POLLUTION MICROBIOLOGY

A LABORATORY MANUAL

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This volume is dedicated to the founders of the Department of Environmental Sciences at Rutgers University who had the vision, fifty years ago, to see what is now obvious to all.

PREFACE

Microorganisms play many roles in pollution. They may warn of unsatisfactory conditions, or the microorganisms may themselves be pollutants. They may contaminate the environment with noxious metabolic products. Microorganisms consume oxygen, sometimes deoxygenating waters, but in so doing destroy much of the wastes with which man burdens the environment. Obvious signs of gross pollution may be perceived without the aid of a microscope - an unpleasant odor, dead fish, a swimming-prohibited notice. But these often result from microbial activities, as would the restoration of the environment to a clean state. We call this study area pollution microbiology.

This manual is designed for use in the laboratory phase of courses dealing with microbial aspects of pollution. The presentation is at the advanced undergraduate and beginning graduate student level. An introductory course in microbiology (or bacteriology) including laboratory instruction is assumed, as is familiarity with the basic techniques of analytical chemistry. Some knowledge of sewage treatment processes would be helpful. In the latter third of the twentieth century it seems reasonable to expect genuine student interest in the causes and in the prevention of pollution.

The manual attempts to cover the subject area broadly, as indicated by the four categories delineated in the table of contents. Principles are stressed, rather than specific modes

of technological applications. Most of the exercises deal with more than one phenomenon to reflect associations as they exist in nature. Although the aquatic environment is emphasized, some consideration of microorganisms in relation to solid wastes and air pollution logically finds its way into this volume.

So that the student might understand clearly why he is being asked to carry out the exercise, a brief introductory essay places each topic into the context of pollution where this might not otherwise be obvious. The topics can be pursued in greater detail through the literature listed at the end of the exercises. It may be necessary to do so to answer some of the questions that follow the experimental sections.

In a single semester it may not be feasible to cover all of the material in the manual. A balanced presentation can be maintained by selecting exercises from each of the sections. Some of the exercises can be abbreviated by omitting selected parameters, or by subdividing the work among the members of the class. Many of the experiments are, unavoidably, of long duration, with resulting overlap in execution. It will frequently be necessary to return to the laboratory several times during the week for continuing observations. This can be kept within reasonable bounds by organizing the class into teams of two or three.

Many students have contributed to this manual as it evolved during the course of several years, and I am indebted to them. Readers familiar with *Experimental Soil Microbiology* (1964, Burgess, Minneapolis, Minn.) by David Pramer and E.L. Schmidt will recognize its influence on the present volume. Associa-

tion with Dr. Joseph V. Hunter is a continuing education that leaves an imprint on these exercises. The Reverend Clarence R. Waldron, Jr., read the manuscript and made numerous valuable suggestions for improvement. Alan Caplans' technical assistance is much appreciated. Miss Donna Novak typed and patiently retyped repeatedly successive versions of the manuscript.

CONTENTS

PART III

Nitrogen, Phosphorus, Iron, and Sulfur as Pollutants

PART IV

Sanitary Bacteriology

Part I

SOME MICROORGANISMS IN CLEAN AND POLLUTED WATERS

Even a cursory microscopic examination of aquatic environments where decay is actively underway reveals a fascinating, and potentially bewildering, variety of organisms. The eye is at first strongly attracted to the larger forms actively swimming, crawling, beating cilia, or otherwise in motion. Among these would be protozoa, rotifers, and nematodes. Also conspicuous are large brightly colored algae, especially filamentous species. Less obvious visually but no less important functionally are nonmotile organisms and smaller forms, including fungi, and the bacteria (which approach the limit of resolution of the light microscope). Below this limit lie the viruses, and to demonstrate their presence special procedures are required.

Representatives of all seven of these biological groups will probably be encountered in the first series of exercises. These groups encompass diverse segments of the biological world. Viruses, at one extreme of biological organization, consist of little more than a genetic endowment in the form of a nucleic acid. Nematodes are at the opposite extreme of the organisms we are likely to observe. These members of the animal kingdom have many of the organ systems characteristic of the most complex of animals. Clearly, a frame of reference is needed to put into a biological perspective the organisms we encounter in clean and polluted waters, and to this end selected readings from several texts are suggested. The reading in *The Microbial World* emphasizes the subcellular component structures of

organisms, the selections from *The Microbes* summarizes salient features of five of the groups of interest, and the indicated section in *Biological Science* is a comprehensive discussion of the classification of all organisms.

LITERATURE

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Exercise 1

VIRUSES

Viruses are the most rudimentary form of life known, consisting of, essentially, a nucleic acid core enveloped by a protective covering during extracellular phases of existence. Viruses lack an energy-yielding metabolism and cannot reproduce independently. Rather, the nucleic acid core penetrates the cell of a susceptible organism and redirects host synthesis into the formation of new viruses. Representatives of the bacteria, algae, plants, and animals are infected by viruses. Viruses are too small to be seen with the light microscope, and they pass through filters that retain most bacteria.

Viruses that infect all the biological groupings have a bearing on water pollution problems. Plant and algal viruses may prevent the unrestricted development of the hosts when conditions are otherwise favorable, as in a lake that has reached an advanced state of eutrophication. It has been suggested that excessive growths of blue-green algae might be controlled by seeding with specific viruses. Similarly, bacterial viruses (bacteriophage) may help control the size and the make-up of bacterial populations through the lysis of susceptible groups. Of special interest is the possible role of bacteriophage in the destruction of bacteria pathogenic to man, and of bacterial indicators of fecal pollution.

The possible importance of water as a medium for the trans-

mission of human viral disease is currently receiving serious attention. Water may have played a small role in the transmission of polio, prior to its elimination as a major hazard through mass public vaccination. The only viral disease which has been conclusively shown to be waterborne is infectious hepatitis. Two routes are known, through contaminated drinking water, and through shellfish harvested from sewage polluted estuaries. It is suspected that other viral diseases may also be waterborne, but conclusive evidence is lacking. Some of the suspect diseases are mild, and reliable epidemiological evidence is difficult to obtain.

In this exercise we take advantage of the relative ease of demonstrating bacteriophages, and their predictable presence in sewage.

MATERIALS

Day 1

Sewages (if possible, provide each team with a different sewage)

Fresh agar slant cultures of *Escherichia coli* and some non-related bacterium common to sewage (*Pseudomonas* or *Achromobacter*, for example)

Nutrient agar (Difco 0001 or equivalent) slants

Two-500 ml flasks

Dehydrated nutrient broth (Difco 0003 or equivalent)

Basal medium components 3 and 4 (see appendix)

Sterile pipettes

Sterile petri dishes

35°C incubator

Day 2

300 ml portions of nutrient agar autoclaved on day of use and held molten at 47°C

Water bath incubator (47°C)

Autoclave sterilized medium components 3 and 4 (see appendix)

Sterile culture tubes (warmed)

Sterile pipettes (warmed)

Centrifuge

Dilution bottles holding 90 ml sterile nutrient broth

Sterile membrane filtering apparatus (Millipore XX11 047 00, or equivalent)

Membrane filters (Millipore HAWG 047 AO, or equivalent)

Smooth-tip forceps (Millipore XX62, 000 06, or equivalent)

Ethanol

Day 3

300 ml portions of nutrient agar autoclaved on day of use and held molten at 47°C

Autoclave sterilized medium components 3 and 4 (see appendix)

PROCEDURE

Day 1

Aseptically transfer *E. coli* and the other test culture to slants for use on days 2 and 3.

In a 500-ml Erlenmeyer flask combine 0.3 g dehydrated nutrient broth, 0.2 ml $MgSO_4$ solution (component 3), 0.1 ml $CaCl_2$ solution (component 4), and 40 ml distilled water. Using the old *E. coli* culture prepare a dense cell suspension by adding 1 ml of broth to the slant and scraping with a loop.

Disperse the cells by slapping the tube against your palm. Add an additional 4 ml of broth. To the broth in the flask add 1 ml of the host cell suspension and 1 ml of sewage.

Similarly, prepare a phage enrichment for the other bacterial test culture. Incubate both flasks overnight at 35°C.

Day 2

Aeseptically assemble the filtering apparatus. To each 300 ml portion of molten agar aeseptically add 2 ml of the $MgSO_4$ solution and 1 ml of $CaCl_2$ solution, mix. Transfer 4 ml portions of the agar to sterile culture tubes. Use warmed pipettes and warmed tubes to keep the agar molten; keep the tubes in the 47°C water bath. Also pour thin layers of the agar (about 8 ml) into petri dishes and allow to harden.

Centrifuge the phage enrichment culture, then pass the supernatant through the membrane filter. Aeseptically dilute the filtrate in nutrient broth to give the following dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. To a series of tubes containing 4 ml molten nutrient agar add 0.5 ml freshly prepared dense pure culture host cell suspension and 1 ml undiluted homologous filtrate, or dilution thereof. Mix thoroughly and pour as the second layer into a dish containing hardened agar. Prepare one double layer plate at a time to avoid solidification of the agar in the tube. After the top layer solidifies invert the plate and incubate overnight at 35°C.

Subsequently, examine the confluent bacterial growth (lawn) for clear areas (plaques) representing bacteriophage colonies. Note the size and shape of the plaques.