data Sheet (Form 1).

QUALITY CONTROL

Inoculate one plate with _Escherichia_ _coli_ and record results on the Culture Q.C. Data Sheet (Form 2).

3.0 SAMPLE ANALYSES

The accurate analysis of any given sample is dependent on many factors including methods of collection (which in turn depend on sampling conditions), preservation and transport of samples, sample tracking within the laboratory, sample manipulation within the laboratory (dilution), and the recording and reporting of results (including appropriate controls). This section will address procedures required to ensure accurate sample analysis.

3.1 Collection

The acquisition of valid data begins with collection of a representative water sample or other environmental material being tested. Samples must be maintained as closely as possible to original condition by careful handling and storage.

All samples derived from or contaminated by a human source must be handled as if they contain potentially pathogenic organisms. These sources include urine, shower water, fecal material or any other material directly or indirectly in contact with humans. In addition to laboratory coats, gloves and face masks (Section 10.4) should be worn when working with these materials.

All water samples must be collected in accordance with Standard Methods for the Examination of Water and Wastewater 17th ed.(SM). In the case of Environmental Control/Life Support System hardware special sampling procedures may be required. In these instances the laboratory must insure that the procedure(s) employed will not affect the integrity of the system hardware. This includes such parameters as component compatibility with any chemical and/or physical sterilants used, introduction of contaminants (microbial, chemical, and particulate) as a consequence of the procedure and any apparatus employed, and stress on system operation. When special procedures are required, they will be written and incorporated as part of this manual in the appendices. Only analysts trained in aseptic sampling techniques should collect samples for microbiological analysis.

3.1.1 Water

1. Collection of samples should be carried out in sterile wide-mouth plastic or non-corrosive glass bottles with non-toxic caps or stoppers. These containers should withstand repeated sterilization and have a capacity of at least 250 ml.

2. When collecting water that contains residual chlorine or other halogens, add 0.1 ml of 10% solution of sodium thiosulfate (see section 6.5 for preparation) for each 125 ml of sample. This amount will reduce 15 mg/l of residual chlorine. The reducing agent is added before sterilization.

3. For samples high in copper, zinc or heavy metals, add a chelating agent. Add 0.3 ml of 15% ethylenediaminetetraacetic acid (EDTA) (Section 6.1) to the collection bottle before sterilization.

4. Sample collection containers are rinsed with distilled water several times and sterilized for 15 minutes at 121 degrees Celsius before use.

QUALITY CONTROL

Select one bottle at random from each sterilized batch and confirm sterility by adding approximately a 25 ml volume of sterile non-selective broth. Incubate at 35 degrees Celsius for 24 hours and check for growth. Record results in sterilization notebook under heading "SAMPLE BOTTLE QC".

5. Immediately prior to collection, remove the bottle covering making sure the top does not contact any surfaces. Allow 2-3 minutes flushing of the port prior to collection. As this is not always possible when testing ECLSS hardware, chemical parameters should be taken by personnel trained in aseptic collection techniques prior to microbial sampling.

6. Avoid splashing while collecting sample and do not allow sample port to contact inner surface of sample bottle. Leave at least a 2.5 cm air space for proper mixing and immediately cap the bottle following sample collection.

7. When collecting tap water samples, be sure the samples are not collected from spigots that leak around their stems, or from spigots that contain aeration devices or screens within the faucet. If collecting from a mixing faucet run the hot water for 2 minutes, then run the cold water for 2-3 minutes prior to collection. Otherwise, allow the water to flow for 2-3 minutes before collection.

8. All samples should be labeled with a clean, waterproof, nonsmearing label of sufficient size to address the following information: date, time, sample description, sample site, test(s) to be run, sequence number, sampler's name.

9. Samples should be shipped on ice with a maximum travel time of 8 hours. The sample should have a Chain of Custody form (provided by requester) with it whenever it is shipped. Samples should be stored and maintained at 2-8 degrees Celsius. The elapsed time between sample collection and analysis should not exceed 30 hours. Samples which fall outside of the specified holding times should be discarded.

3.1.2 Air

Microorganisms are normally collected from the air by using a sterile stainless steel cartridge (Sartorius) fitted with a 0.45 um HAWG membrane filter (Millipore).

1. Insert cartridge onto the Sartorius MD2 or other suitable vacuum pump with the grided surface of the membrane facing outward.

2. Air is sampled for 30 minutes at a rate of 1 m^3 per hour. The filter is then aseptically removed from the cartridge using sterilized forceps (dipped in alcohol and flamed) and transferred to a plated media such as PCA, R_2A or Rose Bengal

3. Samples should be incubated at 28 degrees Celsius for 5 to 7 days.

3.2 Sample Logging

All samples received by the laboratory for analysis should be recorded in the Samples Received notebook located at the receiving station within the lab. Included in the record is the laboratory number which consists of a sequential number for the sample and includes the date received and the laboratory number. For example, a sample received on September 12, 1989 representing the 8th sample received by the lab would be recorded as 9-12-89-008. Also recorded is the time the sample was received, volume received, parameters to be run, and the requester Sample/Chain of Custody number. An individual sample data sheet used to summarize the subsequent results is also made out at this time (Form 3). The analyst who is given the data sheet records the assigned number in his/her personal bound notebook. This notebook will provide a more detailed description of the analysis including raw data, specific problems, and manipulations required.

3.3 Sample Grouping for Analysis

Multiple samples taken from a common or similar source should be analyzed consecutively when performing the membrane filtration technique (Section 4.1.1) or acridine orange direct count technique (Section 4.2.2). Samples suspected of containing high numbers of microorganisms (dirty ports), should be analyzed following analysis of "clean" samples. Brine and urine pre-treatment samples collected from ECLSS testing are always analyzed last.

These strategies are intended to reduce the risk of microbial and chemical carry-over contamination. Exceptions are allowed only if the sample in question is expected to exceed its holding time.

3.4 Dilution Scheme

Dilution of the original sample of water, wastewater or other material is often necessary to reduce the number of bacterial cells to measurable levels or to isolate single cells for purification and identification.

A known quantity of the sample (usually 1.0 ml) is transferred through a series of known volumes of dilution water, usually 99 ml (Section 6.0). This procedure is repeated until the desired bacterial density is reached. After dilution of the sample, the bacteria are enumerated. For ease of calculation and preparation, serial dilutions are usually prepared in succeeding ten-fold volumes called decimal dilutions (see Figure 1). When making dilutions it is important to use a sterile pipette for initial and subsequent transfers from each container and dilution bottle. Each sample tested should be shaken vigorously (25 times in 7 seconds) and the pipette not be inserted more than 2.5 cm below the surface of the sample or dilution.

Calculation of the dilution is as follows:

$$\frac{\text{Vol of Sample}}{\text{Vol of Dilution Blank + Vol of Sample}} = \text{Dilution Ratio}$$

When 1.0 ml is transferred from dilution bottle A to a second dilution bottle B, the dilution ratio for bottle B dilution is the product of the individual dilution as follows:

A x B = Final or Total Dilution Ratio

Volumes of 10 ml can be tested directly from each serial dilution to provide intermediate dilutions.

NOTE: The potential toxicity of phosphate dilution water and the stimulatory effect of peptone dilution water increases rapidly with time. Therefore, diluted samples should be tested as soon as possible after make-up and should not be held for longer than 30 minutes after preparation.

3.5 Recording of Results (Data)

Before release to the customer, the data sheet results are compared to the data recorded in the personal bound notebook by a second analyst. Final release of data is approved by NASA personnel.

4.0 ENUMERATION OF MICROORGANISMS

There are a variety of methods available for the enumeration of microorganisms each having certain advantages and disadvantages. Two of the methods available for use in this lab are cultural and direct counting procedures.

4.1 Cultural Methods

Cultural methods refer to those methods whereby a single organism is able to reproduce in a suitable environment until a visible colony can be observed. The total number of colonies is counted and the result given in terms of colony forming units (CFUs) per volume analyzed. The advantage of this method is that identification of the organism is possible. Disadvantages include incubation time required to visualize colonies (7-21 days in some cases) and inability to optimize all environmental conditions necessary for growth of all organisms present. This would include such variables as incubation temperature, pH and assimilable nutrients. These disadvantages can, however, also be used in the selection of specific types of organisms and will be discussed in Section 4.1.2.

Although a variety of procedures may be employed for the cultural enumeration of organisms, the membrane filtration technique is used routinely due to its high reproducibility, ability to test large volumes of sample, and ease of use. A second procedure, the spread plate technique, is included for the enumeration of anaerobic microorganisms (Section 4.1.3.9).

4.1.1 Membrane Filtration Technique

1. Shake sample bottle vigorously (about 25 times in 7 seconds) to evenly distribute the bacteria. Take care to secure the screw-cap and prevent leakage during shaking.

2. The number of colonies per plate must be limited for the membrane filtration test. Depending on the test used, the ranges are 20-60, 20-80 or 20-100 and are achieved by decimal dilution series (Section 3.4).

3. Media for this test is prepared by dispensing 5 ml of sterile molten media into 50 x 9 mm petri dishes. These plates have a shelf life of 2 weeks.

4. All glassware, filter units, filter holders and utensils should be thoroughly cleaned and presterilized at 121 degrees Celsius for 10 minutes. A sterile 47 mm, 0.45 um, grided membrane filter should be used. Filters are purchased presterilized eliminating a sterilization step.

5. When analyzing potable water, 250 ml of sample should be sufficient for filtration. An ideal sample volume will yield growth of about 20-200 CFU per filter (see Section 3.4). When less than 10 ml of sample (diluted or

undiluted) is to be filtered, approximately 10 ml of sterile dilution water should be added to the funnel before filtration. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

6. A sterile membrane filter (grid side up) should be placed over a porous plate or receptacle. The funnel unit is then carefully placed over the receptacle and locked in place.

7. The sample should be filtered in duplicate through a sterile 0.45 um HAWG filter (Millipore) under a partial vacuum (5-10 psig).

8. With filter still in place, the funnel should be rinsed by filtering three 20-30 ml portions of sterile dilution water.

9. After filtration the funnel is unlocked and removed. The membrane filter should be immediately removed with sterile forceps, and placed on a sterile nutrient pad or agar plate previously equilibrated to room temperature. A rolling motion should be used to avoid entrapment of air. Incubate the plates in an inverted position.

10. Decontaminate the funnels between successive filtrations by using an ultraviolet (UV) sterilizer. Irradiate 2 minutes upside down, invert, and irradiate 3 minutes. Filtration equipment should be sterilized between filtration series. A filtration series ends when 30 minutes or more elapse between filtrations.

11. Count the colonies on the plates as directed in the appropriate procedure and record results as in Section 3.4.

12. If counts from all membrane filters are zero, calculate the number of colonies per 100 ml that would have been reported if there had been one colony on the filter representing the largest filtration volume for that sample. Report results as < (less than) that number of colonies per 100 ml.

13. If colonies are too numerous to count, use the upper limit count from the smallest filtration volume for that sample. Report as > (greater than) that number per 100 ml.

14. If there is no result because of confluency, lab accident, etc., report as "No Result" and specify reason.

Note: Do not expose membrane filter culture preparations to UV radiation leaks from sterilization cabinet. Protect eyes.

QUALITY CONTROL

Prepare two replicate plates for each sample dilution used. Insert a sterile rinse water sample (100 ml) after filtration of a series of 10 samples to check for cross- contamination of contaminated rinse water. Record control results in analyst book.

4.1.2 Spread Plate Method

1. Media for this method is prepared as described in Section 5.19.

2. Dilution water is prepared as described in Section 6.0 and should be held for 2 days under anaerobic conditions prior to use (for anaerobe analysis).

3. Select the dilution(s) so that the total number of colonies on a plate will be between 30-300 (Section 3.4).

4. Use a separate sterile pipette for all transfers. Shake the sample vigorously to disperse the sample. When removing sample do not insert pipettes more than 2.5 cm below the surface of sample or dilution.

5. Label petri dishes beforehand with the sample I.D., date, dilution, media lot number and Initials. Media used is determined by the individual test.

6. Deliver 0.1 ml quantities to the agar surface using a digital pipette.

7. Using a sterile bent glass rod, distribute the inoculum over the surface of the medium by rotating the dish. Let the inoculum be completely absorbed into the media before incubating.

8. Invert the plates and incubate at the specified temperatures and time.

9. Count the plates as directed in the appropriate procedure and record results as described above for the membrane filtration technique (Section 4.1.1).

QUALITY CONTROL

Dilution water used for anaerobic procedures should be maintained in the presence of an anaerobic indicator (BBL Gaspack) to assure a proper environment. A 0.1 ml aliquot from an uninoculated dilution water bottle should be included in the procedure as a negative control.

4.1.3 Media Considerations

The choice of media used in the membrane filtration technique is an important consideration in determining the contents of the sample. Media may be divided into three groups: non-selective, selective and differential. Non-selective implies that the medium is designed such that the growth of any one or more organisms should not be favored. A selective medium favors the growth (isolation) of certain organisms while inhibiting the growth of others. Differential plating media is both selective

of certain organisms and differentiating within the selected group. For example, MacConkey agar selects for gram negative bacteria and inhibits gram positive bacteria. This media further differentiates within the gram negative bacteria those which metabolize lactose. The following provides a brief description of microbiological parameters that may be analyzed for in the laboratory and the procedures employed, including the media used. Preparation of the media may be found in Section 5.0.

4.1.3.1 Total Coliforms

Total coliforms may be defined as all aerobic and facultative anaerobic, gram negative, nonspore-forming, rod shaped bacteria producing a dark metallic sheen colony within 24 hrs at 35 degrees Celsius on an Endo-type medium containing lactose. Presence of these organisms indicates contamination of a water source. Due to false positive reactions noted from _Pseudomonas spp._, confirmation tests must be performed to assure a true positive response.

1. Prepare the m-Endo agar LES as directed in the appropriate procedure in Section 5.6.

2. Select sample volumes to produce 20-80 coliform colonies per filter.

3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.

4. Incubate the inverted agar plates with the filter for 22-26 hours at 34.5-35.5 degrees Celsius in an atmosphere with near-saturated humidity. If tight lidded dishes are used there is no requirement for near-saturated humidity.

5. After incubation remove the dishes and count the golden-green metallic sheen colonies per 100 ml using the following formula:

$$\frac{\text{No. colonies counted}}{\text{Vol. of sample filtered (ml)}} \times 100$$

6. Confluent growth is defined as bacterial growth with or without sheen covering the entire membrane filter. Too Numerous To Count (TNTC) is defined as greater than 200 total bacterial colonies on the membrane filter.

7. Samples resulting in confluent growth or TNTC with less than 5 distinguishable sheen colonies are invalid. Record this as "confluent growth" or "TNTC" and obtain a new sample from the same sampling site for analysis.

8. Samples resulting in confluent growth or TNTC with 5 or more distinguishable sheen colonies should be reported as such and the number of distinguishable sheen colonies included. If possible, a new sample should be taken and analyzed.

9. Stressed organisms may grow relatively slowly with no sheen production until 22 to 24 hours. Non-stressed organisms may lose their sheen after 18 hours. Therefore, sheen colonies should be counted at both 18 and 24 hours and the higher count reported.

Note: Count only the membrane filters with 20-80 sheen colonies.

Confirmation:

Confirm all sheen colonies regardless of the amount of sheen when the number of sheen colonies is 5 to 10 per 100 ml. When the number of sheen colonies exceeds 10 per 100 ml, randomly pick and verify at least 10 colonies representative of all sheen types.

Confirm as total coliforms by transferring growth into parallel tubes of lauryl tryptose broth and brilliant green bile lactose broth: incubate both at 34.5-35.5 degrees Celsius for 48 hours. Gas production in the brilliant green broth by 48 hours verifies the colony as coliform. If only a lauryl tryptose broth produces gas, transfer to a second tube of brilliant green bile lactose broth. Verification requires production of gas in this tube by 48 hour at 34.5-35.5 degrees Celsius.

Alternately, colonies may be identified by biochemical testing (Section 2.2). An oxidase test should be performed initially on each colony. All oxidase negative colonies should be identified by the Enterobacteriaceae test (Section 2.2.4.3). Oxidase positive colonies require identification by the Gram Negative Nonfermentor test (2.2.4.2). Incubation is at 35 degrees Celsius for 24 hours.

4.1.3.2 Fecal Coliforms

This procedure uses an enriched lactose medium and special incubation temperature (44.5 degrees Celsius) for selectivity and gives 93% accuracy in differentiating between coliforms from warm-blooded animals versus other sources (total coliforms).

1. Prepare m-FC medium as directed in the appropriate procedure in Section 5.7.

2. Sample volumes are selected that will produce 20-60 fecal coliform colonies per filter. When the bacterial density of the sample is unknown, filter several decimal volumes to establish fecal coliform density.

3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.

4. Place prepared cultures in waterproof plastic bags (whirl bags) and submerge in a water bath. Anchor dishes below the water surface to maintain critical temperature requirements and incubate for 22-26 hours at 44.3 to 44.7 degrees Celsius. Place all prepared cultures in the water bath within 30 minutes after filtration.

5. Colonies produced by fecal coliform bacteria on m-FC medium are various shades of blue. Normally, few nonfecal coliform colonies will be observed on mFC but those found will be gray to cream-colored.

6. Count only the membrane filters with 20-60 fecal coliform colonies. Densities should be recorded as fecal coliforms per 100 ml (Section 4.1.2.1).

Confirmation:

Verify fecal coliforms by picking at least 10 isolates exhibiting a blue color and transfer to lauryl tryptose broth. Incubate at 35 degrees Celsius for 24 and 48 hours and examine for gas production. Transfer growth from positive tubes to EC broth and incubate at 44.5 degrees Celsius for 24 hours. Verification of fecal coliforms is indicated by gas production in the broth.

Alternately, colonies may be identified by biochemical testing using the Enterobacteriaceae test (Section 2.2.4.3). Incubation is at 35 degrees Celsius for 24 hours.

4.1.3.3 Heterotrophic Plate Count

A wide variety of organisms comprise this group which may be defined as those aerobic and facultatively anaerobic organisms requiring an organic carbon source. The media used can be considered for practical purposes as non-selective. Of the two types of media listed, R_2A contains a lower overall nutrient content that aids in the recovery of some stressed organisms from suboptimal water conditions.

1. Prepare R_2A and/or Plate Count Agar as directed in Sections 5.13 and/or 5.14

2. Select volumes that will give 20-200 colonies on the membrane surface.

3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.

4. Incubate the inverted agar plates with the filter for 7 days at 27.5-28.5 degrees Celsius.

5. Plate counts should be recorded on day 7.

6. Count only those plates containing 20-200 total colonies.

7. Compute the density using the formula in Section 3.4.

8. Record densities as total heterotrophs per 100 mL.

4.1.3.4 Fungi

These eukaryotic microorganisms can be distinguished more readily than bacteria based on their morphology. They may be divided into two groups: filamentous fungi called molds, and non-filamentous fungi called yeasts. Considered ubiquitous in nature these microorganisms may be found on surfaces, in the air, and in waters usually containing a low pH. The media used for their isolation (Emmons) is a low nutrient base containing antibiotics and Rose Bengal to reduce the number of bacteria that may otherwise overgrow the plates. Unlike bacteria, these organisms can be readily distinguished by colonial and/or cellular morphological observation.

1. Prepare Emmons medium (Section 5.9) as directed.

2. Select volumes that will give 20-200 colonies on the membrane surface.

3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.

4. Incubate the inverted agar plates with the filter for 3 days at 19.5-20.5 degrees Celsius.

5. Count only those plates containing 20-200 yeast and mold.

6. Compute the density using the formula in Section 3.4.

7. Record densities as total yeast and mold per 100 mL.

Confirmation:

Confirm molds by colonial morphology under a binocular dissecting microscope (10X). Yeasts may be confirmed based on cellular morphology.

4.1.3.5 Non-Saprophytes

This diverse group of organisms has complex nutritional requirements (fastidious) and thus require a medium which will fulfill their specific needs. The medium employed here contains lysed sheep red blood cells which provide hemin and pyridine to improve the isolation of this group, especially members of the genus Haemophilus.

1. Prepare chocolate agar as directed in Section 5.4.
2. Select volumes that will give 20-200 colonies on the membrane surface.
3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.
4. Incubate the inverted agar plates with the filter for 2 days at 35 degrees Celsius.
5. Count only those plates containing 20-200 total colonies.
6. Compute the density using the formula in Section 3.4.

4.1.3.6 Gram Negative

Gram negative bacteria are defined based on the structure of their cell wall which will not retain the primary stain in the Gram reaction (Section 2.1.2.1). The medium chosen here contains bile salts which inhibit the growth of gram positive organisms while favoring isolation of gram negative, primarily enteric bacilli.

1. Prepare MacConkey agar as directed in Section 5.10.
2. Select volumes that will give 20-200 colonies on the membrane surface.
3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.
4. Incubate the inverted agar plates with the filter for 5 days at 35 degrees Celsius.
5. Count only those plates containing 20-200 colonies.
6. Compute the density using the formula in Section 3.4.
7. Record densities as total gram negatives per 100 mL.

Confirmation:

Colonies on MacConkey agar may be confirmed as gram negative by the gram stain (Section 2.1.2.1).

4.1.3.7 Gram Positive

In contrast to gram negative bacteria, organisms of this group retain the primary stain in the Gram reaction. Phenylethanol agar (PEA) used in their isolation inhibits growth of gram negative bacteria by the presence of phenylethanol.

1. Prepare PEA as directed in Section 5.12.

2. Select volumes that will give 20-200 colonies on the membrane surface.

3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.

4. Incubate the inverted agar plates with the filter for 3 days at 28 degrees Celsius.

5. Count only those plates containing 20-200 colonies.

6. Compute the density using the formula in Section 3.4.

7. Record densities as total gram positives per 100 mL.

Confirmation:

Colonies on phenylethanol agar may be confirmed as gram positive by the gram stain (Section 2.1.2.1).

4.1.3.8 Legionella

The genus Legionella consists of both pathogenic and non-pathogenic species, the most notable pathogen being L. pneumoniae. These organisms are found naturally in the environment and apparently pose health risks only under conditions that selectively favor their growth such as water temperatures between 38-46C and interaction with specific algae, protozoa, and other bacteria. A non-selective plating medium is used with an agar enrichment to isolate and cultivate these organisms. This genus can be distinguished from others present on the plate by their characteristic ground glass appearance. Colonies may also appear white to yellow or grey-blue in color. Background organisms can also be reduced by the addition of antibiotics. Verification of Legionella pneumophila is accomplished by a fluorescent antibody technique currently performed by the Center for Disease Control (CDC). Other species may be identified by their fatty acid composition using gas chromatography.

1. Prepare legionella agar + supplements as directed in Section 5.18.

2. Select volumes that will give 20-80 colonies on the membrane surface.

3. Follow procedure 4.1.1 for detailed instructions on membrane filtration.

4. Incubate the inverted agar plates with the filter for at least 10 days at 35 degrees Celsius in an atmosphere containing 5.0% CO_2, at 90% relative humidity.

5. Examine plates daily for blue to grey-blue colonies exhibiting ground-glass appearance.

6. Compute the density using the formula in Section 3.4.

NOTE Since legionellosis appears to be acquired via airborne routes, generation of aerosols should be avoided. A biological safety cabinet should be used in the handling and examination of plates.

Confirmation:

Colonies suspected of being Legionella spp. should be gram stained (Section 2.1.2.1) and subcultured to fresh Legionella Agar plates, one without L-cysteine. Gram negative organisms which grow on a Legionella Agar plate containing L-cysteine but not on a plate lacking it are presumptively identified as Legionellae.

4.1.3.9 Anaerobes

Anaerobes consist of those microorganisms that grow best under conditions of little or no oxygen. They may be divided into those that are destroyed in the presence of oxygen (obligate) and those that can tolerate but do not necessarily prefer its presence (facultative). Collection and testing of waters for anaerobes is more complicated in that anaerobic conditions must be maintained, especially for obligate anaerobes. The procedure given below is based on the presence or absence of anaerobes as performed by the Kennedy Space Center on shuttle water. Although enumeration is not necessarily obtained by this method it can provide useful information as to the bacterial composition of the water. Because facultative anaerobes may also grow on media maintained in an aerobic environment, separation of obligate and facultative species is included.

1. Prepare Fluid Thioglycollate medium as directed in Section 5.19.

2. Add 100 ml of water to the sample bottle and maintain in an anaerobic state (anaerobic hood or bell jar) at 35 degrees Celsius for 10 days.

3. If growth is observed, as indicated by turbidity in the bottle, perform spread plate technique (Section 4.1.2) using Brewers Anaerobic Agar (Section 5.20).

4. Isolated colonies should be picked and streaked on duplicate plates of Brewers Anaerobic Agar, one of which is maintained aerobic and the other anaerobic. Obligate anaerobes will only grow under the anaerobic condition.

4.2 DIRECT MICROSCOPIC METHODS

Direct microscopic methods allow for the enumeration of single organisms, eliminating extended incubation times found in cultural methods. Also, environmental conditions that affect cultural methods of enumeration (pH,temperature,nutrients) are not factors in these methods. Direct microscopic methods also indicate the total population, living and non-living, in a given sample which in some instances may actually be a disadvantage. These methods also have the disadvantage of not allowing for the identification of enumerated organisms.

4.2.1 Petroft-Hauser Chamber Method

1. Clean chamber and coverslip thoroughly, blot and let air dry.

2. Dilute sample 1:1 with 0.1N HCL and fill chamber with 5 ul of sample.

3. Let set 2-3 minutes and observe under phase contrast microscope.

4. Count the cells lying within the small squares. Optimally, the number of bacteria per square is 5-15. Score the cells that cross the boundaries of a square on the upper or right side but not the lower or left side.

5. At least 600 total organisms should be counted and the number of cells calculated by the following formula:

$$\frac{\text{Total bacteria counted} \times \text{dilution factor} \times 4 \times 10^{8}}{\text{number of small squares counted} \times \text{thickness (in um)}}$$

4.2.2 Acridine Orange Direct Count

1. Collect sample to be analyzed and add sufficient formalin (37%) to give a final concentration of 3.7% formalin.

2. Mix a 4 ml volume of sample with 0.4 ml acridine orange (0.1%) for 3 minutes and filter using a 0.2 um black polycarbonate filter (Nucleopore). Rinse funnel with phosphate buffer dilution water (Section 7.1) and using sterile forceps, place the filter on a microscope slide containing a drop of immersion oil. Place a drop of immersion oil on the filter and add a coverslip.

3. Place under a fluorescent microscope and observe on oil objective.

4. Count up to 200 fluorescing particles or 50 fields.

5. Calculate bacterial densities by using the following equation:

$$N = \frac{21.46^{*} \times n}{m \times v}$$

where,

N = total number of cells/100 ml
n = mean count of cells present per area of filter delineated by the eyepiece reticule
m = area enclosed by the eyepiece reticule, mm^2 at magnification
v = volume of liquid sampled, liters

* effective surface area of the filter (mm^2).

5.0 MEDIA PREPARATION

Whenever possible, dehydrated media are employed to ensure consistent product. For quality control of media inoculate as indicated below. A media log form (Form 4) is used to record the results of positive and negative control cultures. Included on this sheet are overnight sterility check results (at 25 degrees Celsius) listed as number of contaminated plates and/or tubes per total number prepared, pH, batch and manufacturer lot numbers, amount weighed, volume prepared, preparer's initials and any comments. For plated media, prepare clean borosilicate flasks by washing in Alconox (Alconox, Inc.) and thoroughly rinsing with distilled water (Section 8.10). Follow directions for preparation and sterilization according to the Difco Manual, 10th ed. unless otherwise indicated. Allow to cool to 50 degrees Celsius and dispense into sterile petri dishes. For screw-cap tubes and caps prepare as above and dispense into tubes using a Cornwall dispenser. Sterilize with caps slightly ajar, remove, and let cool slightly before tightening caps. For agar slants, place sterilized tubes on incline rack and leave at room temperature until agar solidifies.

The recommended shelf life for prepared media is as follows:

agar or broth in loose-cap tubes; 4C	1 week
membrane filter agar in plates with tight fitting covers; 4C	2 weeks
poured agar plates with loose fitting covers in sealed plastic bags; 4C	2 weeks
agar or broth in tightly closed screw-cap flasks; 4C	3 months

5.1 AK AGAR (BBL Microbiology Systems)

This agar is used to promote sporulation in spore-producing microorganisms.

1. Suspend 30.8 grams in 1 L deionized water.

2. Mix thoroughly and heat to dissolve agar.

3. Dispense and autoclave at 121 degrees Celsius for 15 minutes.

QUALITY CONTROL

Dehydrated powder: light tan and free flowing
Prepared medium: amber, slightly opalescent
Positive control: _Bacillus_ sp.
Negative control: _Pseudomonas_ sp.

5.2 BRAIN HEART INFUSION MEDIA BROTH/AGAR

This medium is useful for cultivating Streptococci spp.

1. Dissolve 37 grams in 1 L deionized water and dispense. For agar base add 15.0 grams agar base and heat to dissolve.

2. Adjust pH to 7.4 +/- 0.2 at 25 degrees Celsius.

3. Dispense and autoclave at 121 degrees Celsius for 15 minutes.

QUALITY CONTROL
Dehydrated powder: light tan and free flowing.
Prepared medium: light to medium amber and clear.
Positive control: Streptococcus pyogenes
Negative control: Neisseria sp.

5.3 BRILLIANT GREEN BILE LACTOSE BROTH

This medium is used in the confirmation of total coliforms.

1. Suspend 40 grams in 1 L deionized water and warm slightly to dissolve completely.

2. Adjust pH to 7.2 +/- 0.2 at 25 degrees Celsius.

3. Dispense into test tubes and autoclave for 15 min. at 121 C.

QUALITY CONTROL
Dehydrated medium: greenish beige and free flowing.
Prepared medium: emerald green and clear.
Positive control: Escherichia coli
Negative control: Staphylococcus aureus

5.4 CHOCOLATE AGAR

This medium provides growth requirements for fastidious organisms associated with the human body.

1. Suspend 71 grams of Difco Chocolate Agar in 1 liter distilled water and heat to boiling.

2. Autoclave at 121 degrees Celsius for 15 minutes.

3. Cool to 50 degrees Celsius in a water bath and aseptically add supplement VX. Mix thoroughly.

4. Dispense under laminar flow hood into sterile 50x9mm dishes.

QUALITY CONTROL

Dehydrated medium: tan and free-flowing
Prepared medium: chocolate brown
Positive control: Haemophilis hemolyticus
Negative control: not applicable

5.5 EC BROTH

This medium is used in the confirmation of fecal coliforms.

1. Suspend 37 grams in 1 L deionized water and warm slightly to dissolve completely.

2. Adjust pH to 6.9 +/- 0.2 at 25 degrees Celsius.

3. Dispense into test tubes and autoclave for 15 min at 121C.

QUALITY CONTROL
Dehydrated medium: light beige and free flowing
Prepared medium: light amber and clear
Positive control: _Escherichia coli_
Negative control: _Streptococcus faecalis_

5.6 mENDO AGAR LES

This medium is used for the detection and enumeration of total coliform organisms.

1. Suspend 51 grams in 1 L Type I water containing 20 mL ethanol and heat to boiling to dissolve.

2. Adjust pH to 7.2 +/- 0.2 at 25 degrees Celsius.

3. Cool to 50 degrees Celsius and dispense.

QUALITY CONTROL
Dehydrated medium: purple and free flowing.
Prepared medium: red with possible precipitate.
Positive control: _Escherichia coli_
Negative control: _Staphylococcus aureus_

5.7 mFC AGAR

This medium is used for the detection and enumeration of fecal coliform organisms.

1. Suspend 52 grams in 1 L Type I water and heat to boiling to dissolve completely.

2. Add 10 mL 1% Rosolic Acid in 0.2N NaOH and continue heating for 1 minute.

3. Adjust pH to 7.4 +/- 0.2 at 25 degrees Celsius.

4. Cool to 50 degree Celsius and dispense.

QUALITY CONTROL
Dehydrated medium: beige and free flowing.
Prepared medium: cranberry red and slightly opalescent.
Positive control: _Escherichia coli_
Negative control: _Streptococcus faecalis_

5.8 LAURYL TRYPTOSE BROTH

This medium is used in the confirmation of coliform bacteria.

1. Suspend 35.6 grams in 1 L deionized water and warm slightly to dissolve completely.

2. Adjust pH to 6.8 +/- 0.2 at 25 degrees Celsius.

3. Dispense into test tubes and autoclave for 15 minutes at 121 degrees Celsius.

QUALITY CONTROL

Dehydrated medium: light tan and free flowing.
Prepared medium: light amber and clear to slightly opalescent.
Positive control: *Escherichia coli*
Negative control: *Staphylococcus aureus*

5.9 EMMONS MEDIUM

This medium is used for the enumeration of yeasts and molds.

1. Suspend 30 grams Sabouraud Dextrose agar in 1 liter distilled water.

2. Add 0.05 grams rose bengal and heat to boiling.

3. Remove and adjust pH to 7.0 +/- 0.2 at 25 degrees Celsius.

4. Autoclave at 121 degrees Celsius for 15 minutes.

5. Cool to 50 degrees Celsius and immediately add rehydrated antibiotic (Bacto Antimicrobic Supplement C + 2 ml ethanol) aseptically.

6. Dispense under laminar flow hood into sterile 50x9mm petri dishes.

Note Antibiotic should be stored refrigerated in the dark. Vials that have been rehydrated should be used within 24 hours.

QUALITY CONTROL

Dehydrated medium: light beige and free flowing.
Prepared medium: light pink to amber
Positive control: *Saccharomyces cerevisiae*
Negative control: *Pseudomonas aeruginosa*

5.10 MacCONKEY AGAR

This differential plating medium is used for the isolation and differentiation of lactose-fermenting and lactose nonfermenting gram negative bacteria.

1. Suspend 50 grams in 1 L deionized water and heat to boiling.

2. Adjust pH to 7.1 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius.

4. Cool to 45-50 degrees Celsius and dispense.

QUALITY CONTROL
Dehydrated medium: pinkish beige and free flowing.
Prepared medium: reddish-purple and clear.
Positive control: *Escherichia coli*
Negative control: *Staphylococcus aureus*

5.11 NUTRIENT AGAR

This is a slightly acidic nutrient agar used in the water suitability test (Section 9.1.2).

1. Suspend 23 grams in 1 L deionized water and heat to boiling.

2. Adjust pH to 6.8 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and dispense.

QUALITY CONTROL
Dehydrated medium: beige and free flowing.
Prepared medium: light amber and clear to slightly opalescent.
Positive control: *Enterobacter aerogenes*
Negative control: not applicable

5.12 PHENYLETHANOL AGAR

This is a selective medium used for the isolation of gram positive organisms.

1. Suspend 35.5 grams in 1 L distilled water and heat to boiling

2. Adjust pH to 7.3 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius, let cool to 45-50 degrees Celsius, and dispense.

QUALITY CONTROL
Dehydrated medium: beige and free flowing.
Prepared medium: light to medium amber and slightly opalescent.
Positive control: *Staphylococcus aureus*
Negative control: *Escherichia coli*

5.13 PLATE COUNT AGAR (Tryptone Glucose Yeast Agar)

This medium is used for enumeration of bacteria in water and wastewater.

1. Suspend 23.5 grams in 1 L deionized water and heat to boiling to dissolve completely.

2. Adjust pH to 7.0 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and dispense.

QUALITY CONTROL

Dehydrated medium: light tan and free flowing.
Prepared medium: light amber and slightly opalescent.
Positive control: _Escherichia coli_
Negative control: not applicable

5.14 R_2A AGAR

This medium is used for the enumeration of bacteria requiring initially low nutrient concentrations for growth. The recipe listed below is identical to that commercially available except for the reduction of agar base from 1.5% to 1% which appears to aid in the recovery of stressed organisms.

1. Use the following formula to prepare the medium:

Yeast Extract	0.5 g
Proteose Peptone #3	0.5 g
Casamino acids	0.5 g
Dextrose	0.5 g
Starch	0.5 g
Sodium Pyruvate	0.3 g
Potassium Phosphate,Dibasic	0.3 g
Magnesium Sulfate	0.05 g
Agar	10 g

2. Adjust pH to 7.2 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and dispense.

QUALITY CONTROL

Dehydrated medium: light tan and free flowing.
Prepared medium: amber and opalescent.
Positive control: _Escherichia coli_
Negative control: not applicable

5.15 STAPHYLOCOCCUS MEDIUM 110

This is a selective medium used for the isolation of pathogenic strains of <u>Staphylococcus</u>. It is also a suitable medium for performance of the coagulase test (Section 5.1.4).

1. Suspend 149 grams in 1 L deionized water and heat to boiling to dissolve completely.

2. Adjust pH to 7.0 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 10 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and dispense.

QUALITY CONTROL

Dehydrated medium: beige and free flowing.
Prepared medium: light amber and opalescent with heavy precipitation.
Positive control: <u>Staphylococcus aureus</u>
Negative control: <u>Escherichia coli</u>

5.16 TRYPTOSE GLUCOSE YEAST AGAR

See Section 5.13

5.17 TRYPTIC SOY AGAR OR BROTH

This is a general purpose medium used for the cultivation of both fastidious and nonfastidious organisms.

1. Suspend 40 grams in 1 L deionized water and heat to boiling to dissolve completely. For preparation of agar add 15 grams agar base to the broth.

2. Adjust pH to 7.3 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and dispense.

QUALITY CONTROL

Dehydrated medium: light beige and free flowing.
Prepared medium: light amber and slightly opalescent.
Positive control: <u>Escherichia coli</u>
Negative control: not applicable

5.18 LEGIONELLA AGAR

This media is used for the isolation and cultivation of *Legionella* and consists of an agar base containing yeast extract, charcoal, ACES buffer and alpha-ketoglutarate. The yeast extract provides nutritional requirements, the charcoal appears to serve as a carbon dioxide collector or detoxifier, the ACES buffer maintains proper pH, and the alpha-ketoglutarate provides an additional nutrient source. Agar is added as a solidifying agent. Also included is a separate enrichment source consisting of L-cysteine and ferric pyrophosphate which provide growth factors.

1. Suspend 37 grams agar base in 1 L distilled water.

2. Adjust pH to 7.1-7.2 with 1N KOH.

3. Autoclave for 15 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and aseptically add 2 vials rehydrated enrichment (rehydrated with 5 ml distilled water each).

4. Add 1 vial rehydrated antibiotic suspension (BBL PAV).

5. Mix thoroughly and pH to 6.85-7.0 with 1N HCL or 1N KOH.

6. Maintain agitation during dispensing to prevent settling.

NOTE Enrichment and antibiotic vials should be stored at 2-8 degrees Celsius and protected from light. Rehydrated vials should be used immediately.

NOTE Since legionellosis appears to be acquired via airborne routes, generation of aerosols should be avoided. A biological safety cabinet should be used in the handling and examination of plates.

QUALITY CONTROL

Dehydrated medium: grey-black and free flowing.
Enrichment vial: beige with absence of caking
Antibiotic vial: beige with absence of caking
Positive control: *Legionella gormanii* ATCC 33297
Negative control: *Escherichia coli*

5.19 FLUID THIOGLYCOLLATE MEDIUM

This media is used to determine the presence or absence of anaerobic bacteria. Thioglycollate provide low oxidation-reduction potentials i.e. takes up electrons and thereby reduces the environment. In this way, oxygen is removed from that environment.

1. Suspend 59.6 grams of Bacto Fluid Thioglycollate medium (Difco) in 1 liter distilled water and heat to boiling.

2. The media should be adjusted to pH 7.1 +/- 0.2 at 25 degrees Celsius and 100ml aliquots dispensed into 250ml nitrogen purged bottles.

3. Autoclave for 15 minutes at 121 degrees Celsius.

4. After autoclaving and while media is still hot, insert a sterile gas purge apparatus and purge with nitrogen until media cools.

5. Store in the dark at 15-30 degrees Celsius.

QUALITY CONTROL

Dehydrated medium: not applicable
Prepared medium: straw colored, upper 10% or less pink
Positive control: _Bacteroides melaniogenicus_
Negative control: _Pseudomonas cepacia_

NOTE A positive control is indicated by good to excellent growth within 48 hours incubation at 35 degrees Celsius. Stored media exhibiting more than a 20% pink upper layer should be placed in a boiling water bath to drive off the oxygen.

5.20 BREWERS ANAEROBIC AGAR

This media is used in the confirmation of anaerobic microorganisms and in the differentiation between obligate and facultative species (Section 4.1.2.9).

1. Suspend 58 grams in 1 L distilled water and heat to boiling to dissolve completely.

2. Adjust pH to 7.2 +/- 0.2 at 25 degrees Celsius.

3. Autoclave for 15 minutes at 121 degrees Celsius, cool to 45-50 degrees Celsius, and dispense under laminar flow hood into petri plates.

4. Place plates in anaerobic jar with indicator and maintain this environment until use

QUALITY CONTROL

Dehydrated medium: beige and free flowing.
Prepared medium:
Positive control: <u>Bacteroides melaniogenicus</u>
Negative control: <u>Pseudomonas cepacia</u>

6.0 REAGENT PREPARATION

6.1 Ethylenediaminetetraacetic acid (EDTA)

To prepare EDTA add 372 mg of the disodium salt of EDTA to a volumetric flask and bring up to 1 liter using distilled water.

6.2 Phosphate Buffered Dilution Water

<u>phosphate buffer stock</u>		<u>magnesium chloride stock</u>	
potassium dihydrogen phosphate	34.0 g	magnesium chloride hexahydrate	81.1 g
distilled water	500 ml	distilled water	1 L

1. Dissolve potassium dihydrogen phosphate in distilled water, adjust the pH to 7.2 using 1 N NaOH, and bring the volume to 1 L. This is the phosphate buffer stock.

2. Dissolve magnesium chloride hexahydrate in 1 L distilled water. This is the magnesium chloride stock.

3. Add 1.25 mL phosphate buffer stock solution and 5.0 mL magnesium chloride stock to 1 L distilled water.

4. Dispense in amounts that will provide 99 +/- 2 ml after autoclaving for dilution purposes or as needed for rinsing (500 ml).

5. Dilution bottles should be loosely capped and sterilized for 15 minutes at 121 degrees Celsius. Cool and tighten caps and store in a cool place.

6. Rinse water and stock solutions are autoclaved for 30 minutes at 121 degrees Celsius. Rinse water is likewise stored in a cool place.

7. After sterilization of the stock solution, store in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the stock solution should be discarded and a fresh solution prepared.

6.3 Peptone Dilution Water

Prepare a 10% solution of peptone in deionized water. Dilute a measurable volume to provide a final 0.1% solution. Adjust the final pH to 6.8.

Dispense in amounts to provide 99 +/- 2.0 mL after autoclaving for 15 minutes at 121 degrees Celsius.

NOTE: Phosphate buffer and peptone water are now available commercially and are approved for use in the laboratory.

6.4 1 N Sodium Hydroxide

Dissolve 40 grams of solid NaOH in 500 mL distilled water and bring final volume to 1000 mL in a volumetric flask. Store in a borosilicate glass bottle with stopper.

To make 0.1 N NaOH, dissolve 4 grams in 1 liter or prepare a 1:10 dilution of the 1N stock. To make 0.01 N NaOH, dissolve 0.4 grams in 1 liter or prepare a 1:100 dilution of the 1N stock.

6.5 Sodium Thiosulfate, 10% wt/wt

Dissolve 100 grams of solid sodium thiosulfate in 1000 ml of distilled water. Add 0.1 ml of this solution for each 125 ml of sample volume to sample bottle before sterilization. This concentration will neutralize approximately 15 mg/liter of residue chlorine.

6.6 Disinfectant

Add 1 gallon water to a one gallon jug. To this add 28 ml of Roccal II (10%) (Sterling Drug Inc.). Invert the jug several times to mix. Dispense into plastic squeeze bottles.

6.7 Bromthymol Blue

Add 16 mls of 0.01 N NaOH to 0.1 gram of bromthymol blue. Dilute to 250 mls with distilled water and label as 0.04% solution. Store in a glass bottle at room temperature.

6.8 Kinyoun Acid Fast Stain

1. To prepare the 20% Kinyoun Carbolfuchsin, place 4 grams of Basic Fuchsin in 20 ml of 95% ethanol.

2. Place 8 grams of phenol crystals in 100ml distilled water.

3. Mix these two solutions together and store in a plastic stain bottle that is labeled with the name and concentration, the preparation date and the initials of the preparer. The expiration date is 3 months from preparation.

4. To prepare the 3% acid alcohol, place 3 ml of concentrated hydrochloric acid into 97 ml of 95% ethanol. Do not add alcohol to acid. Store in a plastic stain bottle labeled with the name and concentration, the preparation date and the initials of the preparer. The expiration date is 3 months from the date of preparation.

5. To prepare the 3% Methylene blue counterstain, place 0.3 grams of Methylene blue into 100 ml of distilled water. Store in a plastic stain bottle labeled with the name and concentration, the preparation date and the initials of the preparer. The expiration date is 3 months from date of preparation.

6.9 Poly-B-Hydroxybutyrate (PHBA) Stain

1. To prepare the 0.3% Sudan Black B, place 0.3 grams of Sudan Black B into 100 ml of 70% ethanol (70% ethanol can be made by adding 73.6 ml of 95% ethanol and bringing the volume to 100 ml using distilled water).

2. Shake the solution thoroughly and allow it to stand overnight before use.

3. Store the stain in a plastic stain bottle and label with the name and concentration, the preparation date and the initials of the preparer. The expiration date is 3 months from date of preparation.

6.10 Capsule Stain

1. To prepare the 1% nigrosin solution, add 1 gram of nigrosin granules to 100 ml of distilled water.

2. Store in a plastic bottle and label with the name and concentration, the preparation date and the initials of the preparer. The expiration date is 3 months from date of preparation.

6.11 Algicide for Water Baths

Laboratory algicide (Polyscience Corporation) is added to water baths with each refilling. The Fisher brand water bath requires 3.7 ml algicide. The Precision and Lab Line water baths require 2.6 ml.

7.0 STORAGE AND MAINTENANCE OF MICROORGANISMS

Laboratory-acquired contamination of laboratory personnel are largely avoidable through proper equipment use and/or correct laboratory practices and techniques. A discussion of currently available equipment in the laboratory and proper use can be found in Section 8.0. The following section describes proper manipulation of microorganisms within the laboratory and should be adhered to whether the organism is of human or environmental origin. It is also recommended that all laboratory personnel read the pamphlet Biosafety in Microbiological and Biomedical Laboratories produced jointly by the Centers for Disease Control and National Institute of Health, May 1988. This pamphlet is located in Room 1038A of the Microbial Ecology laboratory.

7.1 Lyophilization of Cultures

Lyophilization, or freeze drying, of cultures consists of removing all water from the cell under the influence of a vacuum. Organisms prepared in this way can theoretically remain dormant indefinitely. Reactivation usually requires only the addition of a suitable liquid medium and incubation at the proper temperature. All cultures acquired by the laboratory are stored in a freeze-dried state for future use. Two vials of each organism are retained and stored in the low temperature freezer (Revco) at -40 degrees Celsius. The following procedure is used to prepare these cultures for storage.

1. The lyophilization vials to be used are stoppered with cotton and sterilized by autoclaving prior to use.

2. Cultures to be lyophilized are inoculated into a suitable broth for growth and incubated for 18-24 hours.

3. Fill a Dewar Flask 2/3 full with crushed dry ice. Add enough acetone to produce a slushy consistency.

4. Aseptically transfer 0.5 ml of the broth culture to a sterilized lyophilization vial.

5. Turn on the lyophilizer refrigeration unit.

6. Check that all port valves are set to VENT.

7. When the temperature is below -40 degrees Celsius, turn on the vacuum pump.

8. The unit is ready to use when the vacuum is below 25 microns of mercury.

9. Place the vial in the dry ice mixture to freeze it. Swirl the vial so that the frozen broth lines the side of the vial.

10. Connect the pre-frozen samples one at a time to the vacuum valves.

11. Turn the vacuum valve to the VAC position.

12. Allow the system pressure to return to a vacuum sufficient to prevent melting of the sample before adding additional samples (usually 25 microns of mercury).

13. Allow samples to freeze dry (about 3 hours).

14. When all of the frost has disappeared from the outer surface of the sample container and no cold spots can be detected by handling the container, the sample is nearly dry. To be certain of a low final moisture content, dry the sample for several more hours.

15. Once the sample is dry, the ampules may be sealed while connected to a vacuum valve using a propane torch. Apply the torch to the ampule neck until it turns red, then gently pull the base of the ampule away from the neck.

16. To remove the ampule neck, turn the vacuum valve to the VENT position. Discard into an autoclave receptacle.

17. Remove all vials and ampules from the unit.

18. Turn the vacuum pump off.

19. Turn the refrigeration unit off.

20. Take the top off the lyophilizer and clean the inside with disinfectant (Section 6.6).

21. Open the drain tube to remove accumulated water.

22. The oil in the vacuum pump should be changed quarterly or as needed using #19 Premium Grade Mechanical Vacuum Pump Oil (Fisher). Record oil changes (date and person) on the sticker attached to the pump.

7.2 Opening of Lyophilized Cultures

It must be remembered that the inside of a lyophilization tube is under vacuum and that when broken may eject the freeze-dryed organisms out at the technician. The following steps will eliminate this risk to the individual and should be performed on a previously disinfected surface (not under a hood). Gloves should be worn during the operation.

1. If the ampule is not previously scored, do so with a metal file.

2. Dampen heavy gauze with disinfectant (Section 6.6) and wrap the gauze around the ampule.

3. Break ampule at the scored spot by pushing away from you.

4. Add sufficient liquid medium to the ampule to dissolve pellet (1 ml) using sterile pasteur pipette.

5. Using the same pipette, withdraw the contents and transfer to 10 ml of a suitable liquid medium and incubate as required.

6. Disinfect both sections of the ampule and discard into autoclave bag.

7. Disinfect working area when completed.

7.3 Low Temperature Storage

Cultures may be stored and maintained in the presence of liquid nitrogen (-196 degrees Celsius). The same precautions apply as described in the introduction to Section 7.2.

1. Aseptically transfer 2 ml of a 24 hour broth culture to a sterile cryogenic tube and add 15% sterile glycerol to stabilize the culture.

2. Tighten the cap to prevent liquid nitrogen from entering the tube.

3. Place the tube in a vertical storage rack and immerse in a liquid nitrogen tank containing liquid nitrogen at the level recommended by the manufacturer.

4. Liquid nitrogen should be monitored weekly to insure proper levels. During periods of frequent use the levels should be monitored daily.

5. If the liquid nitrogen tank does not have a level gauge, a wooden ruler of sufficient size to reach the bottom of the tank may be used. Insert the ruler into the tank, remove and shake. A visible frost line will form on the ruler indicating the level.

7.4 Transfer of Microorganisms

Removing the stopper or cap from a tube may produce an aerosol of infectious material. This may be reduced by incubating the tubes with the caps slightly ajar. When transferring liquid from one tube to another, always use a sterile pipette with bulb or mechanical suction. **UNDER NO CIRCUMSTANCES SHOULD YOU MOUTH PIPETTE.** Disinfect the area prior to and after use, discarding all contaminants into an autoclave receptacle.

All cultures should be considered pathogenic. In case of spills, saturate the area with disinfectant prior to clean up.

If glassware containing the organisms is broken disinfect as above and carefully remove large pieces of glass with heavy gloves and forceps. Very small pieces may be picked up with a large piece of wet cotton. Discard glass in broken glass receptacle. Report all spills to senior laboratory personnel.

Cultures of bacteria can be maintained on agar slants (Section 5.0) provided they are subcultured periodically to fresh media. In general, organisms may be subcultured every 2-3 weeks. An exception is Pseudomonas cepacia which must be transferred weekly. Tryptic soy agar (Section 5.17) is normally the medium of choice but it is advised that for commercially obtained organisms the recommended media be employed.

1. Allow all media to come to room temperature prior to transferring cultures.

2. Flame an inoculating loop to redness and let cool briefly. Remove the slant cap, flame the lip of the tube and touch the loop into the growth on the slant. Remove the loop and reflame the lip of the tube. Recap.

3. Remove the slant cap of the fresh media, flame the lip of the tube and insert the loop containing the organism into the bottom of the slant. Slowly move the loop in a back and forth motion up the agar slant.

4. Remove the loop and flame it. Reflame the lip of the tube and recap.

Note To reduce "splattering" of the organism after subculturing place the loop in the uppermost part of the flame and gradually move down.

8.0 LABORATORY MAINTENANCE

Periodic maintenance of laboratory equipment reduces cost and ensures correct operation of the instrumentation. Specific maintenance requirements may be found in the operating manual of each instrument which is on file in Room 1038B. A general guideline is provided below.

8.1 Autoclave

Residue left within the chamber will quickly bake into the surface and make cleaning difficult. Immediately clean any spills or overflows within the chamber using a mild abrasive cleaner. Using a sponge, rinse thoroughly but do not allow water to flow through the drain filter. The filter should also be cleaned daily. Periodically check the gasket for deterioration.

NOTE: ALWAYS USE GLOVES WHEN OPENING THE AUTOCLAVE AT THE END OF A CYCLE. OPEN DOOR GRADUALLY AND STAND BACK FROM THE DOOR WHEN FIRST OPENING TO AVOID CONTACT WITH ESCAPING STEAM.

8.2 Balances

The balances and balance work areas should always be kept clean. Insure that the balance is arrested before cleaning the balance pan. Use a soft brush when cleaning the pan. Balance doors should be kept closed except to load and unload materials.

8.3 Centrifuges

Because centrifuges are one of the major sources of contamination in the laboratory it is important to properly maintain them. Brushes and bearings should be checked at the time intervals specified by the manufacturer. Properly balancing the sample loads will prevent premature wear of the parts. Using appropriate containers with tight fitting lids will prevent breaks/leaks while in operation. NO SAMPLES CONTAINING ORGANISMS SHOULD EVER BE CENTRIFUGED WITHOUT APPROPRIATE LIDS. Centrifuge tubes used in angle-head centrifuges should never be filled to the point that liquid is in contact with the lip of the tube when placed in the rotor. If spilling of contents occurs, immediately clean the drum with disinfectant (Section 6.6).

IF GLASS BREAKS OCCUR, SATURATE THE AREA WITH DISINFECTANT USING A SQUEEZE BOTTLE BEFORE PICKING UP THE BROKEN GLASS WITH HEAVY GLOVES AND FORCEPS. DISPOSE OF THE GLASS IN GLASS DISPOSAL CONTAINERS.

8.4 Glassware

Inspect all glassware prior to use for chipped edges, cracks and etched inner surfaces. Damaged glassware should be discarded in glass disposal containers. Properly maintained glassware is cleaned thoroughly with Alconox, rinsed with hot water and rinsed 3X with distilled water. Periodically test glassware for chemical or detergent residue by adding several drops of bromthymol blue indicator (Section 6.7). A yellow-bluegreen-blue color (pH 6.5-7.3) is acceptable. Rewash or discard (in glass disposal container) if unacceptable.

8.5 Incubators/Refrigerators/Freezers

Incubator, refrigerator and freezer chambers should be cleaned with disinfectant (Section 6.6) as necessary. This should also include the removal and cleaning of racks, where applicable. Gaskets should be examined periodically for deterioration and loss of sealing capability. Refrigerators with freezer compartments and freezers should be defrosted on an as needed basis making sure arrangements have been made for storing contents during the process.

8.6 Laminar Flow Hood

The laminar flow bench area should be kept free from any particulate matter that may blow into the room when the unit is turned on. This may be maintained by wiping the surface with disinfectant (Section 6.6) prior to turning the blower on and after use.

NOTE: NEVER TRANSFER BACTERIAL CULTURES UNDER THIS HOOD

8.7 Microscopes

General maintenance requirements for microscopes pertain mainly to keeping the instruments and surrounding areas clean. Microscopes should be covered when not in use. If the Nikon epifluorescent attachment has been used do not cover the instrument until the lamp housing has cooled. Clean lens and eyepieces with lens paper only. If an oil immersion objective has been used, remove all oil from the surface of the lens and stage with lens paper before storing.

8.8 PH Meter

The pH meter should be in stand-by position when not in use, with the electrode in pH 7.0 buffer. This will prevent drying out of the electrode. When placing the electrode into a different solution rinse it thoroughly with distilled water and promptly place in new solution to avoid drying out.

8.9 Water Baths

Water baths should be drained and cleaned periodically with a disinfectant (Section 6.6). Prior to refilling of the bath an algicide (Section 6.11) should be added. Use only distilled water to refill to reduce the build-up of scale within the bath.

8.10 Water Distillation Unit

To insure uninterrupted use, the pretreatment cartridges should be changed as indicated by the panel light. At the end of the day all water should be drained from the boiler. This will reduce scale build-up on the heater elements. When necessary, the boiler and heater elements can be cleaned with 5-10% acetic acid solution.

8.11 Nanopure Water System

Water produced by the distillation process may be further processed by the Nanopure System to provide a higher quality water source (Type I). Cartridges should be replaced when resistivity falls below its preset range (usually 18 Mohm) as indicated on the display panel.

9.0 ANALYTICAL CONTROL

Unlike most analytical laboratories, microbiology laboratories do not lend themselves to usual control checks such as "spiking " of samples. Therefore, the laboratory must provide preventative analytical control measures to insure accuracy in reporting of data. These measures include monitoring of laboratory cleanliness, equipment and personnel.

Recommended laboratory methods for evaluating control efficiency are presented below. Many of these methods have been developed by the Environmental Protection Agency (EPA) for the certification of laboratories to perform total coliform counts on drinking water supplies. Because the laboratory is not directed toward this specific goal it may be determined during the evolution of the laboratory that all of these methods are not critical to its normal operation. In some cases, modifications of the recommendations may be sufficient. Any revisions require the approval of the laboratory director and senior laboratory personnel and is included in the appendices of this manual.

9.1 Methods for Monitoring Laboratory Cleanliness

9.1.1 Laboratory Water

Laboratory water, more commonly termed reagent grade water, is divided into specific types, each of which are defined by maximum chemical and bacterial levels allowed. Type II water comprises most of the water processed in the laboratory and is used in the preparation of media, dilution and rinse volumes,and for filling water baths. This water is produced in the laboratory by distillation and can be sterilized by autoclaving. Type I water is produced by further processing the distillate through a series of polishing beds and a microbial filter (Nanopure System). This water is intended for critical microbiological analysis such as preparation of reagents susceptible to high temperatures and trace contaminants e.g. antibiotics. Type I water may also be sterilized by autoclaving when necessary.

The quality of water used in microbiology testing is monitored in terms of both bacterial and chemical compositions. Parameters for each are specified by the EPA for Type II water and are outlined below. In addition, bacterial analysis specifications for Type I water is also included.

9.1.1.1 Bacterial Analysis

Water used for routine laboratory procedures (Type II) is tested weekly for bacterial contamination using the membrane filtration technique (refer to Section 4.1.1 of this manual). Heterotrophic plate count media (4.1.2.3) should be employed for the analysis. Samples are taken directly from the water storage container. The number of colony forming units should be below 1000 CFU/mL of water tested . If the counts exceed this limit,

clean the water storage container. If the counts still remain too high, descale the still and/or change the prefilter(s). Results should be recorded in the Analytical Control Notebook, Section 1.

Frequency of testing of water produced by the Nanopure System is dependent upon usage. During periods of high demand (> 12 liters/week) the water should be tested as outlined above for distillation storage tank water. Otherwise, monthly testing will be sufficient. The number of colony forming units should be below 10 CFU/ml. If the counts exceed this limit, the final filter should be replaced. Results should be recorded in the Analytical Control Notebook, Section 1.

9.1.1.2 Chemical Analysis

The following tests should be run on Type II water to assure its purity. Results should be recorded in the Analytical Control Notebook, Section 3.

Parameter	Requirements	Frequency
Conductivity	>0.5 megaohm resistance	Monthly
Total Chlorine Residual (Hach Method)	Non-detectable	Monthly
Quality of Laboratory Pure Water (Water Suitability Test, Section 9.1.2)	Ratio 0.8-3.0	Annually

If water does not meet quality requirements, the following tests have been recommended by the EPA to determine possible causes:

Parameter	Limits
Pb,Cd,Cr,Cu,Ni,Zn	Not greater than 0.5 mg/L
Total metals not limited to those above	Equal to or less than 1.0 mg/L
pH	5.5-7.5

9.1.2 Water Suitability Test

This test is used for the determination of toxic or stimulatory effects of distilled water on bacteria. This is done annually or when there is a new source of Type II water in the laboratory.

1. Preparation of Reagents:

   The sensitivity of the test depends on the purity of the reagents used. Use reagents of the highest purity and prepare them in freshly distilled water.

   Sodium Citrate Solution: Dissolve 0.29 g of sodium citrate in 500 ml of distilled water. This is reagent 1.

   Ammonium Sulfate Solution: Dissolve 0.60 g of ammonium sulfate in 500 ml of distilled water. This is reagent 2.

   Salt Mixture Solution: Dissolve 0.26 g of magnesium sulfate, 0.17 g of calcium chloride, 0.23 g of ferrous sulfate, and 2.50 g of sodium chloride in 500 ml of distilled water. This is reagent 3.

   Phosphate Buffer Solution: Stock phosphate buffer solution, diluted 1:25 in distilled water. See Section 6.2 of this manual. This is reagent 4.

   Boil all reagent solutions 1-2 minutes to kill vegetative cells. Reagents may be stored in sterilized glass-stoppered bottles in the dark at 5 degrees Celsius for up to 3 months but should be checked for sterility prior to use.

2. The following media should be prepared for use in the test:

   Plate Count Agar (Section 5.13)
   Nutrient Agar Slants (Section 5.11)
   Sterile Buffered Dilution Water-99 ml (Section 6.2)

3. A 24 hour culture of Enterobacter aerogenes (ATCC 13048) is used as the control organism.

4. Collect 200 ml each, of the unknown Test Water and Control Water (EPA Reference water or other suitable ultrapure water source) in sterile 500 ml screw cap flasks. Boil for 2 minutes and cool to room temperature.

5. Label five sterile 200 ml screw cap flasks A, B, C, D, and E. Add the Test Water, Control Water, and reagents to each flask as described in the following table.

	Flasks (ml)				
Reagents	A	B	C	D	E
1	2.5	2.5	-	2.5	-
2	2.5	2.5	-	-	2.5
3	2.5	2.5	2.5	2.5	2.5
4	1.5	1.5	1.5	1.5	1.5
Test Water	-	21.0	21.0	21.0	21.0
Control Water	21.0	-	5.0	2.5	2.5
Total Volume	30.0	30.0	30.0	30.0	30.0

6. Perform a Standard Plate Count on prepared reagents, Control Water and Test Water as a check on contamination.

7. The day before the test inoculate a strain of <u>E. aerogenes</u> onto a nutrient agar slant. Incubate for 18-24 hours at 35 degrees Celsius.

8. To harvest viable cells, pipette 1-2 ml of sterile dilution water from a 99 ml water blank onto the culture. Emulsify the growth on the slant by gently rubbing the bacterial film with a pipette, being careful not to tear the agar. Pipette the suspension back into the original 99 ml water blank.

9. Make a 1:100 dilution of the original bottle into a second water blank, a further 1:100 dilution into a third water blank, and a 1:10 dilution of this into a fourth water blank, shaking vigorously after each transfer.

10. Transfer 1.0 ml of the fourth dilution into each of Flasks A, B, C, D and E. This procedure should result in a final dilution of the organisms to a range of 30-80 cells from each mL of test solution.

14. Calculate and record the results using the following formula:

(a) For growth-inhibiting substances:

$$\text{Ratio} = \frac{\text{colony count/ml flask B}}{\text{colony count/ml flask A}}$$

A ratio of 0.8 to 1.2 shows no toxic substances; a ratio of less than 0.8 shows growth-inhibiting substances in the water sample.

The following formulas are used to calculate any stimulatory effects of the Test Water:

(b) For Nitrogen and carbon sources that promote growth:

$$\text{Ratio} = \frac{\text{colony count/ml flask C}}{\text{colony count/ml flask A}}$$

(c) For nitrogen sources that promote growth:

$$\text{Ratio} = \frac{\text{colony count/ml flask D}}{\text{colony count/ml flask A}}$$

(d) For carbon sources that promote bacterial growth:

$$\text{Ratio} = \frac{\text{colony count/ml flask E}}{\text{colony count/ml flask A}}$$

Do not calculate ratios b, c or d when ratio a indicates a toxic reaction. Ratios b, c, or d, in excess of 1.2 indicate an available source for bacterial growth. Results are recorded in the Analytical Control Notebook, Section 3.

9.1.3 Ultra Violet Light Check

This test is done quarterly or when a new lamp is installed, to monitor the effectiveness of the ultraviolet (U.V.) rays used to disinfect membrane filtration equipment.

1. Using E. coli, prepare a set of dilutions so as to obtain 200-250 colony forming units in 0.5 ml. Inoculate three Plate Count Agar plates with 0.5 ml of the desired dilution and spread with a glass rod.

2. Place two plates under the U.V. light source, remove the covers and expose the plates to U.V. light for two minutes. Place the remaining plate under ordinary light to use as a positive control.

3. Replace the covers and label the plates with test, location, and date. Incubate the plates at 35 degrees Celsius for 24h.

4. Count the colonies and record the results in the Analytical Control Notebook, Section 2. A count reduction of less than 99% requires replacement of the lamp.

9.2 Calibration Procedures

All calibration procedures performed by or for the laboratory are recorded in the Calibration Notebook. Specific sections are provided for each instrument that requires calibration.

9.2.1 Calibration Check for Autoclave

Calibration of the autoclave is performed yearly by MSI, a NASA subcontractor.

9.2.2 Calibration Check for Balances

The Calibration Facility at NASA is responsible for the calibration of all balances twice yearly and whenever a balance has been moved.

9.2.3 Thermometer Calibration Check

The Calibration Facility at NASA is responsible for the calibration of all thermometers twice yearly.

9.2.4 Calibration Check for Safety Cabinet

Calibration of the safety cabinet is performed by a laboratory certified according to NIH, NCI, and NSF standards. A performance contract is arranged with that agency based in terms of cost, availability and capability. Cabinets should be recertified to factory standards every 12 months.

9.2.5 Volumetric Check for Digital Pipettors

All pipettes are checked quarterly to assure proper delivery of desired volume. The analyst should use the following procedure to check each pipette's delivery volume.

1. Place a small weighing boat on the analytical balance pan and tare it to zero.

2. Select the desired volume to be delivered from the pipette. Using megapure water, pipette the desired volume onto the weighing boat. Record the results in the pipette calibration log book.

3. Repeat step 2 nine times and record the readings. Each time, subtract the previous result from the current result and record it.

4. Convert each weight into volume using the following formula:
   $$1 \text{ microliter } H_2O = 1 \text{ milligram } H_2O$$

5. Delivery volumes should be within 1% of the expected volume.

9.2.6 Calibration Check for Laminar Flow Hood

See Section 9.2.4.

9.2.7 Calibration Check for Incubators

The Calibration Facility at NASA is responsible for the quarterly calibration of all incubators in service.

9.3 Personnel

Before any procedure is performed or instrument used, the analyst should be approved by senior laboratory personnel as capable of performing the task required. Certification should be based on mental and physical proficiency for each instrument or procedure, the criteria for each being established by the laboratory director and senior laboratory personnel.

10.0 SAFETY

Specific safety guidelines pertaining to a particular operation or procedure have been incorporated into the manual under each section. General safety guidelines are addressed below.

10.1 Decontamination/Sterilization

The decontamination or sterilization times of various materials is provided in the table below and includes the time necessary for the safe disposal or use of materials.

Item	Time (minutes)
Membrane filters, pads	10
Carbohydrate containing media	12-15
Contaminated materials	30
Membrane filter assemblies	15
Sample collection bottles	15
Individual glassware	15
Dilution water blank	15
Rinse water (up to 1 L)	30

10.2 Hand Washing

One of the most important general safety measures is the frequent washing of hands. It is considered good practice to wash hands before and after completion of an operation involving sampling, sample analysis or manipulation of microorganisms. Liquid hand soap is provided in squeeze bottles at each sink in the laboratory.

10.3 Laboratory Appearance

A clean and orderly laboratory not only reduces the potential for contamination but also provides a safe working place. Prompt cleaning of reagent, media and chemical spills reduces the risk of carry-over contamination to the next person using the work area and helps maintain a clean appearance in the lab. Boxes should be stored under counters and away from passageways and exits. Each workstation is equipped with the necessary supplies to carry out the operations designated for that area. It is the responsibility of all laboratory personnel to monitor the availability of supplies at each workstation and alert the responsible individual(s) when low.

10.4 Personal

In accordance with the Centers for Disease Control and National Institutes of Health, eating, drinking, smoking, and the applying of cosmetics are not permitted in the work area. Likewise, contact lenses should not be applied in the work area. Food should be stored outside the work area.

Any clothing, including laboratory coats, that become contaminated should be autoclaved prior to laundering. Shoes should not be open-toed or raised such as to cause instability to the wearer.

Gloves and face masks should be removed promptly after completion of the operation and discarded into a autoclave receptacle. Gloves should be removed prior to handling the telephone, lab reports, notebooks, etc. If necessary, a paper towel may be used in the gloved hand when objects must be handled.

Visitors to the laboratory should notify the laboratory director at least one day prior to their visit. All visitors should be escorted by laboratory personnel while in the laboratory. The doors located on the south side of the laboratory should only be used in case of an emergency.

10.5 Safety Equipment

The laboratory contains a eyewash station at the left hand sink. All present and in-coming personnel should be familiar with the operation of the eyewash station prior to performing any task within the laboratory. The location of first aid kits, fire extinguishers (inspect annually) and fire blankets should also be indicated to all personnel. The laboratory director is responsible for ensuring that the individual is made aware of these items.

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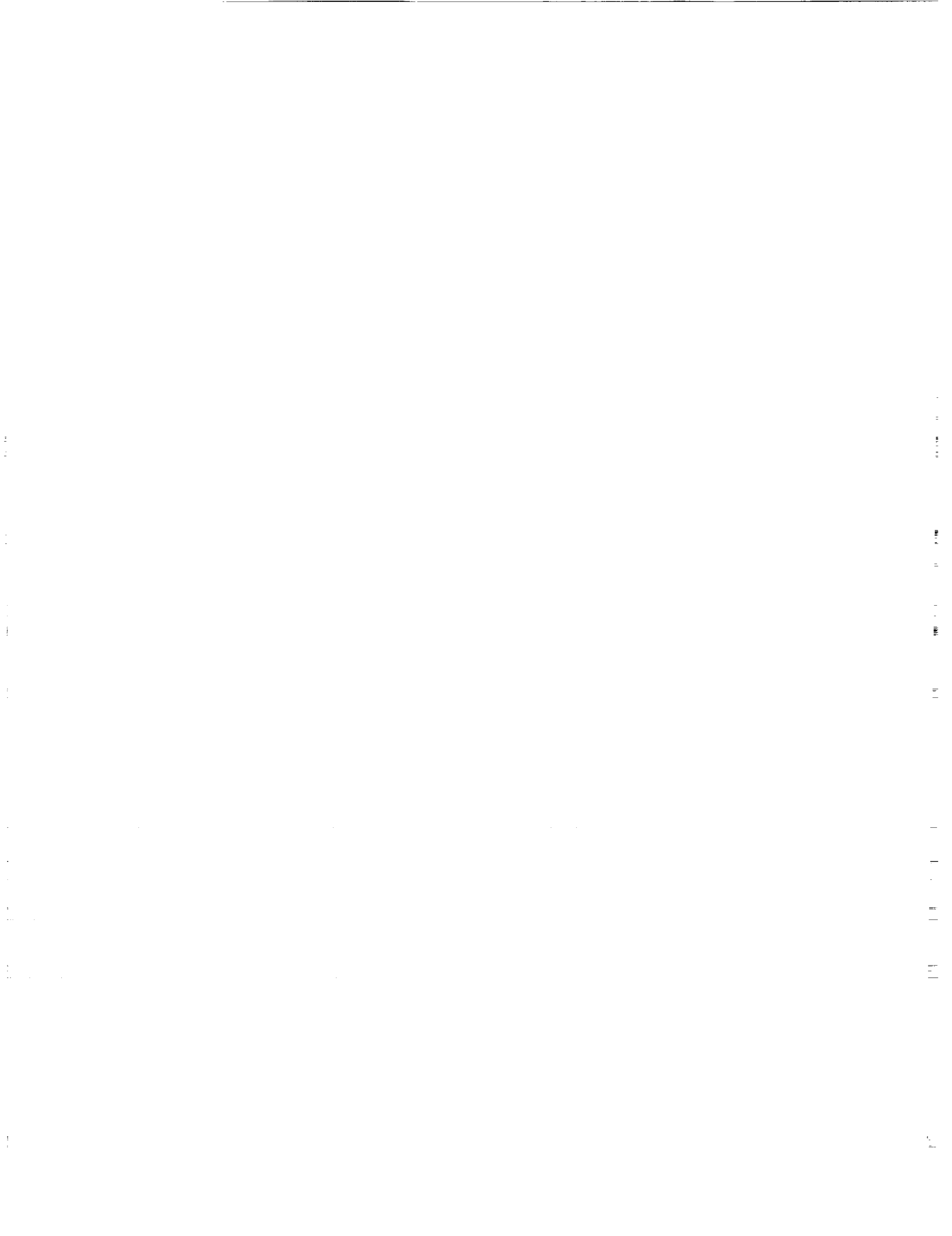
APPENDIX A



FORM 1
CULTURE DATA SHEET

Reference Number: ______________ Isolation Date: ____________
Lab Number: ____________________ Isolation Medium: ___________
Culture Number: ________________ Source: ____________________

Colony Morphology: 18hr 24hr 48hr Medium: ________________

Form: punctiform circular rhizoid irregular filament
Surface: smooth rough dry moist dull glistening
Elevation: flat raised convex pulvinate
Edge: entire undulate lobate filament
Consistency: butyrous viserid brittle
Color: white off-white yellow brown pink red

Cell Morphology: 18hr 24hr 48hr Media: ________________

Form: sphere long/short rod filament comma spiral
Arranged: single pairs fours cubes chains clusters
Spores: present (central,terminal,sub) absent
Granules: present absent

Staining Reaction:

Gram: ______ Slide #: ___________ Control: __________
Other __________: ______ Slide #: ___________ Control: __________
Other __________: ______ Slide #: ___________ Control: __________

Physiology:
Tests:

Catalase	________	24hr	48hr	72hr	other	______
Oxidase	________	24hr	48hr	72hr	other	______
O/F	________	24hr	48hr	72hr	other	______
Motility	________	24hr	48hr	72hr	other	______

Identification:

Minitek Test: Gram positive Enterobacteriaceae Non-fermenter
Profile #: ______________
I.D.: ____________________________________
Probability: _________

Special Tests: Results:

__________________ __________________
__________________ __________________

Comments: ____
____

Storage Location: ______________

Signed: ____________________ Date: ___________

FORM 2
CULTURE QUALITY CONTROL WORKSHEET

Date Tested: ____________ Technician: ________________

Culture Number:

____________	____________	____________
____________	____________	____________
____________	____________	____________
____________	____________	____________

Controls:

Oxidase	_Pseudomonas aeruginosa_	________
	Enterobacter cloacae	________
Catalase	_Staphylococcus aureus_	________
	Streptococcus faecium	________
O/F	_Enterobacter cloacae_	________
	Pseudomonas aeruginosa	________
Minitek		
Enterobacteriae	_Enterobacter cloacae_	________
Nonfermenter	_Proteus vulgaris_	________
Gram Positive	_Streptococcus faecium_	________

FORM 3
SAMPLE DATA SHEET

Chain of Custody/
Sample Number: ____________ Date Received: ____________
Lab Number: ____________ Time Received: ____________
Requestor: ____________

Description: ____________

Sample Condition: ____________

PARAMETER	RESULT/UNIT	ANALYST	DATE	REFERENCE

Comments: ____________

APPENDIX B

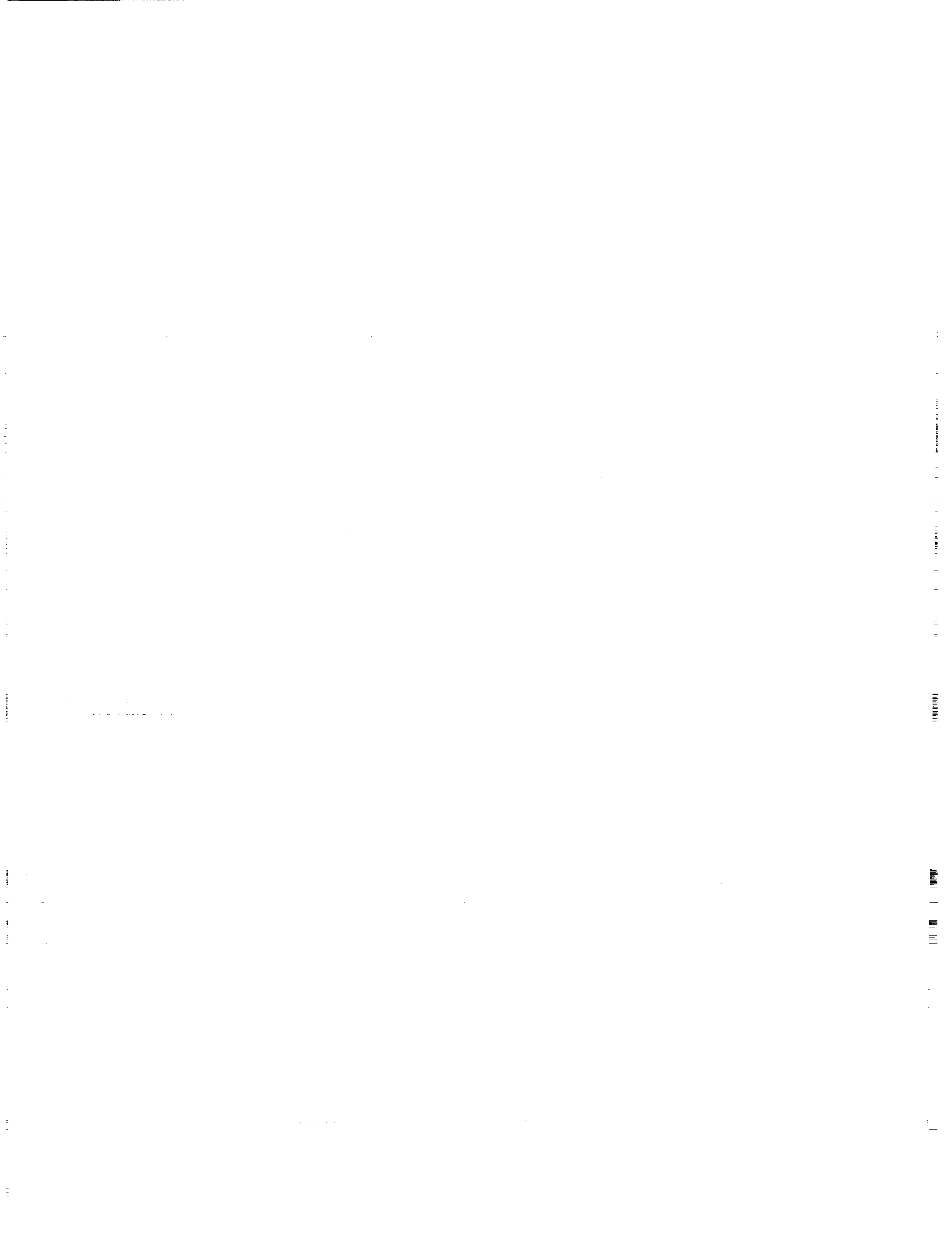


TABLE 1

MICROBIAL ECOLOGY LABORATORY CONTROL ORGANISMS

Organism	ATCC Number
Acinetobacter calcoaceticus	19606
Bacillus subtilis	6633
Citrobacter freundii	8090
Enterobacter aerogenes	13048
Enterobacter cloacae	23355
Escherichia coli	25922
Klebsiella pneumoniae	13883
Proteus vulgaris	13315
Pseudomonas aeruginosa	27853
Pseudomonas cepaciae	35254
Pseudomonas diminuta	19146
Pseudomonas picketti	27511
Salmonella typhimurium	14028
Serratia marcescens	8100
Shigella flexneri	12022
Shigella sonnei	25931
Staphylococcus aureus	25923
Staphylococcus epidermidis	12228
Streptococcus faecalis	19433
Streptococcus pyogenes	19615

TABLE 2

Illustrations of terms used to describe colonial growth

Form

Elevation

Edge

APPENDIX C

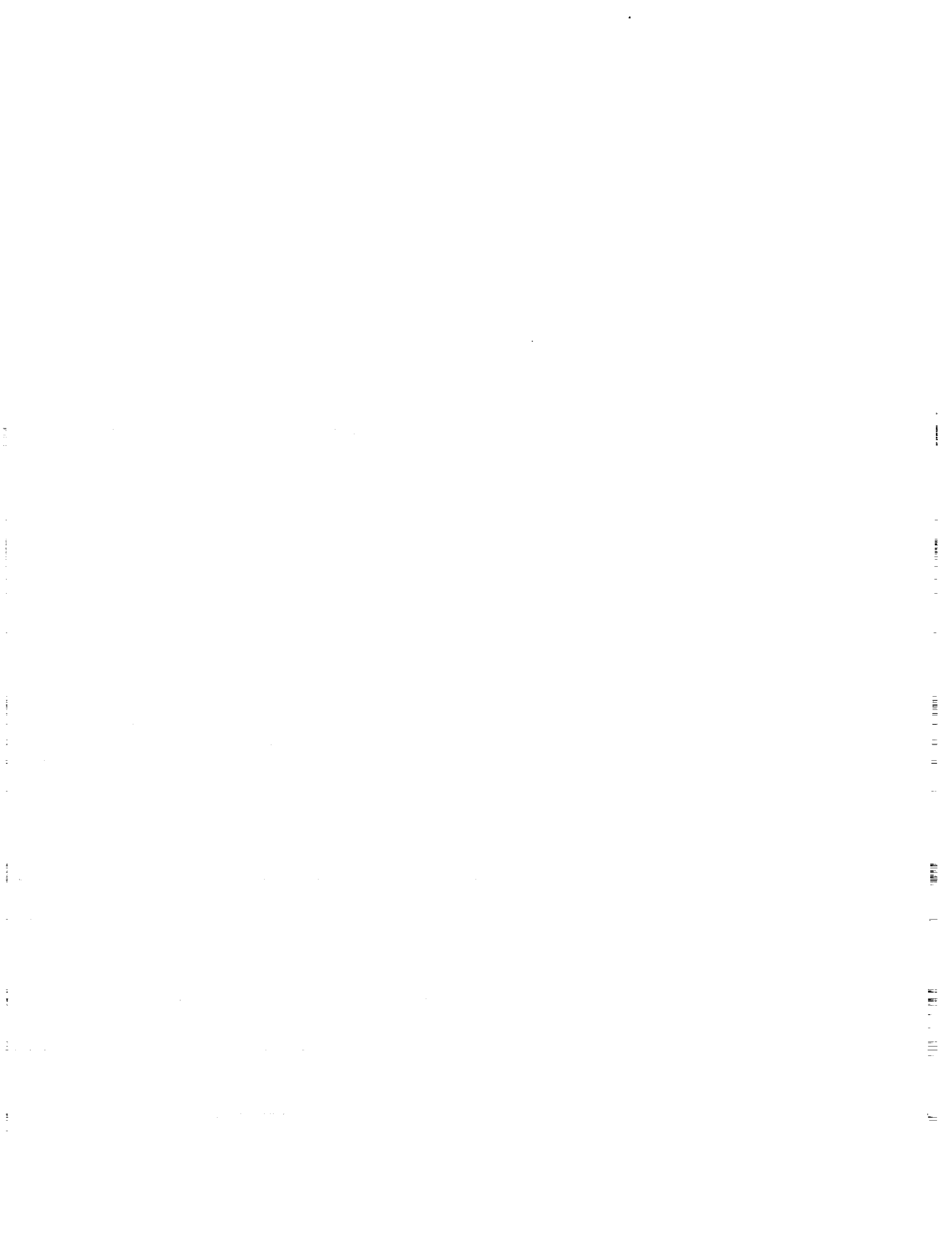
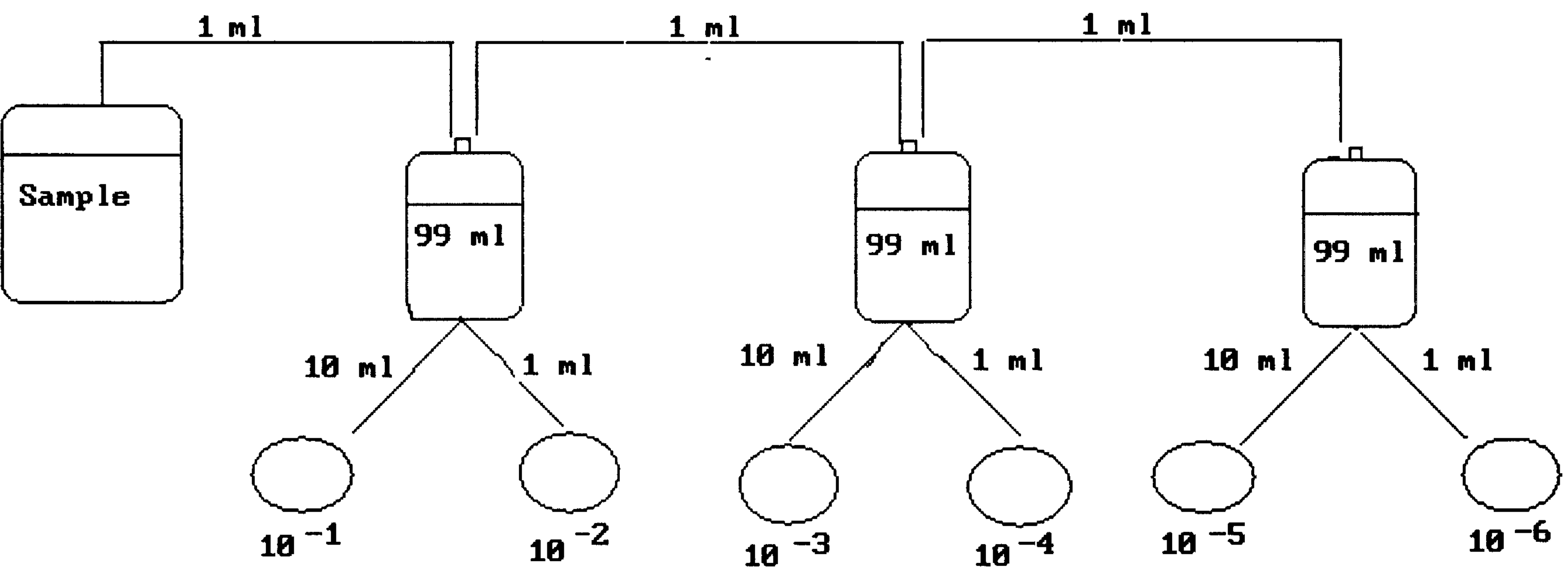


FIGURE 1

DECIMAL DILUTION



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