Long-term Programme for Pollution Monitoring and
Research in the Mediterranean Sea
(MED POL Phase II)

MICROBIOLOGICAL METHODS FOR COASTAL WATER
QUALITY MONITORING

Third report on the joint WHO/UNEP meeting

Tunis
12-16 November 1984

Note

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PREFACE

Within the framework of the Plan of Action for the Mediterranean Sea, adopted at Barcelona in February 1975 by the Coastal States of the Region, and under the terms of Article 10 of the Convention for the Protection of the Mediterranean Sea against Pollution, the contracting parties undertook to establish, in close cooperation with the competent international organizations, a system of pollution monitoring for the Mediterranean Sea region.

The details of this monitoring programme were adopted by the contracting parties at their second ordinary meeting (Cannes, 2-7 March 1981), within the framework of the Long-term Programme for Pollution Monitoring and Research in the Mediterranean Sea (MED POL Phase II), which was adopted at the same meeting. The majority of the Mediterranean countries have already submitted their national monitoring programmes, or are in the course of completing preparation of these programmes, which comprise the monitoring of various bacteriological parameters in coastal waters used for recreational purposes and also in water used for shellfish farming.

To promote the standardization of methods and to ensure the comparability of results, reference methods for sampling and analysis have been prepared for the use of Mediterranean laboratories participating in this programme. The World Health Organization and the United Nations Environment Programme have developed, tested and reviewed methods of determination of total coliforms, faecal coliforms and faecal streptococci in seawater by the membrane filtration culture (MF) method and determination of faecal coliforms in bivalves by the multiple test-tube method – the most probable number (MPN) method. Reference methods for the determination of total coliforms, faecal coliforms and faecal streptococci in seawater by the multiple test-tube methods are at present being developed and will be available shortly.

To recheck the comparability of results and of quality control operations, a series of intercalibration exercises was commenced in 1983, after completion of a preparatory exercise in Rome in 1982. These exercises were attended by specialists responsible for the monitoring programme from the host country's laboratories and also from a number of laboratories in other countries. They have been held alternately in the English and French languages and combined with consultation meetings to analyse the results obtained and discuss matters relating to the MED POL programme.

The first exercise in the series (in French) was held in Barcelona from 7 to 11 November 1983 and the second (in English) in Athens from 25 to 29 June 1984. The present exercise (in French) has been organized by WHO and UNEP, in collaboration with the Institut Pasteur, Tunis, within the framework of Phase II of the MED POL programme and of other Tunisian monitoring programmes. Like its predecessors, it was mainly intended to enable participants to determine the bacteriological parameters in identical seawater samples, using the recommended methods, finalized after the Rome meeting in November 1982. These methods were:

- determination of total coliforms in seawater by the MF method (UNEP/WHO, Reference Methods for Marine Pollution Studies, No. 2, Rev. 1);

- determination of faecal coliforms in seawater by the MF method (UNEP/WHO, Reference Methods for Marine Pollution Studies, No. 2, Rev. 1);

- determination of faecal streptococci in seawater by the MF method (UNEP/WHO, Reference Methods for Marine Pollution Studies, No. 4, Rev. 1);

- determination of faecal coliforms in bivalves by the MPN method (UNEP/WHO, Reference Methods for Marine Pollution Studies, No. 5, Rev. 1).

In view of the need for an exhaustive comparison of the two principal methods of bacteriological analysis used, namely the MF and MPN methods, a further aim of the exercise was to make parallel determinations of the total coliforms, faecal coliforms and faecal streptococci in seawater by both methods, using the same samples.

It was also proposed that the consultation meeting should:

- review the results of this exercise and those of previous ones in the series;

- discuss the microbiological parameters used for monitoring the sanitary quality of coastal waters, and the methods for their determination and interpretation;

- formulate any necessary recommendations for subsequent exercises in the series;

- make appropriate recommendations on relevant aspects of the long-term monitoring and research programme.

Representatives of Tunisian institutes participating in the microbiological monitoring process within the framework of MED POL Phase II and other Tunisian monitoring programmes were invited to take part in the intercalibration exercise and consultation meeting; representatives of institutes in other Mediterranean countries participating in MED POL Phase II (Algeria, France, Italy, Morocco and Yugoslavia) were also invited to participate. To facilitate the eventual application of the reference methods in other regions, representatives from two non-Mediterranean institutes, situated in Portugal and West Africa, were also invited to take part in the exercise and consultation meeting.

The following organizations and institutions were also invited to send representatives; the Food and Agriculture Organization (FAO), the United Nations Educational, Scientific and Cultural Organization (UNESCO), the Intergovernmental Oceanographic Commission (IOC), the World Meteorological Organization (WMO) and the International Atomic Energy Agency (IAEA).

1. Opening of the meeting (agenda item 1)

The intercalibration exercise and consultation meeting, organized by WHO and UNEP, in collaboration with the Institut Pasteur, Tunis, was held from 12 to 16 November 1984. It was attended by 26 temporary advisers from Tunisian institutes, from other Mediterranean countries and from Portugal. The WHO Regional Office for Europe also sent a representative. A list of participants is attached as Annex 4.

Dr L.J. Saliba, Senior Scientist, Coordinating Unit of the Plan of Action for the Mediterranean, WHO Regional Office for Europe, declared the consultation meeting open in the name of Dr Leo A. Kaprio, WHO Regional Director for Europe, and of Dr M. El Gezairy, WHO Regional Director for the Eastern Mediterranean. After listing briefly the work done within the framework of the MED POL programme which had led up to the present exercise, he paid tribute to the activities undertaken and the facilities provided by the Institut Pasteur. He pointed out with satisfaction that this was the first exercise within the framework of the MED POL programme to take place in the North African country and that, again for the first time, the meeting had the honour of being welcomed by the Minister of Health in person.

Professor A. Chadli, Director of the Institut Pasteur in Tunis, welcoming the participants, said that the meeting was a landmark in the campaign which many countries were waging against the pollution of the seas in general and of the Mediterranean Sea in particular. Mr M. Mzali, Prime Minister and Minister of the Interior of Tunisia, had shown his close interest in the work of the committee by accepting the appointment of honorary president of the meeting. After reminding participants that the wellbeing and the physical and moral health of the Tunisian people were one of the main concerns of the Tunisian head of state, he described briefly the characteristics of the Mediterranean Sea and its present degree of pollution, after which he described the activities of the Institut Pasteur in the field of public health and in particular within the framework of the MED POL project, in many cases in collaboration with other Tunisian institutes. In conclusion, he wished the intercalibration exercise all the success that the participants were entitled to expect.

Mrs S. Lyagoubi-Ouahchi, Minister of Public Health, also extended a welcome to the participants. She said that it was indeed auspicious that a meeting of this nature should be held for the first time in a North African country and that the Institut Pasteur in Tunis, which had been a regular and active participant since 1976 in the meetings held in various countries in the Mediterranean Region, should have been chosen to host this exercise. She thanked WHO and UNEP for their assistance in holding these meetings and pointed out that the choice of the Institut Pasteur brought out clearly the continuing efforts of the Tunisian Government to institute a two-way collaboration between the northern and southern coasts of the Mediterranean Sea in the field of scientific research, for the greater benefit of all. Environmental problems were also her concern as Minister of Public Health. Her Government attached the very greatest importance to the resolute pursuit of a higher standard of sanitary hygiene in general, with the aim of achieving more effective health protection for all Tunisian citizens, since the human being was the essential element in national development policy. In conclusion, she thanked Professor Chadli and his team for their work in organizing this exercise at the Institut Pasteur and wished the meeting all success.

2.   Scope and aims of the meeting (agenda item 2)

     Dr Saliba described briefly the scope and aims of the intercalibration exercise and consultation meeting.[a] He emphasized the importance of following strictly the instructions prepared for the determination of the various parameters, so as to ensure comparability of the results obtained.

3.   Election of officers (agenda item 3)

     Professor Chadli was elected Chairman, Dr A. Nejjar Vice-Chairman and Dr P. Bernard Rapporteur. Dr Saliba acted as Secretary of the meeting.

4.   Adoption of the agenda (agenda item 4)

     The provisional agenda was adopted unanimously.

5.   Organization of the programme of work (agenda item 5)

     The Chairman explained the programme of practical work. The instructions given to the participants relating to the organization of the work are attached at Annex 2.

6.   General discussion on the bacteriological parameters used for monitoring the sanitary quality of coastal waters, and on the methods for their determination and interpretation (agenda item 6)

     Professor S. Jekov presented a paper entitled "The comparability of the MF (MF) and multiple test-tube (MPN) methods for determining total coliforms, faecal coliforms and faecal streptococci in seawater". This paper, which was greatly appreciated by all the participants, is reproduced at Annex 1.

7.   Review of the results of previous meetings (agenda item 7)

     The reports of the intercalibration exercises held in Rome from 22 to 26 November 1982 and in Barcelona from 7 to 11 November 1983 were presented to the participants in their final form. The summary report of the exercise held in Athens from 25 to 29 June 1984 was also circulated to the participants. The results obtained in these exercises were described briefly.

8.   Analysis of results of the present exercise (agenda item 8)

     The results of the present exercise are given later in the report (Annex 3).

     Comparison of the analyses of the bacterial concentration in a single seawater sample (three different types of seawater having been analysed) by the MF and MPN methods yielded satisfactory results, allowing for the human factor. There were, however, reservations about comparing a method which had been tested 24 times during the present exercise (MPN method) with one tested only eight times (MF method).

     The results of the MF method as applied to a single seawater sample were similar for all participant groups, although two groups obtained differing results for total coliforms and faecal streptococci. This can only have been due to individual variation.

     The reproducibility of the MPN method was found fully satisfactory.

     The following comments were made by the participants during the exercise:

-   some difficulties arose over the reading and interpretation of the number of total coliform colonies on Endo agar and over the faecal streptococcal count on Enterococcus agar by the MF method;

-   the MPN method was easier to interpret than the MF method;

-   some countries found the cost of membrane filters to be high;

-   WHO and UNEP should notify participating laboratories of the reference methods one month before the exercise, at the same time circulating the reports of preceding exercises.

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[a] Document ICP/CEH 001/m03/2.

9. Future action and recommendations (agenda item 9)

In addition to the individual recommendations on items of the agenda, the participants made the following general recommendations.

(1) The series of intercalibration exercises on microbiological reference methods for monitoring the quality of coastal waters should be continued on a regular basis. A new programme should start at the beginning of 1986. This programme should include other microbiological indicators of pollution.

(2) On the basis of the results obtained in the last two exercises (Athens, 25-29 June 1984, and Tunis, 12-16 November 1984), a significant correlation is evident between the MF method and the MPN method. Consequently, the MPN method should also be recommended as a reference method for determining the degree of pollution of coastal waters. In this regard, the definitive versions of the reference methods for determining total coliforms, faecal coliforms and faecal streptococci in seawater by the MPN method should be completed as soon as possible.

(3) It was desirable that other methods be developed for determining pathogenic organisms and pollution indicator organisms in wastewater.

(4) In the research component of MED POL Phase II, the activities dealing with epidemiological studies that correlate the bacteriological quality of seawater with health effects should be extended. In this regard, the participants noted the intention of WHO/UNEP to convene a consultation meeting on this subject in 1985 and stressed the necessity of developing a standard protocol to render comparable future work, which should be carried out on the widest possible basis.

Annex 1

COMPARABILITY OF THE MEMBRANE FILTRATION CULTURE (MF) AND MULTIPLE
TEST-TUBE (MPN) METHODS OF DETERMINING THE CONCENTRATION OF TOTAL
COLIFORMS, FAECAL COLIFORMS AND FAECAL STREPTOCOCCI IN SEAWATER

by
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Before describing the basic criteria used for comparison of the two methods, the membrane filtration culture (MF) and the multiple test-tube (MPN) methods (MPN = most probable number) for the detection and simultaneous counting of coliforms and enteroccoci (faecal streptococci) in seawater, it is useful to give the historical background of the development of colimetry:

- the use of these microorganisms as faecal pollution indicators;

- the diversity of views on the sanitary interpretation, let alone the significant values in epidemiological prognosis, of the different taxonomic species of coliforms;

- the correct choice of the most suitable method of determining coliforms in a seawater medium.

In earlier times, although the bacterial origin of infectious diseases had not yet been discovered, it was already known that faecal pollution of watercourses was a contributory factor to the propagation of cholera and typhoid epidemics among populations living along the banks.

At a later stage, when the natural process of the mineralization of organic matter and its end products (nitrites, nitrates and chlorides) became known, it was accepted that research workers could use specific faecal pollution indicators to reveal the insalubrious properties of water. At the same time, laboratory procedures for the detection of these chemical parameters were being developed and implemented in sanitary control systems.

In 1881, T. Escherich isolated from the stools of adults and children a microorganism which he designated Bacterium coli commune.

Since this microorganism was detected consistently and in large numbers in human stools and in the excreta of warm-blooded animals, the conclusion was drawn that human and animal organisms were its only natural hosts.

B. coli commune was therefore regarded as the principal bio-indicator of faecal pollution in view of its specificity and sensitivity to the chemical parameters referred to above.

In addition to these two criteria (specificity and sensitivity), the bio-indicator must have a longer survival time than any pathogens that may be present in the faecal material responsible for pollution of the water.

At that time, only some strains of Shigella, Salmonella and vibrios had a comparable survival time to that of B. coli commune, and it was generally accepted that B. coli commune survived longer in water than pathogenic microorganisms. Therefore, in addition to its usefulness as an indicator of faecal pollution, B. coli commune was in general regarded as an indirect indicator of the possible presence of pathogens in polluted water.

Vincent (1889), Eijkman (1904) and Bulir (1907) then developed multiple tube culturing processes for the determination of B. coli commune in soft water (for coliforms, Vincent used a selective broth with the addition of 0.85% phenol).

Subsequently, however, other research workers (Levine, 1921; Koser, 1926) discovered in the soil, and even in plants, bacteria identical with B. coli commune in terms of their morphology and the biochemical criteria used by Escherich.

These workers believed B. coli commune to be ubiquitous in nature, so that there was no reason to regard it as a specific faecal pollution indicator.

After the introduction of new differential parameters, strains isolated from the soil and identified as B. coli commune were found on application of the "citrate" test to be citrate-positive, whereas those derived from human stools were constantly citrate-negative.

The ability of the microorganisms to make use of citrates introduced into the culturing media (Koser broth or Simmons agar) as a sole source of carbon came to be used subsequently as a means of classifying B. coli commune strains into two categories: citrate-negative coliforms which are normally found in the intestinal microflora and therefore signify faecal pollution, and citrate-positive coliforms, which belong to the normal microflora of the environment and in consequence have no sanitary significance.

This classification into two categories - citrate-negative and citrate-positive - was a first attempt at a sanitary classification of coliforms. After testing a wider range of biochemical criteria, including in particular the IMVIC tests,[a] it was found that two clearly distinguishable biotypes existed within these two coliform categories.

In their taxonomic classification, coliforms belong to the Enterobacteriae family. They make up the tribe of Escherichia, within which they are divided into genera (Escherichia, Citrobacter, Enterobacter, Klebsiella and Levinea (not universally accepted)), and then into species.

The classification is being continuously modified, however, in respect of the taxonomic nomenclature and the distribution of coliforms into genera and species. This lack of precision is responsible for the confusion surrounding the sanitary interpretation of coliforms. The different principles on which current laboratory techniques have been based have also contributed to the state of confusion.[b]

It is worth remembering in connection with coliform determinations that the word "coliform" is not a taxonomic term. It is currently used to designate all intestinal bacteria belonging to the tribe Escherichia, which share the characteristics of being gram-negative bacilli, non-sporulating, most frequently mobile and metabolizing lactose with a release of gas at 37°C.

Faced with the taxonomic diversity of the coliforms, at the practical level, attention has to be concentrated on the species Escherichia coli (B. coli commune discovered by Escherich), Citrobacter freundii and Enterobacter aerogenes.

Over and above the taxonomic classification of coliforms, sanitary monitoring, which is interested in coliforms solely as faecal pollution indicators, makes use of a so-called "sanitary" classification, the first version of which (referred to above) distinguished between two coliform categories: the citrate-negative category (represented by E. coli) and its biotypes, which are of faecal origin, and citrate-positive coliforms which are only found in the environment.

At the present time, after intensive research and many years of intensive argument between research workers, the sanitary classification of coliforms still consists of two categories or, to be more exact, two tests, which are used for the detection of faecal coliforms and total coliforms, distinguished as in the initial classification on the basis of a single criterion: their ability to cause lactose fermentation at 44.5°C.

It is accepted (according to the American school) that coliforms, the representative species of which is Escherichia coli, are undoubtedly of faecal origin. The definition of faecal coliforms[c] is based on their common characteristics of being gram-negative, aerobic and facultative anaerobic bacteria, in the form of bacilli, non-sporulating and capable of fermenting lactose with gas production in less than 24 hours in cultures at 37°C and 44.5°C.[d] They produce indole in tryptonated water (containing tryptophan) when cultured at 44.5°C. In some laboratories, coliforms which are indole-negative at 44.5°C are also regarded as "faecal" in

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[a] The IMVIC tests (I = indol, M = methyl red, V = Voges-Proskauer, IC = sodium citrate) are only useful at the present time for identifying genera but not coliform species.

[b] It is to be hoped that current studies on coliform classification based on new criteria and more sophisticated methods, possibly including a numerical, genetic and molecular taxonomic system, will produce a more suitable classification for monitoring a marine medium and more consistency overall.

[c] United Nations Environment Programme. Determination of faecal coliforms in bivalves by the multiple test-tube method. Reference methods for marine pollution studies, No. 5, Rev. 1, 1983 (in cooperation with WHO).

[d] In the MF method, the differential criterion for coliforms (lactose degradation, manifested by acidification followed by a release of gas) has been reduced to the parameter "acidification".

routine practice.[a] Other laboratories consider the terms "E. coli" and "faecal coliforms" as synonymous. In our own experience, the need to differentiate E. coli can only be met by the IMVIC tests, in which case indole production at 44.5°C is an obligatory criterion.

The total coliform test reveals both faecal coliforms and coliforms which are not capable of fermenting lactose at 44.5°C, but research workers are not agreed on the interpretation of the total coliform test for sanitary purposes.

To reconcile current definitions and interpretations, which often differ considerably, the authors of the International standards of drinking water (Geneva, World Health Organization, 1962) accepted that all coliforms might be of faecal origin[b] so that their presence is deemed to indicate pollution.

We may quote Leclerc (1984) in this connection, who states that high-temperature tests to reveal the presence of a microorganism population or group, which are significant from the hygiene point of view, are:

(a) significant in the sense that the bacteria detected (at 44°C) are in most cases E. coli or bacteria of definitely faecal origin;

(b) restrictive and relative in the sense that many faecal intestinal bacteria are probably incapable of multiplying at this temperature; this involves accepting the existence of coliforms which are incapable of multiplication at 44°C, without disputing their faecal origin.

According to Farmer (1977), only 20% of all intestinal bacteria of faecal origin can be identified as a known species and 30% as a given genus without specifying the species, whereas 50% escape classification altogether.

Coliforms detected in the total coliform test, the faecal origin of which is disputed, are at present attributed to an earlier faecal contamination, which is less important from the point of view of epidemiological prognosis, whereas faecal coliforms are regarded more particularly as indicators of a recent faecal contamination or, in more exact terms, of a contamination by fresh faecal material.[c]

Colimetry (coliform determination) in a marine medium

The theoretical concepts, methods of analysis and forms of sanitary interpretation of coliforms, used as faecal pollution indicators, constitute a special field in microbiology of the medium (sanitary microbiology), known in particular as colimetry.

The aim originally was to improve sanitary control, and the relevant methods were designed and tried out in freshwater.

Later, these principles and tests, developed for colimetry in freshwater, were applied directly in a marine medium, without taking account of the fact that the latter is a highly dynamic and highly diversified ecosystem, for which there is no possible basis for comparison with the human biotope of coliforms or the conditions of life which the latter experience in the continental environment.

There are therefore some weaknesses in colimetry as currently used for monitoring the marine environment, especially seawater at seaside resorts.

On introduction into the marine environment, in order to survive, coliforms have to adapt to conditions completely different from their natural habitat and modify their metabolism to fit in with the marine ecosystem. New characteristics may then appear or one or more characteristics considered to be specific to the species may disappear, e.g. the loss (by E. coli) of the ability

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[a] Modification of the Eijkman thermophile tests at 46°C.

[b] This practice has been confirmed in our experience, since we have detected in stools sent to the laboratory for routine examination, in parallel with E. coli, the presence of citrate-positive coliforms in 40% of the samples.

[c] In the normal version of the MF method, the two tests may be distinguished in the following way: total coliform count/100 ml at 37°C for the total coliform test and total coliform count/100 ml at 44°C for the faecal coliform test.

to ferment lactose and produce indole at 44°C.[a] These are provisional adaptations, and sooner or later the microorganisms degenerate and die. This may explain the variability and instability of the biochemical characteristics of coliform strains isolated directly from the marine environment.

The interpretation of sanitary and epidemiological purposes, which has been based at present on survival in freshwater and on comparisons with certain microorganisms of the <u>Shigella</u> or <u>Salmonella</u> genus and certain vibrios, is no longer in line with the present state of knowledge in epidemiology and microbiology.

It is now known that faecal contamination introduces into the marine environment not only pathogenic intestinal bacteria, but also the agents responsible for various viral diseases (<u>Poliovirus</u>, the virus of infectious hepatitis B, the <u>Coxsackie virus</u>, etc.) and also of parasitic diseases, the <u>survival time of which is undoubtedly longer than that of E. coli</u>, the coliform species recognized today as the main faecal pollution indicator. <u>In consequence, absence of E. coli and faecal coliforms in general (during sanitary monitoring) in no way signifies that seawater is free from pathogenic agents, nor is the E. coli determination, including the faecal coliform test in any way a better method, in terms of sensitivity and specificity, of measuring the quality of bathing water than the total coliform test.</u>

In our experience, seawater samples taken at points remote from any source of pollution have been found to be free of coliforms;[b] in other words, if coliforms are detected in seawater, their presence may be regarded, irrespective of the species, as an indicator of pollution of man-made origin; we understand this term to mean the pollution of the marine environment by wastewaters, in a broader sense than faecal contamination. Coliforms in the marine environment are in fact transmitted by wastewaters (drainage waters, river waters receiving wastewaters and waters flowing through urban zones) which carry, together with pathogenic microorganisms, physical and chemical pollutants. Travellers and ships' crews may be responsible for part of the faecal load on the marine environment. This load may, however, be calculated on the basis of the number of travellers, by the equivalent inhabitant/day test.

Since at the present time industrial wastes are collected and in most cases discharged on the "everything down the drain" principle through the public sewer system, coliforms may be used not only to assess the risks of infectious diseases transmitted by seawater, but also as an aid to sanitary engineering, since they help us to follow the diffusion of wastewater and to establish the path followed by chemical contaminants carried by wastewaters. Colimetry can help in assessing the self-purification capacity of the marine environment and in siting a drainage outfall; it may even be used as an aesthetic parameter.

These different applications of coliform determination raise the question of which of the two indices (MPN/100 ml) (total coliforms or faecal coliforms) is the more specific and more sensitive indicator for the purposes listed above.

Before considering priorities and the disadvantages inherent in the two methods for comparison, the point has to be made that the MPN method has not yet been standardized. There are various modifications of the method with different sensitivities. It is therefore essential to know in advance which of these modifications is to be compared with the MF method. In the first place, the MPN method makes use in its preliminary phase of different lactose broths, three of which are particularly recommended: lactose broth, free from inhibiting components; MacConkey broth, with the addition of sodium taurocholate as inhibitor; and brilliant green lactose bile broth, the bile and the brilliant green being added as inhibitors. These two latter are therefore regarded as selective broths in relation to coliforms.

We have already pointed out that the marine environment does not favour coliform survival. Their vitality decreases progressively and the least resistant individuals pass into a state of "stress". If cultured in broths with brilliant green and bile salts, the inhibitor concentrations also exert an inhibiting action on the coliforms (Table 2), whereas broth with the addition of lactose favours the resuscitation of coliforms under "stress" in the same way as the tryptonated water used as a pre-enrichment medium in food analysis techniques.

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[a] This faculty has not yet been adequately studied, but it has to be regarded as an ability to adapt. <u>Escherichia coli</u> psichrophiles have been discovered (by Leclerc) which are capable of growing at 4°C.

[b] A study of the visceral microflora of fish caught in the open sea has shown to be free of coliforms.

To establish which of the three liquid media rendered the MPN method most sensitive, we determined in 1982, within the framework of the MED POL Phase II project, the MPN/100 ml for total coliforms, faecal coliforms and E. coli, using simultaneously the three broths referred to above.

The study was carried out on 72 seawater samples, consisting of three groups of 24 samples each taken in three different stations (one heavily polluted by outfalls of wastewaters, the second moderately polluted and the third assumed to be relatively clean). The samples were chosen on the basis of routine monitoring data.

The samples were inoculated in series of five 10 ml, 1 ml and 0.1 ml tubes for each of the three lactose broths in the presumptive phase of the MPN method (in the confirmatory phase, culturing was carried out at 44°C on brilliant green lactose bile broth and in tryptonated water).

Comparison of the MPN values obtained for coliforms (total, faecal and E. coli) for each of the three lactose broths in each sample enabled the lactose broths to be classified into three sensitivity levels: high, medium and limited.

The highest sensitivity level was found with the lactose broth,[a] for which the highest MPN values were found, i.e. the lactose broth proved to be the most sensitive.

The results of this evaluation are shown in Tables 1 and 2, from which it is evident that the "high sensitivity" percentages for total coliforms, faecal coliforms and E. coli are significantly higher in the case of the broth with a lactose additive. This broth was therefore more sensitive and of higher quality than the other two. The percentages in Table 2, relating to the brilliant lactose bile broth, clearly indicate its inhibiting action on coliforms.

In the second phase of our study, we compared the sensitivity of the MPN technique using lactose broth with that of the MF technique using MF agar. The results are shown in Table 3.

The overall results appear to indicate that the MPN method using lactose broth is a more sensitive means of determining the faecal pollution of a marine zone.

The bacteriological parameters relating to the two methods have already been discussed during the intercalibration exercise and consultation meeting in Rome (WHO/UNEP, 22-26 November 1982). After discussion of the comparability of the two methods at that meeting, neither method was found to be superior to the other. Each one had advantages and disadvantages. This finding was in agreement with the data in the relevant literature.

The results of the intercalibration exercises held at Rome (in 1982) and at Barcelona (in 1983)[b] cast no further light on the comparability of the two methods.

The different working groups used the MF method for determination of the coliform and enterococcal concentrations in the three types of seawater sample (A1, B1 and C1). The results are accordingly only significant for verifying the precision and sensitivity of the MF method, applied to a single sample by different working groups.

On this basis, the concentration ranges in colonies per 100 ml in the intercalibration exercise in Rome and even more so in the Barcelona exercise (75 000 000, 45 000 for total coliforms; 4 000 000, 12 900 for faecal coliforms; and 1 730 000, 57 000 for enteroccoci) show the MF method to be insufficiently exact provided, of course, that the method was correctly carried out by all experimenters. A similar calibration of the MPN method is obviously necessary for comparison of the precision of the two methods.

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[a] For coliform determinations in the marine environment, we use lactose broth with the addition of bromothymol blue as a pH indicator of lactose acidification without the release of gas. This medium, which yielded very encouraging results, has the advantage of enabling cultures from the presumptive phase of the MPN method to be used for determining the MPN/100 ml of faecal streptococci by reinoculation on Slanets agar-agar or on a potassium tellurite agar-agar (in the confirmatory phase).

[b] See "Report on a joint WHO/UNEP meeting", Barcelona, 1983.

Table 1. Comparison of the sensitivity (in %) of three lactose broths
in the determination of the Escherichia coli concentration in the analysed samples

Type of broth used	MPN/100 ml total coliforms	MPN/100 ml faecal coliforms	MPN/100 ml Escherichia coli
Lactose	79.41	71.01	76.81
MacConkey	41.17	49.27	46.37
Brilliant green broth	19.11	30.43	40.57

Table 2. Comparison of the sensitivity (in %) of the multiple test-tube technique
depending on the type of lactose broth used for the determination of the MPN of coliforms

Type of lactose broth	Sensitivity								
	MPN/100 ml total coliforms			MPN/100 ml faecal coliforms			MPN/100 ml Escherichia coli		
	High	Medium	Limited	High	Medium	Limited	High	Medium	Limited
Lactose	79.41	14.71	5.88	71.01	24.63	2.89	76.81	20.27	2.89
MacConkey	41.17	47.11	11.76	49.27	28.98	21.73	46.73	33.33	20.28
Brilliant green broth	19.11	45.58	35.29	30.43	47.82	21.73	40.57	43.47	15.98

Note. A single sample was examined on each of the three lactose broths and compared in three MPN values obtained in relation to each test.

Table 3. Comparison of sensitivity (in %) of the two techniques in relation to
the numbers of samples analysed

Type of sample	MPN technique superior to the membrane filtration technique	MF technique superior to the MPN technique	Equivalence of the two techniques
Clean	42.80	33.33	23.8
Contaminated	70.58	29.41	0.0
Polluted	87.50	12.50	0.0

Disadvantages and advantages of the MPN method (multiple test-tube technique on lactose broth) in relation to the MF method

1. Disadvantages

The MPN method was originally designed as a means of assessing the "colititre" test - based on the smallest volume of analysed water in which the presence of coliforms is detected.

Subsequently, this method was artificially adapted to become the MPN test by mathematical conversion of the results of the coliform determination (colititre), using statistical tables[a] to determine the MPN as a function of the results observed.

The position is therefore that the MF method enables a direct determination of the coliform concentration to be made by counting the colonies, whereas in the MPN method the concentration is arrived at by statistical means.

Where systematic control checks are required on seawater samples from sectors believed to be free from faecal pollution and suspended matter, the MPN method is technically less rapid and requires more equipment (stove, laboratory benches), space and staff than the MF method.

2. Advantages

It is possible to obtain simultaneously, from the presumptive phase onwards, the results of the three MPN tests: for total coliforms, faecal coliforms and E. coli.

The MPN determination can be carried out on constant inoculation volumes (5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml), irrespective of the assumed level of faecal pollution in the seawater for analysis.

The method has a higher technical specificity, being based on the ability, specific to coliforms, to ferment lactose with a release of gas, whereas the MF method is based solely on lactose acidification.

A better bacterial cell "regeneration" is obtained (in the case of coliforms under "stress").

Seawater samples rich in suspended matter can be more easily analysed.

The method can be applied in all laboratories, the apparatus and glassware (stove, stands, graduated pipettes, test-tubes, etc.) being available in any operational laboratory.

The method can be used without the need for specialized materials (filtration membranes, special apparatus, etc.).

Reading off is easier and yields more standardized results. The method is also less time-consuming, especially in the case of samples taken in coastal sectors with an outfall of wastewaters, where several filtration membranes would have to be used.

The method is less costly, especially for countries requiring to import filtration membranes. "The MPN technique is the method of choice mainly because one does not know in advance the level of pollution and in consequence the number of filtration membranes which it will be necessary to use" (Leclerc, 1984).[b]

Conclusions

In sanitary hygiene monitoring, the finding of faecal pollution is only a presumptive stage, an alarm signal, necessitating subsequent investigations to establish the origin of the pollution, to track down the mechanisms of transmission and establish its path and survival range, so as to be able to work out and plan measures to "clean up" the marine environment.

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[a] These tables are not complete since they do not include results for a number of combinations which are designated "paradoxical cases" or "false reactions".

[b] During the intercalibration exercise at Barcelona (1983), coliform determinations by the MF method necessitated the filtration of 520 ml of each of six dilutions of each type of seawater, so that a large number of membranes had to be used. Geldreich (1975) reduced this number to three, corresponding to three different selected volumes on the basis of the assumed degree of pollution.

E. coli has a short characteristic survival time and the MPN/100 ml test for faecal coliforms cannot in general meet the specific requirements.

The MPN/100 ml index of total coliforms appears to be more suitable for detecting and assessing the degree of man-made contamination. Differentiation of the coliform category is unnecessary with this index. All species, irrespective of the arguments with regard to their faecal or non-faecal origin, are equally capable of indicating man-made contamination, since the different types of coliform found in the marine environment all originate from effluent outfalls.

The MPN/100 ml test for total coliforms should therefore be preferred for colimetry in the marine environment.

The MPN method, using multiple test-tube culturing on lactose broths, is superior to the MF method for colimetry in a marine environment, whether for routine monitoring or specific investigations.

Annex 2

ORGANIZATION OF PRACTICAL WORK

1. Working groups

    Participants will be divided into three groups. Each group will be designated by a Roman numeral (group I, group II, group III, etc.). The members of each group will be distinguished by an Arabic figure (1, 2 and 3). Participants' place and group numbers as shown on the laboratory benches must not be altered throughout the intercalibration exercise.

2. Samples

    Each participant will find on his bench three 1 litre screw-top flasks containing three samples of coastal water having different pollution levels:

    - a sample "A" (red label) of highly polluted coastal water;
    - a sample "B" (yellow label) of moderately polluted coastal water;
    - a sample "C" (white label) of faintly polluted coastal water.

    Each group will also find on its bench a sample of bivalves, clams (tapes decussatus).

3. Laboratory equipment

    Each participant will find on his bench, or in his pigeon hole, the following equipment, intended for performance of the MPN test, using the multiple test-tube method:

    - three identical stands, each for 15 tubes, containing five tubes with concentrated lactose broth and ten tubes with normal lactose broth;

    - three identical stands, each for 15 tubes, containing five tubes of concentrated Rothe medium and ten tubes of normal Rothe medium.

    These test-tubes are to be used for analysis of the 3 seawater samples A, B and C, using MPN determinations of total coliforms, faecal coliforms and faecal streptococci (in the presumptive phase).

    - One stand for 15 tubes, containing five tubes of concentrated lactose broth and ten tubes of normal lactose broth.

    These tubes are to be used for the MPN determination of faecal coliforms in the bivalve sample.

    - 10 x 10 ml graduated sterile pipettes
    - 10 x 1 ml graduated sterile pipettes
    - one set of test-tubes with brilliant green lactose bile broth
    - one set of test-tubes with peptonated water
    - one flask of Kovacs reagent.

    The equipment listed below will be used for the confirmatory phase of the faecal coliform determination.

    - one batch of test-tubes with Litsky medium;

    - one set of Petri dishes containing agar-agar for enteroccoci (Enterococcus agar) for the confirmatory phase on faecal streptococci;

    - one set of Petri dishes containing Endo agar-agar for the confirmatory phase on total coliforms;

    - Pasteur pipettes for reinoculation; one set for the two groups on a single bench.

    Each group will be issued with the following equipment, to be used for the total coliform, faecal coliform and faecal streptococcal counts in samples A, B and C, using the MF method:

- equipment for the MF method, namely a vacuum pump and two filtration units (funnel and membrane support);

- sterile filtration membranes, packed separately;

- sterile graduated pipettes (3 x 50 ml, 3 x 20 ml and 20 x 10 ml pipettes);

- 60 Petri dishes containing different types of agar-agar: 20 Petri dishes with Endo agar-agar for membrane filtration culturing of total coliforms, 20 Petri dishes with MF agar-agar for membrane filtration culturing of faecal coliforms and 20 Petri dishes with Enterococcus agar for membrane filtration culturing of faecal streptococci;

- 24 x 90 ml flasks, each containing buffered water (green label) for use in preparing decimal dilutions (10^{-1}, 10^{-2} and 10^{-3}) of the water for analysis.

4. Labelling

Each participant will be required to mark on the tubes of liquid media and on the Petri dishes of agar-agar medium:

- his group number in Roman numerals (from I to VIII);

- his place number in Arabic figures (1, 2 or 3);

- information on the sample: A, B, C or bivalves;

- the volume of undiluted filtered water in ml - 50 ml, 20 ml or 5 ml;

- or the volume of diluted water - 20 ml and 5 ml - also indicating the degree of dilution: 10^{-1}, 10^{-2} and 10^{-3}.

5. Procedure

Monday, 12 November 1984: p.m.

The participants will carry out determinations of the total coliforms, faecal coliforms and faecal streptococci, using the MPN method on the three samples A, B and C. They will also determine the MPN of faecal coliforms in the bivalve sample.

Tuesday, 13 November 1984: a.m.

Each group will carry out total coliform, faecal coliform and faecal streptococcal counts using the MF method on samples A and B in accordance with the following scheme.

In each group:

participant 1	sample B	total coliforms
participant 2	sample B	faecal coliforms
participant 3	sample B	faecal streptococci

In each group:

participant 1	sample A	faecal coliforms
participant 2	sample A	faecal streptococci
participant 3	sample A	total coliforms

Tuesday, 13 November 1984: p.m.

In each group:

participant 1	sample C	faecal streptococci
participant 2	sample C	total coliforms
participant 3	sample C	faecal coliforms

All the participants are requested to refer to the general programme for activities on Wednesday, 14 and Thursday, 15 November 1984.

6. <u>Recording of the results</u>

For recording their results, the participants will be provided with the following two forms and with a table for calculating the MPN per 100 ml coastal water:

- form 1 for recording the data on the MPN method;
- form 2 for recording the data on the MF method.

The procedure for determining the MPN of faecal coliforms in the bivalve samples will be found in Instruction 5, Rev. 1, page 10.

The table for calculating the MPN per 1 g bivalve flesh will be found in Reference Method 5, Rev. 1, page 12.

7. <u>Important notice</u>

Before starting their operations, the participants should read carefully Reference Methods No. 2, Rev. 1, No. 3, Rev. 1, No. 4, Rev. 1, and No. 5, Rev. 1, published by UNEP (Regional Seas) in 1983. These instructions are included in their folders.

On completion of each practical work session, the participants are requested to leave the test-tubes and Petri dishes which they have used in the laboratory washing unit.

Annex 3

RESULTS OF THE INTERCALIBRATION EXERCISE

Introduction

The aim of the exercise was to enable participants to carry out actual determinations of bacteriological parameters by uniform methods, using identical seawater samples, and to compare the results obtained (a) between individuals for each parameter, and (b) between the two methods (the MF and MPN methods) in relation to the relevant parameters.

Organization and method

The participants were divided into eight groups of three workers. Three seawater samples were analysed by the MF method, which was used by each group, and by the MPN method, which was used by each participant individually; the samples were as follows: sample A of heavily polluted coastal water, sample B of moderately polluted coastal water, and sample C of coastal water polluted to a slight extent only. Determination of the total coliforms, faecal coliforms and faecal streptococci was carried out on each sample. In addition, the MPN method was used by each group for the analysis of a bivalve sample (tapes decussatus clams) originating from a heavily polluted environment.

Participants used Reference Methods Nos 2, 3, 4 and 5 for determination of the bacterial concentrations in seawater by the MF method and in the bivalves by the MPN method. For determinations in seawater with the MPN method, participants used methods devised by the Institut Pasteur on the basis of the 13th edition of Standard methods for the examination of water and wastewater (American Association of Public Health).

Results and discussion

The results obtained by participants in their determinations of total coliforms, faecal coliforms and faecal streptococci in seawater samples are presented below in tabular form (Table 1). The comparability of the results obtained by different individuals may in general be regarded as satisfactory. The variation between the values obtained by some participants or some participant groups and the mean values recorded are doubtless due to the level of experience of each worker. Some participants were using the MF method for the first time. Another possible source of variation in results may have been the slight but clearly evident differences in the evacuation pressure of the apparatus used by the different groups.

The comparability of the results obtained for identical samples by the two methods may also be regarded as satisfactory, especially with regard to the interpretation of these results.

All the participants obtained identical results on the heavily polluted bivalves, namely a faecal coliform count of 2400 or more per 100 g.

Table 1. Bacterial counts per 100 ml seawater

	Total coliforms				Faecal coliforms				Faecal streptococci			
	MF	MPN			MF	MPN			MF	MPN		
Group		1	2	3		1	2	3		1	2	3
Sample A												
1	1200	1600	2400	2400	446	920	350	1600	50	240	17	540
2	2500	2400	2400	2400	500	2400	2400	2400	150	240	350	350
3	1250	2400	2400	2400	400	350	1600	1600	0	240	350	34
4	3200	–	2400	1600	900	–	2400	1600	500	170	280	927
5	1275	2400	2400	2400	400	2400	2400	2400	2000	1600	540	540
6	3300	920	920	350	500	920	920	200	350	920	2400	34
7	5400	1600	2400	1600	167	1600	1600	1600	258	350	540	350
8	2150	2400	350	350	1200	2400	350	350	0	540	12	170
Sample B												
1	1100	130	220	350	180	33	94	130	50	5	4	4
2	850	70	79	79	60	31	49	49	35	4	4	4
3	250	110	130	110	60	79	7	33	0	2	2	2
4	740	–	130	240	35	–	49	79	5	2	2	4
5	620	79	130	180	106	49	130	33	33	6	4	34
6	800	94	70	49	100	24	70	22	160	4	4	6
7	3460	130	110	356	105	130	49	79	7	2	2	4
8	660	220	350	350	375	110	26	33	0	4	2	4
Sample C												
1	35	11	14	140	9	8	2	21	3	2	2	2
2	14	17	23	17	0	2	5	11	5	2	2	2
3	2	17	23	17	0	13	2	7	0	2	2	2
4	8	–	27	13	2	–	2	0	0	–	2	2
5	523	170	9	11	97	130	9	6	57	2	2	2
6	150	17	33	17	15	13	13	5	12	2	2	2
7	30	11	11	23	0	7	4	2	0	2	2	2
8	24	17	17	22	10	5	2	2	0	2	2	2

Table 2. Calculation of the MPN/100 ml test in coastal water

Number of test tubes exhibiting a positive reaction, out of			MPN index per 100 ml	95% confidence limits	
5 x 10 ml tubes	5 x 1 ml tubes	5 x 0,1 ml tubes		Lower	Upper
0	0	0	2		7
0	0	1	2	0,5	7
0	1	0	2	0,5	7
0	2	0	4	0,5	11
1	0	0	2	0,5	7
1	0	1	4	0,5	11
1	1	0	4	0,5	11
1	1	1	6	0,5	15
1	2	0	6	0,5	15
2	0	0	5	0,5	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	0	1	17	5	46
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	3	0	27	9	80
4	3	1	33	11	93
4	4	0	34	12	93
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	110
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	150
5	2	0	49	17	130
5	2	1	70	23	170
5	2	2	94	28	220
5	3	0	79	25	190
5	3	1	110	31	250
5	3	2	140	37	340
5	3	3	180	44	500
5	4	0	130	35	300
5	4	1	170	43	490
5	4	2	220	57	700
5	4	3	280	90	850
5	4	4	350	120	1000
5	5	0	240	68	750
5	5	1	350	120	1000
5	5	2	540	180	1400
5	5	3	920	300	3200
5	5	4	1600	640	5800
5	5	5	2400		

Source: Standard methods for the examination of water and wastewater, 3rd ed. Washi..on, DC, AAPH, 1971.

Form 1

WHO/UNEP Intercalibration exercise

Date: ....

RESULTS
(MPN/100 ml)

Sample: ....   Group No.: ....   Participant No.: ....

Stages \ Volumes	10	10	10	10	10	1	1	1	1	1	0.1	0.1	0.1	0.1	0.1
Presumptive phase (lactose broth)															
Confirmation on Endo agar-agar at 37°C															
Confirmation on brilliant green broth at 44°C															
Confirmation with indole/peptonated water at 44°C															

(a) MPN/100 ml total coliforms: ....   (positive cultures formula ....)
(b) MPN/100 ml faecal coliforms: ....   (positive cultures formula ....)

Symbols to be used for recording the results:

0	Growth with release of gas
+	Growth (turbid) without release of gas
X	Sterile culture (no turbidity)
Δ	Positive indole culture
−	Negative indole culture

Form 2

WHO/UNEP Intercalibration exercise

Date: ....

RESULTS
(filtration membranes)

Sample: ....   Group No.: ....   Participant No.: ...

Volume of filtered water in ml	Dilutions used	Number of colonies per membrane		
		Total coliforms	Faecal coliforms	Faecal streptococci
50	1	....	....	....
20	1	....	....	....
5	1	....	....	....
20	10^{-1}	....	....	....
5	10^{-1}	....	....	....
20	10^{-2}	....	....	....
5	10^{-2}	....	....	....
20	10^{-3}	....	....	....
5	10^{-3}	....	....	....
Bacterial concentration in colonies/100 ml		....	....	....

Remarks:

Form 3

WHO/UNEP Intercalibration exercise

Date: ....

RESULTS
(MPN/100 ml)

Sample: ....   Group No.: ....   Participant No.: ...

Stages	Volumes	10	10	10	10	10	1	1	1	1	1	0.1	0.1	0.1	0.1	0.1

Presumptive phase (Rothe medium)

Confirmation (Litsky medium)

Confirmation on Enterococcus agar

MPN/100 ml streptococci: ....   Positive cultures formula: ....

Annex 4

LIST OF PARTICIPANTS

REPRESENTATIVES OF LABORATORIES PARTICIPATING IN THE EXERCISE

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