INTERNATIONAL STANDARDS FOR DRINKING-WATER

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WORLD HEALTH ORGANIZATION

PALAIS DES NATIONS

GENEVA

1958

PRINTED IN SWITZERLAND

CONTENTS

PREFACE

The World Health Organization has long appreciated the need for some form of international agreement and co-operation on the requirements for safe and potable water supplies. This problem becomes particularly pertinent with the great increase in travel, especially air travel, where common carriers must be watered at many points in the world, and the traveller must be furnished with acceptable drinking-water that will not produce unfavourable effects on his health.

The status of water treatment and quality in the Member States of the World Health Organization was the subject of a questionnaire circulated in 1953. The replies clearly indicated the magnitude of the problem and the need for attention by the World Health Organization.

As a result, groups of experts in sanitation and water treatment were convened in several of the regions of WHO to consider all problems related to standards of water quality. Since standards of quality are dependent upon the techniques used in the laboratory, the recommendations as to the specific methods to be used were an integral part of these discussions.

The reports of the regional groups proved a valuable basis for the deliberations of a further study group, composed of experts from these regions and from elsewhere, which met in Geneva in 1956. The standards proposed by that group make up the present publication.

In considering their assignments, the study group felt that the term "standards" should be applied to the suggested criteria of water quality, even though these are considered to be tentative and subject to modification after experience in their application. With laboratory methods, on the other hand, it was believed that these should not be designated as "standard methods", since it is certain that with further use and study these may well be changed in major as well as minor details. Thus the laboratory methods prepared are proposed as "approved methods".

In the preparation of the material for this publication, particularly in the sections devoted to laboratory techniques, full use was made of many sources, chiefly The Bacteriological Examination of Water Supplies *of the Ministry of Health and the Ministry of Housing and Local Government for England and Wales, and* Standard Methods for the Examination of Water, Sewage, and Industrial Wastes, *10th edition, of the American Public Health Association.*

This publication is presented by WHO with the aim of stimulating further investigations of the problem and immediate consideration of the function of criteria of water quality in the control and improvement of water treatment and the provision of safe and potable water to all people.

It is only by constructive criticism based on broad experience with these recommendations that their value may be assessed. WHO would therefore welcome all such critical observations.

The membership of the several study groups which contributed towards the standards given on the following pages is listed in Annex 6.

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1. INTRODUCTION

That water intended for human consumption must be free from chemical substances and micro-organisms in amounts which would provide a hazard to health is universally accepted. Supplies of drinking-water should not only be safe and free from dangers to health, but should also be as aesthetically attractive as possible. Absence of turbidity, colour and disagreeable or detectable tastes and odours is important in water-supplies intended for domestic use. The location, construction, operation and supervision of a water-supply—its sources, reservoirs, treatment and distribution—must exclude all potential sources of pollution and contamination.

Some countries in the world have established standards of quality which are applicable to their respective areas and have developed a certain degree of uniformity in methods of analysis and in the expression of the results of such analyses. Other countries, however, lack official or recognized standards of water quality and have no accredited procedures for the examination of water to assess its quality and safety. During regional and international conferences sponsored by the World Health Organization, the problems of standards of quality for a safe and acceptable water-supply, and of accredited or approved methods for the examination of water, have been fully discussed by groups of expert hygienists and engineers concerned with matters of water sanitation. Great improvement in water quality can be achieved throughout the world if various treatment processes are made easily comparable by the adoption of uniform methods for the examination of water and for the expression of results of such examinations. Further, outbreaks of water-borne disease could be avoided through stricter control by the responsible water-supply and health authorities of the quality of water distributed for drinking purposes. The World Health Organization has therefore conducted a study of these problems, in collaboration with Member States and with the assistance of a number of experts, in an effort to offer technical guidance to health and sanitation administrations wishing to revise their regulations on water-quality control.

1.1 Purpose

Although this publication may be of assistance to operators of water-supplies and others involved in the treatment and distribution of water, it is intended primarily to apply to water as it is supplied to the public,

and it is hoped that it will be of particular value to health authorities who are concerned with ensuring that the supplies of water which reach the public are safe and potable.

1.2 Scope

This publication is concerned with the minimum standards of chemical and bacteriological quality of public supplies of water for domestic use. Although it is desirable that the quality of water for individual and small supplies should not be inferior to that supplied to the public in large communities, it is not considered that all small supplies could reasonably be expected to conform to the standards suggested for larger communities. However, the standards recommended here are applicable to all communal supplies serving a group of the population and for which control of treatment and distribution is essential for a safe and sanitary quality.

Conditions differ widely throughout the world. Some countries are fortunate in having an abundant supply of water from deep wells and underground springs, while others must make extensive use of rivers, lakes and other sources of surface water. It is felt, however, that the recommendations which follow should be applicable whatever the original source of the water or its treatment may be.

It is not envisaged that the standards of physical, chemical and bacteriological quality or the various laboratory methods recommended here will be the final word on the subject. New methods are constantly being proposed and developed, and it is anticipated that the methods suggested and the standards of quality will be revised from time to time. There are certain matters, such as the problems of pollution from radioactive material and from chemical products used in industry and agriculture (hydrocarbons, detergents, pesticides) and the possibility of the presence of pathogenic viruses in water, on the importance of which there is as yet insufficient knowledge. It is possible also that, in the control of pollution and in epidemiological investigations, the study of bacteriophages and plankton may yield pertinent information. These are matters on which further investigation is needed, and additional knowledge may lead to modifications in the chemical and bacteriological standards suggested at the present time.

1.3 Arrangement of Material

In view of the importance of uniformity in the methods of expressing the results of physical, chemical and bacteriological examination of water, it has been thought advisable to define, in a preliminary paragraph, the terms in which it is recommended that these results should be expressed.

This publication is concerned primarily with the protection of communal supplies of drinking-water from dangers to the health of the consumers. It has been divided into four main parts, dealing with bacteriological, chemical and physical, biological, and radiological requirements. In Part 2, on bacteriological requirements, consideration has been given to the choice of organisms that should be used as indicators of pollution; to methods that it is suggested should be used for the detection of these organisms; to standards of bacteriological quality that might reasonably be established for communal drinking-water supplies; to the frequency with which samples should be taken for bacteriological examination; and to the precautions that should be observed in the collection, storage and transport of samples for bacteriological examination. Details of the technical procedures are given in Annex 3 (page 52). Tables required for computation of " most probable number " values are given in Annex 2 (page 45).

In Part 3, on chemical and physical requirements, attention has been devoted primarily to the limits of concentration for certain toxic substances which may constitute an actual danger to health. Consideration has also been given to the approximate critical concentrations at which other chemical substances may affect the health of the consumer. Chemical substances which affect the quality and acceptability of water have also been listed in permissible and excessive concentrations. These substances are not necessarily a menace to the physical well-being of the consumer, but they render the water aesthetically undesirable for domestic use or cause trouble in the distribution system. The additional chemical substances that provide ancillary indication of pollution are also given. Methods for the analysis of water to detect these chemical constituents of water are given in detail in Annex 4 (page 69).

In Part 4, biological requirements related to studies of water quality, including their use in the detection of pollution, are discussed in general. Technical procedures are described in Annex 5 (page 133).

Part 5, on radiological requirements, deals with the problems of pollution of water by radiological wastes and the standards of radioactivity which may be tolerated in water intended for domestic uses.

The important question of laboratory services in the control of water quality is discussed in Part 6.

1.4 Expression of Results

In accordance with international agreement, results of chemical analyses should, wherever possible, be expressed in terms of milliequivalents per litre (mEq/l), as this enables a balance to be established between

anions and cations. Milligrams per litre (mg/l) are retained in this publication, as this method of expression is used in various countries. It is considered, however, that the expression "parts per million" (p.p.m.) should be progressively abandoned. Wherever possible, chemical components should be expressed in ions. Turbidity should be expressed in units of turbidity, and colour in units of colour based upon the platinum-cobalt scale. Volumes should be expressed in millilitres (ml), and temperature should be measured in degrees Centigrade (°C). In bacteriological examinations, the total number of micro-organisms developing on solid media should be expressed in significant figures as colonies per millilitre of water, the medium, time and temperature of incubation being stated. Estimates of the numbers of coliform organisms—*Escherichia coli* and other micro-organisms indicative of pollution—should be given in terms of "most probable number" per 100 ml (MPN/100 ml).

In reporting chemical analyses, the sensitivity, accuracy and precision of the method should be indicated. This includes the proper use of significant figures and the expression of confidence limits.

1.5 Sanitary Surveys

The importance of a sanitary survey of sources of water cannot be over-emphasized. With a new supply, the sanitary survey should be made in conjunction with the collection of initial engineering data as to the suitability of a given source and its capacity to meet existing and future demands. The sanitary survey should include the detection of all potential sources of pollution of the supply and the assessment of their present and future importance; it should be made by persons trained and competent in public health engineering and the epidemiology of water-borne diseases. In the case of an existing supply, the sanitary survey should be made at a frequency compatible with the control of the pollution hazards and the maintenance of a good sanitary quality. The information furnished by the sanitary survey is essential to complete interpretation of bacteriological and chemical data and must always accompany the laboratory findings (see Annex 1, page 43).

1.6 Treatment of Water

The treatment of water required to ensure satisfactory sanitary quality is not considered. However, where the term "treatment" is employed, it covers storage for periods in excess of one month, coagulation, sedimentation, filtration, disinfection, distillation, and other physical or chemical processes, or any combination thereof.

Although treatment of water was not considered to be within the scope of this publication, this fact in no way minimizes the fundamental importance of adequate and intelligent control of treatment processes.

It is fully realized that the establishment of standards will not alone improve the quality or safety of any water-supply. Rather, such standards must be employed as a target towards which treatment should be directed, and should serve basically to develop improved treatment operations. Similarly, approved laboratory methods for the examination of water should be used as the essential tools for control of the operation of treatment processes. Unless standards of water quality and approved laboratory methods can be made to function in the improvement of water treatment, they will be of little value in public health administration.

1.7 Records

The maintenance of accurate and complete records is an integral part of any programme for the control of water quality or for the examination of water to determine its conformity with established standards of quality and safety.

In the operation of any water-supply, pertinent information on the construction and location of the water-supply and its structures, on the results of inspections of sanitary and operating conditions, on the details of treatment and operation, and on sampling are essential and should be recorded promptly and accurately for future reference. Pertinent complaints as to quality and as to the occurrence of unusual conditions, of climatic or other origin, are equally valuable.

In carrying out analyses of water by approved methods, all details of the actual specific determinations should be recorded. These would include burette and other instrument readings, weights, and all the calculations required to obtain the final result. All this information should, of course, remain as laboratory records, and only the final result of the determination should be reported. Without such data, review of results of analyses to ensure their accuracy—an important factor when unusual findings may be questioned—would be impossible.

Record-keeping should be intelligently and carefully planned so that only pertinent material is kept. The design and preparation of forms or cards providing spaces for entering the desired data will greatly ease and facilitate the accuracy of keeping records.

Whereas records are essential for adequate and intelligent operation of waterworks processes and for laboratory activities, the keeping of records should not become an end in itself, but should rather be part of a greater aim—the control of water quality and safety. If they are to be worth the keeping, records must be used, and should be essential tools in laboratory examinations and in the operation of water-supplies.

Records should be retained or stored for as long as they may be useful—a factor depending upon the type of record and various local requirements —and must be stored in such a manner as to be protected from rapid deterioration and yet readily available for reference or other purposes.

2. BACTERIOLOGICAL REQUIREMENTS

No bacteriological examination of water, however exact, can take the place of a complete knowledge of the conditions at the sources of supply and throughout the distribution system. Every water-supply should be regularly inspected from source to distribution taps, and sampling should be repeated under varying climatic conditions, especially after heavy rainfall. It should be emphasized that when sanitary inspection indicates a water, as distributed, to be subject to pollution, the water should be condemned irrespective of the results of bacteriological examinations. Contamination is often intermittent and may not be revealed by the examination of a single sample. The examination of a single sample can indicate no more than the conditions prevailing at the moment of sampling; a satisfactory result cannot guarantee that the observed conditions will persist in the future. The quality of a water-supply can be assessed only by a series of samples over a definite period of time.

2.1 Indicators of Pollution

2.1.1 *Organisms indicative of faecal pollution*

The major danger associated with drinking-water is the possibility of its recent contamination by sewage or by human excrement, and even the danger of animal pollution must not be overlooked. If such contamination has recently occurred and if, among the contributors, there are cases or carriers of such infectious diseases as enteric fever or dysentery, the water may contain the living micro-organisms of these infections, and the drinking of such water may result in additional cases of the disease. Although modern bacteriological methods have made it possible to detect pathogenic bacteria in sewage and sewage effluents, it is not practicable to attempt to isolate them as a routine procedure from samples of drinking-water. When pathogenic micro-organisms are present in faeces or sewage, they are almost always greatly outnumbered by the normal excremental organisms, and these normal intestinal organisms are easier to detect in water. If these organisms are not found in the water, it can be inferred that disease-producing micro-organisms are also absent. Thus, the use of normal excremental bacteria as indicators of faecal pollution introduces a margin of safety.

The organisms most commonly employed as indicators of pollution are *Escherichia coli* and the coliform group as a whole. *E. coli* is of undoubted faecal origin, but the precise significance of the presence in water of other members of the coliform group has been much debated.[3, 4, 23, 25] All the members of the coliform group may be of faecal origin, and the worst possible interpretation should, therefore, be attached to their presence in water. Quite apart from the question of their being indicative of faecal pollution, organisms of the coliform group are foreign to water and must be regarded as indicative of pollution.

The search for faecal streptococci, of which the most characteristic type is *Streptococcus faecalis*, and for anaerobic spore-forming organisms, of which *Clostridium welchii* is particularly typical, may be of value in confirming the faecal nature of pollution in doubtful cases.

Faecal streptococci regularly occur in faeces in varying numbers, which are usually considerably smaller than those of *E. coli*. In water they probably die and disappear at approximately the same rate as *E. coli*, and usually more rapidly than other members of the coliform group. When, therefore, organisms of the coliform group, but not *E. coli*, are found in a water sample, the finding of faecal streptococci is important confirmatory evidence of the faecal nature of the pollution.

Anaerobic spore-forming organisms are also present in faeces, though generally in much smaller numbers than *E. coli*. The spores are capable of surviving in water for a longer time than organisms of the coliform group and usually resist chlorination at doses normally used in water-works practice. The presence of *Cl. welchii* in a natural water suggests that faecal contamination has occurred, and, in the absence of organisms of the coliform group, suggests that the contamination occurred a long time ago.

Examination for faecal streptococci and anaerobic spore-forming organisms may also be of value when water samples are examined at infrequent intervals, or when a new source of supply is being considered and as much information as possible is required about the sanitary quality of the water.

2.1.2 *Total content of micro-organisms*

Plate counts on nutrient agar at 35-37°C and on nutrient agar or gelatin at 20°C are not infrequently used in the bacteriological examination of water. The plate count alone is of little value in detecting the access of faecal pollution, since organisms of all types capable of growing at these temperatures will be counted. A series of plate counts from a source such as a deep well or a spring may be of considerable value—a sudden increase in the plate count from such a source may give the earliest indication of the access of pollution. Plate counts frequently repeated from a series of points in a treatment plant are of considerable value in the control

of waterworks treatment; they are also of value when a new source of supply is being considered and as much information as possible about the quality of the water is being collected.

An isolated plate count is rarely of value, and from raw surface-waters even a series of plate counts is of little value, because of the wide variations which occur, due, for example, to changes in climatic conditions.

2.2 Sampling

Scrupulous care in the collection of samples for bacteriological examination is necessary to ensure that the sample is representative of the water under examination, and to avoid accidental contamination of the sample during collection.

When several samples are collected on the same occasion from the same source, the sample for bacteriological examination should be taken first, to avoid the danger of contamination of the sampling point during the collection of other samples.

2.2.1 *Sample containers*

Sterilized (neutral) glass bottles provided with ground-glass stoppers should be used for collection of samples for bacteriological examination. The stopper and neck of the bottle should be protected by a paper or parchment cover, or by thin aluminium foil.

If the water to be sampled contains, or is likely to contain, traces of residual chlorine or chloramine, it is necessary to add to the sampling bottle, before sterilization, a sufficient quantity of sodium thiosulfate ($Na_2S_2O_3$, $5H_2O$) to neutralize these substances. It has been shown [9] that 0.1 ml of a 3% solution of crystalline sodium thiosulfate (3.0 mg) in a 170-ml bottle has no significant effect on the coliform or *Escherichia coli* content of unchlorinated water during storage. This proportion of sodium thiosulfate is sufficient to neutralize more than 5 mg/l of residual chlorine. It is, therefore, recommended that this proportion of sodium thiosulfate solution be added to all bottles used for the collection of samples for bacteriological examination. When samples of chlorinated water are taken, it is desirable to determine the content of residual chlorine at the sampling point.

2.2.2 *Sampling procedures*

The sampling bottle should be kept unopened until the moment at which it is required for filling. The stopper should be removed with care to eliminate soiling, and during sampling the stopper and neck of the bottle should not be handled and should be protected from contamination.

The bottle should be held near the base; it should be filled without rinsing and the stopper should be replaced immediately.

If a sample of water is to be taken from a tap on a distribution system, it should be ascertained that the tap chosen is supplying water from a service pipe directly connected with the main, and not, for instance, one served from a cistern or storage tank. The tap should be cleaned and opened fully and the water allowed to run to waste for two to three minutes or a sufficient time to permit clearing the service lines. The flow from the tap should then be restricted to permit filling the bottle without splashing. Leaking taps which permit water to flow over the outside must be avoided as sampling points.

In collecting samples directly from a river, stream, lake, reservoir, spring or shallow well, the aim must be to obtain a sample that is representative of the water which will be taken for purposes of supply to consumers. It is therefore undesirable to take samples too near the bank or too far from the point of draw-off, or at a depth above or below the point of draw-off. In a stream, areas of relative stagnation should be avoided.

Samples from a river, stream, lake or reservoir can often be taken by holding the bottle near its base in the hand and plunging it, neck downwards, below the surface. The bottle should then be turned until the neck points slightly upwards, the mouth being directed towards the current. If no current exists—as in a reservoir—a current should be artifically created by pushing the bottle horizontally forward in a direction away from the hand. If it is not possible to collect samples from these situations in this way, a weight may be attached to the base of the bottle, which can then be lowered into the water. In any case, damage to the bank must be guarded against, otherwise fouling of the water may occur. Special apparatus which permits mechanical removal of the stopper of the bottle below the surface is required to collect samples from the depths of a lake or reservoir.

If the sample is to be taken from a well fitted with a hand-pump, water should be pumped to waste for about five minutes before the sample is collected. If the well is fitted with a mechanical pump, the sample should be collected from a tap on the discharge. If there is no pumping machinery, a sample can be collected directly from the well by means of a sterilized bottle fitted with a weight at the base; in this case, care should be taken to avoid contamination of the sample by any surface scum.

2.2.3 *Data relative to samples*

All samples of water should be accompanied by complete and accurate identifying and descriptive data (see Annex 1, page 43). Samples not so identified should not be accepted for examination.

2.2.4 *Preservation and storage of samples*

The bacteriological examination of samples of water should be initiated immediately after collection. However, such a requirement is seldom practical, and more realistic ones must be established.

It is, therefore, recommended that, with samples intended for bacteriological examination, the technical procedures should be started as soon as possible, preferably within one hour after collection; the time elapsing between collection and examination should in no case exceed 24 hours. During the period elapsing between collection and examination, the temperature of the sample should be maintained as closely as possible to that of the source of the sample at the time of sampling. The time and temperature of storage of all samples should be recorded and should be considered in the interpretation of the laboratory results.

When local conditions necessitate delays in excess of 24 hours, consideration should be given to providing for a field examination of samples, by making use, for example, of the microfilter technique or of temporary laboratory facilities at the site.

2.2.5 *Size of samples*

The volume of a sample of water should be sufficient for carrying out all the tests required, and preferably not less than 100 ml for samples intended for bacteriological examination.

2.2.6 *Frequency of sampling*

The frequency of bacteriological examinations for the control of the sanitary quality of a supply, and the location of the sampling points at pumping stations, treatment points, reservoirs and booster pumping stations, as well as in the distribution system, should be such as to enable proper supervision of the bacteriological quality of the water-supply to be maintained. Inspection of the entire water-supply system from source to consumers' premises is of the utmost importance, and the authority responsible for operation and safety of the water-supply should have the services of an expert adviser in deciding on the sampling points and the frequency with which samples from each point should be collected.

The frequency of sampling should be established according to the magnitude of the problem involved.[12, 27] It would seem reasonable that the frequency of examination of routine samples of water from the distribution system, and of routine samples of the water entering the distribution system, should be based on the size of the population served. These examinations should be spaced over a period of time, according to the risks of pollution, geographical location and protection of the source of supply. When the safety of a water is dependent upon disinfection or other treatment processes, a constant check on the bacteriological quality

of the water entering the distribution system is necessary, and bacteriological examination of such water should, in principle, be carried out at least daily.

Treated water, as it enters the distribution system from each treatment point, should be examined bacteriologically at least once a day. The absence of such control in many small plants is to be regretted. When safety depends upon chemical disinfection, bacteriological examination is recommended at a frequency of not less than once a week. There should be a check on any chemical disinfection process several times a day, and the results of these examinations should be recorded for permanent reference. This control should be supplemented at least twice a year by an inspection *in situ* by engineering and sanitation experts acting on behalf of the responsible authority. A complete and accurate plan of the water-supply system should be maintained and placed at the disposal of the experts.

For *untreated water entering the distribution system*, the following maximum intervals between successive routine examinations are proposed:

Population served	*Maximum interval between successive samplings*
Up to 20 000	One month
20 001– 50 000	Two weeks
50 001–100 000	Four days
More than 100 000	One day

On each occasion samples should be taken from all the points at which the water enters the distribution system.

With regard to *samples to be collected from the distribution system, whether the water has been subjected to treatment or not*, the following maximum intervals between successive samplings and minimum numbers of samples to be examined in each month are proposed:

Population served	*Maximum interval between successive samplings*	*Minimum number of samples to be taken from entire distribution system*
Up to 20 000	One month	One sample per 5000 of population per month
20 001– 50 000	Two weeks	
50 001–100 000	Four days	
More than 100 000	One day	One sample per 10 000 of population per month

It is considered justifiable to reduce the minimum number of samples to one sample per 10 000 of population per month when the population exceeds 100 000, since in systems serving populations of that size some samples would be examined each day.

The samples should not necessarily be taken from the same points on each occasion. The experts referred to above should determine the points on the distribution system from which samples should be collected.

It should be emphasized that in routine control it is more important to examine numerous samples by a simple test than occasional samples by a more complicated test or series of tests.

It should be borne in mind that these are the minimum frequencies recommended for routine bacteriological examination, and, in the event of an epidemic or immediate danger of pollution, or when more stringent control is necessary, much more frequent bacteriological examination will be required.

2.3 Standards of Bacteriological Quality applicable to Drinking-Water Supplies

2.3.1 *General considerations*

Water circulating in the distribution system, whether treated or not, should not contain any organisms which may be of faecal origin. The presence of the coliform group, as defined below, should be considered an indication of recent or remote faecal pollution. The presence of *Escherichia coli*, as defined below, should be considered a definite indication of recent faecal pollution and hence of a hazardous or dangerous condition requiring immediate remedial action.

Coliform group includes all aerobic and facultative anaerobic Gram-negative non-spore-forming rods capable of fermenting lactose with the production of acid and gas at 35-37°C in less than 48 hours.

Escherichia coli, for the purpose of the sanitary examination of water, is defined as a Gram-negative non-spore-forming rod which is capable of fermenting lactose with the production of acid and gas at 44°C in less than 48 hours; which produces indole in peptone water containing tryptophane; which is incapable of utilizing sodium citrate as its sole source of carbon; which is incapable of producing acetylmethylcarbinol; and which gives a positive methyl-red test.

2.3.2 *Recommended standards of bacteriological quality*

Some public drinking-water supplies are chlorinated or otherwise disinfected before being distributed; others are not. Effective chlorination yields a water which is virtually free from coliform organism, i.e., these organisms are absent in 100-ml portions; if communal supplies which are distributed without treatment or disinfection cannot be maintained to the bacteriological standard established for treated and disinfected water, steps should be taken to institute chlorination or disinfection, or other treatment, of these supplies.

A standard demanding that coliform organisms be absent from each 100-ml sample of water entering the distribution system—whether the water be disinfected or naturally pure—and from at least 90% of the samples

taken from the distribution system can be applied in many parts of the world. Although there is no doubt that this is a standard that should be aimed at everywhere, there are many areas in which the attainment of such a standard is not economically or technically practicable.

In these circumstances there would appear to be economic and technical reasons for establishing different bacteriological standards for public water-supplies which are treated or disinfected and for those which are not treated. The following bacteriological standards are recommended for treated and untreated supplies for present use throughout the world, with the hope that improvements in economic and technical resources will permit stricter standards to be adopted in the future.

The standards described below are based on the assumption that frequent samples of water will be taken, in accordance with recommendations in section 2.2.6 (page 18). For each individual sample, coliform density is estimated in terms of the " most probable number " in 100 ml of water, or " MPN " index.[24] The dilution-tube technique to be employed for estimating coliform density in terms of MPN is described in section 2.4.2 (page 22). The use of the MPN index is recommended as the basis of quantitative estimation of coliform density after full recognition of its limitations. However, the value of the index is sufficiently enhanced by the use of data from a series of samples to warrant its use in the recommended standards.

2.3.2.1 *Treated water*

In 90% of the samples examined throughout any year, coliform bacteria shall not be detected or the MPN index of coliform micro-organisms shall be less than 1.0. None of the samples shall have an MPN index of coliform bacteria in excess of 10.

An MPN index of 8-10 should not occur in consecutive samples. With the examination of five 10-ml portions of a sample, this would preclude three of the five 10-ml portions (an MPN index of 9.2) being positive in consecutive samples.

In any instance in which two consecutive samples show an MPN index of coliform bacteria in excess of 8, an additional sample or samples from the same sampling point should be examined without delay. This is the minimum action that should be taken. It may also be desirable to examine samples from several points in the distribution system and to supplement these with samples collected from sources, reservoirs, pumping stations and treatment points. In addition, the operation of all treatment processes should be investigated immediately.

2.3.2.2 *Untreated water*

In 90% of the samples examined throughout any year, the MPN index of coliform micro-organisms should be less than 10. None of the samples should show an MPN index greater than 20.

An MPN index of 15 or more should not be permitted in consecutive samples. With the examination of five 10-ml portions of a sample, this would preclude four of the five 10-ml portions (an MPN index of 16) being positive in consecutive samples. If the MPN index is consistently 20 or greater, application of treatment to the water-supply should be considered.

In any instance in which two consecutive samples show an MPN index of coliform organisms greater than 10, an additional sample or samples from the same sampling point should be examined immediately. It may also be desirable to examine samples from several points in the distribution system and to supplement these with samples collected from sources, reservoirs and pumping stations.

When accurate and complete data concerning the sanitary conditions at the source of an untreated water-supply, covering all possible points of pollution, are available and indicate that indices higher than the established maximum may bear little relation to potential health hazards, the local health and water-supply authorities should be responsible for ruling that such higher indices do not constitute need for treatment of the water.

2.4 Methods for the Bacteriological Examination of Water

Approved laboratory methods for the bacteriological examination of water are given in Annex 3 (page 52).

2.4.1 *Temperature of incubation* [22]

The temperature of incubation to be employed in the bacteriological examination of water should be between 35°C and 37°C. This temperature range will provide for the maximum growth-rates of coliform bacteria and will permit the minimum fluctuations in incubator temperatures. Temperatures of incubation beyond this range must not be tolerated even for very short periods of time.

2.4.2 *Technique*

The dilution-tube technique, employing definite volumes of the samples and definite numbers of portions of each volume tested, is the most satisfactory procedure for determining conformity with established standards of quality. After consideration of the need for simplicity and practicability, and since a series of samples will be studied, the use of five 10-ml portions of each sample for each test is recommended as the minimum procedure. The use of larger portions (such as 100 ml), a greater number of replicate portions and a larger series of fractional volumes will enhance

the precision of the estimate of coliform densities. Appropriate tables of MPN values per 100 ml and of confidence limits are included in Annex 2 (page 45) for these more extensive tests as well as for the minimum acceptable procedure using five 10-ml portions.

2.5 The Detection of Coliform Organisms and Escherichia coli

The first essential for the detection and estimation of coliform organisms in water is that every coliform organism should have the maximum possibility of developing in the medium into which it is inoculated. It is apparent, therefore, that if selective media are used for the detection of coliform organisms, care should be taken to ensure that they are not inhibitory, but encourage the growth of these organisms.

2.5.1 *Presumptive coliform test*

The basis of the presumptive coliform test is the inoculation of the water to be tested into tubes containing a suitable liquid medium which are then incubated and examined after the appropriate period of time. The test is called presumptive because the reaction observed in the tube may occasionally be due to the presence of some other organism or combination of organisms, and the presumption that the reaction is due to coliform organisms has to be confirmed.

The proportion of false positive reactions obtained depends both on the bacterial flora of the water under examination and on the medium used.

A variety of different media are used in different countries for the presumptive coliform test. At the moment no standard medium can be proposed. Up to the present time, either lactose broth, or MacConkey's broth, with bromcresol purple [25] as an indicator and a standardized concentration of bile salts [5]—incubated at not less than 35°C nor more than 37°C for not more than 48 hours—, seems to be the most suitable media for presumptive tests.

2.5.2 *Confirmatory tests*

The presumptive coliform test must be followed by at least *rapid confirmatory tests* for coliform organisms and for *E. coli*. The most practical procedure is the subculture of each presumptive positive tube into two tubes of brilliant-green/lactose bile broth or of MacConkey's broth,[a] one

[a] If MacConkey's broth is used for confirmatory tests, it is recommended that presumptive positive tubes derived from chlorinated waters be plated on to a solid medium to confirm the presence of coliform organisms, since false reactions in MacConkey's broth both at 35-37°C and at 44°C may be caused by spore-bearing anaerobic organisms.[8]

of which should be incubated at 35-37°C for up to 48 hours for confirmation of the presence of coliform organisms, and the other incubated at 44°C and inspected after 6 and 24 hours to determine whether or not *E. coli* is present. Further confirmation of the presence of *E. coli*, if desired, can be obtained by testing for indole production at 44°C.

Where *complete confirmation* is necessary, presumptive positive tubes can be plated on to a solid medium, such as lactose agar, Endo's medium, eosin/methylene-blue agar, or MacConkey's agar, and individual colonies picked off for identification by the indole, methyl-red, Voges-Proskauer and citrate-utilization tests and by testing for fermentation of lactose at 35-37°C and at 44°C.

2.5.3 *Field tests : the microfilter technique* [13, 19, 26]

The microfilter technique—or, as it is frequently designated, the membrane-filter technique—is considered to have potential value for the quantitative detection of coliform bacteria. It presents a promising development as a field test, particularly for areas in which excessive time would be required to send water samples to a central laboratory. Further research on details of the test, including the culture media to be employed and the time of incubation, is needed before this technique can be recommended as a standard procedure. Without doubt, the present membrane-filter technique, when used in full recognition of its limitations, is a valuable tool in the control of water-supply quality.

2.6 The Detection of Faecal Streptococci and Anaerobic Spore-forming Organisms

On those occasions when it is considered desirable to supplement the examination for coliform organisms and *E. coli* by examination for faecal streptococci or anaerobic spore-forming organisms, the following methods can be recommended.

2.6.1 *Faecal streptococci*

Methods commonly used for the detection and estimation of the number of faecal streptococci are :

(1) The inoculation of multiple portions of water into sodium-azide broth, in tubes which are then incubated at 44-45°C for 48 hours. Only a microscopical confirmation of the tubes which become positive within 18 hours is required, but those which become positive later require more complete confirmation.

(2) The inoculation of multiple portions of water into tellurite-lactose broth, in tubes which are then incubated at 35-37°C for 48 hours. Presumptive positive tubes, indicated by a black deposit, may be confirmed by subculture into further tubes of tellurite-lactose broth incubated at 44-45°C for 24-48 hours; tubes positive at this temperature should be submitted to further confirmatory tests. Tellurite media have the disadvantage that potassium tellurite is decomposed on heating, and therefore the solution has to be added to the tubes of medium after sterilization.

2.6.2 *Anaerobic spore-forming organisms*

A method commonly employed for the detection and estimation of the number of *Cl. welchii* is the inoculation of multiple portions of water into freshly boiled litmus milk. The mixtures are then heated at 80°C for 10-15 minutes to destroy vegetative organisms. The inoculated tubes may be incubated anaerobically, but this is not strictly necessary. The tubes should be incubated at 35-37°C for at least five days, but a positive reaction ("stormy clot") may appear in 24-72 hours.

3. CHEMICAL AND PHYSICAL REQUIREMENTS

Chemical analysis has a wide range of uses in the investigation of water-supplies and of water quality. The following sections are concerned primarily with the protection of users of public water-supplies from dangers to health, and therefore attention is largely directed to the detection and estimation of certain toxic chemical substances, of those which may affect health, of chemical compounds which may impair the acceptability of the water for domestic purposes, and of certain chemical substances which are ancillary indicators of pollution. The frequency of general systematic chemical examination should be governed by local circumstances, but frequent chemical analysis may be required for the control of water-treatment processes.

3.1 Sampling

3.1.1 *Collection, transport and storage of samples*

For general chemical examination, a sample of at least 2 litres is required; it should be collected in a chemically clean bottle made of good quality (neutral) glass, practically colourless, and which should be fitted

with a ground-glass stopper. The bottle should be rinsed out at least three times with the water that is to be sampled before the bottle is filled. Polyethylene bottles should be substituted for glass bottles for the special purpose of measurement of radioactivity in a sample.

In collecting samples for chemical analysis, the general recommendations given for the collection of samples for bacteriological examination (see section 2.2, page 16) should be followed.

In the collection of samples from mineralized sources, the bottle should be completely filled and the stopper securely fastened.

Samples should be transported to the laboratory with as little delay as possible and should be kept cool during transport. Chemical analysis should be started as soon as practicable after the collection of the sample, and in any case should not be delayed for more than 72 hours.

3.1.2 *Data relative to samples*

A record should be made of every sample collected, and every bottle should be identified, preferably by attaching an appropriate tag or label. The record should include sufficient information to provide positive identification of the sample at some later date (see Annex 1, page 43), e.g.: name of the sample collector; date, hour and exact location of sampling; water temperature; details of any treatment of the water; and any data which may be needed for future reference, such as weather conditions, water level, stream flow. Sampling points should be fixed by detailed description, by maps, or with the aid of stakes, buoys, or landmarks, in such a manner as to permit their identification by other persons.

3.1.3 *Frequency of sampling*

Collection of samples of both raw and treated water for examination for toxic substances should be carried out at least once every three months, and more frequently when sub-tolerance levels of toxic substances are known to be generally present in the source of supply, or where such potential pollution exists—as, for example, in an area where industries may be discharging toxic wastes into sources of a water-supply.

Samples for general systematic chemical examination should be collected at least once every three months in supplies serving more than 50 000 inhabitants, and at least twice a year in supplies serving up to 50 000 inhabitants. More frequent sampling for chemical examinations may be required for the control of water-treatment processes.

Sampling of new sources of supply. Frequent examinations both for toxic substances and for general chemical analysis will be required for new or proposed sources, depending on local circumstances, which may necessitate establishing special periods for sampling.

3.2 Standards of Chemical and Physical Quality

3.2.1 *Toxic substances*

There are certain substances which, if present in supplies of drinking-water at concentrations above certain levels, may give rise to actual danger to health. A list of such substances and of the levels of concentration which should not be exceeded in communal drinking-water supplies is given below:

Substance	*Maximum allowable concentrations*
Lead (as Pb)	0.1 mg/l
Selenium (as Se)	0.05 mg/l
Arsenic (as As)	0.2 mg/l
Chromium (as Cr hexavalent)	0.05 mg/l
Cyanide (as CN)	0.01 mg/l

The presence of any of these substances in excess of the concentrations quoted should constitute grounds for the rejection of the water as a public supply for domestic use.

It is realized that the limiting value for chromium is well below the known toxic concentration, but it is considered that this element should not be present in drinking-water, and a minimum limiting concentration has been set to provide rejection of a water-supply.

3.2.2 *Specific chemical substances which may affect health*

There are certain chemical substances which, if present in drinking-water at concentrations greater than definite limits, may be injurious to health. Some of these chemical substances are regarded as essential constituents in drinking-water and, if they are not present at sufficient concentration levels, human health is affected adversely.

3.2.2.1 *Fluorides*

Fluorides occur naturally in many public water-supplies and, if present in drinking-water in excess of 1.0-1.5 mg of fluorine per litre, they may give rise to dental fluorosis in some children. When present in much higher concentrations, they may, eventually, cause endemic cumulative fluorosis with resultant skeletal damage in both children and adults.

In assessing the safety of a water-supply with respect to the above limits of fluoride concentration, special consideration should be given to the total daily fluoride intake by the individual. Apart from variations in climatic conditions, it is well known that in some areas certain food

substances contain fluorides; consequently, due attention should be given to both these factors. It should be emphasized, therefore, that in those areas where fluoride-containing foods are ingested, the lower limit of the above concentration range should be the guiding factor as to the chemical quality of the water.

Fluoride is also regarded as an essential constituent of drinking-water, particularly with regard to the prevention of dental caries in children. If the fluoride concentration in the drinking-water of a community is less than 0.5 mg/l, a high incidence of dental caries is likely to occur. To prevent the development of dental caries in children, a number of communal water-supplies are fluoridated to bring the fluorine concentration to 1.0 mg/l.

3.2.2.2 *Nitrates*

Nitrates occur naturally in many water-supplies and may also find access to them directly or indirectly through, for example, the discharge of raw sewage, purified sewage effluent, or barn-yard drainage.

The danger of nitrates to human health is limited to some infants under one year of age. The ingestion of water which contains nitrates in excess of 50 to 100 mg/l (as NO_3) may give rise to infantile methaemoglobinaemia.

In view of the small quantity of water consumed by infants directly as drinking-water, or indirectly as prepared food, it should not be difficult to find an alternative source of water with low nitrate content.

In the light of present knowledge, no single and economic method for the removal of excessive amounts of nitrates from water is available. It is necessary for health authorities, therefore, in areas in which nitrate content of water is known to be in excess of the limiting concentrations, to be vigilant and to warn the population of the potential dangers of using the water for infant feeding and to inform them of alternative sources of water that may be used with safety.

3.2.3 *Chemical substances affecting the potability of water*

The following criteria are important in assessing the potability of water. In view of the wide variations in the chemical composition of water in different parts of the world, rigid standards of chemical quality cannot be established. The limits thereafter designated " permissible " apply to a water that would be generally acceptable by consumers; values greater than those listed as " excessive " would markedly impair the potability of the water.

However, these limiting concentrations are indicative only and can be disregarded in specific instances.

	Permissible	*Excessive*
Total solids	500 mg/l	1500 mg/l
Colour	5 units *	50 units *
Turbidity	5 units **	25 units **
Taste	unobjectionable	—
Odour	unobjectionable	—
Iron (Fe)	0.3 mg/l	1.0 mg/l
Manganese (Mn)	0.1 mg/l	0.5 mg/l
Copper (Cu)	1.0 mg/l	1.5 mg/l
Zinc (Zn)	5.0 mg/l	15 mg/l
Calcium (Ca)	75 mg/l	200 mg/l
Magnesium (Mg)	50 mg/l	150 mg/l
Sulfate (SO_4)	200 mg/l	400 mg/l
Chloride (Cl)	200 mg/l	600 mg/l
pH range	7.0-8.5	Less than 6.5 or greater than 9.2
Magnesium + sodium sulfate	500 mg/l	1000 mg/l
Phenolic substances (as phenol)	0.001 mg/l	0.002 mg/l

3.2.4 *Chemical and physical indicators of pollution*

Chemical constituents or properties of water other than those previously listed are important as indicators of pollution. They have no effect on health or on the acceptability or attractiveness of the water. Their inclusion in the general chemical analysis of water is important because of the significant data furnished as to the degree of pollution by wastes. Since concentrations of these substances vary widely in waters from different sources, no limiting amounts can be given. Increase above the normal concentration range for each of these characteristics provides an indication of pollution, and the magnitude of such increases should be a reasonable measure of the degree of pollution.

These indicators are :

Total organic matter	Total nitrogen
Biochemical oxygen demand	Nitrite
Albuminoid nitrogen	Phosphate [a]

3.3 Methods for the Chemical Analysis of Water

3.3.1 *General*

Recommended procedures for the analytical determination of each of the chemical substances listed in the preceding sections are given in Annex 4 (page 69).

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* Platinum-cobalt scale
** Turbidity units
[a] Metaphosphates are frequently added in water treatment for the control of corrosion and for other purposes.

Where specific sampling procedures are required for a particular examination, they are given in Annex 4 as part of the directions for the examination. The precautions to be taken to ensure that the estimations are as accurate as possible are also given in Annex 4. Many analytical procedures are subject to interference from unknown constituents which may be present in a sample of water.

Any sudden change in the results of the chemical analysis of a supply which has been of constant quality, any off-colour observed in a colorimetric test or during a titration, any unexpected turbidity, odour, or other laboratory finding, is cause for suspicion. These phenomena may be due to a normal variation in the relative concentrations of expected constituents, but they may also be caused by the introduction into the water of an unknown interfering substance.

A few substances, such as chlorine, chlorine dioxide, alum, iron salts, silicates, copper sulfate, ammonium sulfate, and polyphosphates, are so widely used in water treatment that they deserve special mention as possible causes of interference. Of these, chlorine is probably the worst offender, in that it bleaches or alters the colours of many of the sensitive organic reagents which serve as titration indicators and as colour developers for photometric methods. Among the methods which have proved effective in removing chlorine residuals are: the addition of small amounts or sodium sulfite, sodium thiosulfate, or sodium arsenite; exposure to sunlight or artificial ultra-violet light; and prolonged storage.

Whenever interference is encountered or suspected, and no specific recommendations are given for overcoming it, the analyst must endeavour to determine procedures to eliminate the interference without adversely affecting the analysis itself. If different techniques yield markedly different results, it is probable that interference is present. The effect of certain interfering substances becomes less marked on dilution, or upon use of smaller samples; any tendency of the results to increase or decrease in a consistent manner with dilution indicates the possibility of interference.

3.3.2 *Precision and accuracy of the methods*

"Precision" and "accuracy" are not synonyms, although the words are sometimes incorrectly used as synonymous. In the purely scientific sense, "precision" means reproducibility, "accuracy" means truth. A precise method may also be accurate, but not necessarily. A method may be very precise and always yield the same answer, and yet be inaccurate because the answer may be consistently too low or too high. It is also possible for a method to be relatively accurate, in that the average of many determinations yields an answer close to the true value, and yet have poor precision because individual determinations vary over a wide range.

In any method used for chemical analysis of water, the precision and accuracy should be determined and recorded. Procedures for ascertaining precision and accuracy are available for reference.

3.3.3 *Essential precautions to be taken*

Care must be exercised to ensure that the analyses are representative of the actual composition of the water sample. Important factors affecting the results are the presence of turbidity, the method chosen for the removal of turbidity, and the physical and chemical changes brought about by storage or aeration. Each sample showing turbidity must be treated individually with regard to the substances to be determined, the amount and nature of the turbidity present, and other conditions which may influence the results.

It is impossible to give directions covering all conditions which will be encountered, and the choice of the proper technique must be left to the analyst's judgement. In general, any significant amount of suspended matter should be separated by decantation or centrifugation, or by an appropriate filtration procedure. Often a slight amount of turbidity can be tolerated if experience shows that it will cause no interference in gravimetric or volumetric tests, and that a correction can be made for it in colorimetric tests, where it has potentially the greatest interfering effect. Wherever pertinent, the analyst should state whether or not the sample has been filtered.

4. BIOLOGICAL REQUIREMENTS

The biological examination of water—or, as it is sometimes called, the microscopical examination—provides a useful tool for the control of water quality and treatment. It is of particular value in the rapid detection of pollution and conditions which render water unacceptable for dietetic purposes, and in controlling necessary treatments to improve such quality.

These procedures include a qualitative analysis of the types of organism present, together with a quantitative estimation of their number or bulk. In addition, particulate inorganic and organic solids will be detected. The living organisms found by microscopical examination include many different types and forms and are generally designated as "plankton". The term "plankton" is used in a broad sense to include microscopic and near-microscopic free-floating forms as well as minute attached organisms which develop on the shores of lakes or reservoirs or are attached to rocks or structures.

4.1 Purpose

Biological examinations of water will find application in:

(1) determining the causes of objectionable tastes and odours in water and controlling remedial treatments;

(2) aiding in the interpretation of various chemical analyses;

(3) permitting identification of a specific water when it is mixed with another of different composition;

(4) explaining the causes of clogging of distribution pipes and filters or other treatment units;

(5) rapidly detecting the organic pollution of water, contamination with toxic substances, and cross-connexions of distribution systems with polluted waters.

4.2 Sampling

4.2.1 *Sample containers*

In general, a clean glass (neutral) bottle of at least 2-litre capacity, wide-mouthed and equipped with a ground-glass stopper, should be used. It is not essential that this bottle be previously sterilized. In special conditions, smaller or larger containers may be required, but the minimum size of a sample is usually 200 ml. The bottle should not be completely filled, but a small air space should be left under the stopper to provide oxygen for the respiration of the organisms in the sample.

4.2.2 *Sampling procedures*

Points for the collection of samples for biological examination should be carefully chosen and should correspond, whenever possible, to those used for the samples for bacteriological and chemical examinations. Precautions must be observed in the collection of samples to provide for a representative dispersion of the living organisms and to prevent contamination with floating scum or bottom sediments. Many types of sampler have been developed for the collection of samples for microscopical examination. In most instances, satisfactory samples can be obtained by dipping, in the manner used in the general collection of samples for bacteriological examinations (see section 2.2.2, page 16).

4.2.3 *Data relative to samples*

All samples of water should be accompanied by complete and accurate identifying and descriptive data (see Annex 1, page 43). Samples not so identified should not be accepted for examination.

4.2.4 *Preservation and storage of samples*

The sample should be maintained as closely as possible to its original temperature. When examination for living organisms is to be made, the sample should not be stored in sunlight and dissolved oxygen should not be exhausted. A sample may be iced when maintenance of its original temperature is difficult.

If the sample is not to be examined at once or within a short period (not more than one hour), it should be preserved immediately after collection. Preservation is best accomplished by the addition of 3-5 ml of formalin (containing 37-42% formaldehyde and neutralized to pH 7.0 or slightly higher) to each 100 ml of sample. Such preservation is not ideal and may produce severe effects, such as contraction of the cells of the organisms.

As with all other analyses of water, the sample should be transported to the laboratory and the examination started with as little delay as possible.

4.2.5 *Frequency of sampling*

The circumstances of each specific water-supply will determine the frequency with which sampling should be undertaken. Where seasonal growths of plankton are known to be a regular occurrence, samples would probably need to be taken for microscopical examination at weekly or even shorter intervals, in order to determine the type of treatment essential to maintain acceptable quality. During treatment operations, samples for examination would need to be taken at short intervals, probably daily. At other times, or when growths of plankton would not be anticipated, sampling may be set on a monthly or less frequent basis. With many supplies, microscopical examination would be unnecessary or could be on a seasonal basis only. In tracing possible entrance of pollution into water sources, or more particularly into distribution systems, special frequencies, determined by experience, will be indicated.

4.3 Methods of Biological Examination

The biological examination of water requires special laboratory equipment and procedures. These are described in Annex 5 (page 133). It is impracticable to give these methods in detail in this publication, but general directions are indicated.

Since the plankton will be dispersed in the entire volume of the sample, direct examination cannot be made. The sample must, therefore, be concentrated, using special techniques which are an integral part of the examination procedures. In carrying out these examinations, not only numbers of the living organisms should be determined, but the amount

of debris or amorphous matter should also be estimated as an important factor in the biological content of the water.

These methods are applicable to the investigation of the acceptability of a water-supply for use as a public supply, to the rapid detection of pollution and to the control of treatment processes.

4.4 Expression of Results

The content of plankton may be reported in various ways. In many instances a simple numerical count can be used to give the concentration of organisms per ml of sample. Occasionally, the use of figures expressing the results in milligrams per litre (mg/l) or parts per million (p.p.m.) is preferred. This mode of expression is based on the assumption that the specific gravity of the organisms is 1.0.

The more usual means of reporting results is in terms of areal standard units or volumetric standard units. A "standard unit" is a square or cube 20 microns (μ) on each side. Thus an "areal standard unit" is equivalent to 400 square microns and a "volumetric standard unit" to 8000 cubic microns. The use of either of these standard units permits a direct comparison of the number of organisms to the size. This is important, particularly in assessing taste and odour conditions due to plankton, since one large organism may produce more trouble than many small ones. When such standard units are used, they should be designated as areal units or volumetric units per ml of sample. The use of these units involves counting the numbers of individual types and multiplying them by average factors based upon the area or volume occupied by each specific type of organism.

In the biological examination of water, it is not essential to identify each different species present. For routine work all the living organisms should be counted and reported in terms of areal or volumetric standard units of plankton per ml of sample.

Amorphous matter or debris, including particulate organic or inorganic solids, should be determined and reported as such in terms of areal or volumetric standard units.

For the estimation of the degree of pollution, a simple statement of the numbers of significant organisms per ml is sufficient.

4.5 Interpretation of Results

It is impracticable to place definite significance upon the concentration of plankton found in any water-supply. Local conditions and general experience will dictate the exact interpretation of the results of such examinations.

Individual organisms differ widely in their capacity to produce tastes and odours in water. Thus, for example, the detection of a single *Synura* per ml of sample will provide a sure indication of potential serious taste and odour conditions. Conversely, large numbers of standard units, areal or volumetric, of many types of plankton can be tolerated without the development of taste and odour conditions. General experience suggests that when the total plankton count is 300 areal standard units per ml, or greater, the development of objectionable taste and odour conditions can be expected. Counts below this value but above normal low values provide a definite warning that remedial treatment is required.

The effectiveness of treatment to reduce plankton growth in reservoirs and storage units will be indicated by a sharp decrease in plankton counts and a corresponding increase in amorphous matter concentration, resulting from accumulation of the dead cells following treatment. Since destruction of one group of plankton is frequently followed by rapid growth of other forms, the examination of additional samples immediately after treatment is essential to ensure full control of taste and odour conditions in the water-supply.

4.6 Detection of Pollution by Biological Examination

The biological examination of water provides a valuable and sensitive means for the rapid detection of pollution of water by sewage, organic wastes and toxic substances. It is particularly adapted to detecting pollution in distribution mains due to cross-connexions of polluted water and potable supplies. Numerous techniques have been developed for this purpose. These depend upon the susceptibility of fresh-water plankton to the destructive effects of polluting substances. Most of these organisms are very sensitive indicators of pollution because of the narrow range of pollution concentrations which they can tolerate. Thus, by biological or microscopical examination the degree of pollution may be estimated.

4.6.1 *Procedure*

A sample of 500-1000 ml should be collected, concentrated and examined as soon as possible and without the use of any method of preservation. A count is made, using the established techniques, of the individual unicellular organisms, dividing them into two groups:

A = chlorophyll bearing

B = non-chlorophyll bearing

4.6.2 *Biological index of pollution*

The biological index of pollution (BIP) is then calculated by the following formula:

$$BIP = \frac{B}{A + B} \times 100$$

Values of BIP: 0-8 = clean water
8-20 = slightly polluted water
20-60 = polluted water
60-100 = grossly polluted water

This index is a rapid means of detecting pollution, requiring only a short time to complete. It yields information comparable to that given by the general plate count of bacteria and the several chemical tests for pollution.

5. RADIOLOGICAL REQUIREMENTS

5.1 General

Pollution of water-supplies by radioactive material represents an increasing hazard with regard to water quality.

It is important to keep radioactivity in drinking-water to a minimum and it is therefore recommended that radioactive wastes should not be admitted to sources of potable water. It is realized that in certain instances this is not possible, and therefore limiting concentrations of radioactivity are suggested for water to be used for domestic purposes.

The figures given for radioactivity are based on the recommendations of the International Commission on Radiological Protection,[18, a] but the safety factor of 10 proposed by the Commission for water which is to be supplied to large communities has been taken into account in the figures given. These figures are not intended to be used as values which, if exceeded, would render the water unfit for drinking purposes. They are merely intended to indicate that, if the levels are lower than those given, then the water is safe for use without further investigation; if, however, the levels exceed these figures, radiochemical analysis will be required to determine the nature of the radionuclides present before deciding on the safety of the water for use as a public supply. The figures given are intended to include naturally occurring radioactivity, as well as any radioactivity that may reach the water from effluents and fall-out. It is at least possible that naturally occurring radioactivity may be just as dangerous to health as that which may be discharged in effluents from nuclear reactors or other sources, and it is considered that, in drinking-water, it is the total radioactivity that is of importance from the point of view of possible

[a] These recommendations can be obtained from the British Institute of Radiology, 32 Welbeck St., London W.1.

danger to the health of the community. It is appreciated that the figures given for radioactivity are near the limits of measurement by the instruments at present in use.

5.2 Sampling

Samples should be collected not only at consumers' taps, but also at the sources of supply and at relevant points throughout the system. Samples representative of sources such as reservoirs and collecting basins can best be obtained from the bottom, since many radioactive substances settle rapidly to the bottom, from which they may later be released and find their way throughout the system. Such samples should normally be of at least 1-litre volume; should be collected in polyethylene bottles to eliminate the possibility of adsorption of the radioactivity by the container; and should be examined with as little delay as possible.

5.3 Upper Limits of Concentration

In the absence of more precise information, the following are tentatively acceptable as upper limits in drinking-water as supplied to consumers for life-time use for large populations:

alpha-emitters	10^{-9} μc/ml
beta-emitters	10^{-8} μc/ml

(For further information, reference should be made to the recommendations of the International Commission on Radiological Protection.[18]) If these limits are not exceeded, no radiochemical analysis is required. Higher figures than those given above are safe for many radionuclides, and a water with a total radioactivity exceeding the above figures may be safe for use if the absence of the more dangerous radionuclides can be confirmed by radiochemistry.

The figure of 10^{-9} μc/ml for alpha-emitters is to allow for the possibility of all the alpha-activity being due to radium and its daughter elements (otherwise, it too might be 10^{-8} μc/ml). Unless it was found to be practicable for all public supplies of drinking-water to be analysed specifically for radium, it would appear to be wise to retain the level of 10^{-9} μc/ml for alpha-emitters as a check level—in spite of the difficulty of measurement.

In the case of very large populations, consultation should always be made with any national committee responsible for deciding on maximum total population doses for any community.

5.4 Methods of Estimation

Scintillation or internal counters should be used for alpha-activity and Geiger counters for beta-activity. The sample should be evaporated to dryness before examination of the residue.

6. LABORATORY FACILITIES FOR THE EXAMINATION OF WATER

It is essential that all examinations of water in accordance with the recommended standards of quality be performed in a well-equipped laboratory staffed with competent personnel and where the reliability of the work can be ensured. Thus, it is recommended that a laboratory undertaking official examinations of the chemical, bacteriological, biological or physical quality of a water-supply for determining conformity with established standards of drinking-water quality, or for routine supervision of such quality, be accredited by the health authority or other agencies directly responsible for maintenance of water-supply quality. Before granting such approval or accreditation to any laboratory, the agency concerned should make certain that the facilities, quarters, equipment and other physical conditions are adequate for the proper functioning of the laboratory, that the personnel is competent and that the laboratory is under the responsible direction of a person qualified in the fields of water examination, water treatment and sanitation. And further, the agency concerned should, at reasonable intervals, determine that such approved or accredited laboratory is following the methods and techniques recommended for the official examination of water to determine its conformity with standards of drinking-water quality.

7. RESEARCH INVESTIGATIONS

Standards of water quality and accredited methods for the examination of water are not static. They must be under continual study and be modified to meet changing conditions and advances in the science of sanitation and water treatment. Research in both laboratory and field applications of sanitary science is the essential force underlying modifications and developments in the broad problem of acceptable standards. Much of the data on which the present standards of quality and the methods for the examination of water are based were produced in a relatively restricted area of the world—i.e., the United States of America and Europe—and the application of standards and methods to other regions with diverse problems of climate and water resources is not well known. Although laboratories and research facilities may not be available in all parts of the world, nevertheless investigations of the fundamental phases of water-quality control and water treatment should be stimulated as rapidly and as widely as possible. In no other way can the essential information be obtained for complete application of standards of quality to meet local needs and for precise interpretation of the results of

examination of waters by the prescribed standard methods. Such research will require a long time for implementation and for production of the desired information. The development of standards cannot wait for such research, and the establishment of tentative standards now will be an important factor in stimulating the studies essential for progress in the field of sanitary science.

The paucity of precise data about many of the factors concerned with effective standards and methods of analysis has been demonstrated. Many of the recommendations must necessarily remain tentative, and in many instances vague, until much more supporting information can be made available through research and detailed investigations. In the light of the deficiencies in the basic information available at present, a list is reproduced below of suggested research projects in the fields of water quality and water examination which might profitably be undertaken without delay. Not all these projects can be initiated in the immediate future and many of them will require much labour and long periods of time for completion. Active investigations are without doubt already under way in many laboratories for a number of the projects listed below, and some data may already be available or have been published. Extension of such studies would, however, be valuable. The development of research activities by laboratories, government agencies and individuals throughout the world should be encouraged promptly in order that the essential basic data regarding water quality and treatment may become available as soon as possible.

Considerable facilities for such research are already available and there is interest and readiness to undertake without delay certain research activities in the fields of immediate concern. It is anticipated that by such research the basic data for further consideration and amendment of recommended standards of water quality and methods of examination will be obtained.

Suggested Subjects for Research

Sampling

1. Effect of temperature and time of storage on bacterial densities of water.

2. Effect of sodium thiosulfate or other reducing agents on the bacterial population of stored samples of water.

Bacteriological

1. The microfilter technique and its relation to other techniques for estimating densities of coliform bacteria.

2. Technique and significance of total bacterial count or standard plate count.

3. Study of improved media and procedures for isolation and detection of coliform bacteria in water.

4. Evaluation of the special inhibitory culture media proposed for the isolation of the coliform group of bacteria.

5. Investigation of the Eijkman procedure for isolation of *E. coli* in comparison with other proposed techniques.

6. Investigation of techniques for the differentiation of *E. coli* of animal and of human origin.

7. Significance of the coliform group of bacteria and its sub-sections as indicators of pollution under varying climatic and hydrographic conditions.

8. Comparative investigation of the techniques for quantitative detection of bacteria of the coliform group recommended by the Ministry of Health for England and Wales and by the American Public Health Association.

9. Investigation of the possible growth of bacteria of the coliform group, including the faecal *coli* section, in natural waters and soils.

10. Potential significance of other bacterial indicators of pollution, including methods for their quantitative detection in water.

11. Significance of viruses found in sewage in relation to pollution of water, including methods for their quantitative detection.

12. Evaluation of the populations of pathogenic organisms, viruses, bacteriophages and coliform organisms in relation to the epidemiological data.

13. Potential significance of bacteriophages as indicators of pollution of water.

Biological

1. Investigation of the significance and value of various biological indices of pollution of water.

2. Study of the ecological balances of plankton in water.

3. Investigation of the quantitative significance of growths of plankton in relation to water quality.

Chemical

1. Isolation, identification and estimation of odour- and taste-producing substances in water.

2. Analytical determination in water-supplies of certain substances such as DDT, parathion, synthetic detergents and other compounds commonly used in agriculture and industry.

3. Study of methods for the removal of nitrates from water.

4. Development of acceptable techniques for the determination of chemical polluting substances in water.

5. Study of sensitivity, precision and accuracy of chemical methods for the analysis of water.

6. Development of a simple but accurate technique for the determination of fluoride in water.

7. Further studies of the relation of fluoride concentration in water to dental health in tropical and other areas.

Physical

1. Development of techniques for the estimation of small concentrations of turbidity (less than 1.0 unit) in water.

2. Determination of hydrogen-ion concentration (pH) in lightly buffered water.

3. Development of procedures for the measurement of radioactive contamination of water, and consideration of the significance of limiting concentrations of radioactivity.

Annex 1

PARTICULARS TO BE SUPPLIED WITH SAMPLES

1. Name and address of person requesting the examination.

2. Reasons for examination; routine sample or otherwise.

3. Exact place from which sample was taken; if from a house tap, whether drawn through a cistern, or directly from the main.

4. Whether source is a well, spring, stream or public supply.

5. What method of purification or disinfection is used, if any, and at what point it is applied; dose (in mg/l) of disinfecting agent employed.

6. If from a well:

(*a*) Depth of well, and of water surface from ground level.

(*b*) Whether covered or uncovered; nature, material and construction of the cover.

(*c*) Whether newly constructed or with any recent alterations which might affect the condition of the water.

(*d*) Construction:

(i) bricks set dry or in cement;

(ii) cement- or cylinder-lined, and whether puddled outside the lining;

(iii) depth of lining;

(iv) whether bricked above ground surface; if so, height of coping;

(v) presence and extent of apron;

(vi) method of pumping or other means of raising water.

(*e*) Proximity of drains, cesspools, or other possible sources of pollution, and distance from source.

(*f*) Any discoloration of the sides of the well, or other visible indication of pollution.

(*g*) Nature of subsoil and water-bearing stratum.

(*h*) When available, a section or drawing of the well and its general surroundings is desirable.

7. If from a spring:

(*a*) Stratum from which it issues.

(*b*) Whether sample was taken direct from spring or from a collecting chamber; if the latter, type of construction of chamber.

8. If from a river or stream:

(*a*) Depth below surface at which sample was taken.

(*b*) Whether sample was taken from the middle or side.

(*c*) Whether the level of water is above or below the average.

(*d*) Conditions of weather at time of sampling, and particulars of any recent rainfall or flood conditions.

(*e*) Observations with reference to any possible sources of pollution in the vicinity, and approximate distance from sampling point.

9. Does water become affected in appearance, odour or taste after heavy rain?

10. Temperature of water at time of sampling.

11. Date and time when sample was taken and dispatched.

Annex 2

COMPUTATION OF MPN INDEX

(Most probable number of organisms in 100 ml of water) *

TABLE I. MPN INDEX AND CONFIDENCE LIMITS WITHIN WHICH IT CAN LIE, FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 10-ml PORTIONS ARE USED

Number of tubes giving positive reaction out of 5 of 10 ml each	MPN index	Confidence limits	
		Lower limit	Upper limit
0	0	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	Infinite	8.0	Infinite

TABLE II. MPN INDEX AND CONFIDENCE LIMITS WITHIN WHICH IT CAN LIE, FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 10-ml PORTIONS, FIVE 1-ml PORTIONS AND FIVE 0.1-ml PORTIONS ARE USED

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
5 of 10 ml each	5 of 1 ml each	5 of 0.1 ml each		Lower limit	Upper limit
0	0	1	2	< 0.5	7
0	0	2	4	< 0.5	11
0	1	0	2	< 0.5	7
0	1	1	4	< 0.5	11
0	1	2	6	< 0.5	15
0	2	0	4	< 0.5	11
0	2	1	6	< 0.5	15
0	3	0	6	< 0.5	15
1	0	0	2	< 0.5	7
1	0	1	4	< 0.5	11
1	0	2	6	< 0.5	15
1	0	3	8	1	19
1	1	0	4	< 0.5	11
1	1	1	6	< 0.5	15

* According to Swaroop.[24]

TABLE II (continued)

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
5 of 10 ml each	5 of 1 ml each	5 of 0.1 ml each		Lower limit	Upper limit
1	1	2	8	1	19
1	2	0	6	< 0.5	15
1	2	1	8	1	19
1	2	2	10	2	23
1	3	0	8	1	19
1	3	1	10	2	23
1	4	0	11	2	25
2	0	0	5	< 0.5	13
2	0	1	7	1	17
2	0	2	9	2	21
2	0	3	12	3	28
2	1	0	7	1	17
2	1	1	9	2	21
2	1	2	12	3	28
2	2	0	9	2	21
2	2	1	12	3	28
2	2	2	14	4	34
2	3	0	12	3	28
2	3	1	14	4	34
2	4	0	15	4	37
3	0	0	8	1	19
3	0	1	11	2	25
3	0	2	13	3	31
3	1	0	11	2	25
3	1	1	14	4	34
3	1	2	17	5	46
3	1	3	20	6	60
3	2	0	14	4	34
3	2	1	17	5	46
3	2	2	20	6	60
3	3	0	17	5	46
3	3	1	21	7	63
3	4	0	21	7	63
3	4	1	24	8	72
3	5	0	25	8	75
4	0	0	13	3	31
4	0	1	17	5	46
4	0	2	21	7	63
4	0	3	25	8	75
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	2	2	32	11	91
4	3	0	27	9	80

TABLE II (continued)

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
5 of 10 ml each	5 of 1 ml each	5 of 0.1 ml each		Lower limit	Upper limit
4	3	1	33	11	93
4	3	2	39	13	106
4	4	0	34	12	96
4	4	1	40	14	108
4	5	0	41	14	110
4	5	1	48	16	124
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	114
5	0	3	58	19	144
5	0	4	76	24	180
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	154
5	1	3	84	26	197
5	2	0	49	17	126
5	2	1	70	23	168
5	2	2	94	28	219
5	2	3	120	33	281
5	2	4	148	38	366
5	2	5	177	44	515
5	3	0	79	25	187
5	3	1	109	31	253
5	3	2	141	37	343
5	3	3	175	44	503
5	3	4	212	53	669
5	3	5	253	77	788
5	4	0	130	35	302
5	4	1	172	43	486
5	4	2	221	57	698
5	4	3	278	90	849
5	4	4	345	117	999
5	4	5	426	145	1 161
5	5	0	240	68	754
5	5	1	348	118	1 005
5	5	2	542	180	1 405

TABLE III. MPN INDEX AND CONFIDENCE LIMITS WITHIN WHICH IT CAN LIE, FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN ONE 50-ml PORTION AND FIVE 10-ml PORTIONS ARE USED

Number of tubes giving positive reaction out of		MPN index	Confidence limits	
1 of 50 ml	5 of 10 ml each		Lower limit	Upper limit
0	1	1	< 0.5	4
0	2	2	< 0.5	6
0	3	4	< 0.5	11
0	4	5	1	13
1	0	2	< 0.5	6
1	1	3	< 0.5	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40

TABLE IV. MPN INDEX AND CONFIDENCE LIMITS WITHIN WHICH IT CAN LIE, FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN ONE 50-ml PORTION, FIVE 10-ml PORTIONS AND FIVE 1-ml PORTIONS ARE USED

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
1 of 50 ml	5 of 10 ml each	5 of 1 ml each		Lower limit	Upper limit
0	0	1	1	< 0.5	4
0	0	2	2	< 0.5	6
0	1	0	1	< 0.5	4
0	1	1	2	< 0.5	6
0	1	2	3	< 0.5	8
0	2	0	2	< 0.5	6
0	2	1	3	< 0.5	8
0	2	2	4	< 0.5	11
0	3	0	3	< 0.5	8
0	3	1	5	< 0.5	13
0	4	0	5	< 0.5	13
1	0	0	1	< 0.5	4
1	0	1	3	< 0.5	8
1	0	2	4	< 0.5	11
1	0	3	6	< 0.5	15
1	1	0	3	< 0.5	8
1	1	1	5	< 0.5	13
1	1	2	7	1	17
1	1	3	9	2	21
1	2	0	5	< 0.5	13

TABLE IV (continued)

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
1 of 50 ml	5 of 10 ml each	5 of 1 ml each		Lower limit	Upper limit
1	2	1	7	1	17
1	2	2	10	3	23
1	2	3	12	3	28
1	3	0	8	2	19
1	3	1	11	3	26
1	3	2	14	4	34
1	3	3	18	5	53
1	3	4	21	6	66
1	4	0	13	4	31
1	4	1	17	5	47
1	4	2	22	7	69
1	4	3	28	9	85
1	4	4	35	12	101
1	4	5	43	15	117
1	5	0	24	8	75
1	5	1	35	12	101
1	5	2	54	18	138
1	5	3	92	27	217
1	5	4	161	39	> 450

TABLE V. MPN INDEX AND CONFIDENCE LIMITS WITHIN WHICH IT CAN LIE, FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 50-ml PORTIONS, FIVE 10-ml PORTIONS AND FIVE 1-ml PORTIONS ARE USED

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
5 of 50 ml each	5 of 10 ml each	5 of 1 ml each		Lower limit	Upper limit
0	0	1	1	< 0.5	2
0	0	2	1	< 0.5	2
0	1	0	1	< 0.5	2
0	1	1	1	< 0.5	2
0	1	2	1	< 0.5	2
0	2	0	1	< 0.5	2
0	2	1	1	< 0.5	2
0	3	0	1	< 0.5	2
1	0	0	1	< 0.5	2
1	0	1	1	< 0.5	2
1	0	2	1	< 0.5	2
1	0	3	2	< 0.5	4
1	1	0	1	< 0.5	2
1	1	1	1	< 0.5	2
1	1	2	2	< 0.5	4

TABLE V (continued)

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
5 of 50 ml each	5 of 10 ml each	5 of 1 ml each		Lower limit	Upper limit
1	2	0	1	< 0.5	2
1	2	1	2	< 0.5	4
1	2	2	2	< 0.5	4
1	3	0	2	< 0.5	4
1	3	1	2	< 0.5	4
1	4	0	2	< 0.5	4
2	0	0	1	< 0.5	2
2	0	1	1	< 0.5	2
2	0	2	2	< 0.5	4
2	0	3	2	< 0.5	4
2	1	0	1	< 0.5	2
2	1	1	2	< 0.5	4
2	1	2	2	< 0.5	4
2	2	0	2	< 0.5	4
2	2	1	2	< 0.5	4
2	2	2	3	1	7
2	3	0	2	< 0.5	4
2	3	1	3	1	7
2	4	0	3	1	7
3	0	0	2	< 0.5	4
3	0	1	2	< 0.5	4
3	0	2	2	< 0.5	4
3	1	0	2	< 0.5	4
3	1	1	2	< 0.5	4
3	1	2	3	1	7
3	1	3	4	1	9
3	2	0	3	1	7
3	2	1	3	1	7
3	2	2	4	1	9
3	3	0	3	1	7
3	3	1	4	1	9
3	4	0	4	1	9
3	4	1	4	1	9
3	5	0	5	2	12
4	0	0	2	< 0.5	4
4	0	1	3	1	7
4	0	2	3	1	7
4	0	3	4	1	9
4	1	0	3	1	7
4	1	1	4	1	9
4	1	2	4	1	9
4	2	0	4	1	9
4	2	1	4	1	9
4	2	2	5	2	12
4	3	0	5	2	12
4	3	1	5	2	12

TABLE V (continued)

Number of tubes giving positive reaction out of			MPN index	Confidence limits	
5 of 50 ml each	5 of 10 ml each	5 of 1 ml each		Lower limit	Upper limit
4	3	2	6	2	14
4	4	0	6	2	14
4	4	1	7	3	17
4	5	0	7	3	17
4	5	1	8	3	19
5	0	0	4	1	9
5	0	1	4	1	9
5	0	2	6	2	14
5	0	3	7	3	17
5	0	4	8	3	19
5	1	0	5	2	12
5	1	1	6	2	14
5	1	2	7	3	17
5	1	3	9	3	21
5	2	0	6	2	14
5	2	1	8	3	19
5	2	2	10	4	23
5	2	3	12	4	28
5	2	4	15	5	37
5	2	5	18	6	53
5	3	0	9	3	21
5	3	1	11	4	26
5	3	2	14	5	34
5	3	3	18	6	53
5	3	4	21	7	66
5	3	5	25	8	78
5	4	0	13	5	31
5	4	1	17	6	47
5	4	2	22	7	70
5	4	3	28	9	85
5	4	4	35	11	101
5	4	5	43	14	118
5	5	0	24	8	75
5	5	1	35	11	101
5	5	2	54	18	140
5	5	3	92	27	218
5	5	4	161	39	424

Annex 3

APPROVED METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF WATER

1. Laboratory Equipment

1.1 Laboratory quarters

A laboratory used for the examination of water should have ample space for the work to be undertaken, and should be well lighted and ventilated. The laboratory should be equipped with the essential furniture, including work tables, cupboards, shelves, storage space and other ancillary units.

1.2 Equipment

1.2.1 General

It is essential for accurate and satisfactory laboratory work that good equipment in proper working order be provided. Thus, the minimum laboratory equipment listed must be available in an approved laboratory and all items should meet the minimum requirements given. Additional items of equipment not listed will be required in an approved laboratory and they should meet similar standards of quality and operation.

1.2.2 Incubators

Incubators should maintain a uniform and constant temperature (35-37°C) at all times in all parts. This can be accomplished by the use of a water-jacketed or anhydric type of incubator, with thermostatically controlled low-temperature electric heating-units properly insulated and located in or adjacent to walls or floor of chamber, and preferably equipped with mechanical means of circulating air.

Incubators should also be provided with shelves spaced to ensure uniformity of temperature throughout the chamber. The inside dimensions of the chamber should be at least 50 × 50 cm at the base and 60 cm high, to accommodate a maximum of 200 Petri dishes ; 2.5-cm space should be provided between adjacent stacks of plates and between walls and stacks.

Accurate thermometers, with bulb continuously immersed in liquid (glycerine, water, or mineral oil), should be maintained within the incubator and daily readings of the temperatures should be recorded. In addition,

it is desirable to maintain a maximum and minimum registering thermometer within the incubator on the middle shelf to record temperature variations over a 24-hour period. Temperature variations within the incubator filled to maximum capacity should be determined at intervals. It is recommended that a recording thermometer be installed in every incubator whenever possible, so that a permanent record of temperature variations within the incubating chamber may be kept.

Incubators equipped with high-temperature heating-units are unsatisfactory, since such sources of heat frequently cause localized overheating. Incubators so heated may be made to operate satisfactorily by replacing the high-temperature units by suitable wiring, arranged to operate at a lower temperature, and by installing mechanical air circulation. It is desirable, where ordinary room temperatures vary excessively, that laboratory incubators be kept in special rooms which may be maintained at a few degrees below the recommended incubator temperature.

1.2.3 Water-baths

Water-baths are useful for carrying out the 44°C fermentation test. They should be capable of maintaining a temperature of 44°C ± 0.25°C. They should be equipped with mercury-toluol or other reliable thermostats for sensitive regulation of the temperature, and should be adequately insulated against heat loss. An accurate thermometer should be provided, with its bulb placed at the level of the medium in the fermentation tubes. A continous-recording thermometer is advisable.

1.2.4 Sterilizers

1.2.4.1 *Ovens*

Hot-air sterilizing ovens should be of sufficient size to prevent crowding of the interior, constructed to give uniform and adequate sterilizing temperatures, and equipped with suitable thermometers capable of registering accurately in the range 160-180°C. The use of a temperature-recording instrument is optional.

1.2.4.2 *Autoclaves*

Autoclaves should be of sufficient size to prevent crowding of the interior, and constructed to provide uniform temperatures within chambers up to and including the sterilizing temperature of 121°C. They should be equipped with pressure gauges, properly adjusted safety valves, and accurate thermometers with bulb properly located on exhaust line, so as to register minimum temperature within sterilizing chambers (temperature-recording instrument optional). In emergencies, a pressure-cooker may

be substituted for an autoclave, if results have previously been demonstrated to be satisfactory with this method.

1.2.5 Glassware

1.2.5.1 *Pipettes*

Pipettes may be of any convenient size, provided it is found by actual test that they deliver accurately the required amount in the manner in which they are used. The error of calibration should not exceed 2.5%. Pipettes with unbroken tips and with graduations distinctively marked should be used. Pipettes with damaged tips should be repaired or discarded.

1.2.5.2 *Dilution bottles*

Bottles or tubes of resistant glass, preferably Pyrex, closed with glass stoppers, rubber stoppers, or screw caps equipped with liners that do not produce toxic or bacteriostatic compounds on sterilization, should be used. Cotton plugs shall not be used as closures. Graduation levels should be indelibly marked on the side.

1.2.5.3 *Petri dishes*

Petri dishes 100 mm in diameter, with the side wall at least 15 mm high, should be used with glass or porous tops, as preferred. The bottoms of the dishes should be free from bubbles and scratches and should be flat, so that the medium will be of uniform thickness throughout the plate.

1.2.6 Refrigerators

An approved laboratory should have a refrigerator, ample in size for the required work load and capable of maintaining a continuous temperature of between 0°C and 5°C. When available, an electrically operated refrigeration unit will provide the most efficient service.

1.2.7 Colony counter

An effective device for examining colonies, providing a magnification of 3 ×, should be available. In general, a Quebec or similar colony counter will be suitable for this purpose.

2. Culture Media

The use of dehydrated media is strongly recommended to provide uniformity. Such dehydrated media should be of known and approved quality.

2.1 Materials

2.1.1 Distilled water

Distilled water tested and found free from traces of dissolved metals and bacteriocidal and bacteriostatic substances should be used in the preparation of all culture media.

2.1.2 Beef extract

Any brand of beef extract known to give satisfactory results should be used.

2.1.3 Peptone

Any brand of peptone known to be satisfactory for the growth of bacteria should be used.

2.1.4 Sugars

Only sugars of known degree of purity and suitable for bacteriological purposes should be used.

2.1.5 Agar

Either chipped or granulated agar of bacteriological grade should be used.

2.1.6 Dyes

Only dyes certified by the Biological Stain Commission, Geneva, N.Y., USA, for use in the preparation of media should be used.

2.2 Preparation

2.2.1 Lactose broth [1]

Using an approved dehydrated medium, prepare in accordance with the directions furnished with the product. Adjust the reaction so that the pH after sterilization will be between 6.8 and 7.0, preferably 6.9. Place in fermentation tubes [a] and sterilize in the autoclave at 121°C for 15 minutes, after the temperature has reached 121°C, provided that the total time of exposure to any heat is not more than 60 minutes. Cool rapidly after removal from the autoclave. If the above condition of exposure to heat cannot be fulfilled, sterilize in an Arnold sterilizer, or in the autoclave without pressure.

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[a] A fermentation tube may be a test-tube of any suitable size containing a Durham fermentation tube.

When dehydrated medium is not available, prepare it as follows:

Beef extract	3 g
Lactose	5 g
Peptone	5 g
Distilled water	1000 ml

Heat slowly all the ingredients except the lactose in a water-bath, stirring until dissolved; then add the lactose and make up to 1000 ml with distilled water. Adjust the reaction so that the pH after sterilization will be between 6.8 and 7.0, preferably 6.9.

When fermentation tubes for the examination of 10-ml or 100-ml portions of sample are prepared, the lactose-broth medium must be of such strength that the addition of that volume of sample to the medium in the fermentation tube will not reduce the concentration of ingredients in the mixture below that in the standard lactose-broth medium. When dehydrated medium is used, the proper concentration of ingredients may be obtained by using the amounts of dehydrated product shown in the following tabulation:

Inoculum	Volume of medium in tube	Volume of medium and inoculum	Amount of dehydrated lactose-broth medium used per 1000 ml *
1 ml	10 ml or more	11 ml or more	13 g
10 ml	10 ml	20 ml	26 g
10 ml	20 ml	30 ml	19.5 g
100 ml	50 ml	150 ml	39 g
100 ml	35 ml	135 ml	50.1 g
100 ml	20 ml	120 ml	78 g

2.2.2 MacConkey's broth [7]

2.2.2.1 *Single strength*

Using an approved dehydrated medium, prepare in accordance with the directions furnished with the product. When dehydrated medium is not available, prepare it as follows:

Commercial sodium taurocholate, sodium tauroglycocholate, or other satisfactory bile salt	5 g
Lactose	10 g
Peptone	20 g
Sodium chloride	5 g
Distilled water	1000 ml

* Based on the use of Bacto-lactose broth (Difco), manufactured by Digestive Ferment Co. Detroit, Mich., USA.

Mix all the ingredients except the lactose, steam for 2 hours, and transfer to the refrigerator overnight. Add the lactose and, when dissolved, filter through a good grade of filter paper while still cold. Adjust the reaction to pH 7.4, using phenol red as the indicator. Add 1 ml of 1% alcoholic solution of bromcresol purple, or about 5 ml of 1% aqueous solution of neutral red, distribute in 5-ml quantities into 15-cm × 1.5-cm test-tubes provided with Durham fermentation tubes, and sterilize in the autoclave at 115°C for 15 minutes, or in the autoclave without pressure for 30 minutes on three successive days.

2.2.2.2 *Double-strength*

This is prepared in the same way as single-strength MacConkey's broth, using double the quantities given in section 2.2.2.1 (except for water); 10 ml of this double-strength medium is put into each tube (15 cm × 2 cm). If 50-ml quantities of water are to be tested in MacConkey's broth, 50 ml of this double-strength medium should be put into tubes or bottles of greater capacity than 100 ml; 180-ml bottles with proper closure will be found convenient for this purpose. Each tube or bottle should be provided with a Durham fermentation tube of suitable size.

2.2.3 BRILLIANT-GREEN/LACTOSE BILE BROTH [1]

Using an approved dehydrated medium, prepare in accordance with the directions furnished with the product. If dehydrated medium is not available, prepare it as follows:

Dissolve 10 g of peptone and 10 g of lactose in not more than 500 ml of distilled water. Add 20 g of dehydrated ox-gall dissolved in 200 ml of distilled water. The solution of dehydrated ox-gall shall have a pH between 7.0 and 7.5. Make up to approximately 975 ml with distilled water. Adjust the reaction to pH 7.4. Add 13.3 ml of 0.1% solution of brilliant green in distilled water. Make up to 1 litre with distilled water, and filter through cotton.

Distribute in fermentation tubes and sterilize in the autoclave at 121°C for 15 minutes, after the temperature has reached 121°C. When pressure reaches zero, remove and cool quickly.

The pH after sterilization (determined by potentiometric and not by colorimetric methods) should be not less than 7.1 and not more that 7.4.

2.2.4 EOSIN/METHYLENE-BLUE AGAR [1]

Using an approved dehydrated medium, prepare in accordance with the directions furnished with the product. When dehydrated medium is not available, prepare it as follows:

Add 10 g of Bacto-peptone (Difco), 2 g of dipotassium hydrogen phosphate (K_2HPO_4) and 20 g of agar to 1 litre of distilled water. Heat to boiling until all the ingredients are dissolved, and make up loss due to evaporation with distilled water. Adjustment of reaction is not necessary. Place measured quantities, usually 100 or 200 ml, in flasks or bottles and sterilize in the autoclave at 121°C for 15 minutes, after the temperature has reached 121°C.

To prepare plates, melt stock agar prepared as described above, and to each 100 ml add 5 ml of sterile 20% aqueous solution of lactose, 2 ml of 2% aqueous solution of eosin, yellowish, and 1.3 ml of 0.5% aqueous solution of methylene blue. Mix thoroughly, pour into Petri dishes, and allow to harden.

It is permissible to add all the ingredients to the stock agar at the time of preparation, to place in tubes or flasks, and to sterilize. Plates may be prepared from this stock. Decolorization of the medium occurs during sterilization, but the colour returns after cooling.

2.2.5 MacConkey's agar [7]

Using an approved dehydrated medium, prepare in accordance with the directions furnished with the product. When dehydrated medium is not available, prepare it as follows:

Commercial sodium taurocholate, sodium tauroglycocholate, or other satisfactory bile salt	5 g
Peptone	20 g
Sodium chloride	5 g
Washed shredded agar	20 g
Distilled water	1000 ml

Steam until the solids are dissolved. Cool to 50°C, and adjust the reaction at 50°C to pH 7.6-7.8. Add egg white, using the albumen of one egg for every 3 litres of medium. Sterilize in the autoclave at 115°C for 15 minutes, and filter hot through a good grade of filter paper. Adjust the reaction of the filtrate at 50°C to pH 7.3, or at room temperature to pH 7.5. Add 10 g of lactose and 5-10 ml of 1% solution of neutral red. Mix thoroughly, distribute into flasks or screw-capped bottles, and sterilize in the autoclave at 115°C for 15 minutes. For use, melt in the autoclave without pressure and pour into Petri dishes, using 15 ml of medium for each dish.

2.2.6 Endo's medium [1]

Using an approved dehydrated medium, prepare in accordance with the directions furnished with the product. When dehydrated medium is not available, prepare it as follows:

Add 5 g of beef extract, 10 g of peptone, and 30 g of agar to 1 litre of distilled water. Boil until the agar is dissolved and make up lost weight with distilled water. Adjust the reaction so that the pH after sterilization will be 7.4. Clarify if desired, add 10 g of lactose per litre of medium, and dissolve. Place 100-ml portions in small flasks or bottles and sterilize in the autoclave at 121°C for 15 minutes, after the temperature has reached 121°C.

Prepare a 3% solution of basic fuchsin in 95% ethanol. Allow to stand for 24 hours and filter. Melt lactose agar prepared as above, and to each 100 ml add 1 ml of the 3% solution of basic fuchsin and 0.125 g of anhydrous sodium sulfite dissolved in 5 ml of distilled water. The sulfite solution must be freshly prepared. Mix thoroughly, pour into plates with usual precautions against contamination, and allow to harden.

The medium should be light pink when hot and almost colourless when cool. As batches of fuchsin differ somewhat in dye content, it is possible that the medium made according to this formula may be too highly coloured before incubation or may not give the proper reaction when seeded with coliform organisms. In such case, the strength of the solution of basic fuchsin may be varied.

2.2.7 Formate-ricinoleate broth [1]

Add 5 g of peptone, 5 g of lactose, 5 g of sodium formate, and 1 g of sodium ricinoleate to 1 litre of distilled water. Heat slowly on a water-bath, with constant stirring, until dissolved. Make up to 1 litre with distilled water. Adjust the reaction so that the pH after sterilization will be 7.3-7.5. Distribute into fermentation tubes and sterilize in the autoclave at 116-119°C for 15 minutes.

2.2.8 Sodium-azide medium [7, 16]

Peptone	10 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5 g
Potassium dihydrogen phosphate (KH_2PO_4)	2 g
Glucose	5 g
Yeastrel	3 g
Sodium azide (NaN_3)	0.25 g
Bromcresol purple, 1.6% alcoholic solution	2 ml
Distilled water	1000 ml

The pH is 6.6-6.8; no adjustment is necessary. Distribute in 5-ml quantities into test-tubes and sterilize in the autoclave at 121°C for 15 minutes. For use with inocula of 10 or 50 ml of water, a medium of double this strength is prepared and distributed in 10-ml and 50-ml quantities.

a positive confirmed test for the presence of the coliform group as a whole.

3.1.3 "COMPLETED TEST"

The completed test may be used following the confirmed test. It may be applied either to the positive fermentation tubes, or to typical or atypical colonies found upon the plates when solid medium is used (section 3.1.2.2).

3.1.3.1 *Procedure*

Streak one or more Endo's medium or eosin/methylene-blue agar plates from each positive fermentation tube, as soon as possible after appearance of gas. Incubate the plates at 35-37°C for 24 ± 2 hours.

From each of the solid-medium plates used for the confirmed test, or from those made from the positive fermentation tubes, fish one or more typical coliform colonies; or, if no such typical colonies are present, fish two or more colonies believed to consist of organisms of the coliform group, transferring each to a lactose-broth fermentation tube and to an agar slant.

The use of a colony counter is recommended to provide optimum magnification to assist in fishing colonies from the plates. When transferring colonies, care should be taken to choose well-isolated colonies separated by at least 0.5 cm from other colonies. Touch the surface of the colony lightly with the needle in order to minimize the danger of transferring the mixed culture.

The secondary lactose-broth fermentation tubes thus inoculated shall be incubated at 35-37°C until gas formation is noted, the incubation not exceeding 48 ± 3 hours. The tubes shall be inspected after 24 ± 2 hours to determine gas production in that period of incubation.

The agar slants should likewise be incubated at 35-37°C for 24 ± 2 hours and 48 ± 3 hours, and Gram-stained preparations (see section 3.1.4) made from the slants corresponding to the secondary lactose-broth tubes that show gas should be examined microscopically.

3.1.3.2 *Results*

The formation of gas in lactose broth and the demonstration of Gram-negative, non-spore-forming bacilli in the corresponding agar slant shall be considered a satisfactory completed test, demonstrating the presence of a member of the coliform group in the sample examined.

The absence of gas formation in lactose broth or the failure to demonstrate Gram-negative, non-spore-forming bacilli in the corresponding agar slant constitutes a negative test.

When spore-forming organisms are found, the culture should be further examined to ascertain the possible presence of bacteria of the coliform group associated with the spore-bearing organisms. This may be done by transferring the culture to formate-ricinoleate broth and incubating at 35-37°C for 48 ± 3 hours.

If no gas is produced, only spore-forming lactose-fermenting organisms may be considered to be present. If gas is produced in the formate-ricinoleate broth, the probable presence of coliform organisms should be verified by inoculation from the formate-ricinoleate broth on to a plate, and thence to lactose broth and to an agar slant. If, after 48 ± 3 hours, gas is produced in the former and no spores in the latter, the test may be considered "completed" and the presence of coliform organisms demonstrated.

3.1.4 Technique for the Gram-stain [1]

The completed test for coliform organisms (section 3.1.3) includes the determination of Gram-stain characteristics of the organisms isolated. There are a large variety of modifications of the Gram-stain. The following technique is valuable for staining smears of pure cultures.

3.1 4.1 *Reagents*

(1) Ammonium-oxalate/crystal-violet solution: Dissolve 2 g of crystal violet, with 85% dye content, in 20 ml of 95% ethanol (solution A). Dissolve 0.8 g of ammonium oxalate in 80 ml of distilled water (solution B). Mix solutions A and B, ordinarily in equal parts. It is sometimes found, however, that this gives so concentrated a stain that some Gram-negative organisms do not properly decolorize. To avoid this difficulty, solution A may be diluted as much as 10 times, and the diluted solution mixed with an equal quantity of solution B.

(2) Lugol's solution (Gram's modification): Dissolve 1 g of iodine crystals and 2 g of potassium iodide in 300 ml of distilled water.

(3) Counter-stain: Prepare an alcoholic solution of safranin by dissolving 2.5 g of dye in 100 ml to 95% ethanol. Add 10 ml of this solution to 100 ml of distilled water.

3.1.4.2 *Procedure*

Stain the smear for 1 minute with the ammonium-oxalate/crystal-violet solution. Wash the slide in water and immerse in Lugol's solution for 1 minute. Wash the stained slide in water, blot dry. Decolorize with 95% ethanol for 30 seconds, using gentle agitation. Blot, and cover with counter-stain for 10 seconds. Then wash, dry, and examine as usually.

3.1.4.3 *Results*

Cells which decolorize and accept the safranin stain are Gram-negative. Cells which do not decolorize, but retain the crystal-violet stain, are Gram-positive.

3.1.5 "COMPLETED TEST" (ALTERNATIVE METHOD) [7]

3.1.5.1 *Procedure*

From all the positive fermentation tubes of the presumptive test (section 3.1.1.2), a loopful of medium should be transferred and streaked on to a suitable solid medium.

MacConkey's agar plates are ordinarily used. However, other valuable media, such as Endo's medium and eosin/methylene-blue agar, are much employed. It is claimed for some of these media that they enable the observer to distinguish between *Escherichia coli* and *Aerobacter aerogenes* by direct inspection of the colonies. With eosin/methylene-blue agar, differences between the colonies of *E. coli* and *A. aerogenes* can, in fact, often be detected within 24 hours, though in 48 hours differentiation is much easier.

A loopful of the primary culture, or a dilution of the primary culture, is inoculated on to the solid medium in a Petri dish in such a way that isolated colonies are obtained, and the plate is then incubated at 35-37°C for 24 hours.

3.1.5.2 *Appearance of colonies : subculturing for differential tests*

On MacConkey's agar, coliform colonies are usually circular in shape and more or less convex, with a smooth surface and an unbroken edge. They are coloured red, but the depth of tint varies considerably. The normal colony of *E. coli* is deeply coloured and non-mucoid, whereas that of *A. aerogenes* is often paler in colour and mucoid in consistency. No reliance, however, can be placed on colonial appearance alone as a means of distinction. For further examination, two or three typical colonies, as far as possible of different appearance, are selected and subcultured. Should no red or pink colonies appear on the plate within 48 hours, at least one of the predominant variety should be selected for further investigation, bearing in mind the possibility that such atypical colonies may owe their absence of redness to a mere temporary slowing of their action on lactose. Should there be any doubt in the observer's mind whether the colonies selected are those of coliform organisms, film preparations should be made and stained by Gram's method. Only colonies consisting of Gram-negative, non-sporing rods should be subcultured.

Each colony selected should be picked off with a straight wire and inoculated into a tube of peptone water which is then incubated at 37°C. After 4-6 hours' incubation, the culture should be used for the inoculation of 2 tubes of glucose-phosphate medium for the methyl-red and Voges-Proskauer tests, 1 tube of citrate medium for the sodium citrate utilization test, 1 tube of MacConkey's or brilliant-green/lactose bile broth to test for gas production at 44°C, and 1 tube of lactose broth for confirmation of lactose fermentation at 35-37°C. The original peptone-water culture can be re-incubated and used to test for indole formation.

3.2 Differentiation of members of the coliform group

For a satisfactory differentiation within the coliform group of *Escherichia coli*, *Aerobacter aerogenes* and *Escherichia freundii* (or intermediate species), four tests (indole, methyl red, Voges-Proskauer and sodium citrate) are commonly required. These four tests are tentatively recommended for such differential determination. The reactions exhibited by the individual members of the coliform group have been described elsewhere.[1, 4, 7, 23, 25]

3.2.1 Indole differential test

3.2.1.1 *Medium*

Tryptone broth : To 1 litre of distilled water add 10 g of Bacto-tryptone (Difco), or other satisfactory peptone, and heat with stirring to obtain complete solution. Distribute in 5-ml portions into test-tubes and sterilize in the autoclave.

3.2.1.2 *Reagent*

Dissolve 5 g of *p*-dimethylaminobenzaldehyde in 75 ml of amyl alcohol and add 25 ml of concentrated hydrochloric acid. The reagent should be yellow.

Amyl alcohol of reagent quality is recommended. Some brands of *p*-dimethylaminobenzaldehyde are not satisfactory and some good brands become unsatisfactory on ageing. Both products should be purchased in amounts as small as consistent with the volume of work to be done.

3.2.1.3 *Procedure*

Inoculate 5-ml portions of the medium, and incubate at 35-37°C for 24 ± 2 hours. Add 0.2-0.3 ml of reagent and shake. Let the tube stand for about 10 minutes and observe the result. A dark red colour in the amyl alcohol surface layer constitutes a positive indole test; the original colour of the reagent, a negative test.

3.2.2 Methyl-red differential test

3.2.2.1 *Medium*

Peptone medium: [a] To 800 ml of distilled water add 5 g of Proteose-peptone (Difco), or other peptone giving equivalent results, 5 g of dextrose, and 5 g of dipotassium hydrogen phosphate (K_2HPO_4). (A dilute solution of the K_2HPO_4 should give a distinct pink colour with phenolphthalein.) Heat over steam, with occasional stirring, for 20 minutes. Filter through folded filter paper, cool to 20°C, and dilute to 1 litre with distilled water. Distribute 10-ml portions into sterilized test-tubes. Sterilize by the intermittent method for 20 minutes on three successive days.

3.2.2.2 *Reagent*

Methyl-red indicator solution: Dissolve 0.1 g of methyl red in 300 ml of ethanol and dilute to 500 ml with distilled water.

3.2.2.3 *Procedure*

Inoculate 10-ml portions of the medium, and incubate at 30°C for five days. To 5 ml of the culture add 5 drops of methyl-red indicator solution. Record a distinct red colour as methyl-red positive (+), a distinct yellow colour as methyl-red negative (—), and an intermediate colour as doubtful (?).

3.2.3 Voges-Proskauer differential test

3.2.3.1 *Medium*

This test may be made on a 5-ml portion of the medium inoculated for the methyl-red test (section 3.2.2.1), or on a separately inoculated tube of the same medium. The test should be made after 24-48 hours' incubation at 30°C.

3.2.3.2 *Reagents*

(1) α-Naphthol solution: Dissolve 5 g of α-naphthol in 100 ml of ethanol.

(2) Potassium hydroxide solution: Dissolve 40 g of potassium hydroxide in 100 ml of distilled water.

3.2.3.3 *Procedure*

To 1 ml of culture add 0.6 ml of α-naphthol solution and 0.2 ml of potassium hydroxide solution. The development of a crimson to ruby colour in the mixture from 2 to 4 hours after adding the reagents con-

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[a] Use of M.R.-V.P. medium (Difco, dehydrated), with sterilization in the autoclave at 121°C for 12 minutes, is also recommended.

stitutes a positive test. Results should be read not later than 4 hours after addition of the reagents.

3.2.4 Sodium-citrate differential test

3.2.4.1 *Medium*

Dissolve 1.5 g of sodium ammonium phosphate (microcosmic salt), 1 g of potassium dihydrogen phosphate (KH_2PO_4), 0.2 g of magnesium sulfate, and 2.5-3.0 g of sodium citrate crystals in 1 litre of distilled water. Distribute 5-ml portions into test-tubes and sterilize.

3.2.4.2 *Procedure*

The inoculation into this medium should be made with a needle or a standard loop; inoculate lightly. A pipette should never be used, because of the danger of invalidating the result by introduction of nutrient material with the transfer. Incubate at 35-37°C for 72-96 hours, and record visible growth as positive (+), no growth as negative (—).

3.3 Tests for faecal streptococci [7]

3.3.1 Direct azide method

Various volumes of water are inoculated into Hannay & Norton's sodium-azide medium [16] and the tubes are incubated at 44-45°C for 48 hours. The presence of faecal streptococci is indicated by the production of acid in the medium. Hannay & Norton [16] reported that all tubes showing acid contained streptococci, but this has not been confirmed. Mackenzie [20] showed, however, that all tubes becoming acid within 18 hours could be accepted as containing faecal streptococci; it was only in those that became acid later than this that the acidity might have been due to other organisms—most commonly aerobic spore-bearing bacilli. Tubes positive after 24 and 48 hours should be submitted to further confirmatory tests (see section 3.3.3).

When several tubes of various volumes of water are used in this test, as in the presumptive coliform test (section 3.1.1), the most probable number of faecal streptococci in the sample can be calculated from the same probability tables (see Annex 2, page 45), remembering that a short chain of streptococci will be counted as one organism. Here too, the larger volumes of water (50 ml and 10 ml) should be added to double-strength medium, and volumes of 1 ml or less to single-strength medium.

3.3.2 Tellurite-lactose broth method

Various volumes of water are inoculated into tubes of tellurite-lactose broth, which are incubated at 35-37°C for 48 hours. A black deposit

is presumptive evidence that a tube is positive; this may be confirmed by subculture into further tubes of tellurite-lactose broth incubated at 44-45°C for 24-48 hours; tubes positive at this temperature should be submitted to further confirmatory tests (see section 3.3.3).

3.3.3 Confirmatory tests

The presence of faecal streptococci in tubes of azide or tellurite medium in which acid has been produced may be confirmed by plating without delay a heavy inoculum on to MacConkey's agar or on to mannitol/bile-salt/neutral-red agar. The presence of minute red colonies on either of these media is strong presumptive evidence of the presence of faecal streptococci, but individual colonies may be picked for examination if required. *Str. faecalis* produces acid—but no gas—from lactose and mannitol, but not from raffinose, produces an acid caseinogen precipitate in litmus milk, and does not reduce nitrate to nitrite. It is also more resistant to heat and to high concentrations of sodium chloride than other species of streptococcus.

Films made from the tubes of azide or tellurite medium in which acid has been produced will show typically short-chained streptococci when faecal streptococci have been responsible for the acid production. This is a more rapid confirmatory test, but not so reliable as plating the tubes on to a suitable medium.

3.4 Test for Clostridium welchii: litmus milk method [7]

Various quantities of the water should be inoculated into bottles or tubes of freshly boiled litmus-milk medium, and the mixtures heated at 80°C for 10-15 minutes to destroy non-sporing organisms. If the same volumes of water are used as in the presumptive coliform test, an estimate of the most probable number of *Cl. welchii* in the original sample can be made by the use of the same probability tables (see Annex 2, page 45). The inoculated tubes may be incubated anaerobically, but this is not strictly necessary. The tubes should be incubated at 35-37°C for at least five days, but a positive reaction (" stormy clot ") may appear in 24-72 hours. A negative reaction is of little value in assessing the purity of the water.

Annex 4

APPROVED METHODS FOR THE CHEMICAL ANALYSIS OF WATER

1. Laboratory Equipment

A laboratory undertaking chemical analyses should have ample space for the amount and scope of the work to be undertaken, should be well lighted and ventilated, and should be so located as to avoid interference with or by other activities in the neighbourhood. A well-ventilated fume-hood, preferably with exhaust ventilation, is essential. The items of furniture should include adequate storage space, cupboards, shelves and ample working benches of suitable height (1 metre) and with acid-resistant tops. Supplies of hot and cold water, electricity and other services should be provided together with ample drain lines. Included should be the required office and clerical space and suitable provision for a library—indispensable to any type of laboratory.

An approved laboratory must have all the essential items of equipment for chemical analyses, and of a type and precision adapted to accurate chemical determinations. Included must be adequate stocks of chemicals of tested purity and reagent grade for preparing the reagents needed in the various analytical procedures.

Volumetric glassware should be available in sufficient amount and of the type required for the specific analyses made. Such glassware should be calibrated by the analyst either " to contain " or " to deliver ".

Instrumental methods of analysis are frequently described and may be used at the discretion of the analyst. Instrumental methods will not be described in the following recommended procedures. If such methods are employed, it is essential that the instruments be carefully calibrated and the calibrations frequently checked, and that the results of the analyses be periodically checked, using a standard method or by simultaneous examination of a sample of accurately known composition. When instrumental procedures are used, this fact should be recorded with the results of the analysis.

2. Analytical Procedures

For each of the following chemical analyses recommended to determine the conformance of a water-supply to the standards of chemical quality, only a single method is given. For many of these analyses, other satisfactory methods are available and can be found in many text and reference books. The analyst may well substitute other techniques for those outlined below, provided that the substituted procedure is known to have equivalent sensitivity, precision and accuracy.

2.1 Toxic chemical substances

2.1.1 LEAD : DITHIZONE MIXED-COLOUR METHOD [1]

Because of the extreme sensitivity of the dithizone method, it must be emphasized that all reagents and apparatus should be free from contamination by lead and other metals. The use of careful techniques is of the utmost importance. It is urged that, before attempting this method, the chemist should acquaint himself thoroughly with the theory and practice of dithizone procedures.

2.1.1.1 *Principle*

Dithizone dissolved in chloroform will completely extract lead from a slightly basic solution containing citrate. Lead and dithizone form a metal complex, lead dithizonate, which is soluble in chloroform, imparting to it a red colour. Measurement of the intensity of the colour will give a quantitative estimation of the lead present.

2.1.1.2 *Interference*

Bismuth, thallium and stannous tin will interfere with the reaction, but these elements are uncommon in most waters. The analysis should be carried out in diffused light, as bright sunlight tends to destroy dithizone and dithizonates.

2.1.1.3 *Apparatus*

Nessler tubes, matched, 50-ml, tall-form.

2.1.1.4 *Reagents*

(1) Lead-free redistilled water, for preparation of reagents and as dilution water.

(2) Ammonium hydroxide, 28-29% solution, lead-free, or redistilled into ice-cold water.

(3) Ammoniacal cyanide solution : Dissolve 40 g of potassium cyanide in 80 ml of redistilled water. Extract this solution repeatedly with 10-ml portions of 0.005% dithizone stock solution until the last portion remains green. Then wash the solution with chloroform until the chloroform extract remains clear, add 1160 ml of ammonium hydroxide solution, and make up the entire mixture to 2 litres with redistilled water. Store in a glass-stoppered Pyrex bottle.

(4) Chloroform.

(5) Dithizone stock solution, 0.005% : Dissolve 50 mg of diphenylthiocarbazone in 1 litre of chloroform. This solution is stable for several weeks if kept at 4.4°C in the dark.

(6) Dithizone standard solution, 0.001%: Dilute 100 ml of dithizone stock solution to 500 ml with chloroform. Check the strength before use, as it will show progressive loss. This solution is stable for several days if kept at 4.4°C in the dark.

(7) Hydroxylamine hydrochloride solution: Dissolve 20 g of hydroxylamine hydrochloride (NH_3O, HCl) in redistilled water and make up to 100 ml.

(8) Hydrochloric acid, 50%.

(9) Nitric acid, 1% solution.

(10) Sodium citrate solution: Dissolve 10 g of sodium citrate ($Na_3C_6H_5O_7$, $2H_2O$) in 90 ml of redistilled water. Extract with 10-ml portions of 0.005% dithizone stock solution until the last portion remains green. Wash with chloroform to remove excess dithizone.

(11) Lead nitrate standard solution: Dry 0.1598 g of lead nitrate at 110°C. Dissolve and dilute to 500 ml with 1% nitric acid solution. One ml of this solution contains 0.2 mg of Pb. From this, prepare a solution of such strength that 1 ml contains 0.010 mg of Pb.

2.1.1.5 *Procedure*

Take a suitable volume of water containing 0.010-0.050 mg of lead, add 1 ml of 50% hydrochloric acid, and evaporate to about 40 ml. Add 10 ml of sodium citrate solution and 2 ml of concentrated ammonium hydroxide. Mix and transfer to a 125-ml separatory funnel. Extract by shaking vigorously for 30 seconds with 5-ml portions of 0.005% dithizone stock solution until colour in last portion remains unchanged. To the combined extracts add 25 ml of 1% nitric acid solution and shake for 1 minute. Discard the chloroform layer. To the acid extract add 5 ml of hydroxylamine hydrochloride solution, 5 ml of ammoniacal cyanide solution, and exactly 20 ml of 0.001% dithizone standard solution. Shake vigorously for 1 minute and allow layers to separate. Discard the first 2 ml of chloroform extract and transfer the remainder to a 50-ml Nessler tube.

Compare visually with standards prepared in the same way from the lead nitrate standard solution, by looking through the Nessler tubes at right angles to their long axis.

2.1.1.6 *Calculation*

$$\text{Pb (mg/l)} = \frac{\text{mg of Pb} \times 1000}{\text{ml of sample}}$$

2.1.1.7 *Precision and accuracy*

It is estimated that with this procedure an experienced chemist can check his own results to within 0.003 mg and can obtain results within

0.006 mg of the true value, provided that the lead content in the sample taken is in the range 0.010-0.050 mg.

2.1.2 Arsenic: Gutzeit method[1]

2.1.2.1 *Principle*

After concentration of the sample, arsenic is liberated as arsine (AsH_3) by zinc in acid solution in a Gutzeit generator. The generated arsine is then passed through a column of sand and glass wool or a roll of cotton, moistened with lead acetate solution. The generated arsine is allowed to produce a yellow-brown stain on test paper strips impregnated with mercuric bromide. The length of the stain is roughly proportional to the amount of arsenic present.

GUTZEIT GENERATOR

WHO 8209

2.1.2.2 *Interference*

Antimony interferes by giving a similar stain if present in quantities greater than 0.10 mg.

2.1.2.3 *Apparatus*

Gutzeit generator (see figure).

2.1.2.4 *Reagents*

(1) Sulfuric acid solution, approximately 24 N: Cautiously add 2 volumes of concentrated sulfuric acid to 1 volume of distilled water.

(2) Lead acetate solution, 10%: Dissolve 10 g of lead acetate ($Pb(C_2H_3O_2)_2$, $3H_2O$) in 100 ml of distilled water.

(3) Stannous chloride solution: Dissolve 40 g of arsenic-free stannous chloride ($SnCl_2$, $2H_2O$) in 25 ml of concentrated hydrochloric acid and dilute to 100 ml with distilled water.

(4) Potassium iodide solution: Dissolve 15 g of potassium iodide in distilled water and dilute to 100 ml.

(5) Zinc, 500-750-μ (US Standard mesh Nos. 20 and 30), arsenic-free.

(6) Sand or cotton roll:

(*a*) Clean 500-μ (US Standard mesh No. 30) white sand by successive washings with hot 10% sodium hydroxide solution, hot nitric acid, and distilled water, then dry the clean sand; or

(*b*) Cut a roll of dental cotton into 2.5-cm lengths.

(7) Mercuric bromide paper: Use commercial arsenic papers cut into strips of uniform size, about 12 cm long and 2.5 mm wide (papers can be obtained already cut and sensitized). Soak the strips in filtered 3-6%

solution of mercuric bromide in 95% ethanol for 1 hour or longer, and dry by waving in the air. Store in a dry, dark place. For best results, papers should be made up just prior to use.

(8) Arsenic standard solution: Dissolve 0.3949 g of arsenic trioxide in 25 ml of 10% sodium hydroxide solution. Acidify with 6N sulfuric acid solution and dilute to 1 litre with distilled water. One ml of this solution contains 0.30 mg of As.

2.1.2.5 *Procedure*

Take a portion of the sample containing from 0.002 to 0.040 mg of arsenic. Add 5 ml of 24N sulfuric acid solution and 5 ml of concentrated nitric acid, and evaporate to sulfur trioxide fumes. Cool, add 25 ml of distilled water, and repeat to expel oxides of nitrogen. Dilute to 25 ml with distilled water. Dip the cotton roll into the lead acetate solution and put into the glass column in the Gutzeit generator. Alternatively, place a small wad of cotton in the constricted end of the glass column, add 2.5 cm of clean sand, and moisten the sand with the lead acetate solution, removing any excess by light suction. Put the dried narrow glass tube in place and insert the mercuric bromide paper. Make sure that the paper strip is straight.

To the 25 ml of the sample concentrate in the generator, add 5 ml of 24N sulfuric acid solution and cool. Add 5 ml of potassium iodide solution, 4 drops of stannous chloride solution, and, finally, 2-5 g of zinc. Immediately connect the absorption tube to the generator. Immerse the apparatus in a water-bath kept at 20-25°C and leave for 1½ hours. Remove the strip and compute the average length of stains on both sides. Estimate the amount of arsenic present by means of the standard curve.

2.1.2.6 *Preparation of standard curve*

Prepare standards containing 10 ml of 24N sulfuric acid solution and from 0.000 to 0.030 mg of arsenic, in 0.003-mg intervals (from arsenic standard solution), in 25 ml of solution in the generator. Treat in the same manner as described for the sample concentrate. Remove the strip and compute the average length of stains on both sides. Plot length in millimetres against micrograms of arsenic.

2.1.2.7 *Precision and accuracy*

The method is qualitative or roughly quantitative with an accuracy of ± 0.005 mg.

2.1.3 SELENIUM [1]

2.1.3.1 *Principle*

By distillation with hydrobromic acid and bromine in the presence of sulfuric acid, selenium is liberated from its solution as volatile selenium

bromide, which in the aqueous distillate becomes selenious acid. The excess of bromine is reduced with sulfur dioxide and then the selenious acid is reduced to elemental selenium with hydroxylamine hydrochloride:

$$H_2SeO_3 + 2(NH_3O, HCl) \rightarrow Se + N_2O + 4H_2O + 2HCl$$

The selenium is precipitated in the form of a stable red colloidal suspension suitable for colour matching.

2.1.3.2 *Interference*

Where extensive organic matter is present, it may be necessary to oxidize it.

2.1.3.3 *Apparatus*

Distillation assembly, all-glass, including an adapter with interchangeable ground-glass connexions, either standard taper or ball-and-socket joints, and with a flask of suitable size, usually 500 ml.

Nessler tubes, matched, 50-ml, tall-form.

2.1.3.4 *Reagents*

(1) Sodium peroxide: The fresh reagent has a pale yellow colour and is free-flowing. White or caked material indicates deterioration.

(2) Hydrobromic acid, 48%: Reserve for this determination a supply of hydrobromic acid which becomes completely decolorized when subjected to treatment with sulfur dioxide, as described in section 2.1.3.6. If the reagent in stock does not meet this specification, purify it by distillation in an all-glass still, collecting the middle fraction of the distillate.

(3) Hydrobromic-acid/bromine reagent: Mix 15 ml of bromine with 985 ml of 48% hydrobromic acid.

(4) Sulfuric acid, concentrated.

(5) Sulfuric/nitric acid mixture: Cautiously add 1 part of concentrated sulfuric acid to 2 parts of concentrated nitric acid.

(6) Sulfur dioxide, from a small commercial cylinder, that can safely be assumed to be free of selenium.

(7) Gum arabic solution, 5% in water: This solution is subject to bacterial growths and should either be prepared as needed, or be preserved by saturating it with benzoic acid.

(8) Hydroxylamine hydrochloride solution: Dissolve 10 g of hydroxylamine hydrochloride (NH_3O, HCl) in 100 ml of distilled water.

(9) Selenium stock solution: Prepare with selenium dioxide of known high degree of purity, dried to constant weight in an oven at 150°C, and cooled over phosphorus pentoxide in a desiccator. Dissolve in distilled

water the equivalent of 1.405 g of selenium dioxide, add about 80 ml of 48% hydrobromic acid, and dilute to 1 litre with distilled water.

(10) Selenium standard solution: Place 100 ml of selenium stock solution into a 1-litre volumetric flask, add 10 ml of 48% hydrobromic acid, and make up to the mark with distilled water. It is best not to allow the acidity, as determined by titration, to fall below 0.05N, since neutral or very slightly acid solutions of dilute selenious acid tend to lose their titre. One ml of this solution contains 0.100 mg of Se.

2.1.3.5 *Preparation of standards*

For most accurate work, a known quantity of selenium standard solution should be evaporated nearly to dryness, transferred with washings to the distillation flask, and treated in the same manner as the sample. Make up to 100 ml with distilled water and mix thoroughly. Make the desired standards by diluting appropriate amounts of this solution to the mark in Nessler tubes.

2.1.3.6 *Procedure*

Use 1 to 10 litres of water containing not more than 0.5 mg of selenium; add just sufficient amount of fresh sodium peroxide to make the liquid definitely alkaline. Evaporate nearly to dryness on a steam-bath. Evaporation may be hastened by using an electric hot plate, if care is taken not to allow the sample to become dry. Evaporation of the last 100 ml should be done on the steam-bath in any case. If a high concentration of organic matter is present, it may be necessary to digest the residue with a few drops of sulfuric/nitric acid mixture to oxidize the organic matter before the distillation is carried out.

Transfer the residue with washings to the distillation flask and add 50 ml of 48% hydrobromic acid plus 5-10 ml of hydrobromic-acid/bromine reagent; then, while cooling under running water and swirling, slowly and carefully add a volume of concentrated sulfuric acid approximately equal to that of the water present with the transferred residue. Before starting the distillation, arrange the receiver so that a minimum amount of hydrobromic-acid/bromine solution will be needed just to cover the tip of the adapter, otherwise some of the selenium bromide may escape into the air. Distil gradually until all the selenium bromide and most of the hydrobromic acid have passed over. The distillation should take about 30 minutes, and the volume of the distillate should be about 75-90 ml.

(CAUTION: The distillation should be conducted in an efficient fume-hood because of the copious evolution of bromine fumes.)

Transfer the distillate to an appropriate-sized beaker and pass in sulfur dioxide until the yellow colour due to bromine is discharged. Continue treating with sulfur dioxide for 5 seconds. Add 1 ml of gum arabic solu-

tion and 2 ml of hydroxylamine hydrochloride solution, and mix. Cover with a watch glass and allow to stand for 1 hour. Make up to 100 ml, mix well, and transfer to a Nessler tube. Compare visually against standards. The colour is a very pale pink and comparison is best carried out in sunlight. It is difficult to match solutions containing more than 0.5 mg of selenium in 50 ml. The colour comparison is most satisfactory when between 0.01 and 0.10 mg is present.

2.1.3.7 *Calculation*

$$\text{Se (mg/l)} = \frac{\text{mg of Se} \times 1000}{\text{ml of sample}}$$

2.1.3.8 *Precision and accuracy*

This method is said to give low results, but when considering concentrations of the magnitude of 0.005-0.10 mg/l the relative error may not be significant.

2.1.4 CHROMIUM, HEXAVALENT [1]

2.1.4.1 *Principle*

Hexavalent chromium reacts with diphenylcarbazide to produce a reddish-violet colour in a slightly acid solution.

2.1.4.2 *Interference*

In the colour development step, the following substances may cause interference: (*a*) Mercury, both mercurous and mercuric, gives a blue or violet-blue colour, but the reaction is not very sensitive at the acidity employed. (*b*) Iron in concentrations greater than 1 mg/l interferes by producing a yellow colour with the reagent. (*c*) Vanadium interferes in the same manner as iron but more strongly; the colour produced with vanadium fades fairly rapidly and is negligible 10 minutes after the addition of the diphenylcarbazide.

2.1.4.3 *Storage of samples*

Since chromate ions have a tendency to be adsorbed on the surface of the container, and may also be reduced by various agents, precautions should be observed in sample collection and storage. New bottles rather than old etched ones should be used for sample collection. The sample should be tested on the same day it is collected. In any case, storage for more than 2 to 3 days is not recommended.

2.1.4.4 *Apparatus*

Nessler tubes, matched, 50-ml, tall-form.

2.1.4.5 *Reagents*

(1) Redistilled water, free from reducing substances: Distil from an all-glass apparatus.

(2) Diphenylcarbazide reagent: Dissolve 0.2 g of diphenylcarbazide in 100 ml of ethanol, and add 400 ml of 10% sulfuric acid solution. If kept under refrigeration, the solution is stable for about one month. Its colour will change from colourless to tan without affecting its usefulness.

(3) Potassium chromate stock solution: Dissolve 0.374 g of potassium chromate in 1 litre of distilled water. One ml of this solution contains 0.1 mg of Cr.

(4) Potassium chromate working solution: Dilute 10.0 ml of potassium chromate stock solution to 1 litre. Prepare this solution fresh each day. One ml of this solution contains 0.001 mg of Cr^{++++++}.

2.1.4.6 *Procedure*

Use a 50-ml sample or an aliquot diluted to 50 ml with redistilled water. To the sample, clarified by centrifuging if necessary, add 2.5 ml of diphenylcarbazide reagent. Mix and compare visually, using Nessler tubes, against standards prepared from the potassium chromate working solution and containing from 0.003 to 0.20 mg/l Cr. Comparisons should be made at least 5, but not later than 20, minutes after the reagent is added.

2.1.4.7 *Precision and accuracy*

The sensitivity of this method is 0.003 mg/l.

2.1.5 CYANIDE [1]

2.1.5.1 *Apparatus*

Distilling flask, 1-litre (Corning No. 3660 or equivalent).

Graham condenser, to fit the distilling flask, with extension tube to deliver the distillate well below the surface of the alkali in the receiver.

Receiving cylinder, 500-ml, graduated, or equivalent.

Koch microburette, 5-ml capacity.

A pH-meter is desirable, but not necessary, since pH may optionally be determined by indicator solutions.

2.1.5.2 *Reagents*

(1) Tartaric acid solution: Dissolve 15 g of tartaric acid in 100 ml of distilled water.

(2) Sodium hydroxide solution: Dissolve 2 g of sodium hydroxide in 100 ml of distilled water.

(3) Methyl-orange indicator solution: Dissolve 0.5 g of methyl-orange

in 1 litre of distilled water. This indicator is not required if a pH-meter is to be used.

(4) Rhodanine indicator solution: Dissolve 0.02 g of *p*-dimethylaminobenzalrhodanine in 100 ml of acetone.

(5) Silver nitrate standard solution, 0.0192N: Dissolve 3.27 g of silver nitrate in 1 litre of distilled water. Standardize against sodium chloride standard solution, using the Mohr method (see section 2.3.12, page 104) with potassium chromate indicator. One ml is equivalent to 1 mg of CN.

2.1.5.3 *Procedure*

(1) *Distillation*

Titrate a portion of the sample with tartaric acid solution to approximately pH 5. Record the volume of sample and acid required, then discard the portion used. Add the volume of sample to be used for distillation to the boiling flask. If necessary, dilute the sample to 300 ml with distilled water. Pumice granules or glass beads should be added to prevent bumping. Place 50 ml of sodium hydroxide solution in the receiving cylinder and adjust the delivery tube from the condenser so that it releases the distillate within ½ cm of the bottom of the receiving cylinder, i.e., well below the surface of the alkali. Adjust the condenser cooling water.

On the basis of the preliminary titration with tartaric acid solution, calculate the volume of acid necessary to reduce the pH of the sample in the flask to approximately 5. Add to the sample this volume plus an additional 5.0 ml of tartaric acid solution. Stopper the flask immediately and be sure that the joint between condenser and flask is tight.

Heat the flask contents to boiling and carefully distil over exactly 250 ml. The distillate and the sodium hydroxide solution in the receiving cylinder are then mixed and the cyanide content determined by titration.

The first 50 ml of the distillate will contain nearly all the cyanide which can be readily hydrolyzed. If the cyanide compound is stable, the distillation of the entire volume of sample might be insufficient for a high recovery of CN.

(2) *Titration*

Dilute an aliquot of the distillate to 250 ml or some other convenient volume to be used for all titrations. Add 0.5 ml of rhodanine indicator solution.

Titrate with silver nitrate standard solution to the first change in colour from a canary yellow to a salmon hue. Titrate a blank containing the same amounts of alkali and water.

It is advisable to adjust the size of the sample or the strength of the silver nitrate solution so that the titration requires from 2.0 to 10.0 ml. If the analyst has better results with more or less indicator, as a result

of visual sensitivity, the same amount should be used for all titrations. Most analysts find this titration difficult at first, as indicated by a high blank value. As the analyst becomes accustomed to the end-point, blank titrations decrease to 1 drop or less and precision improves accordingly.

2.1.5.4 *Calculation*

$$\text{CN (mg/l)} = \frac{(A - B) \times 1000}{\text{ml of original sample}} \times \frac{250}{\text{ml of aliquot}}$$

where: A = ml of silver nitrate standard solution used for the aliquot
B = ml of silver nitrate standard solution used for the blank.

2.1.5.5 *Precision and accuracy*

With distilled or relatively clear samples, this method of titration, when used at a cyanide level above 1 mg of CN per litre, has a coefficient of variation [a] of 2.0%. Extraction and removal of sulfides or oxidizing agents tend to increase the variation to a degree determined by the amount of manipulation required and the type of sample. The limit of sensitivity is approximately 0.1 mg of CN per litre, but at this point the colour change is indistinct. At 0.4 mg/l the coefficient of variation is 4 times that at a concentration level greater than 1.0 mg/l.

2.2 Chemical substances affecting health

2.2.1 Fluoride: Scott-Sanchis method [1]

2.2.1.1 *Interference*

The important causes of interference are given in Table I.

2.2.1.2 *Apparatus*

Nessler tubes, matched, 100-ml, tall-form.

2.2.1.3 *Reagents*

(1) Zirconium-alizarin solution: Dissolve 0.3 g of zirconium oxychloride octahydrate ($ZrOCl_2$, $8H_2O$)—or 0.25 g of zirconyl nitrate dihydrate ($ZrO(NO_3)_2$, $2H_2O$)—in 50 ml of distilled water contained in a 1-litre glass-stoppered flask. Dissolve 0.07 g of alizarin sodium monosulfonate (use the dye marketed under the name "Alizarin Red S") in 50 ml of distilled water and pour slowly into the zirconium solution, while swirling the flask. The resulting solution clears on standing for a few minutes.

(2) Mixed acid solution: Dilute 112 ml of concentrated hydrochloric acid to 500 ml with distilled water. Add carefully 37 ml of concentrated

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[a] Coefficient of variation = 100 ($s/\bar{x}$), where s = sample standard deviation, $\bar{x}$ = mean.

TABLE 1. INTERFERENCE IN FLUORIDE DETERMINATION

Cause	Concentration (mg/l)	Effect on fluoride reading	
		Increase or decrease	Amount (mg/l)
Al^{+++}	0.25	Decrease	0.1 *a*
Cl^-	2000	Decrease	0.1
* Cl_2 (should be completely eliminated)	—	Increase, if not removed	*b*
Fe^{+++}	2	Increase	0.1
* Mn^{++++}	0.05	Increase	0.1 *b*
($NaPO_3$)	1	Increase	0.1
PO_4^{---}	5	Increase	0.1
SO_4^{--}	300	Increase	0.1
Alkalinity (as $CaCO_3$)	400	Decrease	0.1
* Colour (should be low or compensated for)	—	Either	—
* Turbidity (sample should be clear)	—	—	—

* In these cases, the interference can be eliminated without distillation by treating as described in the procedure (section 2.2.1.5).

a Fluoride and aluminium must both be present for the colour intensification to take place.

b Strong oxidizing agents such as free chlorine and manganese dioxide bleach the zirconium-alizarin lake, whether present alone or in association with fluoride. The colours produced are redder than those produced by fluoride alone. Other strong oxidants may exert a similar bleaching effect under the strongly acid conditions of the test. The analyst should therefore be on the look-out for such interference.

sulfuric acid to 400 ml of distilled water and dilute to 500 ml. After cooling, mix the two acids.

(3) Acid zirconium-alizarin reagent: To the clear zirconium-alizarin solution in the 1-litre flask, add the mixed acid solution up to the mark, and mix. The reagent changes in colour from red to yellow within one hour and is then ready for use. Stored in a refrigerator, the reagent is stable for at least 2 months.

(4) Sodium arsenite solution: Dissolve 1.83 g of sodium arsenite in 1 litre of distilled water. Prepare fresh every six months.

(5) Solutions for the elimination of manganese dioxide:

(*a*) Hydrogen peroxide, 3% solution.

(*b*) Potassium iodide solution: Dissolve 3 g of potassium iodide in 100 ml of distilled water.

(*c*) Sodium thiosulfate solution, approximately 0.1N: Dissolve 2.5 g of sodium thiosulfate ($Na_2S_2O_3$, $5H_2O$) in 100 ml of distilled water.

(6) Sodium fluoride standard solution: Dissolve 0.2210 g of sodium fluoride in distilled water and dilute to 1 litre. Dilute 100 ml of this stock

solution to 1 litre with distilled water. One ml of this solution contains 0.010 mg of F.

2.2.1.4 *Preparation of standards*

Pour the following volumes of sodium fluoride standard solution into Nessler tubes : 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, and 14.0 ml. Make up to 100 ml with distilled water.

2.2.1.5 *Procedure*

(1) *Pre-treatment of sample*

If the sample contains free chlorine, remove it by adding 2 drops (0.1 ml) of sodium arsenite solution for each 0.1 mg of Cl_2. Add 2 drops in excess, and mix.

If the sample contains manganese dioxide, up to 0.5 mg/l Mn can be removed by the following treatment : To a 100-ml sample, add 1 drop (0.05 ml) of 3% hydrogen peroxide solution, and mix. After the amber colour characteristic of manganic ion is discharged, the excess peroxide is eliminated by the addition of 1 drop of potassium iodide solution, followed by 3 drops of 0.1N sodium thiosulfate solution. For accurate results, standards should receive the same treatment. If the sample is turbid, filter it.

(2) *Analysis*

Adjust the temperature of samples and standards so that the deviation between them is not more than 2°C. A temperature near that of the room is satisfactory. A constant temperature should be maintained throughout the determination. To 100 ml of the clear sample, or an aliquot diluted to 100 ml, and to the standards in Nessler tubes, add 5.0 ml of the acid zirconium-alizarin reagent from a volumetric pipette. A reagent that has been refrigerated need not be warmed before use since its cooling effect will be small and constant. Mix thoroughly and compare the samples and standards after one hour.

If the sample is moderately coloured, the colour may be compensated for by " adding " the same colour to the standards in the following way. Place the tube containing the standard above a similar tube containing the sample, acidified with 4.5 ml of the mixed acid solution. Compare against the tube containing the sample plus reagent, placed above a tube containing distilled water. The visual comparison is made in the usual manner ; however, the solutions will have double the usual depth.

2.2.1.6 *Calculation*

$$\text{F (mg/l)} = \frac{\text{mg of F in standard matched by sample} \times 1000}{\text{ml of sample}}$$

2.2.1.7 *Precision and accuracy*

If distillation is omitted, caution must be exercised in interpreting results. Precision and accuracy will depend upon time and temperature control and the effect of interfering substances, as well as upon the application of the usual quantitative techniques. When the procedure is carefully followed and when the interferences are low or compensating, the precision and accuracy will both be ± 0.1 mg of F per litre if a 100-ml sample was taken for analysis. When the concentration of any interfering substance is high, accuracy will be limited and distillation is recommended.

2.2.2 NITRATE: PHENOLDISULFONIC ACID METHOD [1]

2.2.2.1 *Principle*

The basic reaction between nitrate and 2,4-phenoldisulfonic acid produces 6-nitro-2,4-phenoldisulfonic acid, which upon conversion to the alkaline salt yields the yellow colour employed for the colorimetric estimation.

2.2.2.2 *Interference*

Even small concentrations of chloride result in nitrate losses in this method. For this reason, it is important that the chloride content be reduced to a minimum, preferably below 10 mg/l. None the less, the use of silver sulfate presents problems with some water samples owing to the incomplete precipitation of the silver ion, which produces an off-colour and/or turbidity when the final colour is developed. On account of its ability to form complexes with silver, ammonium hydroxide may in some situations possess advantages over potassium hydroxide as the alkali of choice at the end of the determination. Since chloride is a frequent constituent of many waters, its significance as a cause of interference cannot be over-emphasized. Nitrite levels in excess of 0.2 mg of N per litre erratically increase the apparent nitrate concentrations. Coloured ions and materials which physically modify the colour system should be absent.

2.2.2.3 *Storage of samples*

To prevent any change in the nitrogen balance through biological activity, the nitrate determination should be started promptly after sampling. If such a step is impracticable, storage near freezing temperature is advisable. If acid preservation is employed, it is important that the sample acidity be neutralized to at least pH 7 immediately before the procedure is undertaken.

2.2.2.4 *Apparatus*

Nessler tubes, matched, 50- or 100-ml.

2.2.2.5 *Reagents*

All reagents should be prepared from chemicals which are white in colour, and solutions should be stored in Pyrex containers.

(1) Phenoldisulfonic acid reagent: Dissolve 25 g of pure white phenol in 150 ml of concentrated sulfuric acid. Add 75 ml of fuming sulfuric acid (containing 15% free sulfur trioxide), stir well, and heat for 2 hours on a hot water-bath.

(2) Ammonium hydroxide, concentrated.

(3) Potassium hydroxide solution, approximately 12N: Dissolve 673 g of potassium hydroxide in distilled water and dilute to 1 litre.

(4) Aluminium hydroxide suspension: Dissolve 125 g of potassium or ammonium alum—$K_2Al_2(SO_4)_4$, $24H_2O$ or $(NH_4)_2Al_2(SO_4)_4$, $24H_2O$—in 1 litre of distilled water. Warm to 60°C and add slowly, with stirring, 55 ml of concentrated ammonium hydroxide. After it has stood for about one hour, transfer the mixture to a large bottle and wash the precipitate thoroughly by successive decantations with distilled water, until free from ammonia, chloride, nitrite, and nitrate.

(5) Potassium nitrate stock solution: Dissolve 0.722 g of anhydrous potassium nitrate in distilled water and dilute to 1 litre. One ml of this solution contains 0.1 mg of N.

(6) Potassium nitrate standard solution: Evaporate 50.0 ml of potassium nitrate stock solution to dryness on a water-bath, dissolve the residue by rubbing with 2 ml of phenoldisulfonic acid reagent, and dilute to 500 ml with distilled water. One ml of this solution contains 0.01 mg of N or 0.0443 mg of NO_3.

(7) Silver sulfate standard solution: Dissolve 4.40 g of silver sulfate, free from nitrate, in distilled water and dilute to 1 litre. One ml is equivalent to 1.0 mg of Cl.

2.2.2.6 *Preparation of standards*

Introduce in a series of 50-ml Nessler tubes the following volumes of potassium nitrate standard solution: 0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 3.5, 6.0, 10, 15, 20, and 30 ml. (Where it is more convenient to use a total volume of 100 ml, the volumes of standard solution may be doubled.) Make up to the mark with distilled water, after adding to each tube 2.0 ml of phenoldisulfonic acid reagent and a volume of the alkali (concentrated ammonium hydroxide or potassium hydroxide solution) equal to that used for the sample. These standards may be kept for several weeks without deterioration.

2.2.2.7 *Procedure*

If the sample has a colour of more than 10 units, decolorize by adding 3 ml of aluminium hydroxide suspension to 150 ml of sample, stir very

thoroughly, allow to stand for a few minutes, and filter, discarding the first portion of the filtrate.

Determine the chloride content of the water, and treat a 100-ml sample with an equivalent amount of silver sulfate standard solution. Remove the precipitated chloride either by centrifugation or by filtration, coagulating the silver chloride by heat if necessary. Neutralize the clarified sample to approximately pH 7, transfer to a casserole, and evaporate to dryness on a water-bath. Rub the residue thoroughly with 2.0 ml of phenoldisulfonic acid reagent to ensure solution of all solids. If necessary, heat mildly on a water-bath for a short time to dissolve the entire residue. Dilute with 20 ml of distilled water and add, with stirring, 6-7 ml of concentrated ammonium hydroxide or 5-6 ml of potassium hydroxide solution, until maximum colour is developed. Filter any resulting flocculent hydroxides from the coloured solution. Transfer the filtrate to a 50- or 100-ml volumetric flask or Nessler tube, dilute to the mark with distilled water, and mix. Compare visually against the standards.

2.2.2.8 *Calculation*

$$\text{N (mg/l)} = \frac{\text{mg of N} \times 1000}{\text{ml of sample}}$$

$$\text{NO}_3 \text{ (mg/l)} = \text{mg/l N} \times 4.43$$

2.2.2.9 *Precision and accuracy*

The problem of eliminating or compensating for the chloride interference makes it difficult to define the precision and accuracy of the method. With some supplies, precision and accuracy of the order of 0.05 mg/l in the range up to 1 mg/l N can be attained, while with other supplies much greater errors have been demonstrated. Even though the method is capable of a sensitivity somewhat below 0.05 mg/l N, the over-all uncertainties warrant reporting results only to the nearest 0.1 mg/l.

2.3 Chemical substances affecting potability

2.3.1 Residue[1]

2.3.1.1 *Total residue*

Choose a volume of sample which will yield a residue between 25 and 250 mg, preferably between 100 and 250 mg. A rough preliminary calculation from the values obtained in other chemical determinations will usually suffice to estimate the volume to be evaporated. Place the sample, thoroughly shaken and unfiltered, in a weighed evaporating dish. The dish should preferably be made of platinum, but silica, porcelain, or

Pyrex dishes may be used. Place the dish in an oven maintained at 103-105°C and evaporate the sample to dryness. If more convenient, evaporation may be carried out on a steam-bath, but the final drying should be done in the oven. After all the liquid has evaporated, continue to dry the residue to constant weight. If time is no obstacle, allow the residue to dry overnight. After drying, allow the warm dish to cool in a desiccator, and weigh. If the residue is hygroscopic, it may be necessary to weigh the dish in a closed container. Report the increase in weight as total solids or residue on evaporation.

2.3.1.2 *Fixed residue*

Ignite the residue remaining in the dish in a muffle furnace at a temperature of 500°C. In order to obtain maximum reproducibility, it is preferable to have the furnace up to temperature before inserting the samples and to ignite for one hour. After ignition, allow the dish to cool, moisten the residue with a 5% solution of ammonium bicarbonate, and dry at 103-105°C as for total residue. This reconverts oxides to carbonates. Weigh the final residue and subtract the weight of the dish to obtain the weight of the fixed residue. The difference between this figure and that recorded as total residue is reported as loss on ignition.

2.3.1.3 *Calculation*

The following formula is applicable to either of the above determinations:

$$\text{Residue (mg/l)} = \frac{\text{mg of residue} \times 1000}{\text{ml of sample}}$$

In reporting results, the form of residue should be stated.

2.3.2 COLOUR [1]

2.3.2.1 *Apparatus*

Nessler tubes, matched, 50-ml, tall-form.

2.3.2.2 *Preparation of standards*

Dissolve 1.245 g of potassium chloroplatinate (K_2PtCl_6), equivalent to 0.500 g of metallic platinum, and 1 g of crystallized cobaltous chloride ($CoCl_2$, $6H_2O$), equivalent to about 0.25 g of metallic cobalt, in distilled water with 100 ml of concentrated hydrochloric acid, and dilute to 1 litre with distilled water.

If potassium chloroplatinate is not available, it may be replaced by chloroplatinic acid, which should be prepared as follows: Dissolve 0.500 g

of pure metallic platinum in aqua regia with the aid of heat; remove nitric acid by repeated evaporation with fresh portions of concentrated hydrochloric acid. Then dissolve the product with 1 g of crystallized cobaltous chloride, as directed above. Commercial chloroplatinic acid should not be used because it is very hygroscopic and may therefore vary in platinum content (potassium chloroplatinate is not hygroscopic).

The stock solution obtained in this way has a colour of 500 units. To prepare standards having colours of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, and 70 units, dilute 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0 ml respectively of the stock solution with distilled water to 50 ml in 50-ml Nessler tubes. Protect these standards against evaporation and contamination when not in use.

2.3.2.3 *Procedure*

Fill a matched Nessler tube to the 50-ml mark with the sample to be examined and compare its colour with that of standards. The observation should be made by looking vertically downwards through the tubes towards a white or specular surface placed at such an angle that light is reflected upwards through the columns of liquids. If turbidity is present and has not been removed by the procedure given below, the colour is reported as " apparent colour ". If the colour exceeds 70 units, the sample should be diluted with distilled water in known proportions until the colour is within the range of the standards, and the results multiplied by the appropriate dilution factor.

In the presence of turbidity, the true colour should be determined after the removal of turbidity by centrifuging. The sample is placed in a suitable centrifuge tube or tubes and centrifuged until the supernatant is clear. The time required will depend upon the nature of the sample, the speed of the motor, and the radius of the centrifuge, but will rarely be more than one hour. The centrifuged sample should be compared in a Nessler tube with distilled water to ensure that all turbidity has been eliminated. If clear, the sample is then compared with the standards.

2.3.2.4 *Expression of results*

The results of the colour determination are expressed in whole numbers with the following accuracy:

Colour range	*Record to nearest*
1- 50 units	1 unit
51-100 ,, ...	5 units
101-250 ,, ...	10 ,,
251-500 ,, ...	20 ,,

2.3.3 TURBIDITY [1]

2.3.3.1 *Principle*

Turbidity measurements are based on the light path of a suspension which just causes the image of the flame of a standard candle to disappear, i.e., to become indistinguishable against the general background illumination, when the flame is viewed through the suspension. The greater the light path, the lower the turbidity.

2.3.3.2 *Interference*

The method is applicable to any water sample free from debris and coarse sediments that settle out rapidly; dirty glassware, air bubbles, and vibrations which disturb the surface visibility of the sample lead to false results.

2.3.3.3 *Storage of samples*

It is preferable to determine turbidity on the same day the sample is taken. If longer storage is unavoidable, however, samples may be stored in the dark for as long as 24 hours. For even longer storage, treat the sample with 1 g of mercuric chloride per litre. In every case, the samples should be vigorously shaken before examination.

2.3.3.4 *Apparatus*

The standard instrument for making such measurements is the Jackson candle turbidimeter, consisting of a calibrated glass tube, a standard candle, and a support which aligns the candle and the tube. The glass tube and the candle are supported in a vertical position so that the centre line of the tube passes through the centre line of the candle. The candle support consists of a spring-loaded cylinder designed to keep the top of the candle pressed against the top of the support as the candle gradually burns away. The top of the support for the candle should be 7.5 cm below the bottom of the glass tube, which must be polished optically flat. The glass tube must be kept clean and free from scratches. It should be graduated to read directly in turbidity units. Most of the glass tube must be enclosed in a metal tube when observations are being made, both for the sake of protection against breakage, and for the exclusion of extraneous light.

The candle is made of beeswax and spermaceti, and designed to burn within the limits of 7.4-8.2 g per hour. To ensure uniform results, the flame must be kept as near constant size and constant distance from the bottom of the glass tube as possible: this will require frequent trimming of the charred portion of the wick and frequent observations to see that the candle is pushed to the top of its support. All drafts must be elimi-

nated during measurements to prevent the flame from flickering. The candle must not be kept burning for more than a few minutes at a time, for the flame has a tendency to increase in size. Each time before the candle is lighted, any portions of the charred wick that can easily be broken off when manipulated with the fingers should be removed.

2.3.3.5 *Preparation of standard suspensions*

It is best to use natural turbid water from the same source as that to be tested. If this is not available, suspended matter or bottom sediments from the body of water under investigation may be boiled with 50% hydrochloric acid to remove water-soluble material, washed repeatedly by centrifugation and decantation, and made into suspensions. If natural materials are unavailable or unsatisfactory, fullers' earth or wet-ground diatomaceous earth may be used. If the turbidity is caused by materials added during water treatment, then carbon, alum, or whatever material is responsible for the turbidity should be used. Add approximately 5 g of dry material to 1 litre of distilled water, thoroughly agitate, and allow to stand for 24 hours. Withdraw the supernatant without disturbing the sediment at the bottom. Determine the turbidity with the Jackson candle turbidimeter. Dilute portions of the suspension to the turbidity values desired, checking all values above 25 units with the candle turbidimeter. Standard suspensions may be preserved by the addition of 1 g of mercuric chloride per litre. Suspensions must be well shaken before each reading, and must be checked at least once a month with the candle turbidimeter. Suspensions having turbidities below 25 units cannot be checked directly: these must be made up fresh at least once a month by dilution of a more concentrated suspension which has been freshly checked. Dilute suspensions calculated to contain 0, 0.2, 0.4, 0.6, 0.8, 1.0, etc. turbidity units should be made fresh weekly by dilution of the 10-unit suspension. It is preferable to store the suspensions in Pyrex or other resistant glass bottles.

2.3.3.6 *Procedure*

(1) *For turbidities between 25 and 1000 units.* Observations are made by pouring the sample into the glass tube until the image of the candle flame just disappears from view. At this stage the analyst should see a uniformly illuminated field with no bright spots. Addition of the sample should be carried out slowly towards the end. After the image has been made to disappear, the removal of 1% of the sample should again make the flame image visible. It is convenient to employ a pipette to add or remove small amounts of the sample at the end. Care should be taken to keep the glass tube clean both inside and outside, and to avoid scratching the

glass. The accumulation of soot or moisture on the bottom of the tube may interfere with the accuracy of the results.

(2) *For turbidities exceeding 1000 units.* The sample is diluted with one or more volumes of turbidity-free water until the turbidity falls below 1000 units. The turbidity of the original sample is then computed from the turbidity of the diluted sample and the dilution factor. For example, if 5 volumes of turbidity-free water were added to 1 volume of sample, and the diluted sample showed a turbidity of 500 units, then the turbidity of the original sample was 3000 units.

(3) *For turbidities between 5 and 100 units.* The Jackson candle turbidimeter has a lower limit of 25 units. In the range from 5 to 100 units, samples may be compared in sets of bottles with standard suspensions made by diluting more concentrated standard suspensions with turbidity-free water in known ratios. The sample and the standards are placed in bottles of the same size, shape and type, enough empty space being left at the top of each bottle to allow adequate shaking before each reading. The comparison shall be made by viewing the sample and the standards through the sides of the bottles, looking through them at the same object, and noting the distinctness with which the object can be seen. The turbidity of the sample shall be recorded as that of the standard which produced the visual effect most closely approximating that of the sample. Readings may be made conveniently by viewing newsprint or a series of ruled black lines on white paper. It is preferable that articificial light be used, and directed downwards from above, so that no direct light reaches the eye. If a commercial turbidimeter is used, the manufacturer's instructions should be followed, and calibration against the Jackson candle turbidimeter is necessary.

(4) *For turbidities of less than 5 units.* When the turbidity of a sample is less than 5 units, measurement of scattered light rather than transmitted light is employed. Visual instruments which operate on the scattering principle include the Baylis and the St. Louis turbidimeters. Several photoelectric instruments, including nephelometers, are on the market. The tubes to be used with the available instrument should be of clear, colourless glass. They must be kept scrupulously clean, both inside and outside, and should be discarded when they become scratched or etched. They should not be handled at all where the light strikes them, but must be provided with sufficient extra length, or with a protective case, so that they may be handled. The tubes should be filled with samples and standards which have been thoroughly agitated, and then sufficient time allowed for bubbles to escape. In visual instruments, the turbidity should be recorded as that of the most closely matching standard. In using commercial instruments, the manufacturer's instructions should be followed, and calibration against the Jackson candle turbidimeter is necessary.

2.3.3.7 *Expression of results*

Analysts who are familiar with earlier standard procedures will note that the terms " silica scale " and " parts per million " for turbidity have not been used here. The reasons are that reproducible suspensions cannot be made up on a weight basis unless the same batch of silica powder is always used, and that the primary standard for turbidity has always been the Jackson candle turbidimeter, making the use of a scale based on silica unnecessary and anomalous. The present turbidity units have the same numerical value as the former silica-scale units.

Turbidity readings are recorded with the following accuracies :

Turbidity range	*Record to nearest*
0-1 unit	0.1 unit
1-10 units	1 ,,
10-100 ,, ...	5 units
100-400 ,, ...	10 ,,
400-700 ,, ...	50 ,,
Over 700 ,, ...	100 ,,

For comparison of the efficiency of water treatments, it may be desirable to estimate the turbidities more precisely than is specified above ; but because of the uncertainties and discrepancies in turbidity measurements, it cannot be expected that two or more laboratories will duplicate results on the same sample more closely than specified.

2.3.4 TASTE AND ODOUR [1]

2.3.4.1 *Cold odour quality*

Shake about 250 ml of the sample at 20°C in a 500-ml wide-mouth Erlenmeyer flask, and lightly sniff the odour. Avoid vigorous or repeated shaking in order not to dissipate the odour.

2.3.4.2 *Hot odour quality*

Pour about 250 ml of the sample into a 500-ml Erlenmeyer flask, and close the mouth of the flask with a watch glass. Heat the water to approximately 58-60°C, agitate it with a rotary movement, slip the watch glass aside, and sniff the odour.

2.3.4.3 *Expression of results*

Results are reported on both hot and cold samples, and as to both intensity and type. The intensity is reported according to the following scale :

35 to 38%, the sodium acetate solution will have to be adjusted for the particular lot of hydrochloric acid to be used.

(2) Sodium acetate solution: Dissolve 350 g of sodium acetate ($NaC_2H_3O_2$, $3H_2O$) in 500 ml of distilled water and dilute to approximately 1 litre. Standardize a pH-meter with the pH-4.0 buffer. Pipette exactly 2.0 ml of concentrated hydrochloric acid into 100 ml of distilled water. Insert the pH electrodes. Place the sodium acetate solution in a burette and titrate the hydrochloric acid with it until the pH is between 3.2 and 3.3. Add enough distilled water to the remainder of the sodium acetate solution so that 10.0 ml, when added to 2.0 ml of hydrochloric acid and 100 ml of distilled water, will bring the pH to a value between 3.2 and 3.3. Re-check this dilution by trial. This adjustment is valid only for the particular bottle of acid used and must be re-checked for each new batch of hydrochloric acid.

(3) Hydroxylamine hydrochloride solution: Dissolve 10 g of hydroxylamine hydrochloride (NH_3O, HCl) in 100 ml of distilled water.

(4) Phenanthroline solution: Dissolve 0.12 g of 1,10-phenanthroline ($C_{12}H_8N_2$, H_2O) in 100 ml of distilled water by stirring and heating to 80°C; do not boil. This solution should be discarded if it darkens. Store in a cool, dark place.

(5) Iron standard stock solution: Use electrolytic iron wire, or "iron wire for standardizing", to prepare the standard. If necessary, clean the wire with fine sandpaper to remove any oxide coating and to produce a bright surface. Weigh 0.2000 g of wire and place in a 1-litre volumetric flask. Dissolve in 20 ml of 16.7% sulfuric acid and dilute to the mark. One ml of this solution contains 0.2 mg of Fe.

(6) Iron standard working solutions: These should be prepared the day they are to be used.

(*a*) By means of a pipette, introduce 50.0 ml of iron standard stock solution into a 1-litre volumetric flask and dilute to the mark with iron-free distilled water. One ml of this solution contains 0.01 mg of Fe.

(*b*) By means of a pipette, introduce 5.0 ml of iron standard stock solution into a 1-litre volumetric flask and dilute to the mark with iron-free distilled water. One ml of this solution contains 0.001 mg of Fe.

2.3.5.5 *Preparation of standards*

By means of a pipette, accurately measure calculated volumes of the working solutions (the weaker solution should be used for the 0.001-0.010-mg portions), introduce into 125-ml Erlenmeyer flasks, and treat in the same manner as the sample. For visual comparison, a set of at least 12 standards is desirable, ranging from 0.001 to 0.12 mg of Fe in the final 100-ml volume.

2.3.5.6 *Procedure*

(1) *Pre-treatment of sample.* The following types of pre-treatment are to be applied for the various forms of iron to be determined. The determinations should be made as soon as possible after the sample is collected, especially for soluble iron. If the sample to be analyzed for total iron is measured and acidified, it may be safely stored.

(*a*) *Total iron.* The sample is mixed thoroughly, measured, and treated.

(*b*) *Dissolved iron.* Allow the sample to settle, then decant and filter the supernatant through fine filter paper, discarding the first portion. Treat a measured volume of the filtrate.

(*c*) *Suspended iron.* Determine total and dissolved iron and calculate the suspended iron by subtracting the dissolved from the total iron.

(2) *Colour development.* If the sample is expected to contain less than 2.4 mg of Fe per litre, pipette a 50.0-ml aliquot into a 125-ml Erlenmeyer flask. If the sample is expected to contain a higher concentration of iron, accurately measure a smaller aliquot which will contain less than 0.12 mg of Fe, and add distilled water to make the volume about 50 ml. Add 2.0 ml of concentrated hydrochloric acid and a few glass beads. Heat to boiling and boil gently for 5 minutes to bring all the iron into solution. Cool to room temperature, transfer to a 100-ml volumetric flask, and add 1 ml of hydroxylamine hydrochloride solution, 10.0 ml of sodium acetate solution, and 10 ml of phenanthroline solution. Dilute to the mark with distilled water, mix thoroughly, and allow to stand for 15 minutes to permit full colour development. Compare visually with the standards in Nessler tubes.

2.3.5.7 *Calculation*

$$\text{Fe (mg/l)} = \frac{\text{mg of Fe} \times 1000}{\text{ml of sample}}$$

It is advisable to report details of sample collection, storage, and pretreatment together with the iron value obtained, if this will aid in the interpretation.

2.3.5.8 *Precision and accuracy*

The precision and accuracy will depend upon the method of sample collection and storage, the method of colour measurement, the iron concentration, and the presence of interfering colour, turbidity, and foreign ions. In general, visual comparison in Nessler tubes is not reliable to better than $\pm 5\%$—often only $\pm 10\%$. The sensitivity limit for visual observation in Nessler tubes is approximately 0.001 mg of Fe. The variability and instability of the sample may limit the precision and accuracy of this determination more than will the errors of analysis itself. In the

past, serious discrepancies have been reported between different laboratories because of differences in collecting and treating the samples.

2.3.6 MANGANESE: PERSULFATE METHOD [1]

2.3.6.1 *Principle*

Persulfate oxidation of soluble manganous compounds to form permanganate is carried out in the presence of silver nitrate. The resulting colour is stable for at least 24 hours if excess persulfate is present and organic matter is absent.

2.3.6.2 *Interference*

As much as 0.1 g of sodium chloride is prevented from interfering by the addition of mercuric sulfate to form slightly dissociated complexes. Only minute amounts of bromide and iodide may be present. Reasonable amounts of organic matter may be present if the period of heating is increased and more persulfate is added. The effect of turbidity can only be estimated, and no correction for interfering colour is possible.

2.3.6.3 *Storage of samples*

The sample should be analysed as soon as possible after collection, or should be acidified prior to storage.

2.3.6.4 *Apparatus*

Nessler tubes, matched, 100-ml, tall-form.

2.3.6.5 *Reagents*

(1) Ammonium persulfate, solid.

(2) Special solution: Dissolve 75 g of mercuric sulfate in 400 ml of concentrated nitric acid and 200 ml of distilled water. Add 200 ml of 85% phosphoric acid and 0.035 g of silver nitrate, and dilute the cooled solution to 1 litre.

(3) Manganous sulfate standard solution: Dissolve 3.2 g of potassium permanganate ($KMnO_4$) in 1 litre of distilled water. Age for several weeks in sunlight, or heat for several hours near boiling-point, filter through a fritted-glass filter, and standardize against sodium oxalate. Using the following formula, calculate the volume of this solution necessary to prepare 1 litre of a solution containing 50 mg of Mn per litre:

$$\text{Volume of } KMnO_4 \text{ solution} = \frac{4.55}{\text{normality of } KMnO_4 \text{ solution}}$$

To this volume add 2-3 ml of concentrated sulfuric acid, and then 10% sodium bisulfite solution, dropwise with stirring, until the permanganate colour disappears. Boil to remove the excess sulfur dioxide, cool, and dilute to 1 litre with distilled water. This solution may be diluted further in order to measure small amounts of manganese.

2.3.6.6 *Procedure*

To a suitable aliquot of the sample, add 5 ml of special solution. Concentrate to 90 ml by boiling, or dilute to 90 ml. Add 1 g of ammonium persulfate and bring to boiling in about 2 minutes over a flame; do not heat on a water-bath. Remove from the flame for 1 minute, then cool under the tap. Dilute to 100 ml with distilled water free from reducing substances, and mix. Compare visually with standards containing 0, 0.005, 0.010, etc. up to 1.5 mg of Mn, prepared by treating various amounts of the manganous sulfate standard solution in the same way.

2.3.6.7 *Calculation*

$$\text{Mn (mg/l)} = \frac{\text{mg of Mn} \times 1000}{\text{ml of sample}}$$

2.3.6.8 *Precision and accuracy*

The relative error in the determination of 0.05-1.5 mg of Mn, in the absence of interfering substances, is usually not more than 1%. It will be higher for lower amounts of manganese.

2.3.7 COPPER: DIETHYLDITHIOCARBAMATE METHOD [1]

2.3.7.1 *Principle*

Copper ion forms a yellowish-brown colloidal chelate compound with sodium diethyldithiocarbamate. If the copper concentration is excessive, the colloid will be so concentrated that the solution will appear turbid.

2.3.7.2 *Interference*

Sodium diethyldithiocarbamate is not specific for copper. Interfering substances, however, are not common in natural waters, and it is easy to detect their presence in this determination. Zinc, lead, and certain other metallic ions produce white turbidities in the developed sample. If the iron/copper ratio exceeds 50 : 1, the brown colour of the iron complex will completely obscure the colour due to the copper complex. Filtration of the sample is not recommended as copper ion is adsorbed on the filter.

2.3.7.3 *Storage of samples*

Copper ion has a tendency to be adsorbed on the surface of the sample container. To avoid this effect, samples should be analysed as soon as possible after they are collected. If storage is necessary, 5 ml of 10% hydrochloric acid per 100-ml sample will prevent " plating out ". When the acidified samples are analysed, a volume correction must be made for the added acid.

2.3.7.4 *Apparatus*

Nessler tubes, matched, 100-ml, tall-form.

2.3.7.5 *Reagents*

Throughout this procedure, standards and dilutions shall be made up with water redistilled from an all-glass Pyrex still. Ordinary distilled water often contains appreciable copper because the water comes in contact with copper or its alloys in the still.

(1) Sodium diethyldithiocarbamate solution: Dissolve 1 g of sodium diethyldithiocarbamate ($N(C_2H_5)_2 \cdot CS_2Na$—Eastman No. 2596, or equivalent—in 1 litre of redistilled water. A brown bottle is recommended for the solution. This reagent will become turbid in approximately 30 days. A reagent which has become coloured should be discarded, but a slight turbidity may be tolerated.

(2) Ammonium hydroxide, 16.7% solution.

(3) Hydrochloric acid, concentrated.

(4) Copper stock solution: Weigh 0.1000 g of copper metal foil, place in a 250-ml beaker under a hood, add 3 ml of redistilled water and 3 ml of concentrated nitric acid, and cover the beaker with a watch glass. After the metal has all dissolved, add 1 ml of concentrated sulfuric acid and heat on a hot plate to volatilize the acids. Stop heating just short of complete dryness; do not bake the residue. Cool and dissolve in redistilled water, washing down the sides of the beaker and the bottom of the watch glass. Transfer quantitatively to a 1-litre volumetric flask and make up to the mark with redistilled water. This solution is stable indefinitely.

(5) Copper working solution: Quantitatively dilute 50 ml of copper stock solution to 1 litre with redistilled water. One ml of this solution contains 0.005 mg of Cu. This solution is stable indefinitely.

2.3.7.6 *Preparation of standards*

Dilute various volumes of the copper working solution to 100 ml with redistilled water in 100-ml Nessler tubes, as follows:

Copper content (mg/l)	*Volume of copper working solution to be diluted (ml)*	*Copper content (mg/l)*	*Volume of copper working solution to be diluted (ml)*
0	0	0.40	8.0
0.05	1.0	0.60	12.0
0.10	2.0	0.80	16.0
0.20	4.0	1.00	20.0

The standards should be treated exactly as described below for the samples.

2.3.7.7 *Procedure*

All glassware to be used in this test shall first be rinsed with concentrated hydrochloric acid and then with redistilled water.

To a 100-ml sample, or an aliquot diluted to 100 ml, add 5 ml of sodium diethyldithiocarbamate solution, and mix by inverting the tube twice. After at least 5 minutes but within 1 hour after mixing, compare visually the yellow colour with that of the standards prepared at the same time. The colours are the same in slightly acid, neutral, or alkaline solutions.[6] Using Nessler tubes, the minimum detectable amount of copper is 0.05 mg/l.

Callan & Henderson[6] claim that the yellow colours produced in the presence of ammonium hydroxide solution are clearer, sharper, and more easily compared. If desired, add 5 ml of ammonium hydroxide solution just before the addition of the carbamate reagent.

Extraction procedures have been recommended by various authors.[14, 28] Carbon tetrachloride and isoamyl alcohol have been used to extract and, thereby, to intensify the colour and/or to eliminate turbidity. The solvents are added after the colour has developed. The mixtures are then shaken to cause the colour to pass into the organic layer. Care must be taken that the solvents themselves do not contain traces of metals. It is desirable to acidify the aqueous solution immediately prior to the extraction, which is slow from an alkaline solution. Iron interference is not overcome by extraction.

2.3.7.8 *Precision and accuracy*

Standards are based on a 100-ml sample. Correct by a proper dilution factor if a smaller portion is taken. A precision of $\pm 6\%$ may be achieved under optimum conditions and of $\pm 10\%$ under routine conditions.

2.3.8 ZINC[1]

2.3.8.1 *Apparatus*

Nessler tubes, matched.

Separatory funnels (Squibb), 125- to 150-ml capacity, lubricated with pure petroleum or silicone grease.

pH-meter.

2.3.8.2 *Reagents*

(1) Carbon tetrachloride.

(2) Zinc-free double-distilled water, for rinsing of apparatus and preparation of solutions: Redistil ordinary distilled water in an all-Pyrex apparatus.

(3) Dithizone solution A: Dissolve 0.10 g of diphenylthiocarbazone (Eastman No. 3092, or equivalent) in 1 litre of carbon tetrachloride. Store in a brown glass-stoppered bottle in the refrigerator. If the solution is of doubtful quality or has been stored for a long time, the following test for deterioration can be applied: Shake 10 ml with 10 ml of 1% ammonium hydroxide solution. If the lower carbon tetrachloride layer is only slightly yellow, the reagent is in good condition.

(4) Dithizone solution B: Dilute 1 volume of dithizone solution A with 9 volumes of carbon tetrachloride. If stored in a brown glass-stoppered bottle in the refrigerator, this solution is satisfactory for several weeks.

(5) Sodium citrate solution: Dissolve 10 g of sodium citrate ($Na_3C_6H_5O_7$, $2H_2O$) in 90 ml of redistilled water. Extract with 10-ml portions of dithizone solution A until the last extract remains green; then extract with carbon tetrachloride to remove excess dithizone. This reagent is used in the final cleansing of glassware.

(6) Hydrochloric acid solution, approximately 0.02N: Dilute 1.0 ml of concentrated hydrochloric acid to 600 ml with redistilled water. If high blanks are traced to this reagent, dilute concentrated hydrochloric acid with an equal volume of water and redistil it in an all-Pyrex still.

(7) Sodium acetate solution, approximately 2N: Dissolve 68 g of sodium acetate ($NaC_2H_3O_2$, $3H_2O$) in redistilled water and make up to 250 ml.

(8) Acetic acid solution, approximately 2N: Mix 1 volume of glacial acetic acid with 7 volumes of redistilled water.

(9) Acetate buffer solution: Mix equal volumes of sodium acetate solution and acetic acid solution. Purify by dithizone extraction as described for sodium citrate solution.

(10) Sodium thiosulfate solution: Dissolve 25 g of sodium thiosulfate ($Na_2S_2O_3$, $5H_2O$) in 100 ml of redistilled water. Purify by dithizone extraction as described for sodium citrate solution.

(11) Zinc standard stock solution: Dissolve 0.100 g of 500-μ (US Standard mesh No. 30) zinc in a slight excess of 50% hydrochloric acid; about 1 ml is required. Then dilute to 1 litre with redistilled water.

(12) Zinc standard working solution: Dilute 10.0 ml of zinc standard stock solution to 1 litre with redistilled water. One ml of this solution contains 0.001 mg of Zn.

2.3.8.3 *Preparation of standards*

To a series of 125-ml Squibb separatory funnels, thoroughly cleansed with dilute nitric acid, then with redistilled water, and finally with a mixture of sodium citrate solution and dithizone solution A, add 0, 1.0, 2.0, 3.0, 4.0, and 5.0 ml of zinc standard working solution equivalent

to 0, 0.001, 0.002, 0.003, 0.004, and 0.005 mg of Zn. Bring each volume up to 10.0 ml by adding redistilled water. To each funnel add 5.0 ml of acetate buffer solution and 1.0 ml of sodium thiosulfate solution, and mix. The pH should be between 4 and 5.5 at this point. To each funnel add 10.0 ml of dithizone solution B, stopper, and shake vigorously for 4 minutes. Allow the layers to separate. Dry the stem of the funnel with strips of filter paper and run the lower carbon tetrachloride layer into a clean, dry Nessler tube.

2.3.8.4 *Procedure*

If the zinc content is not within the working range, dilute the sample with redistilled water or concentrate it in a silica dish. If the sample has been preserved with acid, an aliquot must be taken to dryness in a silica dish to remove the excess of acid. It is not practical to neutralize with sodium or ammonium hydroxide, as these alkalis usually contain excessive amounts of zinc. Using a pH-meter, and accounting for any dilution, adjust the sample to pH 2 to 3 with 0.02N hydrochloric acid solution. Transfer 10.0 ml to a separatory funnel. Add 5.0 ml of acetate buffer solution and 1.0 ml of sodium thiosulfate solution, and mix. The pH should be between 4.0 and 4.5 at this point. Add 10 ml of dithizone solution B, stopper, and shake vigorously for 4 minutes. Allow the layers to separate. Dry the stem of the funnel with strips of filter paper and run the lower carbon tetrachloride layer into a clean, dry Nessler tube.

It will be necessary to run the samples and standards at the same time. The carbon tetrachloride layers may be compared directly in the separatory funnels if these match in size and shape; otherwise, transfer to matched test-tubes or Nessler tubes. The range of colours obtained with various amounts of Zn may be roughly described as:

0 (blank)	green
0.001 mg.	blue
0.002 mg.	blue-violet
0.003 mg.	violet
0.004 mg.	red-violet
0.005 mg.	red-violet

2.3.8.5 *Calculation*

$$\text{Zn (mg/l)} = \frac{\text{mg of Zn} \times 1000}{\text{ml of sample}}$$

2.3.9 CALCIUM [20]

2.3.9.1 *Reagents*

(1) Titrating solution: Dissolve 4.0 g of disodium dihydrogen ethylenediamine-tetra-acetate dihydrate (sodium versenate) in 1 litre of distilled water. Standardize by titration against the calcium standard solution, using the buffer solution and the procedure outlined below.

(2) Buffer solution: Mix 67.5 g of pure ammonium chloride with 570 ml of ammonium hydroxide (specific gravity, 0.88), and dilute to 1 litre with distilled water.

(3) Indicator solution: Prepare a fresh aqueous solution of murexide (ammonium purpurate).

(4) Calcium standard solution: Dissolve 100 g of pure calcium carbonate (calcite) in a small amount of dilute hydrochloric acid, and dilute to 100 ml. One ml of this solution is equivalent to 1 mg of $CaCO_3$.

2.3.9.2 *Procedure*

To 100 ml or an aliquot of the sample add 2 ml of 1N sodium hydroxide solution and sufficient indicator solution to produce a marked colour. In the total absence of calcium, a purple colour will result. If calcium is present, the colour will be salmon-pink. Titrate (dropwise) with the titrating solution until a purple colour is obtained which does not change by addition of a further drop of titrating solution.

2.3.9.3 *Calculation*

$$\text{Ca (mg/l)} = \text{mg of } CaCO_3 \times 0.4$$

2.3.10 MAGNESIUM [20]

2.3.10.1 *Reagents*

(1) Ammonium oxalate, saturated solution.

(2) Titrating solution: Dissolve 4.0 g of disodium dihydrogen ethylene-diamine-tetra-acetate dihydrate (sodium versenate) in 1 litre of distilled water. Standardize by titration against the calcium standard solution, using the buffer solution and the procedure outlined below.

(3) Buffer solution: Mix 67.5 g of pure ammonium chloride with 570 ml of ammonium hydroxide (specific gravity, 0.88), and dilute to 1 litre with distilled water.

(4) Indicator solution: Dissolve a small quantity of Eriochrome Black T in 20 ml of distilled water, by shaking or warming, and allow to cool. This solution will be stable for several days, but preparation of a fresh solution daily is recommended.

(5) Calcium standard solution: Dissolve 100 g of pure calcium carbonate (calcite) in a small amount of dilute hydrochloric acid, and dilute to 100 ml. One ml of this solution is equivalent to 1 mg of $CaCO_3$.

2.3.10.2 *Procedure*

To 100 ml of the sample add 1.5 ml of the buffer solution and 2.5 ml of saturated solution of ammonium oxalate. Mix and allow to stand with

occasional shaking for 2 hours, or overnight if possible. Filter, using a No. 42 Whatman filter paper. Pipette 26 ml or 52 ml, add indicator solution, and titrate with the titrating solution. Magnesium is determined directly in terms of $CaCO_3$.

If the calcium content of the water is abnormally high, more ammonium oxalate solution will be required. It is advisable to run a blank determination, using the same volumes of reagents in 100 ml of distilled water and filtering as above.

2.3.10.3 *Calculation*

$$Mg\ (mg/l) = mg\ of\ CaCO_3 \times 0.24$$

2.3.11 SULFATE [20]

2.3.11.1 *Reagents*

(1) Titrating solution: Dissolve 4.0 g of disodium dihydrogen ethylenediamine-tetra-acetate dihydrate (sodium versenate) in 1 litre of distilled water. Standardize by titration against the barium chloride standard solution, using the buffer solution and the procedure outlined below.

(2) Buffer solution: Mix 67.5 g of pure ammonium chloride with 570 ml of ammonium hydroxide (specific gravity, 0.88), and dilute to 1 litre with distilled water.

(3) Indicator solution: Dissolve a small quantity of Eriochrome Black T in 20 ml of distilled water, by shaking or warming, and allow to cool. This solution will be stable for several days, but preparation of a fresh solution daily is recommended.

(4) Barium chloride standard solution: Dissolve 2.443 g of barium chloride in distilled water and dilute to 1 litre. One ml of this solution is equivalent to 1 mg of $CaCO_3$, or to 0.96 mg of SO_4.

2.3.11.2 *Procedure*

Neutralize 100 ml of sample with 1N nitric acid, adding a slight excess, and boil to expel carbon dioxide. Add 10 ml, or more if required, of barium chloride standard solution to the boiling sample and allow to cool. Dilute to 150 ml, mix, and allow precipitate to settle. Withdraw 50 ml of clear supernatant, add 0.5 to 1.0 ml of buffer solution and several drops of indicator solution. Titrate with the titrating solution to a blue colour which does not change by addition of further drops of titrating solution.

2.3.11.3 *Calculation*

Assume that a water has a hardness of 250 mg/l and that 10 ml of barium chloride solution are added to a 100-ml sample. If no sulfate is present,

the titration would require 25 ml for hardness and 10 ml for barium chloride, i.e., a total of 35 ml of titrating solution. Assume the actual titration of an unknown sample to require 30 ml. The loss due to the presence of sulfate is $35 - 30 = 5$ ml. This is equivalent to 5 mg of $CaCO_3$, or to $5 \times 0.96 = 4.8$ mg of SO_4, for the 100-ml sample examined, i.e., to 48 mg of SO_4 per litre.

2.3.12 CHLORIDE: MOHR METHOD [1]

2.3.12.1 *Principle*

Chloride, in neutral or weakly alkaline solution containing chromate, is titrated with silver nitrate. Silver chloride precipitates, and at the end-point red silver chromate is formed.

2.3.12.2 *Interference*

Iodide and bromide register as equivalent chloride concentrations. Phosphate, sulfide, and cyanide interfere. Sulfite interferes but can be removed by treating with hydrogen peroxide.

2.3.12.3 *Reagents*

(1) Sulfuric acid, 1.4% solution (approximately 0.5N).

(2) Sodium hydroxide solution, approximately 0.5N: Dissolve 4 g of sodium hydroxide in 200 ml of distilled water.

(3) Hydrogen peroxide, 30% solution.

(4) Aluminium hydroxide suspension: Dissolve 125 g of potassium or ammonium alum—$K_2Al_2(SO_4)_4$, $24H_2O$ or $(NH_4)_2Al_2(SO_4)_4$, $24H_2O$—in 1 litre of distilled water. Warm to 60°C and add slowly, with stirring, 55 ml of concentrated ammonium hydroxide. After it has stood for about one hour, transfer the mixture to a large bottle and wash the precipitate thoroughly by successive decantations with distilled water until free from chloride.

(5) Potassium chromate indicator solution: Dissolve 50 g of potassium chromate in a small quantity of distilled water. Add silver nitrate to produce a slight red precipitate. After it has stood at least overnight, filter and dilute to 1 litre with distilled water.

(6) Phenolphthalein indicator solution: Dissolve 5 g of phenolphthalein in 500 ml of ethanol and add 500 ml of distilled water. Then add dropwise 0.02N sodium hydroxide solution until a faint pink colour appears.

(7) Sodium chloride standard solution, 0.0141N: Dissolve 8.243 g of sodium chloride, dried by fusing at 900°C for half an hour, in 500 ml

of distilled water. Dilute 50.0 ml to 1 litre with distilled water. One ml of this solution contains 0.500 mg of Cl.

(8) Silver nitrate standard solution, 0.0141N: Dissolve 2.396 g of silver nitrate in 1 litre of distilled water. One ml of this solution is equivalent to 0.500 mg of Cl. Using the procedure described below, standardize against the sodium chloride standard sollution.

2.3.12.4 *Procedure*

Use a 100-ml sample or a suitable aliquot diluted to 100 ml. If the sample is coloured, decolorize by adding 3 ml of aluminium hydroxide suspension; stir thoroughly and, after a few minutes, filter and wash with 10-15 ml of distilled water. If sulfites are present, add 1 ml of hydrogen peroxide solution, with stirring.

Either a white porcelain evaporating dish, or an Erlenmeyer flask over white surface should be used. Adjust the sample with either dilute sulfuric acid or sodium hydroxide solution so that it is just colourless to phenolphthalein indicator. Add 1 ml of potassium chromate indicator solution, and titrate with silver nitrate standard solution until a colour change from pure yellow to pinkish-yellow is perceptible. An indicator blank should be determined by titrating distilled water in the same way. This blank, showing the end-point colour, should be placed near the sample being titrated to aid in the detection of the colour change at the chosen end-point.

2.3.12.5 *Calculation*

$$\text{Cl (mg/l)} = \frac{(A-B) \times C \times 35.46 \times 1000}{\text{ml of sample}}$$

where A = ml of silver nitrate solution used for sample
B = ml of silver nitrate solution used for blank
C = normality of silver nitrate solution.

2.3.12.6 *Precision and accuracy*

The precision and accuracy are limited by the accuracy of detection of the end-point, which is usually about 0.2 ml, or 0.1 mg of Cl, and by the presence of interfering substances.

2.3.13 HYDROGEN-ION CONCENTRATION (pH): GLASS-ELECTRODE METHOD [1]

2.3.13.1 *Principle*

Several types of electrode have been suggested for the electrometric determination of pH. Although the hydrogen gas electrode is recognized as the primary standard, the glass electrode in combination with the reference potential provided by a saturated calomel electrode is most

generally used. The glass-electrode system is based on the fact that a change of 1 pH unit produces an electrical change of 59.1 millivolts at 25°C.

2.3.13.2 *Interference*

The glass electrode is relatively immune to interference from colour, turbidity, colloidal matter, free chlorine, oxidizing or reducing substances, as well as high saline content, except for a sodium error at high pH. The error caused by high sodium-ion concentrations at a pH above 10 may be reduced by using special "low sodium error" electrodes. When employing ordinary glass electrodes, approximate corrections for the sodium error may be made by consulting a chart which the manufacturer can furnish for the particular make of electrode. Temperature exerts two significant effects on pH measurements: (1) the electrodes themselves vary in potential with temperature; and (2) ionization in the sample varies with temperature. The first effect can be compensated by an adjustment which is provided on the better commercial instruments. The second effect is inherent in the sample and is taken into consideration by recording both the temperature and the pH of each sample.

2.3.13.3 *Apparatus*

Where flow-type electrodes are not available, or where stirring may be inadequate, as in the case of ordinary immersion-type (dipping) electrodes, the best procedure is to wash the glass electrode 6 or 8 times with portions of the sample, particularly when an unbuffered measurement follows one on a buffered solution. Flow-type electrodes are recommended for the accurate measurement of relatively unbuffered waters with low concentration of mineral solids (total residue less than 20 mg/l). Measurements on buffered waters can be obtained on open samples. Equilibrium should be established between the sample and the electrode system as shown by the absence of drift, before readings are accepted as final. If the water is hot or if the pH is over 10, special glass electrodes should be used and the assembly should be standardized under conditions of temperature and concentration as close as possible to those of the sample, taking into account the manufacturer's recommendations. The analyst should constantly be on the alert for possible erratic results arising from mechanical or electrical failures such as weak batteries, cracked glass electrodes, plugged liquid junction, and fouling of the electrodes with oily or precipitated materials.

2.3.13.4 *Buffer solutions*

Electrode systems are calibrated against buffer solutions of known pH value. Since buffer solutions may deteriorate because of mould growth or through contamination, it may be advisable to prepare them freshly as needed by dissolving dry buffer salts in distilled water. Commercially

TABLE III. EFFECT OF TEMPERATURE ON pH VALUES OF BUFFER SOLUTIONS

Temperature (°C)	pH 4 buffer	pH 7 buffer	pH 9 buffer
0	4.01	7.08	9.46
5	4.01	7.05	9.38
10	4.00	7.02	9.33
15	4.00	7.00	9.27
20	4.00	6.98	9.22
25	4.01	6.96	9.18
30	4.01	6.95	9.14
35	4.02	6.94	9.10
40	4.03	6.94	9.07
45	4.04	6.93	9.04
50	4.06	6.93	9.01
55	4.08	6.94	8.98
60	4.10	6.94	8.96

available buffer tablets or powders of tested quality may also be used. It is good practice to calibrate the electrodes with a buffer whose pH is close to that of the samples, so as to minimize any error resulting from non-linear response of the electrode. In making up buffers from solid salts, it is imperative that all the material be dissolved, otherwise the pH may be incorrect. Polyethylene bottles are preferable for the storage of buffers and samples, although Pyrex glassware may be employed. Limitations of space permit information on the preparation of only three buffer solutions at practically spaced intervals—pH 4, pH 7, and pH 9—for standardization purposes and for checking the linearity of electrode response.

In general, analytical-reagent-grade chemicals are satisfactory for the preparation of these buffer solutions. The pH values of the three buffer solutions at various temperatures are listed in Table III. The tabulated pH values were obtained with salts of the highest purity.

(1) pH 4 buffer solution: Dissolve 10.2 g of anhydrous potassium biphthalate ($KHC_8H_4O_4$), using boiled and cooled distilled water, and dilute to 1 litre.

(2) pH 7 buffer solution: Dissolve 1.361 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4) and 1.420 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4), both of which have been dried overnight at between 110°C and 130°C, and dilute to 1 litre. Use distilled water which has been boiled and cooled.

(3) pH 9 buffer solution: Dissolve 3.81 g of sodium tetraborate decahydrate ($Na_2B_4O_7$, $10H_2O$) (borax), using boiled and cooled distilled water, and dilute to 1 litre.

2.3.13.5 *Procedure*

Because of the differences between the many makes and models of pH-meters which are available, it is impossible to provide detailed instructions for the correct operation of every instrument. In each case, the manufacturer's instructions must be followed. The glass electrode and the calomel electrode should be thoroughly wetted and prepared for use in accordance with the given instructions. The instrument can be standardized against a buffer solution with a pH approaching that of the sample and then the linearity of electrode response can be checked against at least one additional buffer of a different pH. The readings with the additional buffers will afford a rough idea of the limits of accuracy to be expected of the instrument and the technique of operation.

2.3.13.6 *Precision and accuracy*

The precision and accuracy attainable with a given pH-meter will depend upon the type and condition of the instrument employed and the technique of standardization and operation. With proper care, a precision of 0.02 pH unit and an accuracy of 0.05 pH unit can be achieved with the better battery models. Line-operated instruments, on the other hand, are less accurate, 0.1 pH unit representing the limits of accuracy under normal conditions.

2.3.14 PHENOLIC COMPOUNDS [1]

2.3.14.1 *Preliminary screening procedure*

Apparatus

Distillation apparatus, all-glass, such as a 1-litre Pyrex distilling flask with Graham condenser (Corning No. 3360 or equivalent).

Reagents

All reagents shall be prepared with distilled water free of phenols and chlorine.

(1) Copper sulfate solution : Dissolve 100 g of copper sulfate ($CuSO_4$, $5H_2O$) in distilled water and dilute to 1 litre.

(2) Phosphoric acid solution : Dilute 10 ml of 85% phosphoric acid to 100 ml with distilled water.

Procedure

To a 500-ml sample of water, add 5.0 ml of copper sulfate solution, unless this has previously been added as a preservative. Lower the pH of the mixture to below 4.0 with phosphoric acid solution ; 0.7 ml is sufficient for most samples. Place the mixture in the all-glass distillation apparatus and distil over 450 ml. Stop the distillation and, when boiling

ceases, add 50 ml of distilled water to the distilling flask. Continue the distillation until a total of 500 ml has been collected.

2.3.14.2 *4-Aminoantipyrine method*

Apparatus

Nessler tubes, matched, 100-ml, tall-form.

Reagents

(1) 4-Aminoantipyrine solution: Dissolve 2.0 g of 4-aminoantipyrine in distilled water and dilute to 100 ml. This solution should be prepared fresh weekly.

(2) Potassium ferricyanide solution: Dissolve 8 g of potassium ferricyanide ($K_3Fe(CN)_6$) in distilled water and dilute to 100 ml. Filter if necessary. This solution is not stable and should be made up fresh when decomposition occurs, as evidenced by darkening of the solution, usually within a week.

(3) Ammonium chloride solution: Dissolve 50 g of ammonium chloride in distilled water and dilute to 1 litre.

(4) Potassium bromate-bromide solution, approximately 0.1N: Dissolve 2.784 g of potassium bromate in distilled water, add 10 g of potassium bromide, dissolve, and dilute to 1 litre with distilled water.

(5) Potassium iodide, crystals.

(6) Ammonium hydroxide, concentrated.

(7) Chloroform.

(8) Starch solution: Prepare as described under the determination of dissolved oxygen (reagent (5), page 116).

(9) Sodium thiosulfate standard solution, 0.025N: Prepare as described under the determination of dissolved oxygen (reagent (9), page 116).

(10) Phenol solutions:

(*a*) Stock solution: Dissolve 1 g of phenol in distilled water and dilute to 1 litre. Standardize as directed below.

(*b*) Intermediate solution: Dilute 10 ml of stock solution to 1 litre with distilled water. One ml of this solution contains 0.01 mg (10 µg) of phenol.

(*c*) Standard solution: Dilute 5 ml of intermediate solution to 500 ml with distilled water. One ml of this solution contains 0.1 µg of phenol.

Standardization of phenol solutions. Place approximately 100 ml of distilled water in a 500-ml glass-stoppered Erlenmeyer flask and add 50 ml of phenol stock solution. To this add exactly 10.0 ml of potassium bromate-bromide solution followed by approximately 5 ml of concentrated hydrochloric acid. Swirl gently with flask stoppered. If

brown colour of free bromine does not persist, add bromate-bromide solution in exact 10-ml portions until bromine colour does persist. Stopper and allow to stand for 10 minutes, and then add approximately 1 g of potassium iodide. Four 10-ml portions of the bromate-bromide solution are required if the stock solution contains 1000 mg/l phenol.

Prepare a blank in exactly the same manner, using distilled water and 10 ml of potassium bromate-bromide solution. Titrate both blank and sample with 0.025N sodium thiosulfate solution, using starch solution as indicator.

Calculate the strength of the phenol solution by the following formula:

$$\text{Phenol (mg/l)} = \left(\frac{A \times B}{10} - C\right) \times 7.835$$

where: A = ml of 0.025N sodium thiosulfate solution used for blank

B = ml of potassium bromate-bromide solution used for sample

C = ml of 0.025N sodium thiosulfate solution used for sample.

Procedure

After completion of the preliminary screening (section 2.3.14.1), determine by a preliminary check the proper aliquot of the distillate to use for the final determination. This may be done by carrying out the reaction in 50-ml Nessler tubes and comparing against suitable standards. In this case no chloroform extraction is necessary.

Choose an aliquot which contains not more than 0.50 mg of phenol. As a standard, use 50 ml of the intermediate phenol solution. Dilute all solutions, including the blank, to 100 ml in 100-ml Nessler tubes. Treat samples, blank, and standard as follows: Add 2 ml of ammonium chloride solution and adjust with concentrated ammonium hydroxide to pH 10.0±0.2, usually 0.7 to 1.0 ml being required. Mix and add 2.0 ml of 4-aminoantipyrine solution. Mix, add 2 ml of potassium ferricyanide solution, and mix thoroughly. Allow to stand for 3 minutes and extract immediately with chloroform, making three serial extractions. Combine the chloroform extracts, filter, and dilute to 25 ml with chloroform. Compare using Nessler tubes.

2.4 Chemical substances indicative of pollution

2.4.1 Total organic matter (oxygen consumed) [1]

The dichromate reflux method has been selected for the determination of oxygen consumed, because it has been found that this procedure

has a high reproducibility, is applicable to a wide variety of samples, and has manipulative advantages over methods using other oxidants.

2.4.1.1 *Principle*

Straight-chain aliphatic compounds, aromatic hydrocarbons, and pyridine are not oxidized to any appreciable extent, although this method gives more complete oxidation than the permanganate method. The straight-chain compounds are more effectively oxidized if silver sulfate is added as a catalyst; however, silver sulfate reacts with chlorides, bromides, or iodides to produce precipitates which are only partially oxidized by the procedure. There is no advantage in using the catalyst in the presence of aromatic hydrocarbons, but it is essential to the oxidation of straight-chain alcohols and acids.

2.4.1.2 *Interference*

Unstable samples should be tested without delay and samples containing settleable solids should be homogenized by means of a blender for ease of representative sampling. Initial dilutions in volumetric flasks should be made on those samples having a high "oxygen consumed value", in order to reduce the error which is inherent in measuring small sample volumes.

Chlorides are quantitatively oxidized by this procedure when silver sulfate is not used as a catalyst. In this case, a correction can be applied by determining chlorides on a separate sample and subtracting the calculated oxygen consumption of the chlorides from the result. Since 1 mg/l Cl will consume 0.23 mg/l O, the correction is: mg/l Cl $\times$ 0.23. This correction cannot be applied when silver sulfate is used as a catalyst.

2.4.1.3 *Apparatus*

Reflux apparatus: A 300-ml round-bottom flask (Corning No. 4320) with ground-glass neck 24/40 and a Friedrichs condenser (Corning No. 2600).

2.4.1.4 *Reagents*

(1) Sulfuric acid, concentrated.

(2) Silver sulfate, crystals.

(3) Ferroin indicator solution: Dissolve 1.485 g of 1,10-phenanthroline (monohydrate), together with 0.695 g of ferrous sulfate ($FeSO_4$, $7H_2O$), in distilled water and dilute to 100 ml.[a]

(4) Potassium dichromate standard solution, 0.25N: Dissolve 12.2588 g

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a This indicator solution may be obtained from the G. Frederick Smith Company, Columbus, Ohio, USA.

of potassium dichromate ($K_2Cr_2O_7$), previously dried at 103°C for 2 hours, in distilled water and dilute to 1 litre.

(5) Ferrous ammonium sulfate standard solution, approximately 0.25N: Dissolve 98 g of ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$, $6H_2O$) in distilled water. Add 20 ml of concentrated sulfuric acid, cool, and dilute to 1 litre with distilled water. This solution must be standardized daily against the potassium dichromate solution.

Standardization procedure. Dilute 25 ml of potassium dichromate standard solution to about 250 ml with distilled water. Add 20 ml of concentrated sulfuric acid and allow to cool. Titrate against the ferrous ammonium sulfate solution, using 2 or 3 drops of the Ferroin indicator solution.

$$\text{Normality} = \frac{\text{ml of } K_2Cr_2O_7}{\text{ml of } Fe(NH_4)_2(SO_4)_2} \times 0.25$$

(NOTE: With relatively unpolluted waters, a weaker solution of potassium dichromate may be used. The normality of such a solution should be 0.05. The normality of the ferrous ammonium sulfate solution used should also be about 0.05. However, the oxygen consumed values obtained by using a 0.05N potassium dichromate solution are approximately 10% lower than those obtained with the 0.25N solution.)

2.4.1.5 *Procedure*

Place a 50-ml sample, or an aliquot diluted to 50 ml with distilled water, in the round-bottom flask, and add 25 ml of standard potassium dichromate solution. Carefully add 75 ml of concentrated sulfuric acid, mixing after each addition.

(CAUTION: The reflux mixture must be thoroughly mixed before heat is applied. If this is not done, local heating occurs in the bottom of the flask and the mixture may be blown out of the side arm of the condenser.)

Attach the flask to the Friedrichs condenser and reflux the mixture for 2 hours. Pumice granules or glass beads should be added to the reflux mixture to prevent bumping. Cool and then wash down the condenser with about 25 ml of distilled water. In many cases, the 2-hour reflux period is not necessary. Therefore, with particular samples the reflux period necessary to give the maximum oxygen consumed should be determined and the shorter period of refluxing may be permissible.

Transfer the contents to a 500-ml Erlenmeyer flask, washing out the reflux flask 4 to 5 times with distilled water. Dilute the mixture to about 350 ml and titrate the excess potassium dichromate with ferrous ammonium sulfate standard solution, using Ferroin indicator. Generally 2-3 drops of the indicator are used. The colour change is sharp, changing from a

blue-green to a reddish-blue. The end-point, however, will not be as sharp as in the standardization of the reagents because of the lower acid concentration. For this reason it is necessary that the sample be diluted to at least 350 ml before the titration is carried out.

A blank consisting of 50 ml of distilled water instead of the sample, together with the reagents, is refluxed in the same manner.

More complete oxidation of many organic compounds such as straight-chain alcohols and acids may be obtained by the use of silver sulfate as a catalyst. One gram of silver sulfate crystals is added directly to the mixture before refluxing, or the silver may be dissolved in concentrated sulfuric acid at the rate of 1 g for every 75 ml of acid.

2.4.1.6 *Calculation*

$$\text{OC (mg/l)} = \frac{(A-B) \times N \times 8000}{\text{ml of sample}} - C$$

where: OC = oxygen consumed from potassium dichromate
A = ml of $Fe(NH_4)_2(SO_4)_2$ solution used for blank
B = ml of $Fe(NH_4)_2(SO_4)_2$ solution used for sample
C = chloride correction = mg/l Cl × 0.23
N = normality of $Fe(NH_4)_2(SO_4)_2$ solution.

2.4.1.7 *Precision and accuracy*

The method is quite precise and may be used on a wide variety of waters even though the back titration is less than 1 ml. Its accuracy has been determined by Moore et al.[21] For most organic compounds the oxidation is 95-100% of the theoretical value. Using the silver catalyst, short straight-chain alcohols and acids are oxidized to the extent of 85-95% or better. Benzene, toluene, and pyridine are not oxidized by either procedure.

2.4.2 Biochemical oxygen demand (BOD)[1]

2.4.2.1 *Apparatus*

Incubation bottles, 250- to 300-ml capacity with ground-glass stoppers: They should be cleaned with chromic acid mixture, carefully rinsed, and thoroughly drained before use. As a precaution against drawing air into the dilution bottle during incubation, a water seal is recommended. Satisfactory water seals are obtained by inverting the bottles in a water-bath or adding water to the flared mouth of special BOD bottles.

Air incubator or water-bath: One thermostatically controlled at 20°C ± 1°C should be used. Light should be excluded to prevent formation of dissolved oxygen by algae in the sample.

2.4.2.2 *Reagents*

(1) Distilled water of highest purity.

(2) Phosphate buffer solution : Dissolve 8.5 g of potassium dihydrogen phosphate (KH_2PO_4), 21.75 g of dipotassium hydrogen phosphate (K_2HPO_4), 33.4 g of disodium hydrogen phosphate (Na_2HPO_4, $7H_2O$), and 1.7 g of ammonium chloride in about 500 ml of distilled water, and dilute to 1 litre. The pH of this buffer should be 7.2 without further adjustment.

(3) Magnesium sulfate solution : Dissolve 22.5 g of magnesium sulfate ($MgSO_4$, $7H_2O$) in distilled water and dilute to 1 litre.

(4) Calcium chloride solution : Dissolve 27.5 g of anhydrous calcium chloride in distilled water and dilute to 1 litre.

(5) Ferric chloride solution : Dissolve 0.25 g of ferric chloride ($FeCl_3$, $6H_2O$) in distilled water and dilute to 1 litre.

2.4.2.3 *Preparation of dilution water*

Place the desired volume of distilled water, as near 20°C as possible, in a suitable bottle and add 1 ml each of phosphate buffer, magnesium sulfate, calcium chloride, and ferric chloride solutions for each litre of water.

Seed the dilution water, if necessary, by adding from 1 to 10 ml of settled domestic sewage, 24-36 hours old, per litre. If sewage is not available in the laboratory, it is permissible to seed the sample with 1 drop of lactose broth containing an active culture which is known to produce a positive presumptive coliform test. If it is desired to use river water for seeding purposes, from 10 to 50 ml per litre should be added. The amount of seeding material in any case should be chosen in the light of past experience so as to give oxygen depletions in the blanks of at least 0.6 mg/l in five days' incubation. Seeded dilution water should be used the day it is made. Aerate the dilution water with a clean supply of compressed air to saturate with oxygen. Saturation may also be obtained by 2-3 days' storage in partially filled bottles which are closed with a cotton plug.

2.4.2.4 *Procedure*

If high concentrations of residual chlorine are present (0.1 p.p.m.), allow to stand for 1-2 hours or until residual chlorine is dissipated. Excessive amounts of residual chlorine can be neutralized by the addition of the exact concentration of sodium sulfite required.

If deficient in oxygen, the sample should be aerated to bring oxygen concentration up to but not in excess of saturation at 20°C.

Fill two BOD bottles (300-ml, glass-stoppered) full and place one in a 20°C incubator for 5 days. Water-baths with temperature controlled to 20°C ± 1°C may be used as an incubator.

When the BOD value is expected to be in excess of 5.0 mg/l, dilution of the sample will be necessary. Select a definite volume of sample (less than 300 ml), add to BOD bottle, and fill completely with dilution water.

Determine dissolved oxygen in one bottle before incubation and in the second bottle after an incubation period of 5 days at 20°C by the following method :

Determination of Dissolved Oxygen : Winkler Method, Alsterberg (Azide) Modification [1]

Principle

The basic Winkler procedure entails the oxidation of manganous hydroxide in a highly alkaline solution. Upon acidification in the presence of an iodide, the manganic hydroxide dissolves and free iodine is liberated in an amount equivalent to the oxygen originally dissolved in the sample ; the free iodine is titrated with a sodium thiosulfate standard solution, using starch as an internal indicator, after most of the iodine has been reduced. The normality of the thiosulfate solution is adjusted so that 1 ml is equivalent to 1 mg/l dissolved oxygen when 200 ml of the original sample is titrated.

Interference

Nitrites, ferrous and ferric iron, organic matter, sulfides, sulfites, polythionates, hypochlorites, suspended matter, and other oxidizing and reducing substances interfere with the Winkler test either by absorbing or reducing the liberated iodine or by oxidizing iodide to free iodine.

Ferrous iron in amounts of 1 mg/l will result in an apparent loss of about 0.14 mg/l dissolved oxygen. On the other hand, the presence of ferric salts may occasion high results owing to the liberation of iodine from iodides in the final step of the procedure, especially if the titration is delayed. The Alsterberg (azide) modification given here is most suitable for waters containing more than 0.1 mg/l nitrite nitrogen and not more than 1 mg/l ferrous iron. Other reducing or oxidizing materials should be absent. If 1 ml of potassium fluoride solution is added before acidifying the sample and there is little delay in titrating, the method is also applicable in the presence of 100-200 mg/l ferric iron.

Reagents

(1) Manganous sulfate solution : Dissolve 480 g of manganous sulfate ($MnSO_4$, $4H_2O$)—or 400 g of $MnSO_4$, $2H_2O$, or 364 g of $MnSO_4$, H_2O—in distilled water, filter, and dilute to 1 litre. When uncertainty exists regarding the water of crystallization, a solution of equivalent strength may be obtained by adjusting the specific gravity of the solution to a value of 1.270 at 20°C. The manganous sulfate solution should liberate not more than a trace of iodine when added to an acidified solution of potassium iodide.

(2) Potassium fluoride solution : Dissolve 40 g of potassium fluoride (KF, $2H_2O$) in distilled water and dilute to 100 ml.

(3) Alkaline-iodide/sodium-azide reagent : Dissolve 500 g of sodium hydroxide (or 700 g of potassium hydroxide) and 135 g of sodium iodide (or 150 g of potassium iodide) in distilled water and dilute to 1 litre. Potassium and sodium salts may be used interchangeably. The reagent should not give a colour with starch solution when diluted and acidified. Dissolve 10 g of sodium azide (NaN_3) in 40 ml of distilled water and add to 950 ml of the first solution, with constant stirring.

(4) Sulfuric acid, concentrated: The strength of this acid is about 36N; 1 ml is equivalent to about 3 ml of the alkaline-iodide/sodium-azide reagent.

(5) Starch solution: An emulsion of 5-6 g potato, arrowroot, or soluble starch is made in a mortar or beaker with a small quantity of distilled water. Pour this emulsion into 1 litre of boiling water, allow to boil for a few minutes and settle overnight. Use the clear supernatant. This solution may be preserved by the addition of 1.25 g of salicylic acid per litre or of a few drops of toluene.

(6) Potassium bi-iodate standard solution: A stock solution equivalent in strength to 0.1N sodium thiosulfate solution contains 3.250 g of KIO_3, HIO_3 per litre, in accordance with the following reaction:

$$2(KIO_3,\ HIO_3) + 20KI + 11H_2SO_4 \rightarrow 11K_2SO_4 + 12H_2O + 12I_2.$$

A bi-iodate solution equivalent to the 0.025N sodium thiosulfate solution contains 0.8124 g of KIO_3, HIO_3 per litre and may be prepared by diluting 250 ml of stock solution to 1 litre with distilled water.

(7) Potassium dichromate standard solution: Potassium dichromate ($K_2Cr_2O_7$), previously dried at 103°C for 2 hours, may be substituted for KIO_3, HIO_3. A solution equivalent to the 0.025N sodium thiosulfate solution contains 1.226 g of $K_2Cr_2O_7$ per litre.

(8) Sodium thiosulfate stock solution, 1.0N: Dissolve 248.2 g of sodium thiosulfate ($Na_2S_2O_3$, $5H_2O$) in freshly boiled and cooled distilled water, and dilute to 1 litre. Preserve by adding 5 ml of chloroform or 1 g of sodium hydroxide per litre.

(9) Sodium thiosulfate standard solution, 0.025N: Prepare by (*a*) diluting 25 ml of sodium thiosulfate stock solution to 1 litre with freshly boiled and cooled distilled water, or (*b*) dissolving 6.205 g of sodium thiosulfate ($Na_2S_2O_3$, $5H_2O$) in freshly boiled and cooled distilled water and diluting to 1 litre. Preserve by adding 5 ml of chloroform or 0.4 g of sodium hydroxide per litre. One ml of this solution is equivalent to 0.2 mg of O.

(*a*) *Standardization with potassium bi-iodate.* In an Erlenmeyer flask, dissolve approximately 2 g of potassium iodide, free from iodate, in 100-150 ml of distilled water, add 10 ml of 10% sulfuric acid solution, followed by exactly 20 ml of 0.025N potassium bi-iodate standard solution. Dilute to 200 ml with distilled water and titrate the liberated iodine with the 0.025N sodium thiosulfate standard solution, adding starch solution towards the end of the titration, when a pale straw colour is reached. Exactly 20 ml of sodium thiosulfate solution should be required when the solutions under comparison are of equal strength. It is convenient to adjust the solution to exactly 0.025N.

(*b*) *Standardization with potassium dichromate.* Same as under (*a*), except that 20 ml of 0.025N potassium dichromate standard solution are used in place of the bi-iodate. Place in the dark for 5 minutes, dilute to approximately 400 ml with distilled water, and titrate with the 0.025N sodium thiosulfate standard solution.

Procedure

To the sample as collected in a 250- to 300-ml bottle add 2 ml of manganous sulfate solution, followed by 2 ml of alkaline-iodide/sodium-azide reagent well below the surface of the liquid. Stopper with care to exclude air bubbles completely, and mix by inverting the bottle several times. When the precipitate settles leaving a clear supernatant above the manganese hydroxide floc, repeat the shaking a second time. When settling has produced at least 100 ml of clear supernatant, carefully remove the stopper and immediately add 2.0 ml of concentrated sulfuric acid, allowing the acid to run down the neck

of the bottle, restopper, and mix by gentle inversion until solution is complete. The iodine should be uniformly distributed throughout the bottle before decanting the amount needed for titration. This should correspond to 200 ml of original sample after correction has been made for the loss of sample by displacement with the reagents. Thus, when a total of 4 ml—2 ml each of the manganous sulfate solution and the alkaline-iodide/sodium-azide reagent—is added to a 300-ml bottle, the volume taken for titration should be

$$200 \times \frac{300}{300-4} = 203 \text{ ml.}$$

Titrate with 0.025N sodium thiosulfate solution to a pale straw colour. Add 1-2 ml of freshly prepared starch solution and continue the titration to the first disappearance of the blue colour. If the end-point is overrun, the sample may be back titrated with 0.025N potassium bi-iodate solution added dropwise, or by an additional measured volume of sample. Correction for the amount of bi-iodate or sample should be made. Subsequent recolorations due to the catalytic effect of nitrites or to the presence of traces of ferric salts which have not formed fluoride complexes should be disregarded.

Calculation

Since 1 ml of 0.025N sodium thiosulfate solution is equivalent to 0.2 mg of oxygen, the number of ml of sodium thiosulfate used is equivalent to the quantity of dissolved oxygen in mg/l if 200 ml of original sample are titrated.

If the results are desired in ml of oxygen gas at 0°C and 760-mm pressure, the dissolved oxygen in mg/l should be multiplied by 0.698.

To express the results as percentage of saturation at 760-mm pressure, the solubility data in Table IV may be used. A formula for correcting the solubilities to barometric pressures other than mean sea level is given below the table. These corrections are satisfactory for altitudes under 760 m and for temperatures below 25°C. At higher altitudes and temperatures, the barometric pressure must be corrected for the aqueous vapour pressure.

The solubility of dissolved oxygen (DO) in distilled water at any barometric pressure (P) and temperature (t°C), taking into account the saturated vapour pressure (u) for the given t°C, may be calculated by the following formulae:

$$(1) \text{ between } 0°C \text{ and } 30°C, \quad DO \text{ (ml/l)} = \frac{(P - u) \times 0.678}{35 + t°C}$$

$$(2) \text{ between } 30°C \text{ and } 50°C, \quad DO \text{ (ml/l)} = \frac{(P - u) \times 0.827}{49 + t°C}$$

The solubility data for temperatures from 30°C to 50°C in the last column of Table IV were calculated by formula (2).

Precision and accuracy

The dissolved oxygen in distilled water can be determined with a precision expressed as a standard deviation of 0.043 ml ($n = 3$; 6×10) of 0.025N sodium thiosulfate solution. In the presence of appreciable interferences, the standard deviation may be as high as 0.1 ml. Still greater errors may occur in waters having organic suspended solids or in heavily polluted waters. Errors due to carelessness in collecting samples, delay in the completion of the test, and improper technique should be avoided.

TABLE IV. SOLUBILITY OF OXYGEN IN FRESH WATER, AND IN SEA WATER OF STATED DEGREES OF SALINITY, AT VARIOUS TEMPERATURES WHEN EXPOSED TO WATER-SATURATED AIR AT A TOTAL PRESSURE OF 760 mm Hg * (DRY AIR IS ASSUMED TO CONTAIN 20.90% OXYGEN)

Temperature	Chlorides in sea water (p.p.m.)						Dissolved oxygen in chloride-free water	
	0	5 000	10 000	15 000	20 000	Difference per 100 p.p.m.		
°C	Dissolved oxygen (p.p.m. by weight)						°C	p.p.m.
0	14.62	13.79	12.97	12.14	11.32	0.0165	30	7.6
1	14.23	13.41	12.61	11.82	11.03	0.0160	31	7.5
2	13.84	13.05	12.28	11.52	10.76	0.0154	32	7.4
3	13.48	12.72	11.98	11.24	10.50	0.0149	33	7.3
4	13.13	12.41	11.69	10.97	10.25	0.0144	34	7.2
5	12.80	12.09	11.39	10.70	10.01	0.0140	35	7.1
6	12.48	11.79	11.12	10.45	9.78	0.0135	36	7.0
7	12.17	11.51	10.85	10.21	9.57	0.0130	37	6.9
8	11.87	11.24	10.61	9.98	9.36	0.0125	38	6.8
9	11.59	10.97	10.36	9.76	9.17	0.0121	39	6.7
10	11.33	10.73	10.13	9.55	8.98	0.0118	40	6.6
11	11.08	10.49	9.92	9.35	8.80	0.0114	41	6.5
12	10.83	10.28	9.72	9.17	8.62	0.0110	42	6.4
13	10.60	10.05	9.52	8.98	8.46	0.0107	43	6.3
14	10.37	9.85	9.32	8.80	8.30	0.0104	44	6.2
15	10.15	9.65	9.14	8.63	8.14	0.0100	45	6.1
16	9.95	9.46	8.96	8.47	7.99	0.0098	46	6.0
17	9.74	9.26	8.78	8.30	7.84	0.0095	47	5.9
18	9.54	9.07	8.62	8.15	7.70	0.0092	48	5.8
19	9.35	8.89	8.45	8.00	7.56	0.0090	49	5.7
20	9.17	8.73	8.30	7.86	7.42	0.0088	50	5.6
21	8.99	8.57	8.14	7.71	7.28	0.0086		
22	8.83	8.42	7.99	7.57	7.14	0.0085		
23	8.68	8.27	7.85	7.43	7.00	0.0084		
24	8.53	8.12	7.71	7.30	6.87	0.0083		
25	8.38	7.96	7.56	7.15	6.74	0.0082		
26	8.22	7.81	7.42	7.02	6.61	0.0080		
27	8.07	7.67	7.28	6.88	6.49	0.0079		
28	7.92	7.53	7.14	6.75	6.37	0.0078		
29	7.77	7.39	7.00	6.62	6.25	0.0076		
30	7.63	7.25	6.86	6.49	6.13	0.0075		

NOTE: Under any other barometric pressure, the solubility may be obtained from the corresponding value in the table by the formula:

$$S' = S \times \frac{P}{760} = S \times \frac{P'}{29.92}$$

in which S = *solubility at 760 mm or 29.92 inches*
S′ = solubility at P or P′
P = barometric pressure in mm
P′ = barometric pressure in inches.

The second decimal place in the above table is not accurately known. The average difference from the mean of five different investigators represents 0.07 p.p.m. Until further data are obtained, however, the second decimal place has been retained in the table.

* Data calculated by Whipple & Whipple [29] from measurements by Fox.[11]

2.4.3 TOTAL ORGANIC NITROGEN: KJELDAHL METHOD [1]

2.4.3.1 *Principle*

In the presence of sulfuric acid and copper sulfate catalyst, the amino nitrogen of many organic materials is converted to ammonium bisulfate. The ammonia is distilled from an alkaline medium and determined by nesslerization. Nitrate and nitrite nitrogen are not determined.

2.4.3.2 *Storage of samples*

The most reliable results are obtained on fresh samples. In the event that a prompt analysis is impossible, every precaution should be taken to retard biological activity by storing the samples at a low temperature, preferably just above freezing. Where such a measure is impracticable, the addition of 0.8 ml of concentrated sulfuric acid per litre may serve to maintain the nitrogen balance of the samples.

2.4.3.3 *Apparatus*

Kjeldahl distillation apparatus: Although gas-heated distillation units may be used, electrically heated units often offer smoother operation.

Nessler tubes, matched, 50-ml, tall-form.

2.4.3.4 *Reagents*

The solutions should preferably be stored in Pyrex glassware.

(1) Ammonia-free water: Ammonia-free water may be made by redistillation of distilled water that has been treated with a slight excess of bromine and allowed to stand overnight. The first 100 ml of the distillate should be discarded. For most work the ammonia may be removed from ordinary distilled water by shaking 1 litre of distilled water with 2.6 g of Folin's ammonia Permutit.[a] Ammonia-free water can also be prepared by passing distilled water through a column of glass tubing, 5 cm in diameter, charged with a total of approximately 350 g of the following resins intimately mixed: 2 parts by volume of Amberlite IRA 400 (hydroxyl form) and 1 part of Amberlite IR 120 (hydrogen form), available separately or premixed as Amberlite MB-1.[b] Water prepared by either of these exchange processes often retains about 0.01 mg/l ammonia nitrogen, which none the less is satisfactory for most purposes. Although the resins may be used repeatedly, regular blanks should be run on the ammonia-free water to guard against unsuspected exhaustion and the attendant release of ammonia to the so-called ammonia-free water.[2] Traces of magnesium in some distilled waters cause cloudiness following nesslerization, a condition which may be prevented by the use of Rochelle salt (potassium-sodium tartrate) solution.

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[a] Permutit Company, New York, N.Y., USA
[b] Rohm & Haas Company, Philadelphia, Pa., USA

(2) Nessler reagent: Dissolve 100 g of anhydrous mercuric iodide and 70 g of anhydrous potassium iodide in a small quantity of ammonia-free water; add this mixture slowly, with stirring, to a cool solution of 160 g of sodium hydroxide in 500 ml of ammonia-free water, and dilute to 1 litre with ammonia-free water. Stored in Pyrex glassware and out of the sunlight, this reagent is stable for periods up to one year under normal laboratory conditions. The reagent should give the characteristic colour with ammonia within 10 minutes after addition and should not produce a precipitate with small amounts of ammonia within 2 hours.

(3) Phosphate buffer solution, pH 7.4: Dissolve 14.3 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4) and 68.8 g of anhydrous dipotassium hydrogen phosphate (K_2HPO_4) in ammonia-free water and dilute to 1 litre. A blank ammonia determination should be run on the buffer solution.

(4) Potassium chloroplatinate solution: Dissolve 2 g of potassium chloroplatinate (K_2PtCl_6) in 300-400 ml of distilled water, add 100 ml of concentrated hydrochloric acid, and dilute to 1 litre.

(5) Cobaltous chloride solution: Dissolve 12 g of crystallized cobaltous chloride ($CoCl_2$, $6H_2O$) in 200 ml of distilled water, add 100 ml of concentrated hydrochloric acid, and dilute to 1 litre.

(6) Sulfuric acid, concentrated, low in nitrogen.

(7) Sodium or potassium sulfate, anhydrous.

(8) Copper sulfate solution: Dissolve 100 g of copper sulfate ($CuSO_4$, $5H_2O$) in ammonia-free water and dilute to 1 litre.

(9) Sodium hydroxide solution: Dissolve 500 g of sodium hydroxide in ammonia-free water and dilute to 1 litre.

(10) Phenolphthalein indicator solution: Dissolve 5 g of phenolphthalein in 500 ml of ethanol and add 500 ml of distilled water. Then add 0.02N sodium hydroxide solution dropwise, until a faint pink colour appears.

(11) Ammonium chloride stock solution: Dissolve 3.819 g of anhydrous ammonium chloride in ammonia-free water and dilute to 1 litre. One ml of this solution contains 1.00 mg of N, or 1.22 mg of NH_3.

(12) Ammonium chloride standard solution: Dilute 10.0 ml of ammonium chloride stock solution to 1 litre with ammonia-free water. One ml of this solution contains 0.0100 mg of N, or 0.0122 mg of NH_3.

2.4.3.5 *Preparation of standards*

(1) *Temporary standards.* A practical series of standards can be prepared by introducing in 50-ml Nessler tubes the following volumes of ammonium chloride standard solution: 0, 0.2, 0.4, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 ml, and diluting to the mark

TABLE V. PREPARATION OF PERMANENT STANDARDS FOR THE DETERMINATION OF AMMONIA NITROGEN

Value in ammonia nitrogen (mg)	Approximate volume of potassium chloroplatinate solution (ml)	Approximate volume of cobaltous chloride solution (ml)
0	1.2	0
0.002	2.8	0
0.004	4.7	0.1
0.007	5.9	0.2
0.010	7.7	0.5
0.014	9.9	1.1
0.017	11.4	1.7
0.020	12.7	2.2
0.025	15.0	3.3
0.030	17.3	4.5
0.035	19.0	5.7
0.040	19.7	7.1
0.045	19.9	8.7
0.050	20.0	10.4
0.060	20.0	15.0

with ammonia-free water. Nesslerize the standards and the portions of distillate by adding 1 ml of Nessler reagent to each tube.

(2) *Permanent standards.* Measure into 50-ml Nessler tubes the volumes of potassium chloroplatinate and cobaltous chloride solutions indicated in Table V; dilute to the mark with ammonia-free water and mix thoroughly. The values given in the table are approximate; actual equivalents of the standards thus prepared will differ with the quality of the Nessler reagent, the kind of illumination used, and the colour sensitiveness of the analyst's eye. These standards should be compared with the nesslerized temporary standards and the tints modified as necessary. Such comparisons should be made for each newly prepared Nessler solution and checked by each analyst. These standards may be kept for several months if protected from dust.

2.4.3.6 *Procedure*

Add 10 ml of phosphate buffer solution and a few pumice granules or glass beads to a 500-ml sample (or less) in an 800-ml Kjeldahl flask, and boil off 200 ml. If desired, this fraction can be distilled and analysed for the ammonia nitrogen. Cool and add carefully 10 ml of concentrated sulfuric acid and 1 ml of copper sulfate solution. After mixing, evaporate under a hood until copious fumes of sulfur trioxide are given off and the liquid becomes colourless or a pale straw colour. Digest for an additional 20 to 30 minutes. In resistant cases, where the materials decolorize with

difficulty, the digestion temperature may be raised by the addition of 5 g of anhydrous sodium or potassium sulfate. Cool the flask contents and dilute to 300 ml with ammonia-free water. Add 0.5 ml of phenolphthalein indicator solution and a sufficient volume of sodium hydroxide solution, carefully and with continuous mixing, to make the contents alkaline to the indicator. Connect the flask to the condenser and shake the flask to ensure complete mixing.

Distil the ammonia into a 200-ml flask and finish the analysis by visual comparison. Compare the colours produced in the samples and in reagent blanks against those of the ammonia standards. Temporary or permanent standards may be used. Comparison with permanent standards should be made either 10 or 30 minutes after nesslerization, depending on the reaction time of the nesslerized temporary standards against which the permanent standards were matched (see section 2.4.3.5, paragraph (2)).

2.4.3.7 *Calculation*

$$\text{Organic N (mg/l)} = \frac{[(\text{mg of N in sample}) - (\text{mg of N in reagent blank})] \times 1000}{\text{ml of sample}}$$

2.4.3.8 *Precision and accuracy*

The over-all accuracy of the determination is estimated to be within 5%.

2.4.4 ALBUMINOID NITROGEN [1]

2.4.4.1 *Principle*

After conversion of the unsubstituted amino groups by alkaline potassium permanganate to ammonia, the ammonia is distilled and determined by nesslerization.

2.4.4.2 *Storage of samples*

Samples should be stored as directed for the determination of total organic nitrogen (section 2.4.3.2, page 119).

2.4.4.3 *Apparatus*

Distilling apparatus : A glass distilling flask of 2-litre capacity, attached to a vertical condenser arranged in such a way that the distillate falls directly into the receiver. An all-Pyrex apparatus is recommended.

Nessler tubes, matched, 50-ml, tall-form.

2.4.4.4 *Reagents*

Reagents (1)-(5), (11), and (12) for the determination of total organic nitrogen (section 2.4.3.4, pages 119-120) are required, and in addition :

Alkaline potassium permanganate reagent: In a 3-litre Pyrex beaker dissolve 16 g of potassium permanganate ($KMnO_4$) in sufficient ammonia-free water to effect solution. Add 288 g of sodium hydroxide or 404 g of potassium hydroxide and make up to 2.5 litres with ammonia-free water. Concentrate to 2 litres on an electric hot-plate. Determine the ammonia blank in 50 ml of the reagent and use the result as a basis for correction in subsequent determinations.

2.4.4.5 *Procedure*

The albuminoid-nitrogen determination should be preceded by the removal of ammonia nitrogen by boiling or distillation. For this purpose, an initial sample of 700-1000 ml is taken, neutralized to about pH 7, and placed in a 2-litre distilling flask. After the addition of 10 ml of phosphate buffer solution, 100 to 200 ml of ammonia-nitrogen distillate are collected, depending on the ammonia-nitrogen concentration. Ammonia-nitrogen content of this distillate may be measured by nesslerization. Next, 50 ml of alkaline potassium permanganate reagent are added and distillation is continued until 200-250 ml of albuminoid-nitrogen distillate have been collected in a volumetric flask. To a 50-ml portion of the distillate, or a suitable aliquot diluted to 50 ml with ammonia-free water, add 1 ml of Nessler reagent. The colour developed can be measured by visual comparison as directed for the determination of total organic nitrogen (section 2.4.3.6, page 122).

2.4.4.6 *Calculation*

Calculate the albuminoid nitrogen as described for the determination of total organic nitrogen (section 2.4.3.7, page 122). Report as mg/l albuminoid N.

2.4.4.7 *Precision and accuracy*

The recovery of unsubstituted amino nitrogen in the albuminoid-nitrogen determination is estimated at approximately 80%.

2.4.5 Nitrite [1]

2.4.5.1 *Principle*

The nitrite concentration is determined through the formation of a reddish-purple azo dye produced at pH 2.0-2.5 by the coupling of diazotized sulfanilic acid with 1-naphthylamine (also called α-naphthylamine) hydrochloride. The diazotization method is suitable for the visual determination of nitrite nitrogen in the range from 0.001 to 0.025 mg/l N.

2.4.5.2 *Interference*

By virtue of chemical incompatibility, it is unlikely that nitrite, free available chlorine, and nitrogen trichloride will co-exist in a sample.

Nitrogen trichloride imparts a false red colour when the normal order of reagent addition is followed. Although this effect may be minimized somewhat by adding first the 1-naphthylamine hydrochloride reagent and then the sulfanilic acid reagent, an orange colour may still result when a substantial nitrogen trichloride concentration is present. A check for free available chlorine and residual nitrogen trichloride is advisable under such circumstances. The following ions interfere by virtue of precipitation under the conditions of the test and should therefore be absent: ferric, mercurous, silver, bismuth, antimonous, lead, auric, chloroplatinate, and metavanadate. Cupric ion may cause low results by catalyzing the decomposition of the diazonium salt. Coloured ions which alter the colour system should likewise be absent.

2.4.5.3 *Storage of samples*

The determination should be made promptly on fresh samples to obviate bacterial conversion of the nitrite to nitrate or ammonia.

2.4.5.4 *Apparatus*

Nessler tubes, matched, 50-ml, tall-form.

2.4.5.5 *Reagents*

All reagents must be prepared from chemicals which are white in colour.

(1) Aluminium hydroxide suspension: Dissolve 125 g of potassium or ammonium alum—$K_2Al_2(SO_4)_4$, $24H_2O$ or $(NH_4)_2Al_2(SO_4)_4$, $24H_2O$—in 1 litre of distilled water. Warm to 60°C and add slowly, with stirring, 55 ml of concentrated ammonium hydroxide. After it has stood for about one hour, transfer the mixture to a large bottle and wash the precipitate thoroughly by successive decantations with distilled water, until free from ammonia, chloride, nitrite, and nitrate.

(2) Sulfanilic acid reagent: Completely dissolve 0.60 g of sulfanilic acid in 70 ml of hot distilled water; cool the solution and add 20 ml of concentrated hydrochloric acid. Dilute to 100 ml with distilled water and mix thoroughly.

(3) 1-Naphthylamine hydrochloride reagent: Dissolve 0.60 g of 1-naphthylamine hydrochloride in distilled water to which 1.0 ml of concentrated hydrochloric acid has been added. Dilute to 100 ml with distilled water and mix thoroughly. The reagent becomes discoloured and a precipitate may form after one week, but it is still usable. It should be discarded when the sensitivity or reproducibility is affected. Storage in a refrigerator extends the useful life of the reagent. Filter before using.

(4) Sodium acetate buffer solution, 2M: Dissolve 16.4 g of sodium acetate ($NaC_2H_3O_2$), or 27.2 g of $NaC_2H_3O_2$, $3H_2O$, in distilled water and dilute to 100 ml. Filter if necessary.

(5) Sodium nitrite stock solution: Dissolve 0.246 g of anhydrous sodium nitrite in nitrite-free distilled water and dilute to 1 litre. Preserve by adding 1 ml of chloroform. One ml of this solution contains 0.05 mg of N.

(6) Sodium nitrite standard solution: Dilute 10.0 ml of sodium nitrite stock solution to 1 litre with nitrite-free distilled water. Preserve by adding 1 ml of chloroform and store in a sterilized bottle. One ml of this solution contains 0.0005 mg of N.

2.4.5.6 *Preparation of standards*

A suitably spaced series of colour standards can be prepared by introducing in 50-ml Nessler tubes the following volumes of sodium nitrite standard solution: 0, 0.1, 0.2, 0.4, 0.7, 1.0, 1.4, 1.7, 2.0, and 2.5 ml, and diluting to the mark with distilled water.

2.4.5.7 *Procedure*

If the sample contains suspended solids and colour, add 2 ml of aluminium hydroxide suspension to 100 ml of sample; stir thoroughly, allow to stand for a few minutes, and filter, discarding the first portion of the filtrate. Coagulation may also be made with zinc sulfate and zinc hydroxide.

To 50 ml of clear sample which has been neutralized to a pH of 7, or to an aliquot diluted to 50 ml, add 1.0 ml of sulfanilic acid reagent and mix thoroughly. At this point, the pH of the solution should be about 1.4. After it has been standing for 3 to 10 minutes, add 1.0 ml of 1-naphthylamine hydrochloride reagent and 1.0 ml of sodium acetate buffer solution, and mix well. At this point, the pH of the solution should be 2.0-2.5. After 10 to 30 minutes, compare visually the reddish-purple colour with that of the standards.

2.4.5.8 *Calculation*

$$\text{Nitrite N (mg/l)} = \frac{\text{mg of N} \times 1000}{\text{ml of sample}}$$

$$NO_2 \text{ (mg/l)} = \text{mg/l of nitrite N} \times 3.29$$

2.4.5.9 *Precision and accuracy*

On undiluted samples and in the absence of interference, the precision and accuracy are estimated to be within 0.002 mg/l in the nitrogen range up to 0.025 mg/l.

2.4.6 PHOSPHATE [1]

2.4.6.1 *Orthophosphate*

Principle

In a dilute phosphate solution, the addition of an acidified ammonium molybdate solution produces a light yellow phosphomolybdate, which in turn forms an intensely blue complex compound upon addition of a reducing agent such as aminonaphtholsulfonic acid.

Interference

Erroneous results may be obtained on waters containing a high chromate content; in such case, adsorb the phosphate on an aluminium hydroxide floc, filter, dissolve the floc, and run the analysis on the solution. High concentrations of ferric iron or tannin may interfere. Pentavalent, but not trivalent, arsenic is determined as phosphate.

Apparatus

One of the following is required:

(*a*) Spectrophotometer, for use at 725 mμ, providing a light path of 1-10 cm.

(*b*) Filter photometer, providing a light path of 1-10 cm and equipped with a red filter having maximum transmittance between 600 and 700 mμ.

(*c*) Nessler tubes, matched, 100-ml, tall-form.

Photometric comparison is recommended in preference to visual comparison, because the colour standards change rather rapidly. The following may be used as a rough approximation of the ranges covered with cells of various light paths:

Light path (cm)	*PO_4 content in the final 100-ml volume (mg)*
1	0.3-3
2	0.1-1
5	0.05-0.5
10	0.03-0.3

Reagents

(1) Sulfuric acid solution: Slowly add 120 ml of concentrated sulfuric acid to about 750 ml of distilled water; cool the solution and dilute to 1 litre with distilled water.

(2) Ammonium molybdate strongly acid solution: Dissolve 50 g of ammonium molybdate—$(NH_4)_6Mo_7O_{24}$, $4H_2O$—in 200 ml of distilled water. Cautiously add 310 ml of concentrated sulfuric acid to 400 ml of distilled water; cool, add the molybdate solution, and dilute to 1 litre with distilled water.

(3) Aminonaphtholsulfonic acid solution : Grind 0.50 g of 1-amino-2-naphthol-4-sulfonic acid with 5.0 ml of sodium sulfite solution containing 1 g of anhydrous Na_2SO_3 per 5 ml. Dissolve in 200 ml of sodium metabisulfite solution containing 30 g of $Na_2S_2O_5$, and filter. Keep in a brown, tightly-stoppered bottle at room temperature and prepare fresh every two weeks. The reagent becomes highly coloured as deterioration proceeds. Use a supply of aminonaphtholsulfonic acid (Eastman No. 360 or equivalent) no darker in colour than a flesh shade.[10]

(4) Phenolphthalein indicator solution : Dissolve 5 g of phenolphthalein in 500 ml of ethanol and add 500 ml of distilled water. Then add 0.02N sodium hydroxide solution until a faint pink colour appears.

(5) Potassium phosphate standard solution : Dissolve in distilled water 0.7164 g of potassium dihydrogen phosphate (KH_2PO_4) which has been dried in an oven at 105°C, and dilute to 1 litre. Then dilute 100.0 ml of this solution to 1 litre with distilled water. One ml of the final solution contains 0.05 mg of PO_4.

Procedure

To a 50-ml sample containing not more than 1.5 mg of PO_4, and free from colour and turbidity,[15] add 1 drop of phenolphthalein indicator solution. If the sample turns pink, add dropwise just enough sulfuric acid solution to discharge the colour. Dilute to approximately 70 ml with distilled water. Add, mixing thoroughly after each reagent addition, 10 ml of ammonium molybdate strongly acid solution and 4.0 ml of aminonaphtholsulfonic acid solution ; dilute to 100 ml with distilled water. The rate of colour development and the intensity developed depend on the temperature of the final solution ; hence samples, standards, and reagents should be within 3°C of one another and at a temperature between 20°C and 30°C. After exactly 10 minutes, measure the colour photometrically and compare with a calibration curve, using a distilled-water blank, or compare visually in 100-ml Nessler tubes against standards prepared simultaneously from the potassium phosphate standard solution. A blank should be run on the reagents and distilled water.

Calculation

$$PO_4 \text{ (mg/l)} = \frac{\text{mg of } PO_4 \times 1000}{\text{ml of sample}}$$

Precision and accuracy

The precision with photo-electric measurement is about ± 0.01 mg, or about ± 2% of the result, whichever is the larger numerical value. The precision with visual comparison is somewhat poorer and depends upon the analyst. The accuracy depends upon the apparatus and upon interferences.

2.4.6.2 *Polyphosphates*

Principle

Polyphosphates, such as pyro-, meta-, and tripolyphosphate, are not present in natural waters, but are frequently added in the course of water treatment. The concentration employed depends on the application. This method determines the sum of polyphosphates and orthophosphate present in the sample, as PO_4. Polyphosphates do not respond to tests for orthophosphates but can be converted into orthophosphate by boiling with acid. Then the polyphosphates can be determined as equivalent PO_4, by difference, by subtracting the orthophosphate found before and after boiling with acid.

Interference

Interfering substances are the same as for the method for orthophosphate (section 2.4.6.1, page 126), which is used to complete the analysis. The sample should be analysed as soon as possible after it is collected, as polyphosphates will significantly decrease on long standing or on heating.

Apparatus

The same apparatus as used in the method for orthophosphate (section 2.4.6.1, page 126) is employed. In addition, an autoclave can be used, if desired, for speeding up the acid-treatment step.

Reagents

(1) Sulfuric acid solution: Add cautiously 310 ml of concentrated sulfuric acid to 600 ml of distilled water, cool, and dilute to 1 litre.

(2) Ammonium molybdate half-strength acid solution: Dissolve 50 g of ammonium molybdate—$(NH_4)_6Mo_7O_{24}$, $4H_2O$—in 200 ml of distilled water. Cautiously add 155 ml of concentrated sulfuric acid to 400 ml of distilled water; cool, add the molybdate solution, and dilute to 1 litre with distilled water.

Procedure

To a 100-ml sample, or an aliquot diluted to 100 ml, add 1 drop of phenolphthalein indicator solution. If it becomes pink, add dropwise just enough sulfuric acid solution to discharge the colour. Add 10.0 ml of sulfuric acid solution. Boil for at least half an hour, adding distilled water to keep the volume between 25 and 90 ml. Alternatively, heat for 10 minutes in an autoclave at 126°C. Cool to room temperature and dilute to 100 ml. Determine orthophosphate in 50 ml by following the procedure described in section 2.4.6.1 (page 127). However, the appropriate half-strength acid solution of ammonium molybdate specified for

the present method must be used in place of the strong acid solution specified in the orthophosphate method.

Determine orthophosphate in the original, untreated, sample by the applicable colorimetric method (section 2.4.6.1, page 126).

Calculation

To obtain the polyphosphate content, subtract the orthophosphate result from the total phosphate result and report as mg/l PO_4.

2.5 Residual chlorine [1]

2.5.1 Principle and interference

Within the limitations specified, the orthotolidine-arsenite (OTA) test permits the measurement of the relative amounts of free available chlorine, combined available chlorine, and colour due to interfering substances. In samples containing a high proportion of combined available chlorine, it may indicate more free available chlorine than is actually present. Precision of results depends on strict adherence to the conditions of the test, which are: (*a*) the time intervals between addition of reagents, and (*b*) the temperature of the sample. Precision is also affected by the relative concentrations of free and combined available chlorine in the sample. The temperature of the sample under examination should never be above 20°C, and the precision of the test increases with decreasing temperature.

2.5.2 Apparatus

One of the following is required:

(*a*) Comparator, colour- and turbidity-compensating.

(*b*) French square bottles, 30- or 60-ml capacity.

2.5.3 Reagents

(1) Orthotolidine reagent: Dissolve 1.35 g of orthotolidine dihydrochloride in 500 ml of distilled water. Add this solution, with constant stirring, to a mixture of 350 ml of distilled water and 150 ml of concentrated hydrochloric acid. The use of orthotolidine base in the preparation of this reagent is not recommended.

The orthotolidine solution should be: (*a*) stored in amber bottles or in the dark; (*b*) protected at all times from direct sunlight; (*c*) used for not more than 6 months; (*d*) kept away from contact with rubber; and (*e*) maintained at normal temperatures. At temperatures below 0°C, the orthotolidine will precipitate from solution and cannot be re-dissolved easily. The use of a reagent from which part of the orthotolidine has

precipitated may lead to errors due to a deficiency of active element.

(2) Arsenite reagent: Dissolve 5 g of sodium meta-arsenite ($NaAsO_2$) in distilled water and dilute to 1 litre.

(3) Phosphate buffer stock solution, 0.5M [a]: Dry anhydrous disodium hydrogen phosphate (Na_2HPO_4) overnight at 110°C and store in a desiccator. Dissolve 22.86 g of this salt, together with 46.16 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4), in 1 litre of distilled water. Allow the solution to stand for several days to enable any precipitate to form. Filter before using.

(4) Phosphate buffer standard solution, 0.1M [a]: Filter the buffer stock solution and dilute 200 ml to 1 litre with distilled water. The pH of this solution is 6.45.

(5) Chromate-dichromate strong solution: Dissolve 1.55 g of potassium dichromate ($K_2Cr_2O_7$) and 4.65 g of potassium chromate (K_2CrO_4) in 0.1M phosphate buffer solution and dilute to 1 litre with 0.1M phosphate buffer solution. The final solution corresponds to the colour produced by 10 mg/l Cl_2 by the standard orthotolidine procedure when viewed through a depth of 240 to 300 mm.

(6) Chromate-dichromate dilute solution: Dissolve 0.155 g of potassium dichromate ($K_2Cr_2O_7$) and 0.465 g of potassium chromate (K_2CrO_4) in 0.1M phosphate buffer solution and dilute to 1 litre with 0.1M phosphate buffer solution. Alternatively, dilute 100 ml of chromate-dichromate strong solution to 1 litre with 0.1M phosphate buffer solution. The final solution corresponds to the colour produced by 1 mg/l Cl_2 by the standard orthotolidine procedure when viewed through all cell depths.

2.5.4 Permanent chlorine standards

2.5.4.1 *Preparation*

Permanent chlorine standards may be prepared as follows, according to the modified Scott formula:

(1) *0.01-1.0 mg/l.* The volumes of chromate-dichromate dilute solution indicated in Table VI are pipetted into 100-ml tubes of any uniform length and diameter or into 100-ml volumetric flasks. The volume is then made up to the 100-ml mark with 0.1M phosphate buffer solution. These standards can be read at any cell depth up to 300 mm.

(2) *1.0-10.0 mg/l.* The volumes of chromate-dichromate strong solution indicated in Table VII for the range of cell depths given are pipetted into 100-ml tubes of any uniform length and diameter or into

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a It cannot be emphasized too strongly that precision in the preparation of the buffer solutions is necessary; therefore, the directions must be followed explicitly.

100-ml volumetric flasks. The volume is then made up to the 100-ml mark with 0.1M phosphate buffer solution. These standards can be used for the cell depths listed.

Standards for other cell depths or for other concentrations can be prepared by interpolating between the values stated in Tables VI and VII.

TABLE VI. CHLORINE STANDARDS, 0.01 TO 1.0 mg/l*

Chlorine (mg/l)	Chromate-dichromate dilute solution (ml)	Chlorine (mg/l)	Chromate-dichromate dilute solution (ml)
0.01	1	0.35	35
0.02	2	0.40	40
0.05	5	0.45	45
0.07	7	0.50	50
0.10	10	0.60	60
0.15	15	0.70	70
0.20	20	0.80	80
0.25	25	0.90	90
0.30	30	1.00	100

TABLE VII. CHLORINE STANDARDS, 1.0 TO 10.0 mg/l*

Chlorine (mg/l)	Chromate-dichromate strong solution (ml) at cell depth of			
	25–50 mm	100 mm	200 mm	240–300 mm
1	10.0	10.0	10.0	10.0
1.5	15.0	15.0	15.0	15.0
2	19.5	19.5	19.7	20.0
3	27.0	27.5	29.0	30.0
4	34.5	35.0	39.0	40.0
5	42.0	43.0	48.0	50.0
6	49.0	51.0	58.0	60.0
7	56.5	59.0	68.0	70.0
8	64.0	67.0	77.5	80.0
9	72.0	75.5	87.0	90.0
10	80.0	84.0	97.0	100.0

* These standards are very close visual matches of the chlorine-orthotolidine colour and are preferable to temporary standards which are difficult to prepare accurately.

Variations in the viewing-depth in any set of colour comparison tubes, cells, or bottles should not be more than ± 3%.

2.5.4.2 *Protection of standards*

The tubes should be protected from dust and evaporation by sealing on micro-cover-glasses with collodion, Canada balsam, or similar material.

Apply the material to the top of the comparison tube, cell, or bottle by means of a camel's-hair brush, and immediately put the cover-glass into position with forceps. After it is spot-sealed, reinforce the circumference with additional, brush-applied sealing material until the joining of the tube and cover-glass is complete. The use of rubber stoppers is not recommended. Handy Nessler tubes with special ground-glass caps that permit optical comparison are now on the market. The standards should be neither stored nor used in direct sunlight. They should be renewed whenever turbidity appears.

2.5.4.3 *Commercial standards*

Commercially prepared permanent standards may be used for routine tests, provided the analyst satisfies himself as to their accuracy and checks them frequently.

2.5.5 PROCEDURE

Label three comparator cells, or French square bottles, A, B, and OT. Use 0.5 ml of orthotolidine reagent in 10-ml cells, 0.75 ml in 15-ml cells, and the same ratio for other volumes of sample. Use the same volume of arsenite reagent as of orthotolidine.

To *cell A*, containing orthotolidine reagent, add a measured volume of the water sample. Mix quickly, and immediately, within 5 seconds, add arsenite reagent. Mix quickly again and compare with colour standards as rapidly as possible; record the result. The value obtained (A) represents free available chlorine and interfering colours.

To *cell B*, containing arsenite reagent, add a measured volume of the water sample. Mix quickly, and immediately add orthotolidine reagent. Mix quickly again and compare with colour standards as rapidly as possible; record the result as the B_1 value. After exactly 5 minutes, compare again with colour standards and record the result as the B_2 value. The values obtained represent the interfering colours present in the immediate reading (B_1) and in the 5-minute reading (B_2).

To *cell OT*, containing orthotolidine reagent, add a measured volume of the water sample. Mix quickly and, after exactly 5 minutes, compare with colour standards; record the result. The value obtained (OT) represents the total amount of residual chlorine present and the total amount of interfering colours.

2.5.6 CALCULATION

Total residual chlorine = $OT-B_2$

Free available chlorine = $A-B_1$

Combined available chlorine = total residual chlorine—free available chlorine.

Annex 5

APPROVED METHODS FOR THE BIOLOGICAL EXAMINATION OF WATER

1. Laboratory Equipment

1.1 Filtration equipment

1.1.1 Filtering funnels

A cylindrical funnel is required ; the diameter should be approximately 5 cm at the top, with a straight side for about 23 cm, narrowing over a distance of about 7.5 cm to a bore of 1.3 cm diameter and terminating in a straight portion of this diameter about 6 cm in length. The capacity of this funnel is 500 ml. It should be fitted at the bottom with a tightly fitting one-hole rubber stopper, and the stopper should contain a small glass U-tube, the outer arm of which extends 2.5 cm above the small end of the stopper. This tube prevents complete drainage of water from the filter sand after filtration.

1.1.2 Cloth discs

Cloth discs, about 1 cm in diameter, are needed to support the sand in the funnel. These are cut preferably from silk bolting cloth having about 80 meshes per cm, although nylon or linen cloth may be used.

1.1.3 Filtering sand

White sand is required as the filtering medium. This may be Berkshire sand, Ottawa sand, ground quartz, or white beach sand. The sand should be washed and screened, only that portion being used which passes a 250-μ screen (US Standard No. 60) and is retained on a 125-μ screen (US Standard No. 120).

1.2 Microscopes

1.2.1 Compound microscope

Although most workers prefer the binocular-type compound microscope, the monocular type can be used, and for some individuals it is actually the instrument of choice. Either type of microscope should be equipped with a mechanical stage capable of moving all parts of a counting-

cell past the aperture of the objective. The following eyepieces and objectives should be provided as standard equipment :

(1) *Eyepieces*

7.5× and 10× (paired when binocular microscope is used).

(2) *Objectives*

Type	*Initial magnification* *	*Over-all magnification with 10× eyepiece*
1.8 mm (oil immersion)	97-98×	970-980×
4 mm (high-power, dry)	43-45×	430-450×
8 mm (medium-power)	21×	210×
16 mm (low-power)	10×	100×

* A magnification factor is engraved on each objective.

Unlike the higher-power objectives, the medium-power or 8-mm objective, with a working distance of approximately 1.6 mm, can be used with a standard counting-cell 1 mm deep. Providing, as it does, twice the magnification of the low-power objective ordinarily used in enumerating plankton, this medium-power lens is useful in several ways. For example, small organisms can be examined more carefully during the process of counting by shifting momentarily to the 8-mm objective, and when allowances are made for the reduction in field area it will be found that plankton counts can actually be made with this objective.

1.2.2 Low-power stereoscopic microscope

This consists essentially of two complete microscopes assembled into a binocular instrument to give a stereoscopic view and an erect rather than an inverted image. This microscope is indispensable for the study of organisms which occur in bottom sediments and for counting large organisms such as crustacea in plankton samples. The optical equipment of this microscope should include 9× and 12× paired eyepieces in combination with 1×, 4×, and 8× objectives. This combination of lenses bridges the gap between the hand lens and the compound microscope, making available magnifications ranging from 9× to 96×.

1.2.3 Sedgwick-Rafter counting-cell

A counting-cell is used to provide a known volume and area for microscopic examination and enumeration of organisms. It consists of a brass or glass rim closely cemented to a plate of optical glass, or plate glass. The depth of this cell should be 1 mm. The shape and size of the cell, excepting its depth, are not prescribed ; but a rectangular cell with inside dimensions of 20 × 50 mm and 1-ml capacity is recommended. A coverglass, preferably No. 3, of such size as to cover the cell and its rim, is

required. The inner walls of the cell shall be vertical and corners shall not be rounded for this may interfere with uniformity of distribution and thus influence the accuracy of the count.

1.2.4 MICROMETERS

1.2.4.1 *Whipple micrometer*

For field limitation, the microscope should be fitted with a Whipple disc micrometer. This ocular micrometer, placed in the eyepiece of the microscope, is an accurately ruled square subdivided into 100 squares. One of the small squares near the centre is further subdivided into 25 still smaller squares. Dimensions of the entire square are such that with a 16-mm objective, a 10× ocular, and a correctly adjusted tube length, the area delimited on the stage of the microscope is exactly 1 mm^2. This combination is determined accurately by reference to a stage micrometer, and exact adjustment may be attained by a change in the draw-tube length of the microscope. In many modern microscopes the tube length is no longer an adjustable feature and exact coincidence cannot be obtained. In such cases it is necessary to establish the exact coverage obtained at the tube length which has been established by the manufacturer. If the square exceeds or falls below 1 mm^2, a factor must be used to convert the results obtained to those represented by a standard area.

When the outside limits of the Whipple micrometer cover exactly 1 mm^2 on the stage, the very smallest square will cover an area of 20 × 20 μ, or 400 square microns. This is known as the " areal standard unit ". If this areal standard unit has also a thickness or depth of 20 μ, it becomes a " volumetric standard unit " or " cubic standard unit ", the volume of which is 8000 cubic microns.

If the Whipple micrometer does not exactly coincide with 1 mm^2 on the stage, the volumetric enumeration method may still be used by applying a factor to the cubic standard unit.

If the Whipple micrometer is transferred to another eyepiece, or if the eyepiece is moved to another microscope, recalibration is necessary. This is also true if objectives are changed.

1.2.4.2 *Measuring eyepiece*

A measuring eyepiece is also needed by the microscopist to determine the exact size of specimens which are being examined. A glass disc or reticule bearing a linear scale of arbitrary divisional size and total length is placed engraved side down on the diaphragm of an ordinary eyepiece to convert it to a measuring device. In stereoscopic and binocular microscopes, the measuring reticule is placed in the right eyepiece. If a considerable amount of measurement is done, an eyepiece with a permanent

factory-mounted reticule, or a screw micrometer with a shifting scale, may be desirable.

1.2.4.3 *Stage micrometer*

A stage micrometer is needed for the calibration of the Whipple micrometer and the ocular-measuring device and to establish the diameter of the microscope field for each combination of eyepiece and objective. This micrometer is a standardized, accurately ruled scale mounted on a glass slide. The finest engraved lines are 0.01 mm apart. Calibration of an ocular micrometer, for example, is accomplished by superimposing the images of the stage and the ocular micrometers and noting the point of correspondence of graduations.

2. Procedures

2.1 Calibration of microscope

Microscope eyepieces to be used in counting plankton are fitted with glass discs (reticules) bearing parallel, engraved, equidistant lines, or engraved, subdivided squares which are designed to measure microscopic objects or accurately delimit microscope fields. Before these ocular micrometers can be used, they must be carefully calibrated in combination with each objective.

The ocular micrometers are calibrated by measuring an object of known dimensions, thus determining the value of each subdivision by reference. A stage micrometer or glass slide, on which an accurately ruled scale has been engraved, is placed on the microscope stage and serves as the object of known dimensions.

The procedure consists in determining the number of intervals on the ocular micrometer required to cover one or several intervals on the scale of the stage micrometer. With the ocular and stage micrometers parallel and partly superimposed, a line at one end of the eyepiece scale is selected and matched with a similar line of the stage micrometer scale. If the two scales are then carefully examined along their entire length, it will be observed that the lines also correspond at another point. Since the exact distance between lines on the stage micrometer is known, the linear value of each eyepiece-scale division can now be determined by reference. For example, if the smallest interval on the stage micrometer is 0.01 mm and if 10 of these 0.01-mm divisions are equal to 25 divisions on the eyepiece scale, one eyepiece division is equal to 0.1 mm : 25 = 0.004 mm (4 μ). When this scale is used for measurement, the result is a direct reading of length in microns. Thus an object covering five eyepiece divisions is 20 μ long.

When high-power objectives are calibrated, the stage-micrometer lines are magnified to a point where they have appreciable width. As a result, the calibration procedure must be modified by placing an eyepiece-scale line alongside of, rather than end-to-end with, the stage-micrometer lines. The eyepiece-scale lines used for calibration should both lie on the same side of their stage-micrometer counterparts.

Single observations will not suffice to establish a true calibration of ocular micrometers; the average of a large number of observations must be used. The data obtained will be more readily available if a graph is prepared in which the eyepiece-scale dimensions are plotted against the linear distances they represent on the stage. By reference to this chart, the length in microns of any given object examined may be determined quickly.

If the same eyepiece and objective are used and no change is made in the tube length of the microscope, there will be no need to recalibrate an ocular micrometer.

The Whipple micrometer is usually calibrated by adjusting the tube length of the microscope at low power, such as obtained with a 16-mm objective, until the outer lines of the square coincide exactly with the 0.0 and 1.0 mm lines on the stage micrometer. At higher powers and where the tube length cannot be adjusted, the calibration is carried out in the same manner as for ocular micrometers.

2.2 Concentration of samples

2.2.1 General

If organisms are numerous in the original sample, no concentration is necessary; and in some instances, where a "water bloom" prevails, dilution of the water sample may actually be needed before an enumeration can take place. There is no general agreement among workers in the field regarding the number of organisms needed per unit volume of sample to provide a basis for an accurate count, although it is hoped that current statistical research studies in this field may eventually provide a basis for agreement. However, for practical purposes it is necessary to set some arbitrary limit now. In doing so it can be agreed that organisms are too numerous for an accurate count when they overlap one another in a standard counting-cell. Conversely, organisms are too scarce when the average per Whipple field, under standard procedure, is less than one. It is suggested therefore that the satisfactory sample should contain a concentration of organisms which will produce a count of not less than 10 organisms per field when a Whipple micrometer and a standard counting-cell are used. If this count is not attained, the sample should be concentrated.

If other methods of counting are employed, the corresponding limit can be determined for each particular procedure.

2.2.2 SEDGWICK-RAFTER METHOD

Filtration shall be carried out as soon as possible after collection of the sample. If the sample is kept cool, 3-4 hours may safely intervene; but for longer periods and at high, summer temperatures it is desirable to preserve the sample as directed in section 4.2.4 (page 33).

Prepare the filter for use, first inserting the glass U-tube in the large end of the rubber stopper, then cover the moistened small end with a disc of bolting cloth and place the whole firmly in the lower end of the funnel. The latter should be perfectly clean on the inside.

Pour sand into the funnel to form a layer 1.3 cm deep on top of the disc. Add 5 to 10 ml of distilled water to wash down any sand on the walls of the funnel and to drive the air from the sand. As the distilled water filters through the sand, tilt the funnel from side to side to permit the escape of air.

Mix the sample well, but do not shake it violently. Measure out from 250 to 1000 ml, according to the density of microscopic organisms in the sample, in a graduated cylinder or flask, and pour slowly into the funnel, holding the latter in a slanting position and taking care to leave the sand undisturbed.

Allow the water to filter through the sand. Moderate suction may be used to hasten filtration. Wash down the side occasionally with the waste filtrate water from the sample. If living organisms are being concentrated, keep the temperature of the sample uniform during the filtration period to prevent the lethal effect of heat. After the water has reached the level of the outer arm of the U-tube, disconnect the suction if employed; carefully remove the U-tube from the stopper to allow most of the remaining water to drain through the sand.

As soon as the sand has drained, transfer the funnel to a horizontal position, remove the stopper slowly with a twisting movement, then raise the funnel to a vertical position inside a small beaker. The plug of sand usually falls into the beaker. Wash down the walls of the funnel with 5-20 ml waste filtrate water, the amount varying with the final concentration of sample desired. The water should be measured with a pipette and is finally collected in the beaker containing the sand and organisms. The container is then shaken gently to detach organisms from the sand grains.

Allow a moment for the coarse sand to settle, then decant promptly into a beaker. A second washing of the sand, with an additional 5 ml of water, is usually necessary. If the mixing has been thorough, any water remaining in the sand will have the same concentration of organisms as

the decanted water of the second washing.

If the concentrated sample is to be preserved for future examination, a 3% to 10% solution of formaldehyde may be used to wash down the funnel, or the formaldehyde may be added to the concentrate and the latter made up to a definite volume; some multiple of 5 is convenient.

2.2.3 Selection of aliquot portion for examination

The portion taken from the concentrated sample should be representative and the examination should include a sufficient number of organisms to ensure accuracy.

Shake the concentrated sample gently but in such a manner that complete mixing will occur. Place the cover-glass obliquely across the cell. By means of a pipette, withdraw 1 ml of material from the sample bottle before the motion of the sample induced by mixing has ceased. Introduce half of this volume at each open corner of the cell. When carefully done, this will cause the cover slip to rotate automatically into a position completely covering the cell. After a 4- or 5-minute settling period, the cell is ready for the enumeration procedure. During this interval most organisms settle towards the bottom and a few rise to the surface, coming to rest against the cover slip.

2.3 Differential count of organisms with the Sedgwick-Rafter cell

2.3.1 General

A differential count may be defined as the enumeration of some or all of the different kinds of plankton organisms, distinguishing them qualitatively. It involves identifying, counting, and recording the numbers of individuals of each kind. In contrast, the total count has been defined as an enumeration of all the plankton forms without any attempt to distinguish between different kinds.

In making a differential count, the contents of the cell should be examined under the microscope in three ways. First, the most abundant forms are counted by examining a number of standard fields; next, a strip extending the whole length of the cell is examined for organisms which are less numerous; and, finally, the whole cell is examined for large forms and those very limited in number. In all cases, examine the full depth of the cell to include floating forms.

General rules or suggestions for counting which are applicable to any of the three procedures listed are as follows:

(*a*) It is ordinarily impracticable to count bacteria.

(*b*) If it is difficult to discriminate positively between remains of organisms which were living or dead before the sample was preserved, do not count the doubtful specimens.

(*c*) Objects near the limit of vision of a given combination of eyepiece and objective cannot be adequately counted. Examine them under higher powers.

(*d*) Detritus and objects other than plankton are recorded, since this information may be of use in the final interpretation of results. For example, counts of wood fibres derived from paper mills may be of value in pollution surveys.

(*e*) If a plankton organism lies on the boundary line of the field, count only that portion lying inside and record as a fraction.

(*f*) The length of filamentous forms should be estimated and reported in units of a selected standard length. Some workers report filaments in terms of 100-μ units.

(*g*) Other forms having irregular colonies may be reported in terms of estimated units of volume, selecting arbitrarily a volume which approximates a medium-sized colony.

(*h*) Except as indicated in (*f*) and (*g*), individual organisms should be reported whenever possible, unless colonial groups have a fairly constant number of cells. When a species of the latter type is reported in terms of colony units, it is well to indicate in the record the number of cells which have been considered as representative.

2.3.2 Field count

The field count is made with the 16-mm objective and a 7.5× eyepiece equipped with a Whipple micrometer. When the distribution of organisms in the sample is uniform and organisms are relatively abundant, conditions are ideal for counting and relatively few fields need to be examined.

The exact number of fields which must be examined will vary considerably depending on the accuracy which is necessary and the characteristics of the sample. Careful statistical work is currently being carried on by one research organization to ascertain, on a mathematical basis, the exact number of fields which must be examined in any given sample to attain a preselected degree of accuracy. A method apparently applicable to practical microscopy has been developed. However, before it is adopted as a standard procedure, this method must be tested more extensively.

More or less arbitrarily, microscopists have long used 10 fields as the standard of reasonably good practice. More recently, where highly concentrated samples were involved and organisms were very numerous, certain workers have felt that sufficient accuracy was attained when 5 fields were examined. For routine work it is therefore recommended that, in samples which are adequately concentrated to provide at least 10 organisms per field, not less than 5, and preferably 10, standard fields

should be examined in making a plankton count. When special studies are being made, it may be necessary to increase tenfold the number of fields examined.

The fields selected should be chosen at random and should be well separated from one another. In order that certain parts of the cell may not be inadvertently avoided, some workers divide the area of the counting-cell into four sectors by diagonal lines connecting opposite corners. Fields are selected at random from each of the general areas so delimited.

In making a differential count, certain forms will be too scarce to appear in satisfactory numbers in each field examined. Such organisms must be counted by strip counts or survey counts.

2.3.3 Strip count

This count is made when a species is not abundant enough to be accurately enumerated by the field count. It is also suited to the enumeration of organisms which are too small to be identified or counted properly under the low magnification available in the field-count method. The strip count is essentially the enumeration of a selected group of organisms as they occur within an area represented by the full length of a Sedgwick-Rafter cell (50 mm) and the width of a microscopic field (approximately 0.7 mm). The microscopic field width generally used is that produced by a combination of a 10× eyepiece and an 8-mm objective. The exact width of this strip varies with individual lenses and must be determined by reference to a stage micrometer. In a fully calibrated microscope whose field diameter is known, it is a simple matter to convert strip counts to units corresponding to those obtained with other methods.

In the actual count, enumeration is begun at one end of the cell and all organisms which are to be recorded are counted as the slide is moved past the objective by the mechanical stage. Usually, one trip is made along the long axis of the cell. In some instances it may be desirable to count several strips to obtain the accuracy desired.

2.3.4 Survey count

Large plankton forms such as Microcrustacea and rotifiers, which can be identified rather readily at low magnifications and are less numerous than the smaller organisms, should be counted by the survey method. This involves enumeration of all organisms of this type which are present in an entire cell volume of 1 ml. Ideally, this count is made with a low-power stereoscopic microscope, using its highest power. In routine work, however, most microscopists use the low-power (16-mm) objective in combination with a 10× eyepiece.

By means of the mechanical stage, the entire contents of the counting-cell are moved past the objective for recognition and enumeration of the

organisms which are being counted. In making this traverse it is possible to include smaller plankton organisms if these have special significance and cannot be accurately counted by the other procedures.

If the total number of organisms is still too small for accurate reporting, several cells may be counted or the following supplementary procedure adopted. The entire sample is poured into a Petri dish which is then examined under a stereoscopic microscope. Guide-lines or squares should be ruled on the Petri dish for the purpose of orientation.

The survey count is the method generally applied to silk-net collections, although the other two procedures may also be used.

2.4 Identification of plankton

Wherever possible, the species of an organism should be determined. It is recognized that identification to genus or to a more generalized group may suffice in some instances, but specific identification, when it can be made with assurance, is desirable. Space does not permit a discussion of the principles of taxonomy or the inclusion of suitable keys for the identification of plankton or bottom-dwelling organisms. This information, as well as discussions relating to the use of living organisms as indexes of pollution, are presented by various authors. Illustrations of some of the more common plankton forms will be found in *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes*.[1]

3. Reporting of Results

3.1 Forms

Although it is difficult to design a " bench sheet " which will fit all situations in plankton counting, most workers find forms of this type very useful. Where repeated examinations are made of one sampling point or a series of similar sampling areas, a very satisfactory sheet can be prepared. Since there can be considerable latitude in the preparation of such forms, no exact specifications will be given.

Forms should provide spaces for information relating to the identity and history of each sample. Included should be such items as the exact place and time of collection, the name of the collector, the time of arrival in the laboratory, the laboratory serial number, the date of examination, and the name of the examiner.

The manner of ruling the sheet may vary widely but should facilitate entries when 10 fields are examined and be adaptable to a procedure involving the examination of 5 fields. A form may be adapted to special counts where a larger number of fields is examined by making double entries in a square or by using two or more lines of squares in reporting

the results. Other additions, such as a column for the strip count, will provide a form that may vary slightly with the counting procedure adopted and the type of work being carried on. The form should be fitted to the requirements of the laboratory concerned, for its chief function is to assure an immediate, accurate, and uniform recording of results.

3.2 Reporting in terms of numbers of organisms

Reporting the number of organisms of each species is a straightforward procedure and requires much less time than a method which is based on the volume of each plankton species. In many instances a simple count of this kind is entirely adequate for the practical problems involved and may be used without modification. In other instances, where a fairly uniform plankton prevails and the same locality is examined repeatedly, the direct count can be converted into volume units by applying factors. For example, each species may be assigned a volumetric factor which, for practical purposes, represents the average volume of any organism within the group. By applying this factor to the numerical count, the final report can be given in terms of volume. If desired, this figure can then be converted to its equivalent in volumetric standard units. Some workers have stated that this is a poor way of recording volumetric data, and they prefer to report their counts as parts per million (p.p.m.) or milligrams per litre (mg/l) of living cellular matter. In the latter procedure, the specific gravity of the organisms must be known; ordinarily, this value is assumed to be 1.0, the same as that of water.

When colonial or filamentous forms are counted, they should be recorded in terms of standardized units. *Melosira* or *Oscillatoria*, for example, may be reported as units or fractions of units 100 μ in length. In some colonial forms the number of cells may be enumerated; more often, it is desirable to report the number of colonies. In the latter procedure, especially where colony size may vary considerably, the volume of each colony should be estimated by reference to the Whipple micrometer guide-lines while the count is being made. The report can then be rendered in terms of an equivalent number of standardized medium-sized colonies. The size of colony chosen as a standard may be arbitrary but should fall near the median. Such procedure facilitates comparison of samples and, if desired, provides an easy basis for converting a numerical count to a volumetric equivalent.

In enumerating the organisms, the field count is usually made first, counting all organisms which lie within the limits determined by the Whipple disc. This count is carried out at low power with a 16-mm objective and a 7.5× eyepiece. It is followed by the strip count and survey count, which include organisms too scarce for accurate counting by the random-field technique.

In recording the count a bench sheet is very useful, for the results are simply entered in their proper columns as the enumeration is carried out and are then ready for summation. Certain modifications in the use of a form may be made to fit the situation at the time a count is made. For example, if 10 rather than 5 fields are examined, a double entry may be made in each square by drawing a diagonal line from corner to corner to provide two spaces. When standard lengths of filaments and standard size colonies are reported, suitable entries should be made giving this information. These entries are conveniently made within parentheses following the name of the organism.

3.3 Reporting in volumetric units

The volumetric standard unit has been used for many years and is preferred by some workers as a method for the quantitative expression of results. Where records of long standing have all been in terms of this type of unit, it may be desirable to continue its use although it is more time-consuming than the simple numerical count and not as easily understood as reporting in terms of p.p.m. or mg/l. It is essentially an extension of the areal standard unit method in which the third dimension is measured and volume is expressed in terms of a cube 20 μ on a side. In practical work it is well to obtain a value for the average volume of such organisms as occur repeatedly and are fairly uniform in size. The dimensions of the organisms in the view presented under the microscope are measured directly; the third dimension is estimated from a knowledge of the shape of the organisms on the basis of the measured dimension. This value may be in volumetric standard units for spherical, cubical, and some irregularly shaped species, or it may be in areal standard units (the product of two dimensions) for organisms that have a widely varying third dimension. Representatives of species that are not uniform in size must have their bulk estimated as they appear in the count.

Since a volumetric standard unit represents a cube measuring 20 μ on each side, it is equivalent to 8000 cubic microns, and the volume of each organism in terms of volumetric standard units is obtained as follows:

$$\text{Volume in volumetric standard units} = \frac{\text{volume in cubic microns}}{8000}$$

Record the average size obtained on the counting sheet. If the product of only two dimensions (areal units) is given, designate this. In such cases, the third dimension must be estimated at the time of counting. In species lacking uniformity in size, the bulk is estimated when seen and no value recorded as average size will be necessary.

Proceed with the survey, and on completion record each genus or species, showing the total number of volumetric standard units observed. A record of the number of individual organisms or cells may be kept simultaneously by noting this number on the counting sheet as the numerator of a fraction, the measurements of the cells being recorded as the denominator—e.g., $^4/_3$, where 4 is the number of cells and 3 the size in volumetric standard units or linear units.

Proceed with the total count for 10 random fields. Record for each genus or species and for each field, as in the survey, the total number of volumetric standard units observed. A record of the number of individual organisms or cells may be kept simultaneously, again as in the survey, by noting this number on the counting sheet as the numerator of a fraction, the measurements of the cells being recorded as the denominator.

A record of amorphous matter in volumetric standard units may also be made for each of the 10 fields of the survey examination, and the amount found inserted in the squares of the columns assigned for this purpose.

The computation of volumetric standard units from the individual measurements and estimates is facilitated by the use of a table such as the following, which is commonly included in engineers' and other handbooks.

Diameter or side	Area of circle	Volume of sphere	Volume of cube
0.5	0.196	0.065	0.125
1.0	0.785	0.524	1.000
2.0	3.142	4.189	8.000
3.0	7.069	14.137	27.000
4.0	12.566	33.510	64.000
etc. up to 30 or 40			

3.4 Calculation of results: Sedgwick-Rafter cell count

3.4.1 Volumetric standard units

The following calculation is an example of the procedure followed when reporting in terms of volumetric standard units. (Results may also be reported in terms of the number of organisms per unit volume of sample by a slight change in procedure.)

Record the total number of organisms or of standard units, or both, found by examination of the 10 fields or by mechanical-stage traverses. Convert into terms of volumetric standard units the totals recorded for

each species (this computation is not necessary where the bulk of a species has already been estimated in terms of volumetric standard units).

As each value of volumetric units is obtained, convert this into volumetric standard units per ml of original unconcentrated sample. This latter value is obtained by multiplying the volumetric units by suitable factors secured in the following way:

$$\text{Factor} = \frac{\text{number of fields in 1-ml counting cell (1 mm deep)}}{\text{number of fields counted}} \times \frac{\text{ml of concentrate}}{\text{ml of original sample}}$$

If, for example, there are 1000 fields in the counting cell, 10 fields are counted in the field count, 250 ml of water are filtered, 15 ml is the volume of the concentrate, and the cell holds 1 ml and is 1 mm deep, the formula becomes:

$$\text{Field-count factor} = \frac{1000 \times 15}{10 \times 250} = 6$$

In the survey count the entire cell is examined and 1000 fields are therefore covered. If the other data are the same as those above, the formula becomes:

$$\text{Survey-count factor} = \frac{1000 \times 15}{1000 \times 250} = 0.06$$

Multiplication of the results of the field and survey counts by the respective factors gives the approximate actual volume of each organism in 1 ml of water; this can be converted to volumetric standard units per litre by multiplying the factor by 1000.

Record the volumetric standard units per ml of sample for each species observed in the survey and field counts. The sum of these figures represents the total number of organisms expressed in volumetric standard units per ml of sample. The volume of amorphous matter may be computed and recorded in a similar way.

3.4.2 Number of organisms

The following calculation is an example of the procedure used when reporting in terms of numbers of organisms per unit volume. It is designed to give the number of organisms per litre rather than per ml. (Results may also be reported in terms of volumetric standard units by a slight change in procedure.)

Record the field-count total; this is the total number of organisms found in 5 fields. Calculate the factors needed to convert the survey count,

strip count, and field count to numbers of organisms per litre of original unconcentrated sample. This is accomplished in the following way:

(1) *Survey count*. The entire area of a Sedgwick-Rafter counting-cell or of several cells is examined. If the contents of one cell are examined and counted, the factor is obtained as follows:

$$\text{Factor} = \frac{\text{ml of concentrate} \times 1000}{\text{ml of original sample}}$$

(2) *Strip count*. One or several strips may be examined. If one strip is used, then:

$$\text{Factor} = \frac{\text{area of cell}}{\text{area of strip examined}} \times \frac{\text{ml of concentrate} \times 1000}{\text{ml of original sample}}$$

(3) *Field count*. The total number of organisms in 5 fields is multiplied by a factor obtained as follows:

$$\text{Factor} = \frac{\text{number of fields in counting cell}}{\text{number of fields counted}} \times \frac{\text{ml of concentrate} \times 1000}{\text{ml of original sample}}$$

Annex 6

MEMBERSHIP OF THE STUDY GROUPS

Study Group on International Standards of Drinking-Water Quality

Geneva, 11-16 June 1956

Members :

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Regional Study Groups on Drinking-Water Standards

EASTERN MEDITERRANEAN REGION

Alexandria, Egypt, 23-24 November 1955

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European Region

Geneva, 12-17 March 1956 *

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* This study group met three times; the date given is that of the last meeting.

SOUTH-EAST ASIA AND WESTERN PACIFIC REGIONS

Manila, Philippines, 15-27 April 1956

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