MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

WORLD HEALTH ORGANIZATION

DEVELOPMENT AND TESTING OF SAMPLING AND ANALYTICAL TECHNIQUES FOR MONITORING OF MARINE POLLUTANTS (ACTIVITY A)

MISE AU POINT ET ESSAI DES TECHNIQUES D'ECHANTILLONNAGE ET D'ANALYSE POUR LA SURVEILLANCE CONTINUE DES POLLUANTS MARINS (ACTIVITE A)

Final Reports on Selected Microbiological Projects

Rapports finaux sur certains projets de nature microbiologique

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This volume is the fifty-fourth issue of the Mediterranean Action Plan Technical Report Series.

This series contains selected reports resulting from the various activities performed within the framework of the components of the Mediterranean Action Plan: Pollution Monitoring and Research Programme (MED POL), Blue Plan, Priority Actions Programme, Specially Protected Areas and Regional Marine Pollution Emergency Response Centre for the Mediterranean Sea.

Ce volume constitue le cinquante-quatrième numéro de la série des Rapports techniques du Plan d'action pour la Méditerranée.

Cette série comprend certains rapports élaborés au cours de diverses activités menées dans le cadre des composantes du Plan d'action pour la Méditerranée: Programme de surveillance continue et de recherche en matière de pollution (MED POL), Plan Bleu, Programme d'actions prioritaires, Aires spécialement protégées et Centre régional méditerranéen pour l'intervention d'urgence contre la pollution marine accidentelle.

GENERAL INTRODUCTION

The United Nations Environment Programme (UNEP) convened an Intergovernmental Meeting on the Protection of the Mediterranean (Barcelona), 28 January - 4 February 1975), which was attended by representatives of 16 States bordering on the Mediterranean Sea. The meeting discussed the various measures necessary for the prevention and control of pollution of the Mediterranean Sea, and concluded by adopting an Action Plan consisting of three substantive components:

- Integrated planning of the development and management of the resources of the Mediterranean Basin (management component);

- Co-ordinated programme for research, monitoring and exchange of information and assessment of the state of pollution and of protection measures (assessment component);

- Framework convention and related protocols with their technical annexes for the protection of the Mediterranean environment (legal component).

All components of the Action Plan are interdependent and provide a framework for comprehensive action to promote both the protection and the continued development of the Mediterranean ecoregion. No component is an end in itself. The Action Plan is intended to assist the Mediterranean Governments in formulating their national policies related to the continuous development and protection of the Mediterranean area and to improve their ability to identify various options for alternative patterns of development and to make choices and appropriate allocations of resources.

MED POL - Phase I (1976-1980)

The Co-ordinated Mediterranean Research and Monitoring Programme (MED POL) was approved as the assessment (scientific/technical component of the Action Plan.

The general objectives of its pilot phase (MED POL - Phase I), which evolved through a series of expert and intergovernmental meetings, were:

- to formulate and carry out a co-ordinated pollution monitoring and research programme taking into account the goals of the Mediterranean Action Plan and the capabilities of the Mediterranean research centres to participate in it;

- to assist national research centres in developing their capabilities to participate in the programme;

- to analyse the sources, amounts, levels, pathways, trends and effects of pollutants relevant to the Mediterranean Sea;

- to provide the scientific/technical information needed by the Governments of the Mediterranean States and the EEC for the negotiation and implementation of the Convention for the Protection of the Mediterranean Sea against Pollution and its related protocols;

- to build up consistent time-series of data on the sources, pathways, levels and effects of pollutants in the Mediterranean Sea and thus to contribute to the scientific knowledge of the Mediterranean Sea.

MED POL - Phase I was implemented in the period from 1975 to 1980. The large number of national research centres designated by their Governments to participate in MED POL (83 research centres) from 15 Mediterranean States and the EEC), the diversity of the programme and its geographic coverage, the impressive number of Mediterranean scientists and technicians (about

200) and the number of co-operating agencies and supporting organizations involved in it, qualifies MED POL as certainly one of the largest and most complex co-operative scientific programmes with a specific and well-defined aim ever undertaken in the Mediterranean Basin

MED POL - Phase II (1981-1990)

The Intergovernmental Review Meeting of Mediterranean Coastal States and First Meeting of the Contracting Parties to the Convention for the Protection of the Mediterranean Sea against Pollution, and its related protocols (Geneva, 5-10 February 1989), having examined the status of MED POL - Phase I, recommended that during the 1979/80 biennium a Long-term pollution monitoring and research programme should be formulated.

Based on the recommendations made at various expert and intergovernmental meetings, a draft Long-term (1981-1990) Programme for pollution monitoring and Research in the Mediterranean (MED POL-Phase II) was formulated by the Secretariat of the Barcelona Convention (UNEP), in co- operation with the United Nations Agencies which were responsible for the technical implementation of MED POL-Phase I, and it was formally approved by the Second Meeting of the Contracting Parties of the Mediterranean Sea against pollution and its related protocols and Intergovernmental Review Meeting of Mediterranean Coastal States of the Action Plan held in Cannes, 2-7 March 1981.

The general long-term objectives of MED POL-Phase II were to further the goals of the Barcelona Convention by assisting the Parties to prevent, abate and combat pollution of the Mediterranean Sea area and to protect and enhance the marine environment of the area. The specific objectives were designed to provide, on a continuous basis, the Parties to the Barcelona Convention and its related protocols with:

- information required for the implementation of the Convention and the protocols;

- indicators and evaluation of the effectiveness of the pollution prevention measures taken under the Convention and the protocols;

- scientific information which may lead to eventual revisions and amendments of the relevant provisions of the Convention and the protocols and for the formulation of additional protocols;

- information which could be used in formulating environmentally sound national, bilateral and multilateral management decisions essential for the continuous socio- economic development of the Mediterranean region on a sustainable basis;

- periodic assessment of the state of pollution of the Mediterranean Sea.

The monitoring of, and research on, pollutants affecting the Mediterranean marine environment reflects primarily the immediate and long-term requirements of the Barcelona Convention and its protocols, but also takes into account factors needed for the understanding of the relationship between the socio-economic development of the region and the pollution of the Mediterranean Sea.

As in MED POL-Phase I, the overall co-ordination and guidance for MED POL-Phase II is provided by UNEP as the secretariat of the Mediterranean Action Plan (MAP). Co- operating specialized United Nations Agencies (FAO, UNESCO, WHO, WMO, IAEA, IOC) are responsible for the technical implementation and day-to-day co-ordination of the work of national centres participating in monitoring and research.

The first eight volumes of the MAP Technical Reports Series present the collection of final reports of the principal investigators who participated in the relevant pilot projects (MED POL I - MED POL VIII). The ninth volume of the MAP Technical Reports Series is the final report on the implementation of MED POL-Phase I, prepared, primarily, on the basis of individual final reports of the principal investigators with the co-operation of relevant United Nations Agencies (FAO, UNESCO, WHO, WMO, IAEA, IOC).

From the tenth volume onwards, the MAP Technical Report Series contains final reports on research projects, assessment documents, and other reports on activities performed within the framework of MED POL-Phase II, as well as documentation originating from other components of the Mediterranean Action Plan.

This fifty-fourth volume of the MAP Technical Reports Series contains the final reports of six research projects completed within the framework of MED POL in Activity A - "Development and testing of sampling and analytical techniques for monitoring of marine pollutants".

INTRODUCTION GENERALE

Le Programme des Nations Unies pour l'environnement (PNUE) a convoqué une réunion intergouvernementale sur la protection de la Méditerranée (Barcelone, 28 janvier - 4 février 1975) à laquelle ont pris part des représentants de 16 Etats riverains de la mer Méditerranée. La réunion a examiné les diverses mesures nécessaires à la prévention et à la lutte antipollution en mer Méditerranée, et elle s'est conclue sur l'adoption d'un Plan d'action comportant trois éléments fondamentaux:

- Planification intégrée du développement et de la gestion des ressources du bassin méditerranéen (élément "gestion");

- Programme coordonné de surveillance continue, de recherche, d'échange de renseignements et d'évaluation de l'état de la pollution et des mesures de protection (élément "évaluation");

- Convention cadre et protocoles y relatifs avec leurs annexes techniques pour la protection du milieu méditerranéen (élément juridique).

Tous les éléments du Plan d'action étaient interdépendants et fournissaient le cadre d'une action d'ensemble en vue de promouvoir, tant la protection que le développement continus de l'écorégion méditerranéenne. Aucun élément ne constituait une fin à lui seul. Le Plan d'action était destiné à aider les gouvernements méditerranéens à formuler leurs politiques nationales en matière de développement continu et de protection de zone de la Méditerranée et à accroître leur faculté d'identifier les diverses options s'offrant pour les schémas de développement, d'arrêter leurs choix et d'y affecter les ressources appropriées.

MED POL - Phase I (1976-1980)

Le programme coordonné de surveillance continue et de recherche en matière de pollution de la Méditerranée (MED POL) a été approuvé au titre de l'élément "évaluation" (scientifique/technique) du Plan d'action.

Sa phase pilote (MED POL-Phase I) avait les objectifs généraux ci-dessous, élaborés au cours d'une série de réunions d'experts et de réunions intergouvernementales:

- formuler et exécuter un programme coordonné de surveillance continue et de recherche en matière de pollution en tenant compte des buts du Plan d'action pour la Méditerranée et de l'aptitude des centres de recherche méditerranéens à y participer;

- aider les centres de recherche nationaux à se rendre plus aptes à cette participation;

- étudier les sources, l'étendue, le degré, les parcours, les tendances et les effets des polluants affectant la mer Méditerranée;

- fournir l'information scientifique et technique nécessaire aux gouvernements des pays méditerranéens et à la Communauté économique européenne pour négocier et mettre en oeuvre la Convention pour la protection de la mer Méditerranée contre la pollution et les protocoles y relatifs;

- constituer des séries chronologiques cohérentes de données sur les sources, les cheminements, les degrés et les effets des polluants de la mer Méditerranée et contribuer par là à la connaissance scientifique de cette mer.

La Phase I du MED POL a été mise en oeuvre au cours de la période 1975-1980. Le grand nombre de centres de recherche nationaux désignés par leurs gouvernements pour participer au MED POL (83 centres de recherche de 15 Etats méditerranéens et de la CEE), la diversité du programme et sa couverture géographique, l'effectif impressionnant de scientifiques et techniciens méditerranéens (environ 200) ainsi que la quantité d'organismes coopérants et d'organisations d'appui qui y étaient engagés permettent sans conteste de caractériser le MED POL comme l'un des programmes de coopération scientifique les plus vastes et les plus complexes, comportant un objectif spécifique et bien défini, qui ait jamais été entrepris dans le bassin méditerranéen.

MED POL-Phase II (1981-1990)

La réunion intergouvernementale des Etats riverains de la Méditerranée chargés d'évaluer l'état d'avancement du Plan d'action et première réunion des Parties contractantes à la Convention pour la protection de la mer Méditerranée contre la pollution et aux protocoles y relatifs (Genève, 5-10 février 1979), ayant examiné la situation de la Phase I du MED POL, a recommandé que, durant la période biennale 1979-80, soit formulé un programme à long terme de surveillance continue et de recherche en matière de pollution.

Sur la base des recommandations énoncées lors des diverses réunions d'experts et réunions intergouvernementales, un projet de programme à long terme (1981-1990) de surveillance continue et de recherche en matière de pollution (MED POL - Phase II) a été formulé par le secrétariat de la Convention de Barcelone (PNUE), en coopération avec les organismes des Nations Unies chargés de l'exécution technique de MED POL - Phase I, et il a été officiellement approuvé lors de la deuxième réunion des Parties contractantes à la Convention pour la protection de la mer Méditerranée contre la pollution et aux protocoles y relatifs et réunion intergouvernementale des Etats riverains de la mer Méditerranée chargée d'évaluer l'état d'avancement du Plan d'action, qui s'est tenue à Cannes du 2 au 7 mars 1981.

L'objectif général à long terme de la Phase II du MED POL était de concourir à la réalisation des objectifs de la Convention de Barcelone en aidant les parties contractantes à prévenir, réduire et combattre la pollution dans la zone de la mer Méditerranée ainsi qu'à protéger et améliorer le milieu marin dans cette zone. Les objectifs particuliers étaient de fournir constamment aux Parties contractantes à la Convention de Barcelone et aux Protocoles y relatifs:

- les renseignements dont elles avaient besoin pour appliquer la Convention et les protocoles;

- des indications et une évaluation de l'efficacité des mesures prises pour prévenir la pollution en application de la Convention et des protocoles;

- des renseignements scientifiques qui pourraient servir à réviser et modifier les dispositions pertinentes de la Convention et des protocoles et à rédiger des protocoles additionnels;

- des informations qui pourraient servir à formuler sur les plans national, bilatéral et multilatéral, les décisions de gestion, respectueuses de l'environnement, qui seraient indispensables à la poursuite du développement socio- économique de la région méditerranéenne;

- une évaluation périodique de l'état de pollution de la mer Méditerranée.

La surveillance continue des polluants affectant le milieu marin de la Méditerranée ainsi que la recherche menée à leur sujet répondent en premier lieu aux prescriptions immédiates et à long terme de la Convention de Barcelone et des protocoles y relatifs, mais elles tiennent également compte des facteurs requis pour la compréhension des relations existant entre le développement socio-économique de la région et la pollution de la mer Méditerranée.

Comme lors de la Phase I du MED POL, la coordination et la direction générales de la Phase II étaient assurées par le PNUE, par l'intermédiaire du secrétariat du Plan d'action pour la Méditerranée (PAM). Les organismes spécialisés coopérants des Nations Unies (FAO, UNESCO, OMS, OMM, AIEA, COI) étaient chargés de l'exécution technique et de la coordination quotidienne des travaux des centres de recherche nationaux participant au programme de surveillance continue et de recherche.

Les huit premiers volumes de la Série des rapports techniques du PAM rassemblent les rapports finaux de chercheurs responsables qui ont participé aux projets pilotes correspondants (MED POL I -MED POL VIII). Le neuvième volume de cette même Série se compose du rapport final sur la mise en oeuvre de la Phase I du programme MED POL, établi essentiellement sur la base des rapports finaux individuels des chercheurs responsables avec la coopération des organismes compétents des Nations Unies (FAO, UNESCO, OMS, OMM, AIEA, COI).

A partir du dixième volume, la Série des rapports techniques du PAM, comprend des rapports finaux sur les projets de "recherche", des documents d'évaluation et d'autres rapports d'activités effectués dans le cadre de MED POL-Phase II, ainsi que de la documentation prise dans d'autres domaines du Plan d'action pour la Méditerranée.

Ce cinquante-quatrième volume de la Série des rapports techniques du PAM comprend six rapports finaux exécutés dans le cadre de la Phase II du MED POL, dans l'Activité A - "Mise au point et essai des techniques d'échantillonnage et d'analyse pour la surveillance continue des polluants marins.

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BACTERIOPHAGES OF BACTEROIDES AS INDICATORS OF PATHOGENIC HUMAN VIRUSES IN COASTAL SEAWATERS

by

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1. SUMMARY

Bacteriophages infecting *Bacteroides fragilis* were found in sewage-polluted marine environments, water and sediments, and were absent in non-polluted marine environments. They outnumbered human viruses, such as enteroviruses and rotaviruses. They were also recovered in samples of polluted environments in which human viruses were not. In sediments, when the same methodology of recovery was used for phages and animal viruses, amounts of phages of *B. fragilis* correlated with numbers of both enteroviruses and rotaviruses. Such correlation was not found in water samples. Moreover, they were inactivated at least as slowly as human viruses in seawater. Therefore there are many data showing the usefulness of phages infecting *B. fragilis* as indicators of human viruses in the marine environment.

2. INTRODUCTION

Viruses are contaminants of concern for human health (4, 8). Their detection in environmental samples is complex and very expensive (8). As a consequence, there is a great deal of concern on the need for viral indicators (10, 16, 20). Epidemiological data, in addition to extensive measurements, show that the present bacterial indicators do not serve as indicators for viruses (5, 7, 14, 16).

Several authors have proposed the potential usefulness of coliphages, which are very easily measured in environmental samples, as viral indicators (11, 21, 24). However there are certain aspects of the behavior of such phages that introduce doubts about their validity (17, 27).

A few years ago, we initiated a study in order to explore the possibility of using bacteriophages of *Bacteroides* spp as indicators of human enteric viruses. *Bacteroides* are strict anaerobes and are a major component of human faeces (18). It is unlikely that they occur naturally in the environment, and consequently, the phages infecting them have the potential for being good viral indicators. At an early stage, we obtained data showing that they may be useful (9).

The purpose of this work was to evaluate the utility of such a method in the marine environment. The aims of this study were to evaluate the presence of phages infecting *Bacteroides* in the marine environment, water and sediments; correlate the amounts of such bacteriophages to other faecal microorganisms, mainly viruses; and compare their decay in the marine environment to that of other viruses.

In the experiments performed to obtain these data, it was necessary to adapt some methodologies, that can be of general interest for the study of viruses in the marine environment, mainly sediments, and we have provided some data on the presence of enteroviruses and rotaviruses in the Mediterranean sea.

3. MATERIALS AND METHODS

3.1 Cell cultures

Two continuous cell lines of monkey kidney cells were used: MA-104 (foetal monkey kidney) and BGM (Buffalo green monkey). Both cell lines were grown in Eagle's minimal essential medium (MEM) in Earle's base, supplemented with 5% foetal bovine serum (FBS), 0.03% glutamine, 0.075% sodium bicarbonate, 100 U of penicillin per ml and 100 ug of streptomycin per ml.

3.2 Virus strains

Simian rotaviruses SA11 (ATCC VR-899), polio strain LSc2ab and f2 coliphages (ATCC 15766-B) were used in inactivation experiments. Bacteriophage B40-8 isolated from sewage on *Bacteroides fragilis* 40 (25) was used for the inactivation experiments.

3.3 Viral assays

Strain LSc2ab of poliovirus was quantified by plaque formation in the BGM cell line. SA11 virus was assayed either by plaque assay or by indirect immunofluorescence (22) in the MA104 cell line. Human rotaviruses were quantified by a modification of the indirect immunofluorescence test (IIF) reported by Smith and Gerba (1982) in the MA104 cell line as described elsewhere (2).

3.4 Bacteria, media and growth conditions

Strain HSP40 of Bacteroides fragilis was used as the host (25). It was grown in MBAB (Modified Blood Agar Base) which contains per liter: 40 g of Blood Agar Base N. 2 (Oxoid), 0.05 g of L-cysteinium chloride monohydrate (Merck), 0.12 g of $MgSO_4.7H_2O$ (Merck) and 0.05 g of $CaCl_2$ (Merck). As a liquid medium MBB (Modified Brucella Broth) was used, which contains per liter: 28 g of Brucella-Broth (ADSA), 0.05 g of L-cysteine chloride monohydrate, 0.12 g of $MgSO_4.7H_2O$ and 0.0 g of $CaCl_2$. A soft agar overlay was prepared adding 5 g per liter of agar to MBB. MBAB-S is a modified MBAB, which contains 0.05 % potassium sorbate and adjusted to pH of 5.7, to avoid spore germination.

When solid medium was used, cultures were incubated inside anaerobic jars (GASPAK, BBL). For incubation of liquid cultures, screw-capped tubes were used, thus avoiding the use of anaerobic jars.

E. coli ATCC 15766 was used as the host for f2 bacteriophage. It was grown in nutrient agar or nutrient broth.

3.5 Bacterial indicators

Faecal coliforms and faecal streptococci were determined according to standard methods.

3.6 Phages assays

Enumerations of phages by plaque counts (PFU) were conducted by the double-agar layer technique (1). This method was used in the inactivation experiments.

Phages in environmental samples were enumerated by a modification of the MPN method for coliphage described by Kott (12). Each of three screw-cap tubes (30ml) containing 10 ml of 2.5-strength MBB was inoculated with 10 ml of sample and 10 ml of the host. A second set of three tubes containing 19 ml of single-strength MBB was inoculated with 10 ml of the host and 1 ml of the sample, and a third set of three tubes containing 20 ml of single-strength MBB was

inoculated with 10 ml of the host and 0.1 ml of the sample. The tubes were incubated at 37 °C for 36 to 48 hours. Following incubation, 5 ml from each tube was centrifuged at 2000 x g for 15 min, and the supernatant was mixed with 2 ml of chloroform. Finally, a loopful from each tube was spotted onto overlayed host lawns plated on MBAB-S. Plates were incubated anaerobically at 37 °C for 16 hours. The results of the tests were recorded and computed by the use of the MPN table for coliform bacteria. Sewage and highly polluted samples were diluted, and series of three tubes were inoculated with 1 ml of dilution into single-strength MBB tubes. This method was always used for the enumeration of phages infecting *B. fragilis* in environmental samples.

3.7 Sampling sites

Sewage-polluted water and sediment samples were collected in the metropolitan area of Barcelona. Samples with no know faecal pollutants of human origin were collected in the natural park "Delte de l'Erbe", an important wildlife refuge.

3.8 Collection and processing of samples

Surface water samples were collected into sterile bottles submerged about 20 cm below the water surface. All samples were placed at 4 °C and examined within 6 hours after collection.

Deep sediment samples were collected with a box corer. The top 2-3 cm of sediment were scooped into sterile plastic bags, which were sealed and kept at 4 °C while in transit to the laboratory, where they were processed within 12 hours of collection. For the shallow ones the top layer (2-3 cm) was scooped into sterile plastic bags and processed as above.

3.9 Virus concentration

In environmental water samples enteroviruses were concentrated by adsorption-elution on glass powder (19). However the method was not useful for phages infecting *B. fragilis*. As a part of this project we adapted the concentration using electropositive charged filters as described by Sobsey and Glass (23). Although this method proved to be good for samples seeded with phages, has not been successfully applied to environmental samples.

3.10 Virus elution

Viruses were eluted from 250 g were sediment samples using 750 ml of 0.25 M glycine buffer extract pH 9.5.

3.11 Inactivation experiments

Laboratory studies. Marine water samples were always collected within 4 hours of the initiation of an experiment and held at 4 °C during this period. Replicate 250 ml-flasks, containing 100 ml of the water sample to be tested, were inoculated with viral suspensions as to yield a final titer of approximately 105 PFU/ml. As controls, PBS was incubated with a similar amount of viruses. Zero time samples were removed after 30 sec shaking period to disperse the virus. The flasks were incubated at 20 °C with rotational agitation (120 rpm for 21 days). Alliquots of the samples under test were aseptically removed at different periods of time, placed in test tubes containing 9 ml of PBS and immediately stored at -80 °C, and the different kind of viruses were assayed. Survival of the viruses in the test waters was determined by calculating the log Nt/No where No is the titer of the virus at the time zero and Nt is the virus titer at various time periods post-inoculation. The times required for losses in titer of 90%, 99% and 99.9% of the input virus population are designed T90, T99 and T99.9 respectively, and were used to compare the stability of the different viral strains under the various experimental conditions tested.

"*In situ*" studies . Sterile cellulose dialysis bags were filled with freshly collected seawater and seeded with viruses. The dialysis tubes were mechanically protected by cylindrical mesh

cages and suspended 20 cm deep from a wharf at Garraf. Samples for viral quantifications were collected at days 0, 3, 5, and 7. Temperature of the water was 15 °C. All experimental results described were reproduced in at least two separate experiments.

3.12 Statistical analysis

Statistical analysis of the data were calculated on a computer using the Statistical Package for Social Sciences (SPSS,Chicago, Il).

4. RESULTS AND DISCUSSION

4.1 Occurrence of phages infecting B. fragilis in marine environments

Bacteriophage actives against *B. fragilis* were found in 69.7% of 33 samples of seawater corresponding to an area that receives sewage influence. Eighteen of them were collected in beaches of Barcelona and phages were recovered in the 44.4% of them (table 1). The rest were collected nearby a sewage outlet, where phages were recovered in all the samples (table 2).

On the contrary they were never found in 10 seawater samples corresponding to non polluted waters in which no faecal indicators were found. They were not found either in 6 water samples corresponding to seawater samples corresponding to shallow waters of a bay in which there is an important amount of wildlife (Table 3).

Moreover phages infecting *B. fragilis* were recovered in the 85% of 62 samples of marine sediments corresponding to the area influenced by the city of Barcelona. These results correspond to samples to which different methodologies of virus elution were used. If we consider only 24 samples from which phages were eluted by glycine buffer pH 9.5, shown to be the method useful for the elution of the different viruses of interest (Jofre *et al.* Occurrence of bacteriophages infecting *Bacteroides fragilis* and other viruses in polluted marine sediments. Abstracts of the 17th Biennial Conference & Exhibition on Water Pollution Control. IAWPRC, Brighton, 1988), the percentage of positive samples increases to 92% (Table 4).

However, bacteriophages infecting *B. fragilis* were not isolated in sediments of shallow waters in Delte de l'Ebre (Table 3).

As a consequence, we can conclude that phages infecting *B. fragilis* occur in marine environments that receive sewage of human origin and that they are absent in marine environments without human faecal influence, as it has been shown to happen in freshwaters (26).

Table 1

Counts of microorganisms in marine water samples

	FC/100ml	FS/100ml	FB/100ml	Ent/10ml
Number of samples	18	18	18	18
% positives	100	100	44.4	44.4
Average count	4.8×10^6	1.5×10^6	73.4	0.014
Mediane count	1.1×10^4	9.3×10^3	0	0
Range of counts	$10\text{-}10^6$	$10\text{-}10^7$	0-750	0-0.055
r phages/org[a)]	-0.793	-0.930	1.000	0.823

a) Only r> 0.764 indicates significant ($p<0.05$) correlation
FC = faecal coliforms; FS = faecal streptococci; FB = phages infecting *B. fragilis*; ent = enteroviruses.

Table 2

Counts of viruses in marine water samples collected nearby a sewage outlet

	Enteroviruses (per 100 ml)	Phages actives on *B. fragilis* (per 100 ml)
Number of samples	15	15
% positives	80	100
Average count	0.042	1306.5
Median count	0.020	1100
Range of counts	0-2.4	23-4600
r phages/org	0.294[a)]	1.000

a) Only $r>0.320$ indicates significant ($p<0.05$) correlation
Counts of enteroviruses and phages were calculated as MPN.

Table 3

Mean values per 100 ml or 100 g of bacteria and viruses in water sediments collected in shallow waters in a bay at the "Delte de l'Ebre" natural park

Samples (number)	TC	FC	FS	Phages of Bacteroides	Enterovirus
Water (6)	4587	164	9.3	0	0
Sediment (6)	2087	86.5	439	0	0

TC = Total coliforms; FC = Faecal coliforms; FS = Faecal streptococci

4.2 Relationship of phages infecting B. fragilis to other viruses

In samples of seawater receiving sewage pollution, phages infecting *Bacteroides fragilis* clearly outnumbered enteroviruses both in numbers and in the number of samples from which they were isolated (Tables 1 and 3).

In polluted sediments, phages infecting *B. fragilis* outnumbered enteroviruses and rotaviruses (Table 4) both in amounts and in the number of samples from which they were recovered. Phages outnumbered animal viruses by a factor of around two logs.

Considering all the polluted samples of water and sediments studied only in 2 over 95, enteroviruses were isolated and phages infecting *B. fragilis* were not, whereas the reciprocal situation was very frequent.

Data presented in table 1 show a significant correlation between phages infecting *B. fragilis* and enteroviruses, although these correlation may be too influenced by the high percentage (78%) of samples in which phages and enterovirus were either present or absent. Data

in table 2 do not show a significant correlation among enteroviruses and phages infecting *B. fragilis*. If we consider the data altogether there is not a significant correlation among phages and enteroviruses. Since enteroviruses were concentrated and phages were not, this lack of correlation may be due to the different methodology used.

Data presented on table 4 show a significant correlation among the three kinds of virus in sediments (Table 4 bis). Note that the same methodology has been used to elute all the viruses.

Table 4

Bacteriophages and enteric viruses recovered from 24 sediment samples through elution with glycine buffer pH 9.5

Virus type	Arithmetic mean	Standard deviation	% positive isolations
Phages [a)] (100 g)	1.6×10^3	5.6×10^3	92
Enterovirus (100 g)	20	29	70
Rotavirus (100 g)	5.7	8.3	63

a) Phages infecting *Bacteroides fragilis*

Table 4bis

Multiple correlations among values of phages and enteric viruses eluted with glycine pH 9.5

	Bacteroides phages	Enteroviruses	Rotaviruses
Bacteroides phages	1.000		
Enteroviruses	0.5087	1.000	
Rotaviruses	0.4320	0.4229	1.000

Only $r > 0.4032$ indicates significant ($p < 0.05$) correlation.

4.3 Relationship of phages infecting B. fragilis to bacterial indicators

Bacterial indicators outnumbered phages infecting *Bacteroides fragilis* in the area studied, both in seawater and in marine sediments (Tables 1 and 5). However the ratios faecal coliforms/phages and faecal streptococci/phages decrease significantly in sediment samples as compared to those found in sewage, since according data of tables 4 and 5 the abovementioned ratios are around 10 and 100, whereas in sewage none of them is inferior to 1000 (data not

shown). Therefore we can conclude that the decay rate in seawater of phages infecting *Bacteroides fragilis* is lower than those of faecal coliforms and faecal streptococci.

Neither in seawater nor in marine sediments values of indicator bacteria correlate significantly with values of viruses. These results confirm for phages infecting *B. fragilis* the same behavior that for enteroviruses, for which it has been shown that bacterial indicators do not reflect the virological quality of waters (5, 7, 14, 16).

4.4 Decay rates of phages infecting B. fragilis in seawater

The virus inactivation kinetics with the two bacteriophages, coliphage f1 and phage B40-B infecting *B. fragilis*, and poliovirus 1 and simian rotavirus SA11 in seawater under laboratory conditions was studied at 20 °C. Results show that survival of *B. fragilis* phages was similar to survival of the other viruses tested (fig. 1, table 6).

Moreover, "*in situ*" experiments, water temperature around 15 °C, also show that phage B40-8 of *B. fragilis* survives as long as the other viruses do (fig. 1, table 7). Therefore we can conclude that in seawater the decay rate of phage B40-8 of *B. fragilis* follows the characteristic kinetics of viral inactivations.

Table 5

Bacterial indicators recovered from 24 sediment samples
(the same samples of table 4).
Data of phages of *B. fragilis* are included

	Faecal coliforms (c/100ml)	Faecal streptococci (c/100ml)	Phages of *B. fragilis* (c/100ml)
% positive samples	96	100	92
Average counts	$1.4x10^4$	$1.8x10^5$	$1.6x10^3$
Median count	$1.2x10^3$	$9.6x10^4$	54
Range of counts	$0-9.6x10^4$	$30-9.6x10^5$	0-27600
Correlation phage/org*	0.0328	0.1860	1.000

* Only $r>0.3665$ indicates significant ($p<0.05$) correlation

Table 6

Comparative stability of phages B40-8 and f2,
poliovirus 1 and rotavirus SA11

Virus	T90	T99	T99.9
f2	2	3.7	5.2
B40-8	1.5	3.2	5.3
Poliovirus 1	1	2	2.8
SA11	2.7	4.3	5.8

Table 7

Comparative "*in situ*" stability of phages B40-8 and f1, poliovirus 1 and rotavirus SA11

Virus	T90	T99	T99.9
f2	3.2	4.7	>7
B40-8	3.6	5.7	>7
Poliovirus 1	1.5	3.1	4
SA11	2.1	4.0	>7

Figure 1 Inactivation kinetics of coliphage f2, bacteriophage B40-8, poliovirus 1 and simian rotavirus SA11 in seawater.
a: Seawater laboratory experiments;
b: seawater "*in situ*" experiments. :f2; :B40-8; : poliovirus 1; :SA11

4.5 Other enteric viruses in the area studied

The studies herein reported have provided some data on the presence of human viruses in the marine environment, water and sediments, in the area of the city of Barcelona.

As it has been previously reported, enteroviruses were recovered in not insignificant amounts in seawater and marine sediments (6, 13, 15). Amounts were not very different from those described in other areas in the world, including the Mediterranean (6, 15).

As far as we know, our data on human rotaviruses in marine sediments are the first ones in the Mediterranean. Our data show amounts of rotavirus not very different from the values corresponding to enteroviruses (table 4), as occurs in sewage (3). So far we do not have data on rotaviruses in seawater. This was probably due to some methodological problems in the concentration procedure, as was the case with phages infecting *Bacteroides fragilis*.

Very preliminary results on Hepatitis A virus (HAV), show that they also are found in sediments. Effectively, using a DNA probe specific for HAV, and studying very polluted sediment samples, one of four samples was positive. Considering that the methodology based on DNA probes is not as sensitive as that based on the viral replication, this result may indicate an important concentration of Hepatitis A viruses in marine sediments.

5. CONCLUSIONS

There are three main conclusions that are worth to mentioning in relation to the initial aims of this study.

Bacteriophages infecting *Bacteroides fragilis* were consistently recovered in sewage polluted marine environments and were not in clean environments or in environments where there was no pollution of human origin.

Bacteriophages infecting *B. fragilis* outnumbered enteroviruses and rotaviruses both in the number of samples from which they were isolated and in the numbers of phages that clearly surpass those of enteroviruses and rotaviruses in the vast majority of the samples. The lack of correlation among numbers of phages and enteroviruses in water samples, may be due to several reasons, but does not hamper the potential use of the phages infecting *B. fragilis* as surrogate indicators of human viruses in the marine environment.

Another important point to be considered for a surrogate indicator of human viruses in the environment is whether, once in the environment, the decay rate of the indicator is similar to that of other viruses. Data herein presented indicate that survival rate in seawater of phages infecting *B. fragilis* is similar to that of human viruses.

We understand that these data are very promising, though much research need to be done on different aspects in order to ascertain whether phages infecting *B. fragilis* may be used as indicators of human viruses in the marine environment.

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DETECTION OF HEPATITIS A VIRUS IN SEWAGE, SEAWATER AND SHELLFISH

by

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1. ABSTRACT

Methods to detect HAV in shellfish and seawater are needed to monitor their degree of contamination and to prevent virus transmission to bathers and shellfish consumers. Cell culture adapted HAV in seeded homogenized oyster or mussel tissues was efficiently recovered after two-fold elution by 3% beef extract and PEG 6000 precipitation. The infectious virus was also efficiently recovered from experimentally contaminated seawater by adsorption to an elution from microporous filters followed by PEG 6000 precipitation. A field study was carried out in the Thermaicos Gulf (Thessaloniki) for one year in highly polluted, moderate polluted and unpolluted (recreational) seawater area and in shellfish growing in the highly polluted area. HAAg was detected by SPRIA coupled with a radiocompetition control test. Using these concentration and detection methods, HAAg was detected in 3 of 15 highly polluted seawater samples and in 1 of 13 moderate polluted seawater samples. Virus was not detected in the 15 samples of the recreational places. However, HAAg was found in 6 out of 7 samples of mussels and in 2 out of 12 sewage samples. In 2 samples of highly polluted seawater, one sample of oysters and one sample of sewage contained infectious HAV. The described methods are useful in detecting HAV contamination of seawater and shellfish. These methods should be implemented to monitor HAV contamination of seawater and shellfish as to reduce or prevent outbreaks related to HAV transmission by these routes.

2. INTRODUCTION

There is ample epidemiological evidence to show that water and shellfish are key vectors in the transmission of several viruses to man. This is particularly true with respect to HAV (Papaevangelou, 1984; Cliver, 1985) and several Hepatitis A outbreaks due to consumption of shellfish have been reported (Dienstag *et al.*, 1976; Gerba and Goyal, 1978; Ohara *et al.*, 1983). Thus, methods to detect HAV in shellfish and seawater are needed to monitor their concentration and prevent virus transmission to bathers and shellfish consumers in order to reduce or prevent outbreaks related to raw shellfish consumption. This detection is strictly dependent on an efficient and sensible extraction and/or concentration method, because virus quantities in environmental samples are low.

There are only a few reports concerning the recovery of HAV in polluted or seeded water (Sobsey *et al.*, 1985a; Biziagos *et al.*, 1987b; Jiang *et al.*, 1987: Nasser and Metcalf, 1987; Divizia *et al.*, 1988) and shellfish (Millard *et al.*, 1987; Lewis and Metcalf, 1988) Pietri *et al.*, 1988) after its concentration.

The purpose of the study described in this report was to evaluate the efficiency of concentration methods to recover HAV from experimentally contaminated seawater and shellfish and the effective use of these methods to detect wild strains of HAV from environmental samples in an one-year study in the Thermaicos Gulf area (Thessaloniki, Greece). The study included the

detection of HAV in sewage samples (before their discharge into the sea), as the Gulf is receiving the untreated sewages of the city and surroundings.

3. MATERIALS AND METHODS

Virus detection (tissue culture, laboratory study and solid-phase radioimmunoassay) were performed in Grenoble whereas the field study and the virus concentration procedures from seawater, shellfish and sewage were done in Thessaloniki.

3.1 Virus, cells and virus detection

HAV strain CF 53 has been adapted to growth in PLC/PRF/5 cells by serial passages (Crance *et al.* 1985, 1987). Hepatitis A antigen (HAAg) was detected by a solid-phase radioimmunoassay (SPRIA) as previously described (Biziagos *et al.*, 1987a) and quantified by SPRIA-endpoint titration (Biziagos *et al.*, 1988). Infectious virus was quantified by cell culture titration (Passagot *et al.*, 1987).

For the field experiments, HAAg was detected by SPRIA coupled with a radiocompetition control test that was performed according to Flehmig *et al.* (1978). For this control test, human and chimpanzee preillness (anti-HAV negative) and convalescent (anti-HAV positive) sera were used.

3.2 Laboratory studies : Virus concentration procedures

a. Seawater

HAV concentration from seawater was performed by adsorption to and elution from microporous filters followed by polyethylene glycol 6000 (PEG 6000) precipitation. The nitrocellulose membrane filters (Millipore) were put on a 142 mm dia grid support. The first was a membrane prefilter (AP25 124), the second and the third were two membranes with respectively 8.0 μm (SCWP 142) and 3.0 μm (SSwp 142) porosity, separated by a membrane separator (AP32 124). Cell culture-adapted HAV (strain CF 53) was seeded into 20 l of seawater, the sample was acidified to pH 4.0 and filtered through the microporous filters at a flow rate of about 10 l/min. Adsorbed viruses were eluted from membranes with 80 ml of 3% beef extract (DIFCO) at pH 8.5 and the eluate was adjusted to pH near neutrality. The elution flow rate was about 0.5 l/min. The eluate was further concentrated by polyethylene glycol 6000 (PEG 6000) precipitation: after the addition of 10% PEG 6000 and 1.5% NaCl, the sample was kept overnight at 4 ° C. Precipitated viruses were collected by centrifugation at 10.000 x g for 45 min at 4 ° C and suspended into 3 ml of PBS.

b. Shellfish

Oysters or mussels (from commercial sources) were shucked and the meats were pooled. For each test, a 100 g of pooled meats were used. Samples were blended for 2 min. in a Waring blender, in order to homogenize the meat, and cell-culture adapted HAV was added to each homogenate and subjected to an additional 2 min. homogenization. Then, 200 ml of 3% beef extract in 0.01M borate buffer (pH 9.0) was added and blended at high speed for 1 min. The homogenate was stirred for 15 min. at room temperature to facilitate virus elution. The pH was then adjusted to 9.0, if needed, and shellfish solids were removed by centrifugation at 10.000 x g for 45 C at 4 ° C. The resulting supernatant (containing the virus) was neutralized and subjected to a first PEG precipitation. The resulting pellet was resuspended into 40 ml of the above eluant (pH 9.0), stirred, clarified by centrifugation and the virus from the supernatant was subjected to a second PEG 6000 precipitation. The final pellet containing HAV was suspended into 3-5 ml PBS.

3.3 Field studies

The study was performed for one year (July 1988 - June 1989) in the Thermaicos Gulf area (Thessaloniki). Seawater samples (20 l) from highly polluted (near to the discharge of the sewages), moderate polluted (13 km from the discharge point, but receiving some untreated sewage) and unpolluted (recreational places, 14 km from the discharge point, acceptable for bathing according to EEC regulations) areas were periodically collected. Before the sampling, water temperature and wind direction and velocity were measured. Shellfish samples growing only in the highly polluted area (harvesting not allowed) were also collected. The sewage samples (10 l) were taken before their discharge into the sea. All samples were collected in sterile containers and transported to the laboratory on ice. They were immediately subjected to concentration procedure, as described above. To the sewage samples, after the adjustment of pH to 4.0, 87.75 g NaCl (final concentration of 0.15 M NaCl) were added.

On the same day, the chemical and bacteriological parameters were examined. The chemical analysis of seawater included BOD5 and salinity determinations. The bacteriological examination consisted of plate count (37 ° C/48 h, Plate count agar), total coliforms, *TC*, (MF-method, 37 ° C/24 h, m-Endo agar), faecal coliforms *FC* (MF-method, 44 ° C/24 h, m-FC agar), faecal streptococci *FS* (MF-method, 37 ° C/48 h, m-Enterococcus agar) and sulfite reducing clostridia *SRC* (MPN-method, -DRCM-). The bacteriological examination of the shellfish samples was performed by the MPN method using Lauryl Tryptose Broth (TC), EC Medium (FC), Azide Dextrose Broth (FS) and DRCM (SRC). The sewage samples were not examined as their high bacterial content is given.

4. RESULTS

4.1 Laboratory studies

First we compared the percent HAV recovery in experiments with oyster and mussel tissues. After the HAV addition into homogenized tissues of oysters and mussels and its concentration by the described method, no significant difference was shown between the mean percent recovery of HAV from oyster (69.5%) and that from mussel tissues (75.0%).

Before the use of the concentration methods to detect HAV in environmental samples, these methods were used to concentrate cell culture-adapted HAV from experimentally contaminated seawater and shellfish. The obtained results (Table 1) showed that HAV was efficiently recovered after concentration, from seawater and shellfish. Statistical studies showed that there were no significant differences between the HAAg and infectious HAV recovery efficiencies (t-test; $p>0.05$) for both seawater and shellfish experiments.

Table 1

Recovery of HAV from experimentally contaminated seawater and shellfish tissues

	Recovery (%)*	
	Seawater	Shellfish
HAAg	79.5+11.3	72.3+ 9.6
Infections HAV**	84.3+13.7	65.3+11.4

* After concentration by the described methods. Mean and standard deviation of 10 experiments

** About 1000 TCID50 were present per liter of water or per gram of shellfish tissue

4.2 Field study

From July 1988 to June 1989, 43 seawater samples (15 samples from unpolluted area, 13 samples from moderately polluted and 15 samples from highly polluted areas), 7 samples of mussels growing in the highly polluted area of the Gulf and 12 untreated sewage samples were collected and processed. The samples were tested for HAAg by SPRIA and the positive results obtained were confirmed by a radiocompetition control test (blocking SPRIA) by using preillness and convalescent sera. After concentration by the described methods, HAAg was found in 3 highly polluted and in 1 moderate polluted seawater samples, whereas it was not found in the 15 samples of the recreational places. HAAg was also detected in 2 of the 12 sewage samples and in 6 of the 7 oyster samples. All results from the bacteriological, chemical and physical examinations are shown in tables 2 (recreational seawater), 3 (moderate polluted water), 4 (highly polluted water) and 5 (shellfish). The dotted areas in the tables indicate the positive samples for HAAg. HA virus was isolated in 2 seawater samples from the highly polluted area (13 and 14 in table 4) in 1 from the oysters (6 in table 5) and in 1 from the sewage samples.

5. DISCUSSION

Because of the epidemiological importance of HAV as a waterborne and foodborne virus, the existing methods for enteric virus concentration from water and shellfish were further developed and evaluated for ability to concentrate a laboratory-adapted strain of HAV from seeded seawater and shellfish tissues. Methods for HAV concentration from water and food are needed for further laboratory and field studies on the occurrence, detection, survival, transport and fate of this virus at the low levels likely to be encountered under natural conditions.

Results of laboratory studies indicated that HAV can be efficiently concentrated from seeded seawater by absorption to and elution from electronegative microporous filters. Overall HAV recovery efficiency by this conventional microporous filter method was similar to those usually obtained for other enteric viruses (Farrah *et al.*, 1976; Morris and Waite, 1980; Sobsey *et al.* 1981; Melnick *et al.*, 1984). Extraction and concentration of HAV from seeded homogenized shellfish tissues was obtained by the use of a 3% beef-extract (diluted in borate buffer) eluant and virus concentration by PEG 6000 precipitation; this procedure was repeated twice to obtain the best concentration factor and to diminish the viscosity of the supernatants and their toxicity for cell cultures. By using this method, as for the seawater experiments, HAV was efficiently concentrated from seeded homogenized oyster or mussel tissues. In our laboratory studies, HAV was added directly on the tissue homogenate. This procedure was already used by several authors in laboratory experiments with other enteric viruses (Sobsey *et al.*, 1978; Vaughn *et al.* 1979; Metcalf *et al.* 1980; Richards *et al.*, 1982, Spreis *et al.* 1987; Bemis *et al.*, 1989). Moreover, in comparative studies, oyster flesh was contaminated with HAV either by injection into the digestive tract or by inoculation into the tissue after brief homogenization. In these studies (data not shown) no difference in experimental results due to the mode of virus contamination was observed and the last inoculation procedure was preferred for the offered facilities.

Although the transmission routes of HAV have been well described in epidemiological studies, the virus has not been found routinely in water or shellfish because of the lack of methods for its concentration and direct detection. Consequently, little is known about the prevalence of HAV in surface and groundwaters and nothing in shellfish. HAAg was detected by RIA in one groundwater and two sewage samples in Georgetown, Tex. (Hejkal *et al.*, 1982) and in three estuarine water samples by A-ELISA (Nasser and Metcalf, 1987). Also, HAV was detected in estuarine and freshwater samples collected from a sewage-polluted bay in Houston by the use of single-stranded RNA probes (Jiang *et al.*, 1987). In the present study, HAAg was detected in moderate and highly polluted seawater samples from Thermaicos Gulf and in oysters growing in the highly polluted seawater area. For this detection a SPRIA coupled with a radiocompetition control test were used to avoid the false positive immunological reactions. Without presuming on the infectivity of HAV detected in the above samples, it is reasonable to consider that this Gulf, that receives untreated sewage from the city and surroundings of Thessaloniki, is contaminated by

HAV. The HAAg-positive samples were examined for infectious HAV. Two seawater samples from the highly polluted area, one shellfish sample and one sewage sample were positive for infectious HAV. On this subject, two authors have presented positive results in cell culture for wild strains of HAV isolated from environmental sources. The first (Sobsey *et al.* 1985b) have isolated infectious HAV from water samples implicated in the Georgetown (Texas) outbreak of hepatitis A and the second (Divizia *et al.* 1989) have isolated infectious HAV in three isolates from polluted riverwater of Tiber (Italy).

Although the number of field samples included in this study was small, the results indicate that the described methods can be used to detect HAV contamination of seawater and shellfish. These methods should be implemented to monitor HAV contamination of seawater and shellfish as to reduce or prevent outbreaks related to HAV transmission via these routes.

Table 2

Seawater - Recreational areas

Nr.	Total count per ml	TC per 100ml	FC per 100ml	FS per 100ml	SRC per 100ml	t(water) °C	pH	BOD5 mg/l	Wind velocity m/sec	Salinity ‰
1	14	24	24	29	0	25	7.8	3.15	5 / NW	36.5
2	140	14	14	106	23	25.5	7.9	2	4 / W	35.9
3	120	20	20	40	23	24	7.7	ND	0.5	36.9
4	15	10	15	12	2	20	8	3	3 / NW	35.2
5	18	12	18	14	2	20	8.2	2.7	0	35.8
6	14	6	1	4	18	12	7.9	1.5	3 / N	37.1
7	35	79	25	18	27	8	7.5	4	3 / NW	34.9
8	38	180	150	7	160	9	7.9	1.6	0	37.7
9	4	0	0	3	0	9	7.5	ND	0	37.3
10	10	88	82	100	93	13	7.1	6.9	0	33.2
11	8	76	32	6	15	16	8.1	3.1	0	36.3
12	82	0	0	270	23	17	7.7	3	0	36.8
13	24	8	5	2	4	21	7.7	6	0	34.6
14	14	10	2	1	23	22.5	7.6	3.5	0	36.65
15	88	68	30	70	9	22.5	7.5	3.7	0	36.5
Mean log	1.39	1.22	1.05	1.2	1.04					
Antilog	25	17	11	15	11					
SD	0.46	0.67	0.7	0.7	1.7					
Mean							7.7	3.4		36.09
SD							1.3	1.6		1.2

Table 3

Moderate polluted area

Nr.	Total count per ml	TC per 100ml	FC per 100ml	FS per 100ml	SRC per 100ml	t(water) °C	pH	BOD5 mg/l	Wind velocity m/sec	Salinity ‰
1	580	2100	340	220	150	26	8.1	1.5	0.5	36.2
2	20	160	180	60	93	23	7.8	1.8	0.5 / NW	36
3	122	1000	200	700	20	21	7.7	2.3	3.5 / NW	36.3
4	34	1100	80	0	4	12	8	1.6	3 / E	37.3
5	50	880	75	18	10	9	7.3	1.8	3	37.4
6	20	400	60	30	10	9	7.7	6	0	37.65
7	61	2080	1480	330	90	9	7.7	ND	0	36.7
8	6	500	10	10	400	13	7.5	4.3	0	34.7
9	68	780	390	300	43	16	6.2	3.6	0	35.8
10	12	90	27	8	43	18	7.4	3.7	0	36.9
11	28	140	110	4	240	21	8.2	7.3	0	34.4
12	**280**	**780**	**490**	**88**	**43**	**22.5**	**7.4**	**4.5**	**0**	**36.5**
13	128	232	126	100	23	22.5	7.7	3.6	0	36.4
14	1.7	2.72	2.16	1.63	1.63					
15	51	527	134	44	43					
Mean log	0.55	0.45	0.6	0.85	0.6					
Antilog										
SD										
Mean							7.6	3.5		36.3
SD							0.5	1.8		0.96

Bold areas: positive for HAAg

Table 4

Highly polluted area

Nr.	Total count per ml	TC per 100ml	FC per 100ml	FS per 100ml	SRC per 100ml	t(water) °C	pH	BOD5 mg/l	Wind velocity m/sec	Salinity ‰
1	6300	270000	170000	16500	2400	26	8	5.8	3.5 / NW	35.4
2	640	10200	2200	2500	1400	22.5	7.6	4.7	0.5	36.1
3	3350	300000	300000	30000	3350	24	7	5.4	3.5 / NW	34.8
4	**70000**	**184000**	**128000**	**150000**	**3000**	**21**	**8**	5.4	5 / S	**35.8**
5	7000	64000	24000	18000	35000	21	8	8.1	5 / S	35.8
6	720	40000	27000	15000	1800	12	7.8	5.8	2	37.3
7	616	28000	7000	24000	9000	12	7.8	7	2	35.9
8	3200	96000	89000	106000	9000	8	7.9	ND	1.5 / N	36.7
9	3260	192000	170000	95000	15000	8	8	ND	1.5	31
10	7000	98000	74000	84000	24000	17.5	7	6.8	2 / NW	34.3
11	9600	100000	82000	128000	46000	17.5	6.9	7.2	2 / NW	35.9
12	15000	960000	760000	330000	43000	22.5	7.9	4.6	3.5 / NW	37.95
13*	**5320**	**47000**	**28000**	**18000**	**4000**	**22.5**	**8.5**	7	3.5 / NW	**33.1**
14*	**124**	**1000**	**1000**	**1000**	**4000**	**24.5**	**7.8**	4	0	**38**
15	126	1000	1000	1000	4000	24.5	8	7.8	0	37.8
Mean log	3.4	4.7	4.54	4.45	3.85					
Antilog	2675	46050	34699	28790	7239					
SD	0.76	0.82	0.89	0.8	0.5					
Mean							7.7	6.12		35.7
SD							0.4	1.3		1.9

Bold areas: positive for HAAg
* : positive for HAV

Table 5

Shellfish

Nr	Total count per gr	TC per gr	FC per gr	FS per gr	SRC per gr
1	10300	1600	542	1600	348
2	**9900**	**5420**	**1090**	**5420**	**540**
3	**530**	**5420**	**4100**	**5420**	**1800**
4	**9600**	**5420**	**790**	**5420**	**1090**
5	**4200**	**7090**	**790**	**1300**	**490**
6	**4900**	**4090**	**490**	**1300**	**50**
7	**3500**	**170**	**150**	**2210**	**50**
Mean log	3.63	3.17	2.86	3.42	2.52
Antilog	4293	1495	731	2664	333
SD	0.44	0.6	0.43	0.3	0.61

Bold areas : positive for HAAg
* : positive for HAV

Table 6

Detection of HAAg and infectious HAV from seawater, shellfish and sewage

Nature of samples	Nr of samples	Nr of samples positive for HAAg	Nr of samples positive for HAV
- Seawater			
Recreational areas	15	0	0
Moderate polluted	13	1	0
Highly polluted	15	3	2
- Shellfish	7	6	1
- Sewage	12	2	1

6. ACKNOWLEDGEMENTS

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RESEARCH ON ENTERIC VIRUSES IN AQUATIC ENVIRONMENTS

by

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1. INTRODUCTION

The viral pollution of waters has not been studied extensively world-wide, due to inherent difficulties in the methodology of detection (concentrating and isolating these human pathogens from large volumes of waters (1-3), usually several litres). The main source of viral pollution for such waters is domestic sewage (5-9), which normally carries a large viral load, originating primarily from infected individuals in the community. Among the wide range of viruses that can occur in domestic sewage and other faecally - contaminated waters (Polio-viruses, Coxsackie viruses, Reo-viruses, Echo-viruses, Hepatitis A virus, Rotaviruses, Adeno-viruses, Norwalk agent etc.). the Entero-viruses can be detected most frequently and relatively easily.

Most countries round the Mediterranean sea dispose of their domestic waste waters by traditional methods, which are quite inadequate to destroy viruses and other microorganisms pathogenic to humans (9). Enteric viruses survive in marine or other aquatic environments for prolonged time periods (5), as compared to other pathogens (except for bacterial spores and protozoan cysts), especially if protected by particulate organic matter. Thus, viruses present in domestic sewage, which is discharged into coastal waters, may pose a potential health risk to bathers using such waters, may pose a potential health risk to bathers using such waters for recreation or to consumers of shellfish cultivated in nearby waters (1,3). In the department of Virology of the Hellenic Pasteur Institute, we started to investigate the presence of enteric viruses in sewage in 1982. The methodology of virus detection from waters was established and data on the viral concentration as well as the types of viruses present in sewage effluents from Athens was collected (7-9). Since 1985 the work was integrated into the MED POL Phase II Programme and this report presents the findings of the work performed from January 1985 to June 1987.

2. AREAS STUDIED

During the first year of the programme (1985) the areas studied included the central sewage collector of Athens, before the outfall point, located at Keratsini, and coastal seawater, 100 meters eastward from the sewage outfall. In the second year of the programme, the work was extended to include the locations of (a) Phaliron (slightly polluted) and (b) Alimos and Varkiza (unpolluted recreational areas), as is shown in the attached map in the annex.

3. MATERIALS AND METHODS

Sample volumes of 10-40 litres of coastal-waters were extracted depending on the degree of pollution in each region, whereas from sewage effluents, sample volumes of 1-5 litres were enough to isolate viruses. The concentration method used in all studies was the VIRADEL (Virus-Adsorption-Elution), as recommended by the USEPA manual on water virology (2). In brief, the samples were conditioned by the addition of 0.5 M $AlCl_3$ solution to obtain a final concentration of 5×10^{-4} M, followed by acidification to pH 3.5 with 3N HCl. The acidified samples were filtered through a stack of prefilters and a Millipore filter of porosity 0.45μm, held in disk filter-holder apparatus, at an approximate rate of 500 ml/min. Viral particles, coagulated and held on the

filters, were eluted by soaking and washing the filters with 150ml buffer solution (50mM glycine-NaOH solution supplemented with 1.5% beef extract) at pH 10.5-11.0.

The concentrated samples (buffer eluates) were neutralized with 3N HCl solution, decontaminated with $CHCl_3$ (1/10 the volume of the concentrate), and hydroextracted in dialysis bags with polyethylene glycol 6000, to reduce the volume, before cultivation for virus recovery. Prepared samples were assayed on preformed monolayers of Buffalo-Green Monkey (BGM) and Human epithelial (Hep_2) cell lines, by the plaque forming unit (PFU) or the cytopathic effect (CPE) method. Recovered virus were subcultured once and identified by the microneutralization test using the Lim Benyesh-Melnick pools of antisera for typing enteroviruses. Furthermore, Polio-virus field-isolates were tested with strain-specific cross-adsorbed antisera, in order to determine their origin (4), vaccine derived strains (Sabin-Like) or wild strains.

4. RESULTS AND THEIR INTERPRETATION

During the first year of the work (1985), we examined 48 water samples, 24 originating from sewage effluents and 24 of seawater from Keratsini, for the parallel detection and evaluation of enteric viruses. All 24 sewage effluent samples were positive for virus isolation, whereas 21 seawater samples from Keratsini (sewage receiving region) were positive. The viral loads detected are shown in table 1. The mean virus concentration level for sewage effluents was 267 cytopathic units (CPU)/litre with range values from 32 to 700 CPU/L. On the other hand the mean value in seawater was 35 CPU/L, ranging from 5 to 145 CPU/L. Comparing the viral loads (mean values) recorded at the two sampling locations during 1985, it is evident that viral pollution of seawater, 100 meters eastward from the outfall point of the central sewer, is reduced by a factor of 7.6. This significant reduction is probably due to such factors as the large dilution of rejected effluents, the sedimentation of particulate matter on to which viruses are adsorbed and to the inactivation of viruses by various physicochemical-biological processes taking place in seawater.

Table 1

Enteric Virus concentration levels in sewage effluents and seawater from Keratsini (Period: 1985)

Sample Origin	CONCENTRATION $_{CPUL-1*}$		Positive samples (n = 24)
	Mean	Range	
Keratsini Sewage-Effluents	267	32-700	24 (100%)
Keratsini Seawater	35	5-145	21 (87.5%)

* $CPUL^{-1}$ = Cytopathic units per litre of sample

Concerning the identification of viruses, 489 and 164 randomly selected field-isolates from sewage effluents and coastal seawaters respectively, were tested for identification with the microneutralization test. The results of serotyping the above isolates are shown in Table 2. All three serotypes of Poliovirus were present in both sampling locations, although Poliovirus type 3 was more prevalent in sewage, and type 2 in seawater. Regarding the Coxsackie viruses group B, serotypes CB2, CB4, CB5 and CB6 were present in both types of samples, while CB1 was detected only in sewage. Coxsackie CB5 was the dominant serotype, followed by CB6 and CB4 in sewage effluents, while in seawater CB2 was the second most prevalent serotype. CB1 was

detected only in sewage, while CB3 was totally absent. Finally, five different serotypes of Echoviruses (E3, E7, 15, E19 and E23) were detected, although with low frequencies, except for E7, which was present in both samples (see Table 2).

Table 2

Identified serotypes of Enterovirus field-isolates from Keratsini sewage effluents and seawater (Period 1985)

Virus Serotype	Sewage effluents	Seawater
Polio		
1	27	5
1	56	25
3	63	13
Coxsackie B		
CB1	4	-
CB2	19	15
CB3	-	-
CB4	42	10
CB5	83	38
CB6	49	6
Echo		
E3	4	1
E7	13	2
E15	1	-
E19	1	-
E23	1	-
Other Enteroviruses	70	34
Adenoviruses (untyped)	56	15
TOTAL	489	164

During the second year of the programme (January 1986 - June 1987), the work was extended to include three more sampling areas, those of Phaliron (slightly polluted) Alimos and Varkiza, unpolluted recreational areas. The results are presented in Table 3. Again all sewage samples were positive for viruses (100%), as in the previous year of the study, whereas seawater from Keratsini was positive in 75% of the samples, as compared to 87,5% in the previous year. The mean virus concentration level for sewage effluents was 217 CPU/L with range values of 45 to 655 CPU/L. Both the mean and range values were slightly lower than the values obtained the previous year, 1985. The same was true for seawater from Keratsini, the mean value being 13 CPU/L, and range values 0 to 65 CPU/L.

Five out of 24 (25%) seawaters samples from Phaliron area (slightly polluted waters) were found positive, and the virus levels detected, as well as the different serotypes, were much lower (see table 3), than those detected in Keratsini highly polluted waters. From the other two locations Alimos and Varkiza, which are recreational bathing beaches we had only one positive sample from

Alimos, whereas all Varkiza's samples were negative. The one positive seawater sample from Alimos most probably represents shedding of virus from infected bathers, happened to be at the beach at the time of sampling.

Table 3

Enteric virus concentration levels in waters
with varying degrees of pollution
(Period: January 1986 - June 1987)

Sample Origin	CONCENTRATION $_{CPUL-1*}$		Positive samples (n = 24)
	Mean	Range	
Keratsinis sewage effluent	217	45-655	24 (100%)
Keratsini seawater	13	0-62	18 (75%)
Phaliron seawater	3	0-6	5 (25%)
Alimos seawater	a single positive sample (three virus isolations)		1 (5%)
Varkiza seawater	N E G A T I V E		0

* $CPUL^{-1}$ = Cytopathic units per litre of sample

The identification of the different serotypes of Enteroviruses detected during the second year of the study period is shown in Table 4. From the sewage effluents alone, 14 different serotypes were identified, the three Polio-serotypes, five Coxsackie B (except CB3) and six different Echo serotypes. The new serotype detected for the first time in the second year of the study was Echo 21. This serotype was found not only in sewage but also in seawater from Keratsini. The other serotypes detected in Keratsini seawater remained the same as in the previous year (1985). From the region of Phaliron only four different serotypes were detected, i.e. Polio types 1, 2 and 3 and Coxsackie B5, which was the serotype with the highest frequency of detection throughout the study period. From the single positive sample found in the recreational region of Alimos only 3 Coxsackie B serotypes were isolated. No other serotypes whatsoever were found. All the Varkiza seawater samples were negative for virus isolations.

Finally, the intratypic serodifferentiation test for Polioviruses was performed on 160 field-isolates (50 Polio, 1, 54 Polio 2 and 56 Polio 3 isolates). This test can differentiate those strains of Polioviruses originating from the attenuated Sabin vaccine (Sabin-like strains) from the natural (wild) strains (4), that may still exist in the community. The results of this test on the field isolates of Polioviruses are presented in Table 5. The majority of the strains tested are characterized as Sabin-like (Vaccine derived), except for one Polio 3 isolate, which reacted on Elisa as Non Sabin-like. This strain, was isolated from sewage effluents. Three more Polio 1 strains and two Polio 2 strains reacted as intermediate between the Sabin-like and the Non Sabin-like strains.

Table 4

Identified serotypes of enterovirus field-isolates from different sources (Period: January 1986 - June 1987)

Virus serotype	Keratsini sewage	Keratsini seawater	Phaliron seawater	Alimos seawater	Varkiza seawater
Polio					
1	42	15	3	-	-
2	76	34	1	-	-
3	87	25	6	-	-
Coxsackie B					
CB1	4	-	-	-	-
CB2	21	17	-	-	-
CB3	-	-	-	-	-
CB4	73	15	-	-	-
CB5	114	45	16	3	-
CB6	63	10	-	-	-
Echo					
E3	2	1	-	-	-
E7	11	2	-	-	-
E15	2	-	-	-	-
E19	1	-	-	-	-
E21	7	1	-	-	-
E23	1	-	-	-	-
other enteroviruses	43	17	-	-	-
T O T A L	547	182	25	3	-

Table 5

Intratypic serodifferentiation of poliovirus field-isolates by ELISA (Period: 1985 - 1986)

Characterization	Poliovirus 1	Poliovirus 2	Poliovirus 3
SL	47	52	55
NSL	-	-	1
IM	3	2	-

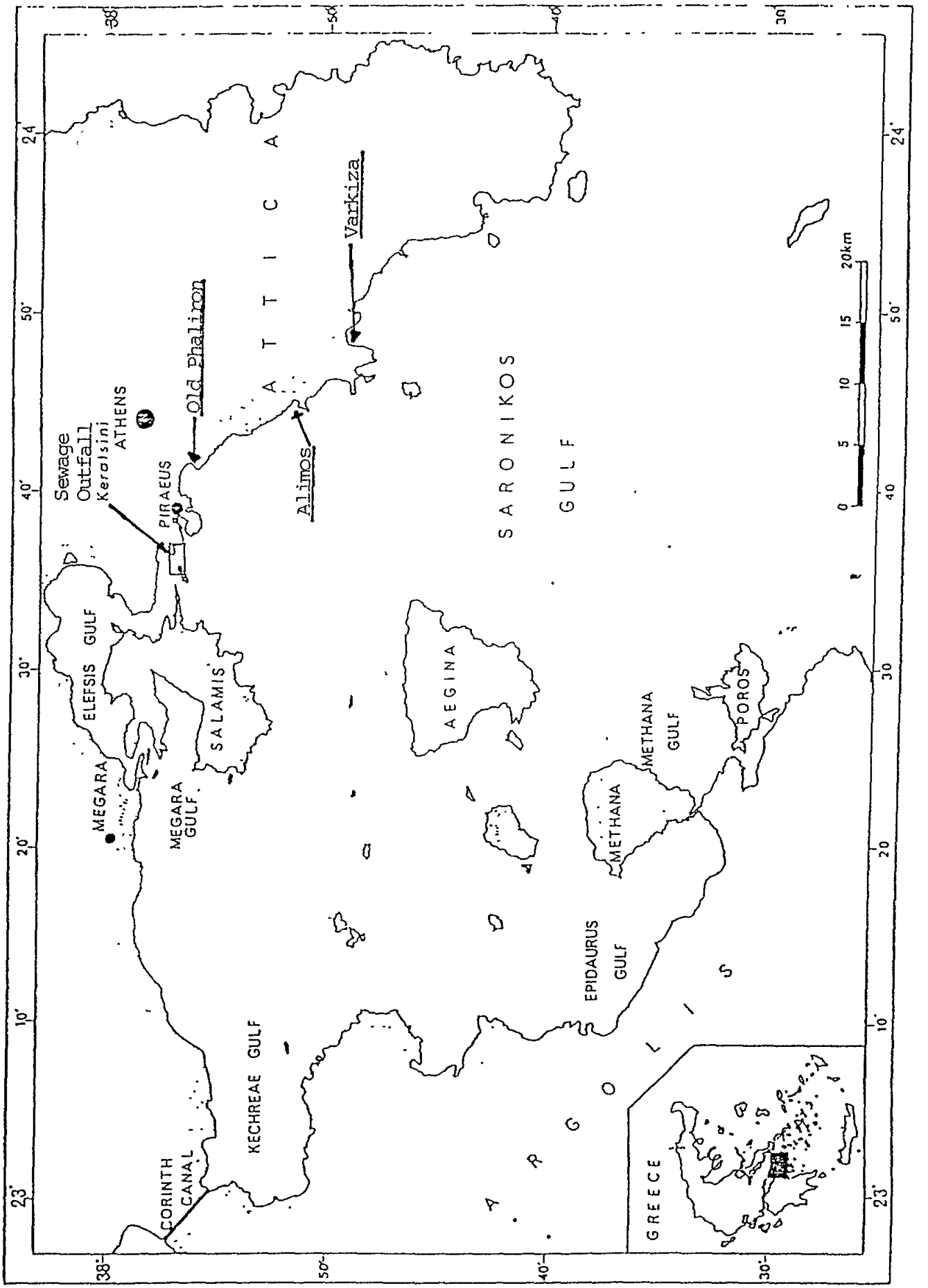

Figure 1. - Locations where sampling was performed (arrows)

5. CONCLUDING REMARKS

- Enteric viruses are regularly found in domestic sewage effluents, as well as in seawaters receiving domestic sewage. The degree of viral pollution in such seawaters depends mainly on the viral loads received and the distance from the polluting source.

- A large number of different serotypes are found in domestic sewage, which serotypes can be detected in seawater as well.

- Non-sewage receiving coastal-waters were practically virus-free. Occasional isolations of viruses from such waters are probably the result of shedding from the bathers themselves.

- The most frequently detected serotypes in our study were the Poliovirus types 2 and 3 and the Coxsackie B5. However, among the Polioviruses, the great majority of the isolates were Vaccine derived or Sabin-like. Only a single isolate was characterized as non Sabin-like strain.

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DETERMINATION OF THE MOST SUITABLE MEDIUM FOR ENUMERATION OF FAECAL STREPTOCOCCI IN SEAWATER

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1. INTRODUCTION

Faecal streptococci (FS) are considered good indicators of faecal pollution, just like faecal coliforms (FC), therefore they are of great sanitary as well as clinical significance. Attempts to find a good selective medium for the isolation of all FS or just enterococci date back to 1918 (18) and the fact that it was no easy task in demonstrated by the 68 media, based on various inhibitory substances, that were proposed for this purpose till 1972, when Povlova *et al.* (18) reviewed the subject. These media were designated for the examination of water, sewage, feces and other clinical material, therefore their efficacy, specificity and selectivity were tested against these environments or bacterial species usually present in them, like Enterobacteriaceae, and the media were found satisfactory (10, 15, 20). Comparative studies between some of the media or modified versions of them (3, 7, 9, 18, 19) were also done on feces, sewage, food and water from rivers, lakes or springs, but very few samples of seawater (7).

KF streptococcus agar (KF) introduced by Kenner *et al.* in 1961 (15) is considered one of the best media for enumeration of FS in water by membrane filtration (MF) and it is recommended in Standard Methods for the Examination of Water and Wastewater (1). It was also adopted for the examination of seawater. However, our experience showed that in some samples the number of FS calculated on the basis of red and pink colonies enumerated on MF incubated on KF exceeded that of the faecal coliforms by 2-3 orders of magnitude, a discrepancy which could not be attributed merely to the longer survival of FS is seawater. This led to isolation and gram staining of some typical colonies, which revealed that many of them were gram negative bacilli or gram positive cocci in clusters. The catalase test showed that they released O_2 from 3% H_2O_2, hence they were not streptococci. Since the non-streptococcal bacteria were the dominant flora in a considerable percentage of the samples examined, and their number exceeded several hundred per 100 ml of water, it was felt that the rate of false positive results on KF should be evaluated in a large number of samples in order to reassess the suitability of this medium for marine water. It was also important to examine other commercially available media like M-Enterococcus agar (ME) and Bile-Esculin - Azide agar (BEA) which is equivalent to PSE (10) and see whether one of them was more suitable for the purpose. This comparative study was therefore undertaken and the results are presented here.

2. MATERIALS AND METHODS

2.1 Media

KF, ME, BEA, Brain-heart infusion (BHI) and Tryptic soy broth (TSB) - all Difco. Nutrient agar with 0,5% NaCl (NA) - Institute Pasteur. Modified KF was prepared from the ingredients, omitting the NaCl (KF^1) and also the Na-glycerophosphate (KF^2).

2.2 Incubation temperature

35 ± 0.5 °C and 42 ± 0.5 °C, 48h for KF and ME, 24h for BEA.

2.3 Membrane filters (MF)

Gelman GN-6, graded.

2.4 Procedure

Samples of seawater from beaches of the central part of Israel, that were brought to the laboratory for routine monitoring of FC were also monitored for FS by the MF method. Portions of 10ml and 50ml of water filtered and the MF were incubated on KF. Later parallel samples were also filtered and incubated on ME and BEA. On KF and ME all pink and red colonies were counted with a magnifying glass and on BEA light to dark brown colonies with brown haloes were counted. Every group of colonies with the same morphological appearance was counted separately and 2-3 colonies from each group were subcultured on NA for observation of colonial morphology on this medium and performance of the catalase test. Well isolated colonies from NA were subcultured in BHI or TSB, gram stained and examined microscopically. The catalase test was performed on microscopic slides with 3% H_2O_2.

According to the colonial and microscopic morphology and the catalase reaction the bacteria that grew on the MF were designated as streptococci, staphylococci and gram negative or gram positive bacilli. All gram positive cocci that did not show any chain formation and were catalase positive were included in the staphylococci group.

The number of each group of bacteria per 100ml of water was calculated. When the representative colonies from each morphological group on the MF showed 2 or 3 different types, the count of that group was divided equally between them (28 cases out of 1332 groups of colonies counted). Selected colonies from each group were identified by the API system (La Balme les grottes, 38390 Montalieu-Vercieu, France).

2.5 Survival experiments

Bacterial strains isolated from MF of seawater or drinking water were identified by the API 20 system. The FS were from MF on KF or ME and two strains from the type collection of the Streptococcus Center in Jerusalem. The FC were blue colonies from MF on M-FC agar incubated at 44.5 °C.

The bacteria were grown on NA slants, harvested and suspended in 500 ML portions of sterile seawater (filtered through MF of 0.45 μ them 0.2 μ porosity) in brown bottles kept at room temperature. Samples were withdrawn at 0 time and 1-7 days interval, diluted if necessary, and filtered. The MF with FS were incubated on KF for 48h and the FC on m-Endo agar for 24h at 35± 0.5 °C. The colonies were counted and the number of CFU per ml was calculated.

3. RESULTS

During a period of two years, 234 samples of seawater from 32 beaches were monitored on KF; 124 were also monitored on ME and 17 on BEA as well. From the first 8 samples on KF an additional set of MF was incubated at 44.5 ± 0.2 °C but growth at this temperature was poor so it was replaced by 42 ± 0.5 °C. Comparison of incubation temperatures (35 ° vs 42 °) was done in 33 samples on KF, in 17 of them also on ME and MEA. From the various MF 2670 representative typical colonies were subcultured gram stained and examined for catalase activity. Identification by the API 20 system was performed on 266 randomly selected colonies, 253 were identified to the species level.

The distribution of bacterial groups that grew on KF according to gram stain and catalase activity is summarized in table 1. It shows that streptococci alone were found in 27.8% of samples, while in 26% (items 8, 9, 10) there were no streptococci among the typical colonies counted as FS. In additional 23.1% (items 3, 5, 7) the streptococci were only a minority and in 23.2% (items 2, 4, 6) they comprised over 50% of the flora. In 23 samples (10%) the number of CFU per 100ml of water exceeded 1000, erroneously indicating high pollution in water that was actually clean, with low FC counts, as illustrated by several samples presented in table 2.

In table 4 the selectivity of KF is compared to that of ME on the basis of the dominant flora obtained on MF incubated on each medium. It shows that the percentage of samples where staphylococci comprised the dominant flora was similar on both media (23% and 28%). However, none of the gram negative and few gram positive bacilli that were isolated from KF were found on ME, which made the latter much more selective, with streptococci comprising the dominant flora in 72% of samples versus 35% on KF. The recovery rate of streptococci on both media is compared in table 4. It seems to be better on KF, but it should be borne in mind that not all streptococci isolated were enterococci or even FS (table 8).

Raising the incubation temperature from 35 °C to 42 °C did not improve the selectivity of the media as shown in table 5. The total number of colonies on KF was higher at 35 °C in 10 samples, at 42 °C in 13 samples and equal in 10 samples. This table also shows that the selectivity of BEA was not better than that of KF, and inferior to ME since some of the gram negative bacilli also produced brownish colonies on it.

Since the most frequently isolated gram negative bacilli on KF were marine holophylic bacteria that require NaCl for growth, it was attempted to improve the KF medium by omitting the NaCl in it (KF^1). When this had no effect and *V. alginolyticus* was isolated from this medium too, the Na glycophosphate was also omitted (KF^2). The recovery rate of gram negative bacilli was smaller, however *V. alginolyticus* was isolated on several occasions. It seems that some strains of this genus can grow without Na^+ ions, therefore the medium should contain substances that are specifically inhibitory to them.

The identification of randomly selected strains of gram negative bacilli isolated from MF on KF and BEA is given in table 6. The dominant species were marine vibrios, mainly *V. alginolyticus* (14) and several *Pasteurella* species. Some strains of oxidase negative vibrios that grew best with 3% NaCl could not be identified, even by the "Centre de Formation API" in Montalieu-Vercieu, France, where several strains were sent. There were also a few strains of gram positive bacilli, probably *Bacillus* spp, which we did not attempt to identify. Some of them also grew better with 3% NaCl.

It was realized that colonial and microscopic morphology, even combined with the catalase test, were not always sufficient to differentiate certain strains of *Streptococcus faecalis* from staphylococci, since on rich media colonies of most group D streptococci are larger than usual and may be confused with staphylococci and micrococci colonies (8). Rarely there is also catalase activity in some strains (8). It was therefore deemed necessary to identify some of the gram positive cocci in clusters which also showed pairs of bacteria to the species level which proved that they were indeed staphylococci as outlined in table 7. They were isolated from all three media.

In order to see whether the streptococci isolated were all FS, 86 strains, most of them from KF, were classified. The results presented in table 8 show that 26 of the 72 strains taken from KF (36%) were not FS.

Survival curves of FS, *E. coli* and other FC in seawater under laboratory conditions are given in figures 1-3. They show that FS survive longer than FC in seawater, over 60-70 days. When the initial rate of pollution is high some FC including *E. coli* can also survive 40 days or more in the winter.

Table 1

Distribution of bacterial flora on membrane filters incubated on KF streptococcus agar at 35 °C for 48h

Bacterial flora	Samples with stated No. of CFU per 100ml of water			
	<100	101-1000	>1000	Total no(%)
1. Streptococci only	42	18	5	65 (27.8)
2. Strep.[1] + staph	21	5	2	28 (12.0)
3. Staph. strept.	5	9	4	18 (7.7)
4. Strept. + gnb [2]	9	3	1	13 (5.6)
5. Gnb. + strept.	6	9	7	22 (9.4)
6. Strept. + staph. +gnb	7	3	3	13 (5.6)
7. Gnb. + Staph. + strept.	2	9	3	14 (6.0)
8. Staphylococci only	10	10	3	23 (9.8)
9. Staph. + gnb	6	5	5	16 (6.8)
10. Gnb only	6	15	1	22 (9.4)
Total	114 (48.7)	86 (36.8)	34 (14.5)	234(100)

(1) - The group written first comprised >50% of the flora.
(2) - Gnb = gram negative bacilli.

Table 2

Examples of discrepancy between actual rate of contamination and false positive results obtained by counting typical red colonies on membrane filters incubated on KE streptococcus agar

Location	Sampling Point	Date	FC[1] CFU/ 100ml	"FS" [2] on KF	
				CFU/100ml	Confirmed results
Nathania	M23	27.5.85	16	2300	100% *Staphylococcus xylosus*
	M24	27.5.85	16	1200	67% *S. xylosus*, 33% gpb[3]
	M29	29.5.85	40	2000	73% gnb[3] (*V. alginolyticus*)
	M31	7.8.85	12	500	90% gnb(*V. vulnificus*)+*Staph*
	M32	7.8.85	<2	360	100% gnb(*V. alginolyticus*)
	M34	7.8.85	<2	320	99% gnb(*V. alg*+ *Pasteurella pneumotropica*
Tel-Aviv	M48	31.7.85	22	2700	75% gnb(*V.parahaemolyticus*)
Bat-Yam	M49	17.6.85	46	3200	90% gnb(*V. alginolyticus*)
	M50	7.8.85	<2	1000	99% gnb(delicate, ox-, not ident.)
Rishon-Lezion	M52	6.8.85	4	1800	99% *S. xylosus*
	M52	17.6.85	<2	1000	99% Nfs[4] (*Gemella haemolysans*)
	M52	1.7.85	2	2800	100% gnb(*V. parahaem.*, + *Plesiomonas shigelloides*
	M53	1.7.85	<2	200	100% gnb(*Pasteurella* sp.)
	M56	17.6.85	2	1100	100% gnb(*V. alg., Past. multocida*)

(1) FC = Faecal coliforms
(2) "FS" = Colonies that would have been considered as faecal streptococci without confirmation
(3) Gpb = Gram positive bacilli, gnb = Gram negative bacilli
(4) Nfs = Non-faecal streptococci

Table 3

Selectivity of KF streptococcus agar (KF) and M-Enterococcus agar (ME) for monitoring faecal streptococci in seawater
Incubation at 35 °C, 48h (no 124)

Total No. of CFU/100ml of seawater	Samples with following dominant flora (>50%)					
	KF			ME		
	Strept.	Staph.	Gnb	Strept.	Staph.	Gnb
<100	20	8	12	62	22	0
101-1000	18	14	31	25	13	0
>1000	5	7	9	2	0	0
Total No. of samples	43	29	52	89	35	0
%	35	23	42	72	28	0

Table 4

Recovery rate of streptococci on KF-Streptococcus agar (KF) and M-Enterococcus agar (ME)

Range of Strept [1] CFU/100ml water	No. of samples							
	KF>ME		KF=ME		KF<ME		Total	
	No	(%)	No	(%)	No	(%)	No	(%)
0 - 20	12	(22)	30[2]	(54)	13	(24)	55	(100)
21 - 50	10	(71)	0	(0)	4	(29)	14	(100)
51 -100	14	(74)	0	(0)	5	(26)	19	(100)
101-500	16	(62)	3	(11)	7	(27)	26	(100)
501-1000	2	(40)	3	(60)	0	(0)	5	(100)
>1000	3	(60)	2	(40)	0	(0)	5	(100)
Total	57	(46)	38	(31)	29	(23)	124	(100)

(1) Confirmed by microscopy and catalase test
(2) In 17 samples the number of CFU was 0.

Table 5

Effect of temperature on selectivity of media

Bacterial flora	No of samples with stated flora					
	KF (n=33)		ME (n=17)		BEA (n=17)	
	35 °C	42 °C	35 °C	42 °C	35 °C	42 °C
Streptococci only	12	11	6	5	2	4
Strept. + staph.	10	12	6	8	8	5
Strept. + Gnb[1]	2	5	0	0	2	4
Staphylococci only	3	2	3	3	3	3
Strept. + Gnb	0	1	0	0	2	0
Gnb only	5	2	0	0	0	1
No growth	1	0	2	1	0	0

(1) Gnb = Gram negative bacilli

Table 6

Gram negative bacilli that produced "typical" red colonies on KF-Streptococcus agar (KF) or brown colonies on bile-esculin-azide agar (BEA), identified by the API 20NE system

Genus	Species	No of strains identified	
		KF	BEA
Vibrio	*V. alginolyticus*	75	5
	V. parahaemolyticus	7	
	V. vulnificus	3	
	V. damsela [1]	1	
Pasteurella	*P. multocida*	6	
	P. pneumotropica	5	
	P. haemolytica	1	
	P. aerogenes	1	
	P. spp.	1	
Aeromonas	*A. hydrophila*	2	1
Plesiomonas	*P. shigelloides*	2	
Moraxella	*M. phenylpyruvica*	1	
Agrobacterium	*A. radiobacter*	2	
Pseudomonas	*Ps. vesicularis*	1	
	Ps. paucimobilis		
CDC gr II B		2	
CDC gr VE [1]		1	
Not identifiable [2]		13	
Total		125	6

(1) Identified in Centre de Formation API, France.
(2) Four of them were designated by the Centre as: Vibrio sp. oxydase (-) Na+ required for growth and one as probably Serratia liquefacients.

Table 7

Staphycolocci that produced "typical" red colonies on KF-streptococcus agar (KF) M-enterococcus agar (ME), or brownish colonies on bile-esculin azide agar (BEA), identified by API 20 staph. system

Species	No of strains identified		
	KF	ME	BEA
S. aureus	8	5	1
S. xylosus 2	13	3	
S. hominis 1	3	4	3
S. saprophyticus	2	2	1
S. epidermidis		1	1
S. warneri	2	2	
S. sciuri	2	1	
S. leutus		1	
Total	30	19	6

Table 8

Classification of streptococci isolated from seawater on membrane filters incubated on KF-streptococcus agar (KF), M.-Enterococcus agar (ME) and bile-esculin-azide agar (BEA), identified by the API 20 strept. system

Genus	Species	No of strains identified		
		KF	ME	BEA
Enterococcus	*E. faecalis* 1	3		
	F. faecalis 3	2		
	E. faecium 1	6	2	1
	E. faecium 2	15	5	2
	E. faecium 3	10		
	E. durans 2	6	2	
	E. avium	3		1
	E. gallinarum	1		
Aerococcus	*A. viridans* 1	2		
	A. viridans 2	10		
	A. viridans 3	9		
Streptococcus	*S. sanguis* I/1	2		
Gemella	*G. haemolysans*	3		
	Total	72	9	5

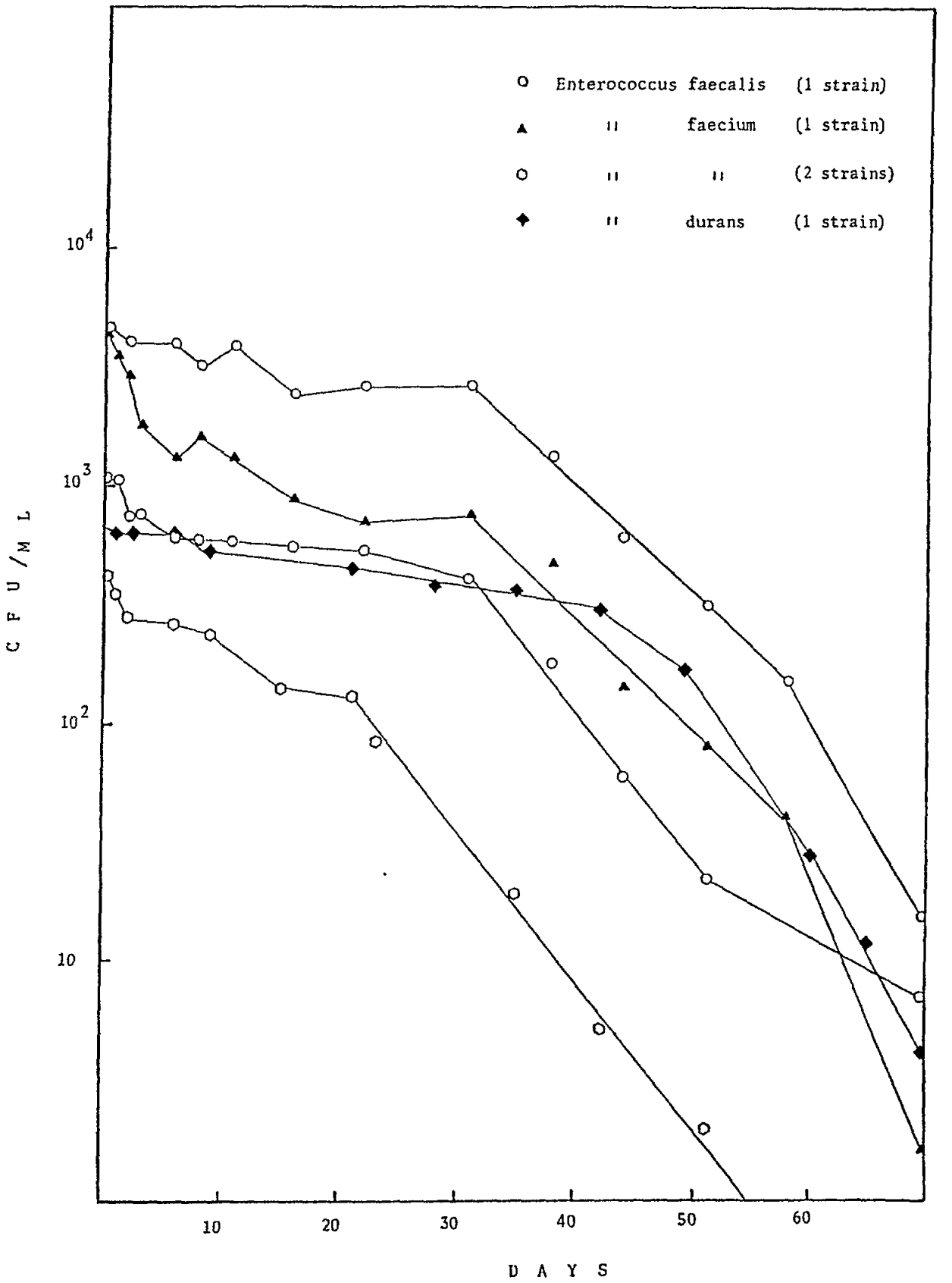

Fig. 1 Survival of faecal streptococci in seawater

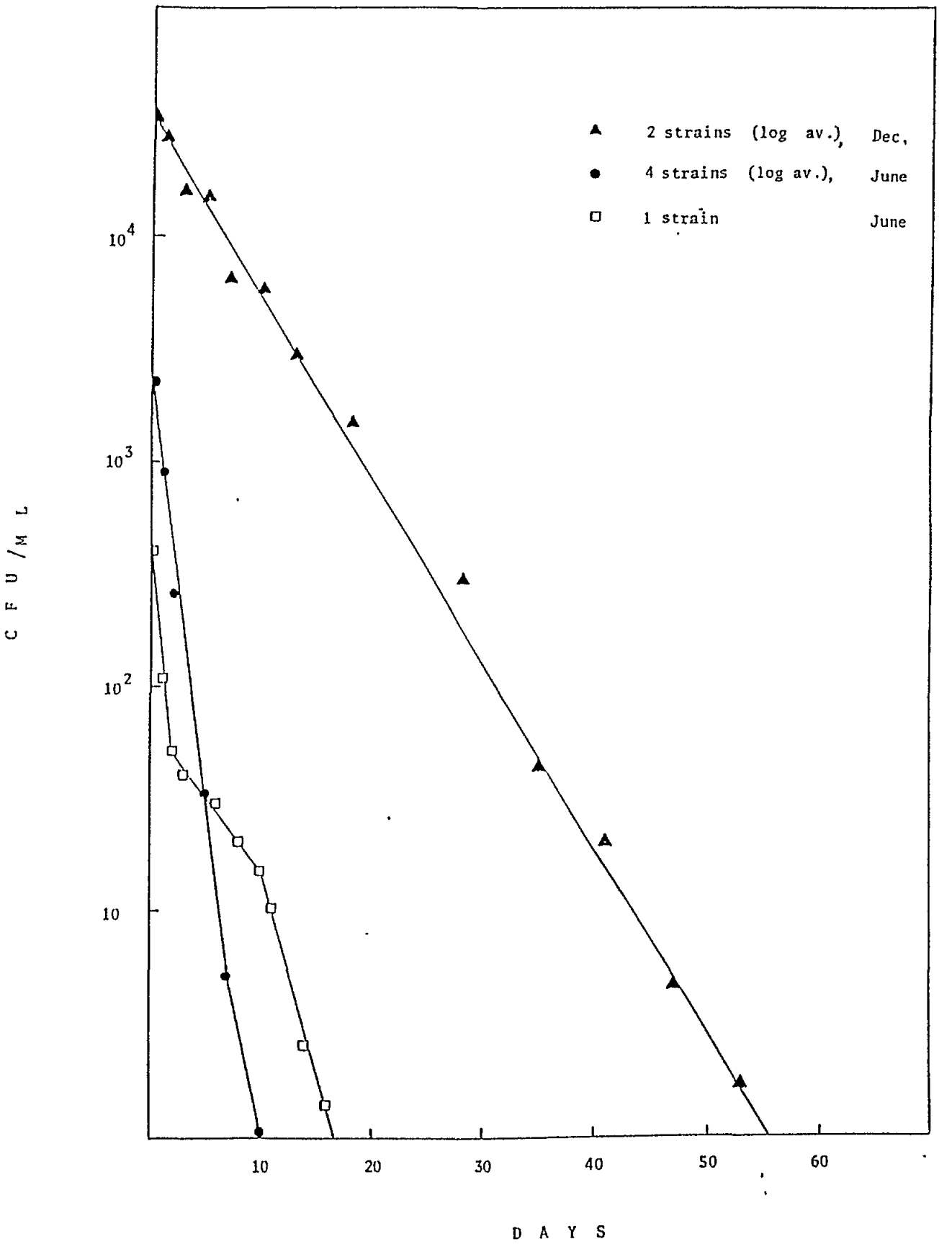

Fig. 2 Survival of *E. coli* in seawater

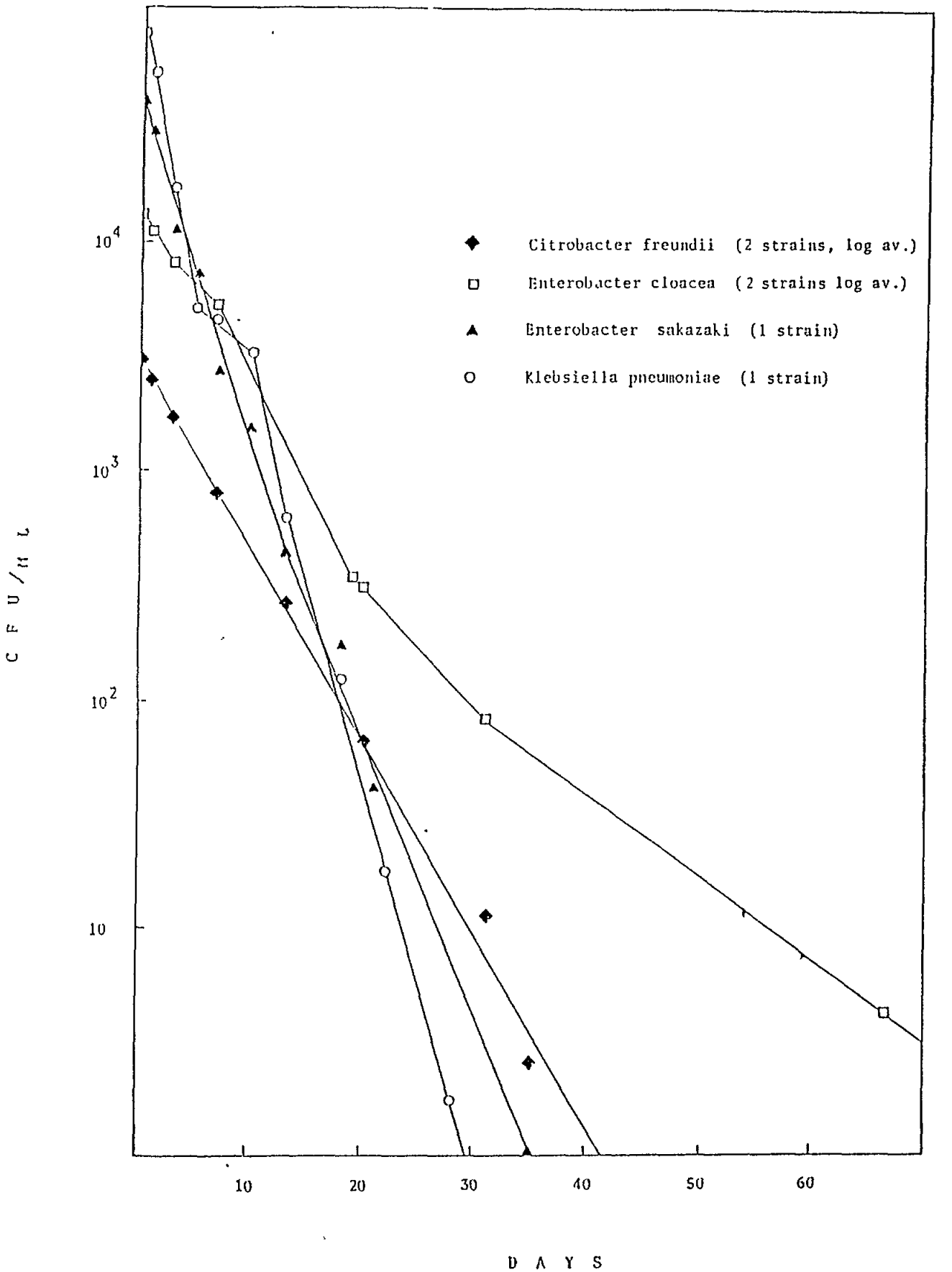

Fig. 3 Survival of coliforms other than *E. coli* in seawater

4. DISCUSSION

Epidemiological studies indicate that the enterococci are the indicator of choice for assessing the quality of marine water (6). It is reasonable to assume that this group of bacteria may eventually replace FC, or at least be of equal sanitary significance in monitoring seawater. It must, therefore, be ascertained that the media employed for enumerating them in seawater is selective against marine bacteria, so that accurate results may be obtained without much additional confirmation.

This study shows that KF-streptococcus agar, which is undoubtedly a very good medium for monitoring FS in other environments is definitely unsuitable for marine water, where the background flora contains many bacteria that grow on it and produce red colonies like those of FS, leading to grossly erroneous results (about 50% in this case). In 25% of the 234 samples, some of them with very high counts, FS were absent and in additional 23.1% they comprised less than 50% of the bacterial flora. The organisms most frequently isolated were marine vibrios, mainly *Vibrio alginolyticus*, which is very common in the marine environment (14). In 1964 Slanetz and Bartley (21) compared KF and ME for recovery of FS from faeces, sewage and water from various sources, including shellfish water and oysters. They reached the conclusion that KF does not appear suitable for selective detection of FS in seawater and seafood. They isolated and identified selected red and pink colonies and found that from salt water and oysters only 28% and 42% of colonies from KF, respectively, were FS. The contaminating organisms were mainly *Pseudomonas*, filamentous gram negative rods and micrococci. Their findings are in good agreement with the present work. The filamentous rods are probably identical to the vibrios isolated here where many filamentous forms were observed, and the rate of streptococcal isolation is also similar. However, they claim that 98,5% and 100% of colonies on ME were confirmed as FS, while we isolated quite a few stains of staphylococci from ME as well as from KF (tables 3 and 7).

Other accepted methods for enumerating FS in water were found inadequate for seawater. Buck (4) found that by the MPN method 45% of the results confirmed in ethyl violet azide (EVA) broth were false positive and the most commonly isolated false positive organism was a gram negative non-pigmented short rod, which was presumed to be a species of vibrio. He also isolated gram positive cocci in clusters and from one sample gram positive bacilli. These findings are also similar to ours on KF. He later (5) increased the concentration of azide to 0.1% and tried Pfizer PSE broth for the examination of 28 samples of coastal and estuarine water. His results indicated that false positive reactions occurred frequently(?) in AD broth and increased azide concentration was inhibitory to streptococci. In PSE broth 3 samples showed false positive tests, in one case rod shaped organisms and in the other two gram positive cocci in clusters.

Bile-aesculin provides a reliable means for identifying group D streptococci (11, 12). Selective media based on hydrolysis of esculin as a marker for FS were found very satisfactory for the examination of clinical specimens, feces, sewage and water from various sources, including some samples of seawater (7, 10, 17, 18). Nevertheless it was shown here that for seawater with the indigenous flora of our region BEA is not selective enough, just like KF. Of the 17 samples examined in this medium some typical light and dark brown colonies were identified as *V. alginolyticus, Aeromonas hydrophila* and several species of staphylococci, (tables 5 and 6). Farmer *et al.* (14) state that only 3% of *V. alginolyticus* hydrolyse esculin, however, according to the API 20 NE scheme for identification of gram negative bacilli 69% of *V. alginolyticus* hydrolyse esculin, as do 95% of *V. vulnificus*, 82% of *A. hydrophila* and 99% to 100% of *Pseudomonas vesiculasis* and *P. paucimobilis* which were all found in our water (table 5). Staphylococci usually do not hydrolyse esculin (2), yet some of the brownish colonies we isolated on BEA were identified as staphylococci (table 7).

The medium of Levin *et al.* (17) which is also based on esculin hydrolysis as a marker for FS, contains actidion and nalidixic acid as inhibitors. The authors examined 2231 colonies isolated from polluted marine and estuarine waters and found that 90% of the typical colonies and 11.7%

of the other colonies were enterococci. It is possible that the marine vibrios so often isolated from our water were absent in the Atlantic Ocean in the New-York vicinity, or that they were inhibited by the 0.24 gr/L nalidixic acid. The 1.5% NaCl in this medium would certainly enhance their growth./ This medium was not included in the present study since it is not available commercially, but the abovementioned points should be clarified before it can be recommended for Mediterranean water.

Staphylococci were isolated from all three media and they included pathogenic and non-pathogenic species (16). Most of the randomly selected streptococci that were identified came from KF and only 64% of them were FS (table 8). Of the 26 non-faecal streptococci, 21 were *Aerococcus viridans*, two were *Streptococcus sanguis* and three were *Gemella haemolysans* (13) which comprised most of the flora in two samples. The 9 strains from ME and the 5 strains from BEA were all enterococci, but their number if too small (compared to the 72 strains from KF) to determine the rate of specificity of these media for FS. Pavlova *et al* (18) identified 721 isolates and found that 28.3% were non-faecal streptococci. Brodsky and Scheiman (3) who examined secondary sewage effluent obtained on KF a confirmation rate of 83% for FS with 54% enterococci. On PSE their confirmation(?) rate was 90% FS with 86% enterococci.

The data of this study show that none of the three media examined is completely selective for FS, however ME seems to be the best since none of the gram negative bacilli grew on it. The recovery rate of streptococci on ME was equal to that of KF when the bacterial counts were low and almost all the colonies on the filters were confirmed. It seems to be somewhat better on KF in the higher counts (table 4), but the results were obtained after additional two days of tedious subculturing and confirmation. This is impractical in routine monitoring, where it is desirable to obtain the best results in the shortest period of time with a minimal amount of work. Volterra *et al.* (22) examined 20 samples of seawater on KF, ME, PSE and MPN at 37° C and 44° C and found that ME and PSE yielded higher counts than KF.

To the best of my knowledge this is the most thorough work on monitoring faecal streptococci in seawater, where over 200 samples were examined and the interfering organisms were isolated and typed to the species level. It leads to the following conclusions:

a) In order to be completely selective for monitoring seawater the medium should contain ingredients inhibitory to marine vibrios and other gram negative bacilli listed in table 6, as well as to staphylococci.

b) Of the three commercially available media evaluated here M-Enterococcus agar seems to be the most suitable for seawater and until a better medium is introduced it is recommended for this purpose.

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ENUMERATION OF FAECAL STREPTOCOCCI IN SEAWATER

by

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1. INTRODUCTION AND AIM OF INVESTIGATION

Our Institution is currently participating in a Marine Pollution Monitoring Programme, as part of MED POL Phase II. Problems have been encountered in the enumeration of Faecal Streptococci (FS) by Membrane Filtration, using KF Streptococcus Medium 36° C/48h (UNEP/WHO Reference Method No. 4, Rev. 1, 1983). Abnormally high FS counts, relative to FC counts, have been obtained. This phenomenon was encountered in other Mediterranean Institutions participating in the MED POL monitoring programme.

On 27 January 1988 our Laboratory was formally asked by the WHO/EURO Project Office for the Mediterranean Action Plan in Athens to participate in a comparative exercise, together with five other Laboratories. The aim of this exercise was to enable WHO and UNEP to arrive at a satisfactory reference method for the enumeration of FS in seawater.

What follows is a report of the exercise. The investigation was coupled with a study of microbial indicator survival in fresh and seawater, and on the behaviour of the FC/FS ratio with time.

2. MATERIALS AND METHODS

62 water and 61 sand/sediment samples were taken from 11 popular bathing sites around the Island of Malta.

Water samples were analyzed for Faecal Coliforms (FC) and Faecal Streptococci (FS) by filtration of 10 ml aliquots, Sand/sediment samples were analyzed by shaking 100 g of the material with 100 ml dilution water, the suspension left to settle for 3 minutes, after which 10 ml aliquots of supernatant were filtered for FC and FS counts.

FS enumeration was attempted on M-Enterococcus Agar at 36° C (M-ENT/36° C), KF Streptococcus Agar at 36° C (KF/36° C) and KF Streptococcus Agar at 44° C (KF/44° C). Incubation time was 48 hr throughout. FC enumeration was carried out on M-FC Agar at 44° C, as per UNEP/WHO Reference Method No. 3 Rev. 1, 1983.

On both M-Enterococcus Agar and KF Streptococcus Agar, all pink to maroon Colony Forming Units (total CFU) were counted. In addition, a note was kept of colony morphology, especially with respect to colony diameter, depth of colour and halo formation. Typical colonies were subcultured onto MacConkey Agar (MA) at 36° C for 48 hours. Growth characteristics on this medium were noted. Further tests were carried out from material which grew on the MA plate. Cultures with the following characteristics were tentatively labelled as FS:

Growth on MA :	pink pin-point colonies
Microscopic observation:	Gram positive cocci in chains
Catalase :	positive

A sample of these isolates was submitted to a specialized laboratory for definite confirmation as Faecal Streptococci.

The following records were kept:

(i) the total CFU on each membrane filter;
(ii) the number of colonies which finally confirmed as FS;
(iii) the fraction of latter colonies as a percentage of total CFU.

Survival tests of FC and FS in fresh and seawater were also carried out. In this exercise, four 300 ml-volumes of tap water and 300 ml-volumes of seawater were inoculated with 10 ml settled sewage and kept in the dark at 10° C, 20° C, 36° C and 44° C respectively. Counts of FS were carried out periodically on M-Enterococcus Agar 36° C/48h.

3. RESULTS

Mean results of analysis on water and sand/sediment samples taken from the selected sites are shown in Tables 1 and 2.

KF/36° C gave the lowest FS counts, both for water and for sand/sediment samples, but the differences between the counts were not statistically significant at the 10% level.

The percentage of total CFU that were confirmed to be FS is shown in Table 3.

Because of the importance of having a "single-step" enumerating technique, i.e. without the need for routine confirmation of colonies, an attempt was made to visually distinguish between true FS colonies and false positives growing on the membrane filter. Table 4 shows the main morphological colony types encountered and the fraction confirming as FS.

Non-FS pink to maroon (false positive) colonies on M-Enterococcus Agar belonged exclusively to Gram positive, catalase positive cocci in clumps which generally could be distinguished on MA from true FS. In many cases, these interfering organisms formed pin-point colonies on M-Enterococcus Agar.

False positive colonies on KF Streptococcus Agar, both at 36° C and at 44° C, were due to Gram positive clumped cocci, as well as to long (occasionally filamentous) Gram negative rods. These formed large, uniformly dark, maroon colonies on KF Streptococcus Agar, but did not grow at all on M-Enterococcus Agar.

The results of indicator survival experiment are shown in Figures 5-8. The straight line of best fit was calculated for each set of points and the decay rate, in terms of logs per day, was obtained from the equation of the line. Figure 9 shows the rate of microbial decay in fresh water and in seawater plotted against temperature.

The change in the value of the FC/FS ratio with time is shown in Figures 9 and 10.

4. DISCUSSION AND CONCLUSIONS

KF Streptococcus Agar is reported to permit the growth of a wider range of Faecal Streptococci, when compared to M-Enterococcus Agar. The poor selectivity of KF Streptococcus Agar, especially when incubated at 36° C, offsets any advantage that this medium may have due to its better sensitivity. The low counts obtained on KF/36° C were due, in fact, to the difficulty incurred in picking FS colonies among the large number of false positives which grew on the medium. A greater degree of interference was noted when working with sand/sediment samples than with water samples.

A good medium for the routine microbial enumeration in environmental samples is expected to show sensitivity for the organisms of interest, coupled with adequate selectivity. It is preferable if counts of the organism of interest are obtained in a single-step procedure, rather than after days of tedious subculturing. None of the microbiological media tested in this exercise was completely selective for FS and difficulty was experienced in attempting to visually distinguish true FS colonies on membranes, especially with respect to colonies with diameters less than 1 mm. However, the above results indicate that until a better medium is put forward, M-Enterococcus Agar may be recommended as the medium of choice for the enumeration of FS in seawater.

The die-off experiments showed that, considering the data obtained at 20° C and 36° C:

(i) FC and FS decay rate increases with increase in temperature;
(ii) FC and FS decay rate is considerably higher in seawater than in fresh water;
(iii) the numerical value of the FC/FS ratio in domestic sewage is initially high (>4), i.e. more FC than FS; the value of the ration decreases with time until it becomes less than 1, i.e. more FS than FC. The exact time at which this occurs is temperature dependent.

Table 1

Mean results of water analysis
(counts expressed to the nearest whole number per 10 ml

		M-ENT/36° C		KF/36° C		KF/44° C	
	FC	TOT.CFU	FS	TOT.CFU	FS	TOT.CFU	FS
SITE 1: MELL							
Mean count	1.0	3.3	2.9	7.9	1.9	4.0	0.7
% of Total CFU		100	87	100	24	100	18
SITE 2: SPB							
Mean count	0.2	6.0	6.0	40.0	0	24.2	0.8
% of Total CFU		100	100	100	0	100	3
SITE 3: MIST							
Mean count	0.4	7.6	7.6	5.2	4.2	7.6	5.6
% of Total CFU		100	100	100	81	100	74
SITE 4: SAL							
Mean count	65.4	28.4	28.4	167.8	10.0	62.0	48.0
% of Total CFU		100	100	100	6	100	93
SITE 5: SGB							
Mean count	18.7	18.6	12.3	530.6	20.4	43.1	40.1
% of Total CFU		100	66	100	4	100	93
SITE 6: FGH							
Mean count	7.9	2.1	2.1	60.8	3.5	10.3	7.0
% of total CFU		100	100	100	6	100	68
SITE 7: STB							
Mean count	6.6	3.2	2.8	660.6	0.6	3.8	0.4
% of total CFU		100	87	100	0	100	11
SITE 8: PB							
Mean count	6.4	3.8	3.8	164.2	1.2	3.6	2.4
% of total CFU		100	100	100	1	100	67
SITE 9: GB							
Mean count	0.3	0.2	0.2	69.2	0.2	9.7	0.2
% of total CFU		100	100	100	0	100	2
SITE 11: GN							
Mean count	0.0	3.4	1.0	1.0	0.0	1.0	0.0
% of total CFU		100	29	100	0	100	0
SITE 12: LAP							
Mean count	1.0	1.0	1.0	1500.0	0.0	1.5	1.5
% of total CFU		100	100	100	0	100	100
Mean % of Total CFU		100	88	100	11	100	47

Table 2

Mean results for sand/sediment analysis
(counts expressed to the nearest whole number per 10 g)

		M-ENT/36° C		KF/36° C		KF/44° C	
	FC	TOT.CFU	FS	TOT.CFU	FS	TOT.CFU	FS
SITE 1: MELL							
Mean count	5.0	21.5	20.2	382.1	0.0	30.8	5.2
% of Total CFU		100	94	100	0	100	17
SITE 2: SPB							
Mean count	4.0	154.00	151.3	2349.7	153.8	551.0	19.7
% of Total CFU		100	98	100	7	100	4
SITE 3: MIST							
Mean count	1.0	1.9	0.7	4310.6	0.0	12.0	0.4
% of Total CFU		100	38	100	0	100	4
SITE 4: SAL							
Mean count	152.0	309.3	309.3	774.4	4.3	414.3	411.4
% of Total CFU		100	100	100	1	100	99
SITE 5: SGB							
Mean count	156.0	275.0	274.8	5385.0	87.5	315.0	315.0
% of Total CFU		100	100	100	2	100	100
SITE 7: STB							
Mean count	8.0	164.00	26.3	1000.0	0.0	153.3	76.7
% of total CFU		100	16	100	0	100	50
SITE 8: PB							
Mean count	3.0	35.0	35.0	506.1	10.1	19.6	16.3
% of total CFU		100	100	100	2	100	83
SITE 9: GB							
Mean count	8.0	60.2	38.5	584.2	11.0	108.1	9.3
% of total CFU		100	64	100	2	100	9
SITE 11: GN							
Mean count	35.0	43.8	42.6	969.7	38.4	43.1	26.4
% of total CFU		100	97	100	4	100	61
Mean % of total CFU		100	79	100	2	100	47

Table 3

Percentage of total colony forming units
that confirmed as faecal streptococci

Medium	MATRIX	
	Water	Sand/Sediment
M-Enterococcus Agar/36° C	88	79
KF Streptococcus Agar/36° C	11	2
KF Streptococcus Agar/44° C	47	47

Table 4

Colony types encountered with fraction that confirmed as FS

	% Confirming as Faecal Streptococci		
Colony morphology	M-ENT/36° C	KF/36° C	KF/44° C
Dark centre, lighter halo	98	96	42
Uniformly dark	94	6	75
Dark, grayish centre	100	50	not occurring
Uniformly light	100	100	100
Pinpoint (<1mm diam), variable morphology	49	23	17

Figure 1

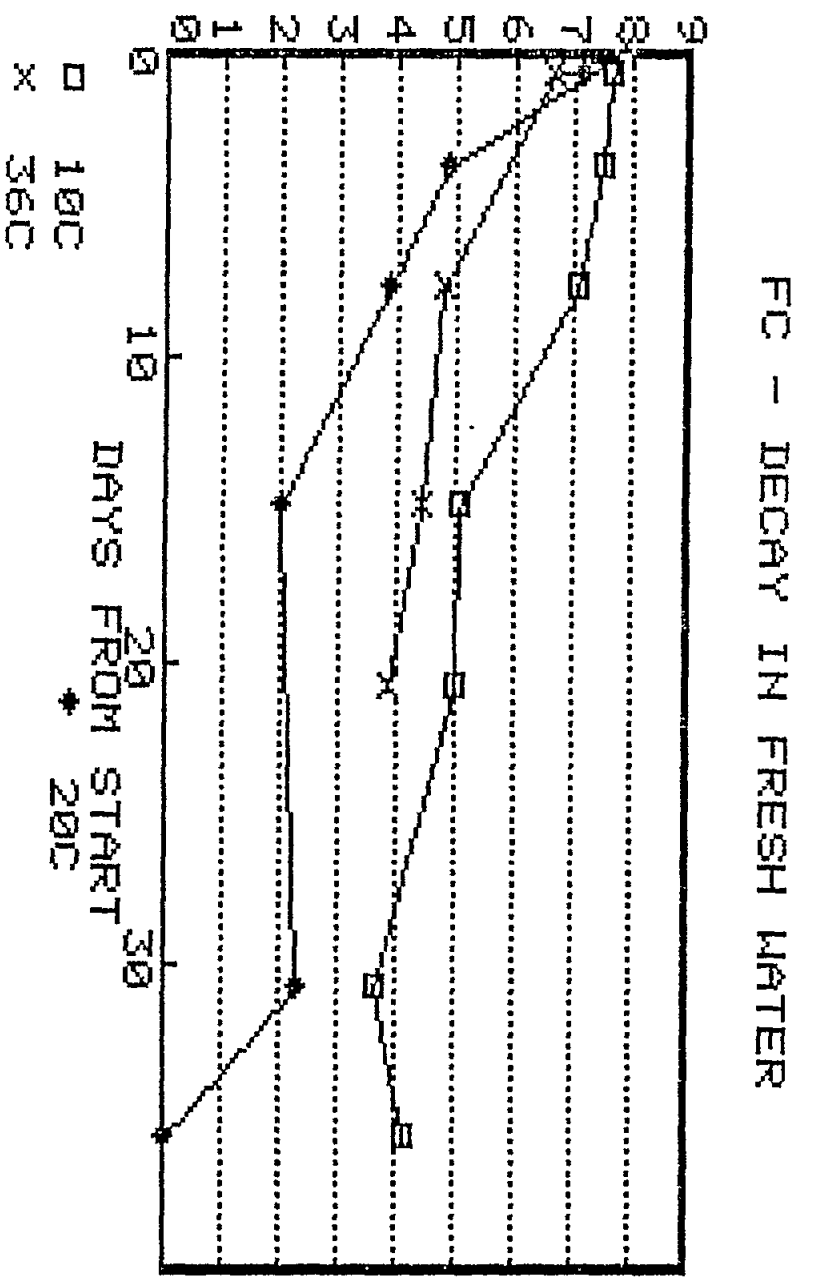

LOG NUMBERS PER 100ML
FC - DECAY IN FRESH WATER
0 1 2 3 4 5 6 7 8 9
0
10
20
30
DAYS FROM START
□ 10C
X 36C
* 20C

Figure 2

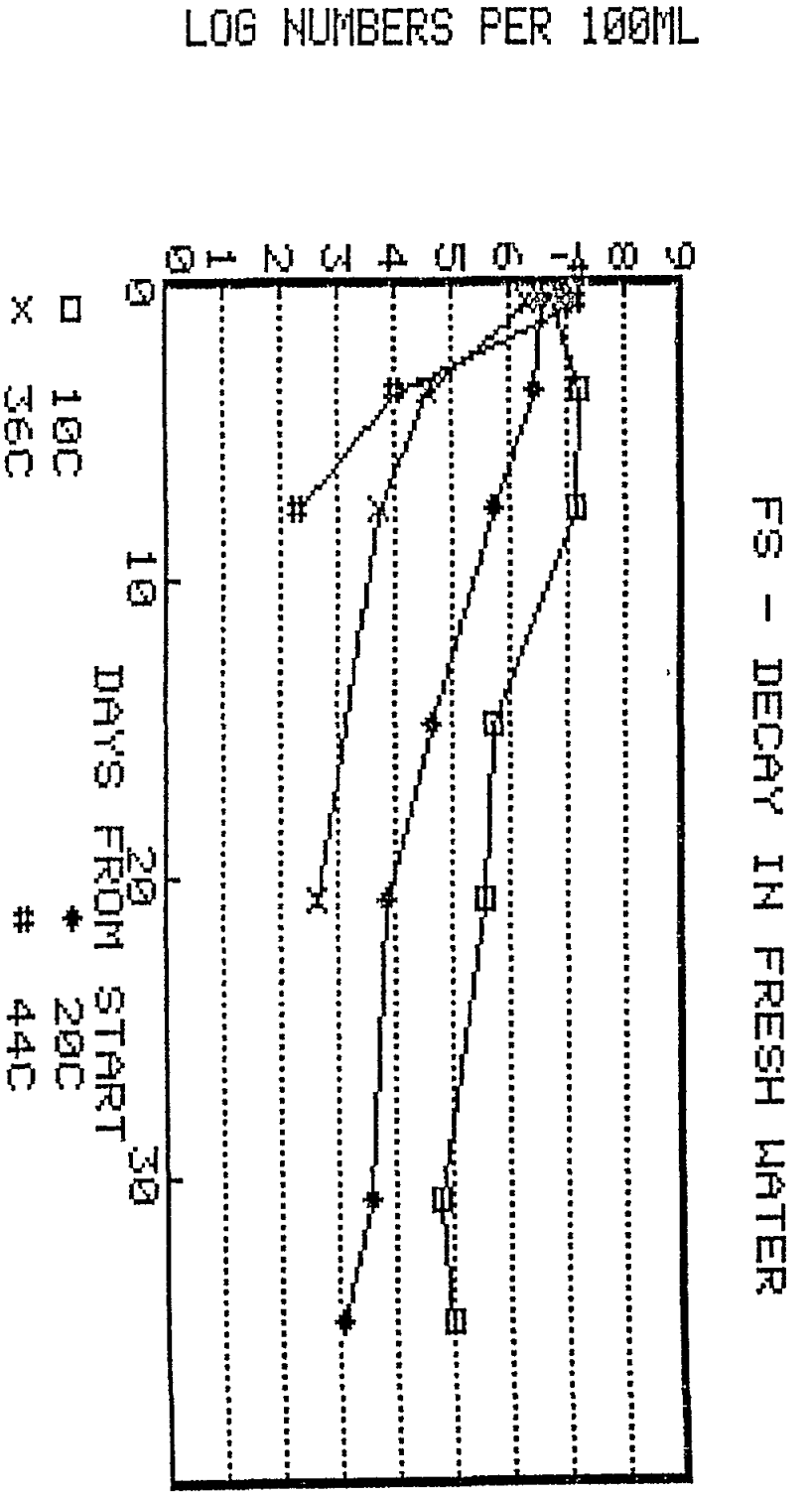

LOG NUMBERS PER 100ML
FS - DECAY IN FRESH WATER
0 1 2 3 4 5 6 7 8 9
0
10
20
30
DAYS FROM START
□ 10C
* 20C
X 36C
44C

Figure 3

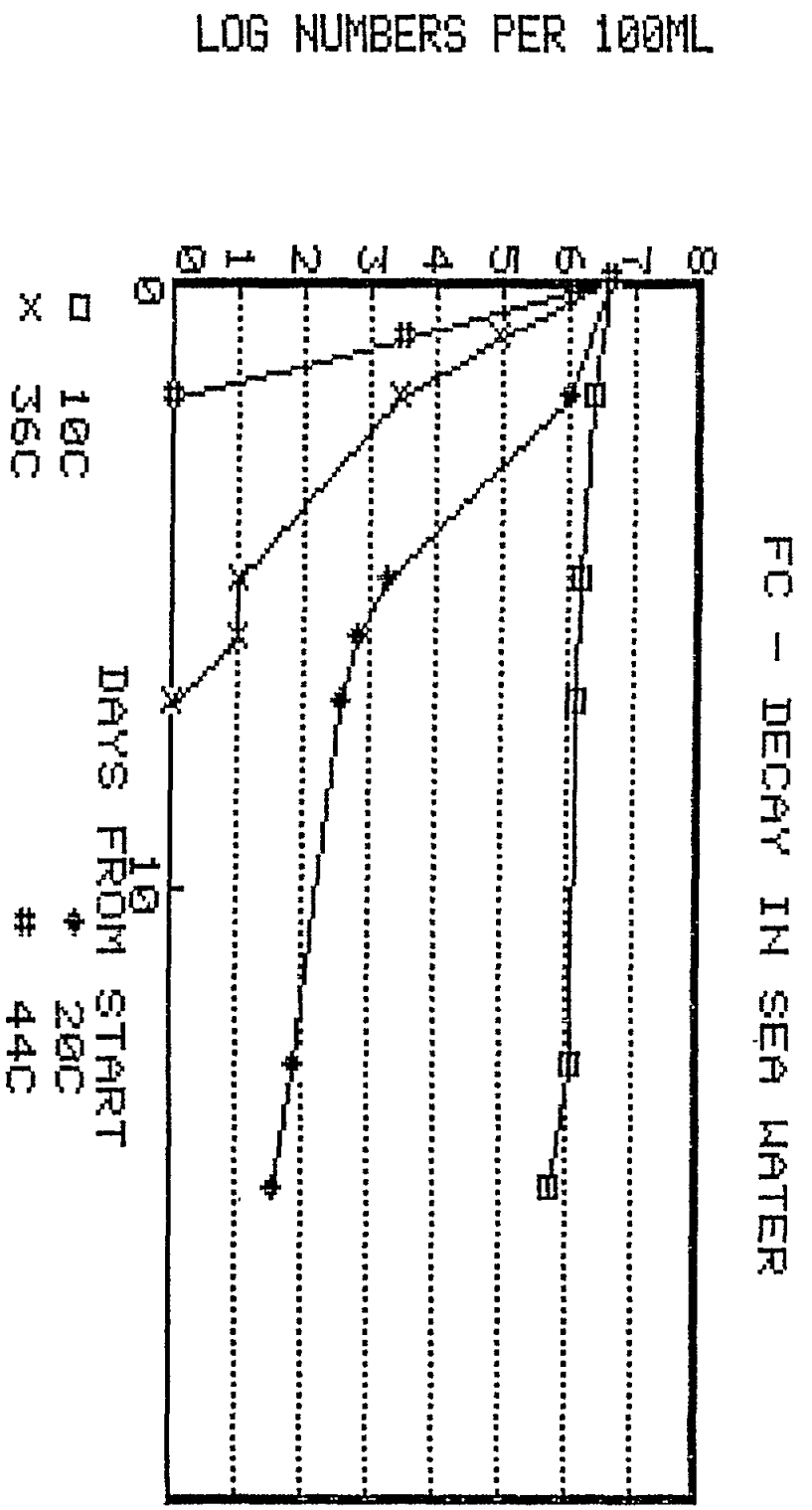

LOG NUMBERS PER 100ML
0 1 2 3 4 5 6 7 8
0
10
DAYS FROM START
□ 10C
X 36C
* 20C
# 44C
FC - DECAY IN SEA WATER

Figure 4

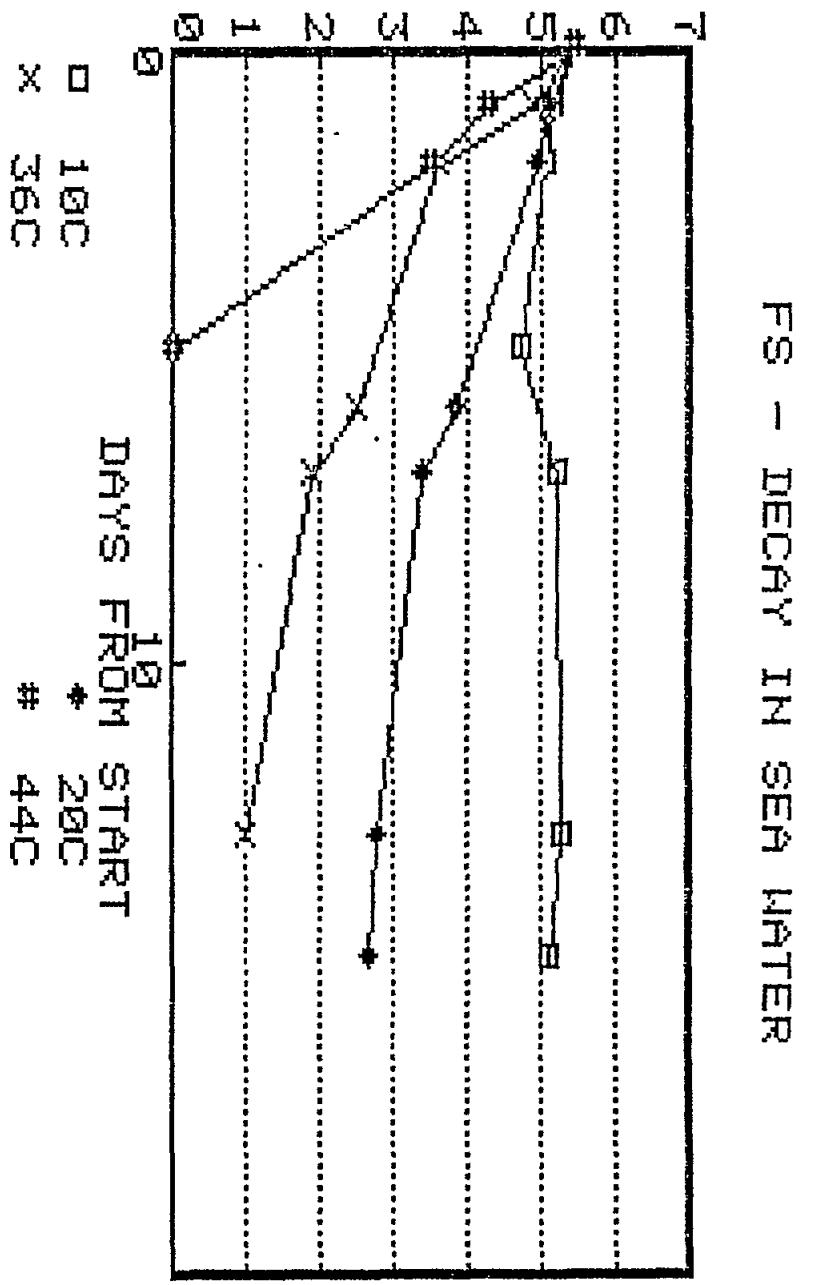

LOG NUMBERS PER 100ML
0 1 2 3 4 5 6 7
0
1
DAYS FROM START
□ 10C
X 36C
* 20C
# 44C
FS - DECAY IN SEA WATER

Figure 5

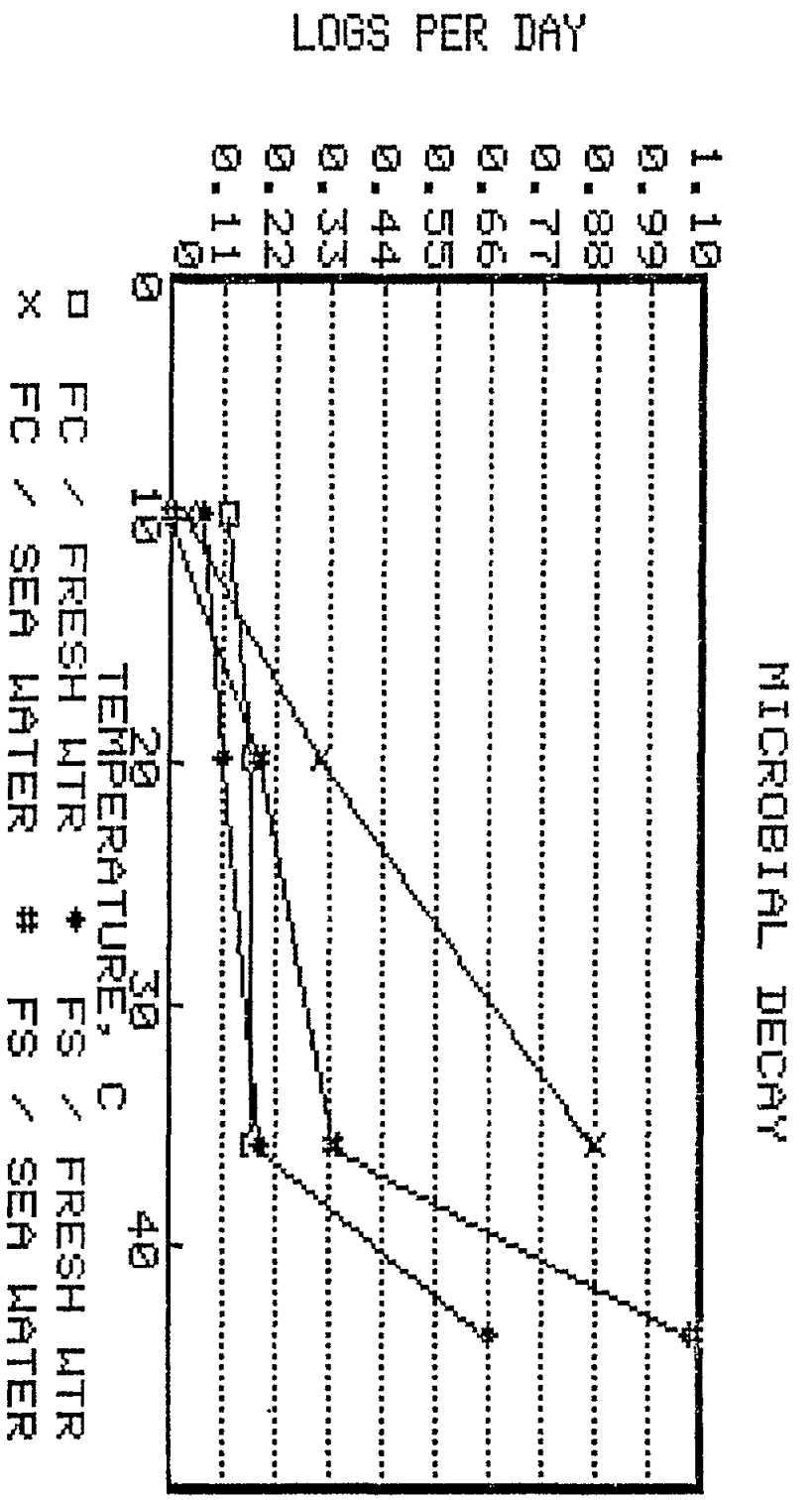

LOGS PER DAY
1.10
0.99
0.88
0.77
0.66
0.55
0.44
0.33
0.22
0.11
0
10
20
30
40
TEMPERATURE, C
□ FC / FRESH WTR * FS / FRESH WTR
X FC / SEA WATER # FS / SEA WATER
MICROBIAL DECAY

Figure 6

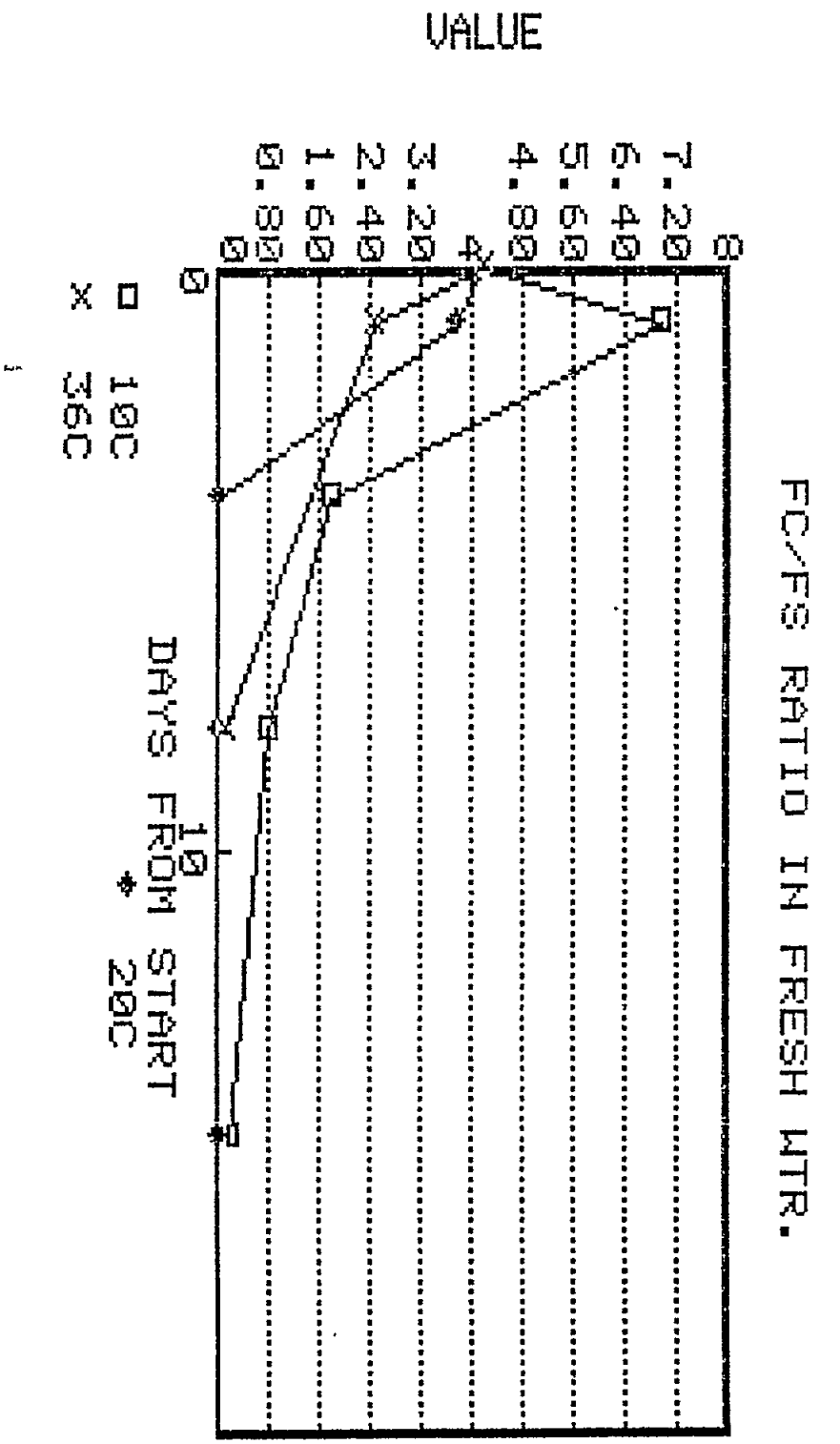
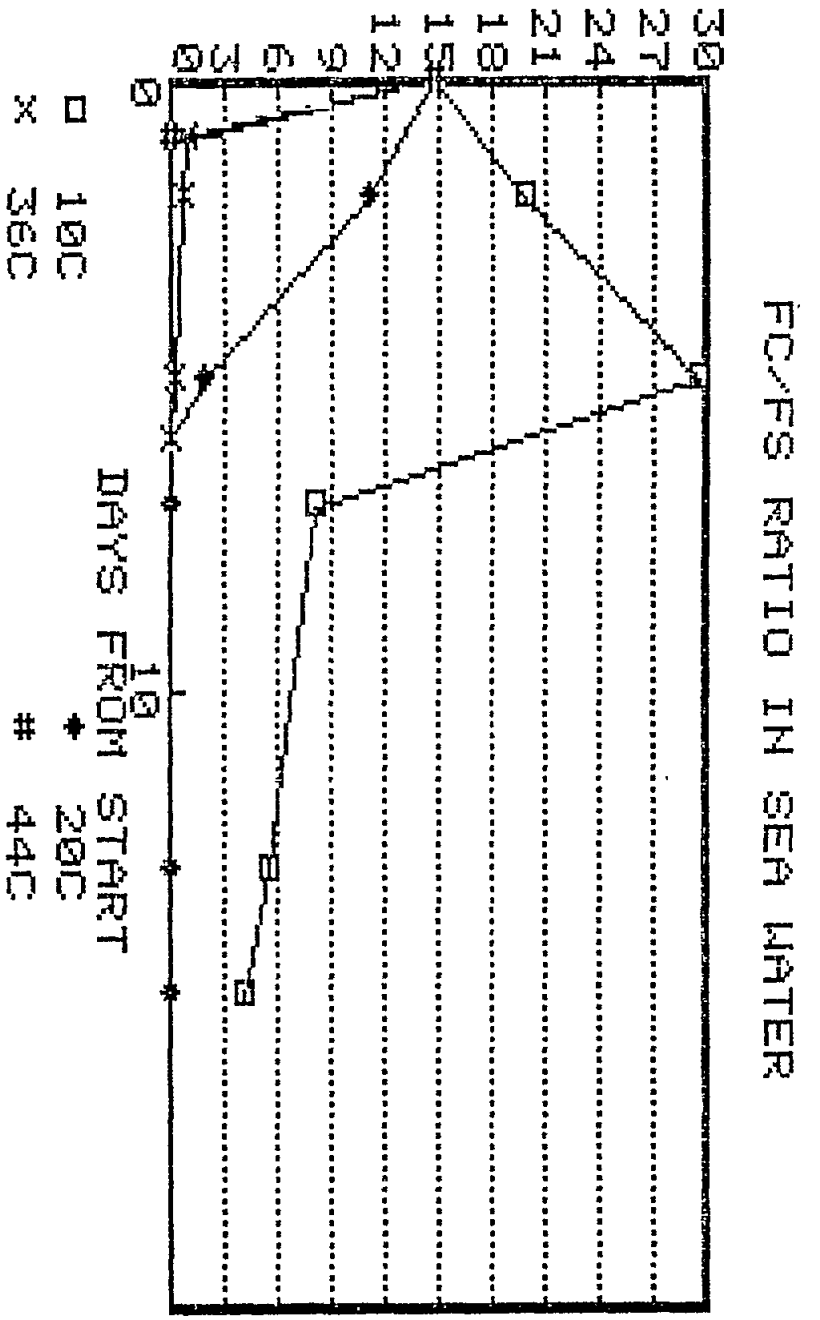

VALUE
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
0
10
DAYS FROM START
□ 10C
X 36C
* 20C
FC/FS RATIO IN FRESH WTR.

Figure 7


VALUE
FC/FS RATIO IN SEA WATER
0
3
6
9
12
15
18
21
24
27
30
DAYS FROM START
10
□ 10C
* 20C
X 36C
44C

COMPARISON OF METHODS FOR ENUMERATION OF FAECAL STREPTOCOCCI IN SEAWATER

by

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1. INTRODUCTION

Previous studies carried out in this laboratory have brought us to the conclusion that faecal streptococci are not reliable indicators of faecal pollution in seawater and shellfish specimens. Media are not sufficiently selective; halophilic bacteria, widely diffused in the marine environment, may grow on them, and the selective power of the available media may differ according to the sample origin.

At 36± 1° C media can support the growth of other microorganisms as *Micrococcus* or marine vibrios. Higher incubation temperature (44° C) generally reduces the number of recoveries. However, as *Streptococcus bovis* does not grow at this temperature, it is advisable to use the intermediate temperature of 42° C in order to have the best selectivity without any loss of faecal streptococci proper.

2. MATERIALS AND METHODS

For the purpose of the present study, which had the objective of comparing different methods for enumeration of faecal streptococci, two different marine areas were selected:

1. in the Adriatic sea, along the coast of the province of Ferrara - 14 sampling stations (Table 1);

2. in the Tyhrrenian sea, along the coast of the province of Rome - 8 sampling stations (Table 1);

The Membrane Filtration (MF) technique was utilized, and m-Enterococcus Agar (MEA) (DIFCO 0746-01) and KF Streptococcus Agar (OXOID CM 701) (KF) used as selective media.

Two incubation temperatures (36± 1° C and 42± 1° C) were used for the Tyhrrenian samples, only one (36± 1° C) for the Adriatic ones.

The brand of membranes used was Millipore HAWG 047 S1 lot H7H82298 C.

Samples were collected without taking meteo-marine conditions into account. Analyses were carried out within 24 hours of collection.

The media were prepared according to the standard instructions for each. For each medium and each sample, 10 ml and 100 ml were filtered.

The Petri dishes, incubated for 2 days at the above cited temperatures, were enumerated according to the manufacturer's recommendations. The membranes were then transferred on Aesculin Iron Agar (EIA) (Aesculin 1 g, Ferric citrate 0.5 g, agar 15 g, distilled water 1,000 ml; pH 7.1± 0.1) before autoclaving at 121° C for 15 min. to assay the aseculin hydrolysis.

After 20 min. at 42° C, the colonies that had produced black haloes on the reverse of the EIA agar were counted. Part of the thus confirmed colonies were isolated and streaked onto plates of Columbia Agar Base supplied with 5% sterile defibrinated sheep blood, and successively examined by the catalase test and by microscopic morphology and gram staining.

A total of 48 samples of seawater from 8 beaches of the Roman coast and 114 from 13 beaches of the Ferrara coast were contemporaneously monitored during the summer of 1988 and analyzed on KF and MEA media (Tables 2 and 3).

3. RESULTS AND CONCLUSIONS

A first difference observed between the two monitored marine areas was the lack of confirmation on EIA medium of the presumptive faecal streptococci in the case of the Ferrara coast-line.

The majority of the colonies grown both on MEA and KF were not able to hydrolyze aesculin. The percentage of confirmation on EIA varied from 0 to 50 (Table 3). On the contrary, along the Roman coast, higher numbers of presumptive faecal streptococci were confirmed according to the aseculin hydrolysis test (the percentage of confirmation on EIA fluctuated from 0 to 10.0).

This result was obviously connected with the difference in the autochtonous bacterial flora from place to place. Moreover it confirmed the unselectivity of the designed media.

In any case, KF agar gave higher faecal streptococci titres (Tables 2 and 3). This appeared both from single values and from the means ($\bar{x}$).

The increase in the incubation temperature (42° C) appeared to improve the KF agar performance (Table 2), but the subsequent EIA confirmation once more provided evidence of its unselectivity (only 18% of samples were totally confirmed on EIA, Table 2).

The appearance of membranes with a diffused bacterial background growth was more frequently observed on KF substratum than on MEA medium (Table 2*).

EIA confirmation tests performed on all the membranes supporting the presumptive faecal streptococci colonies, a test introduced in Italian Law in order to standardize determination of this parameter, proved reliable. Exceptionally, bacteria different from faecal streptococci are able to hydrolyze aesculin too (Table 2**). The thus confirmed faecal streptococci answer to the other characteristics assayed: gram staining, microscopic morphology and catalase test (Tables 2 and 3, last two columns).

In conclusion, the MEA medium provided a better substrate than KF agar in both the two different maine sites selected. The incubation temperature of 42° C could have enhanced the selectivity of the medium. In any case, as even MEA agar is not totally reliable, at least for particular sea areas such as, for instance, the Adriatic Sea, it is considered advisable to include the total test of the membrane for aesculin hydrolysis, when using medium.

Figure 1 - Sites of sampling along the Adriatic coasts (province of Ferrara) and the Tyhrrenian coasts (province of Rome)

Table 1

Stations selected for comparative studies on faecal streptococci
(see Figure 1)

Province	Sampling station	
Ferrara	n.1	Lido di Volano
	n.2	Spiaggia Romea
	n.3	Among Lido di Volano and Lido delle Nazioni
	n.4	Lido delle Nazioni
	n.5	among Lido delle Nazioni and Lido di Pomposa
	n.6	Lido di Pomposa
	n.7	Lido degli Scacchi
	n.8	among Lido degli Scacchi and Porto Garibaldi
	n.9	Porto Garibaldi
	n.10	Lido degli Estensi
	n.11	Lido degli Estensi
	n.12	among Lido degli Estensi and Lido di Spina
	n.13	Lido di Spina
	n.14	Zona di Bellocchio
Rome	n.1	Fiumicino
	n.2	Fiumicino
	n.3	Fiumicino
	n.4	Ostia
	n.5	Ostia
	n.6	Ostia
	n 7	Ostia
	n.8	Ostia

Table 2

Results of the analyses of samples collected along the Tyhrrenian coasts

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Rome - Sampling station N.1 - Fiumicino											
6/5/88	37	12	11	92	11	11	89	10	11	10	7
	42	7	6	86	6	6	59	1	2	1	1
1/6/88	37	17*	16	94	4	4	12*	9	75	4	4
	42	14	12	86	4	4	32	6	19	4	4
14/6/88	37	18	10	56	4	4	NC	4	-	2	2
	42	2	2	100	2	2	8	2	25	2	2
22/6/88	37	42	42	100	4	4	17	7	41	4	4
	42	130	127	98	12	12	30*	30	100	5	5
9/7/88	37	3	3	100	3	3	39	2	5	2	2
	42	1	1	100	1	1	20	1	5	1	1
16/7/88	37	25	4	16	4	4	10	8	80	5	5
	42	6	5	83	5	5	12	0	0	0	0
2/8/88	37	34	16	47	4	4	42	32	76	4	4
	42	6	3	50	3	3	18	13	72	4	4
$\bar{x}$	37	23					72				
	42	23					26				
Rome - Sampling station N. 2 Fiumicino											
6/5/88	37	1	2	100	1	1	112	0	0	0	-
	42	2	2	100	2	2	13*	5	38	5	5
1/6/88	37	18	18	100	4	4	19	10	42	4	4
	42	15	15	100	4	4	24	10	42	4	4
14/6/88	37	52	0	0	0	0	63	2	3	2	2
	42	3	3	100	3	3	0	-	-	-	-
22/6/88	37	7	7	100	5	5	4	1	25	1	1
	42	17	17	100	4	4	29	1	3	1	1
9/7/88	37	5	2	40	2	2	1*	0	0	0	0
	42	5	4	80	4	4	0	-	-	-	-
16/7/88	37	10	9	90	9	9	19	1	5	1	1
	42	11	7	64	7	7	23	0	0	0	0
2/8/88	37	15	15	100	4	4	15	10	67	4	4
	42	35	32	91	4	4	23	8	35	4	4
$\bar{x}$	37	15					33				
	42	13					16				

* backgound bacterial flora

Table 2 continued

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Rome - Sampling station N.3 - Fiumicino											
6/5/88	37	19	19	100	19	19	102	9	9	9	9
	42	14	14	100	14	7	143	9	6	9	9
1/6/88	37	11	6	55	4	4	6	2	33	2	2
	42	3	2	67	2	2	56	1	2	1	1
14/6/88	37	3	0	0	0	0	42	1	2	1	1
	42	3	3	100	3	3	0	-	-	-	-
22/6/88	37	24	24	100	4	4	4**	5**	-	4	4
	42	45	45	100	4	4	87	11	13	4	4
9/7/88	37	0	-	-	-	-	0	-	-	-	-
	42	4	4	100	4	4	2	0	0	0	0
16/7/88	37	13	2	15	2	2	0	-	-	-	-
	42	2	2	100	2	2	5	0	0	0	0
2/8/88	37	41	19	46	4	4	6	5	83	5	5
	42	47	16	34	4	4	17	4	23	4	4
$\bar{x}$	37	16					23				
	42	17					44				
Rome - Sampling station N.4 - Ostia											
6/5/88	37	2	1	50	1	1	153	7	5	7	4
	42	2	2	100	2	2	197	0	0	0	0
1/6/88	37	20	20	100	4	4	21	4	19	4	4
	42	23	17	74	4	4	21	8	38	4	4
14/6/88	37	5	3	60	3	3	12*	2	17	2	2
	42	1	1	100	1	1	1*	0	0	0	0
22/6/88	37	2	2	100	2	1	12*	6	50	4	4
	42	4	4	100	4	4	1*	1	100	1	1
9/7/88	37	0	-	-	-	-	0	-	-	-	-
	42	1	0	0	0	0	0	-	-	-	-
16/7/88	37	5	2	40	2	2	26	2	7	2	2
	42	30	7	23	7	7	61	2	3	2	2
2/8/88	37	1	1	100	1	1	NC	10	-	4	4
	42	2	2	100	2	2	3*	11*	-	4	3
$\bar{x}$	37	5					75				
	42	9					41				

* background bacterial flora
** aesculin hydrolysis of colonies not identified as faecal streptococci on selective media

Table 2 continued

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Rome - Sampling station N.5 - Ostia											
6/5/88	37	0	-	-	-	-	398	4	1	4	3
	42	0	-	-	-	-	416	0	0	0	0
1/6/88	37	33	18	55	4	4	10	2	20	2	4
	42	24	20	83	4	4	29	10	34	4	4
14/6/88	37	5	2	40	2	2	15*	3	20	3	2
	42	2	2	100	2	2	2	2	100	2	2
22/6/88	37	1*	-	-	-	-	42	2	5	2	2
	42	17	17	100	4	4	110	6	5	4	4
9/7/88	37	2	1	50	1	1	2	0	0	0	0
	42	1	1	100	1	1	4	0	0	0	0
16/7/88	37	3*	33	100	3	3	0	-	-	-	-
	42		0	0	0	0	13	3	23	3	3
2/8/88	37	1*	4**	?	4	4	6*	2	33	2	1
	42	1	1	100	1	1	4*	2	50	2	2
$\bar{x}$	37	6					68				
	42	7					83				
Rome - Sampling station N.6 - Ostia											
6/5/88	37	0	-	-	-	-	NC	0	0	0	-
	42	4	1	25	1	1	NC	0	0	0	-
1/6/88	37	7	7	100	4	4	7	2	29	2	2
	42	9	9	100	4	4	16	2	12	2	2
14/6/88	37	7	7	100	3	3	28*	22	79	4	4
	42	19	19	100	5	5	18	14	78	4	4
22/6/88	37	49	49	100	8	8	20**	33**	?	8	8
	42	96	96	100	4	4	86*	51	59	7	7
9/7/88	37	6	6	100	4	4	6	3	50	3	3
	42	4**	6**	?	6	6	13	4	31	4	4
16/7/88	37	0	-	-	-	-	2	0	0	0	0
	42	4	0	0	0	0	4	2	50	2	2
2/8/88	37	63*	58	92	4	4	34**	52**	?	4	4
	42	73	73	100	4	4	132	61	46	4	4
$\bar{x}$	37	21					57				
	42	30					83				

* background bacterial flora

** aesculin hydrolysis of colonies not identified as faecal streptococci on selective media

? bacteria different from faecal streptococci able to hydrolyse aesculin

Table 2 continued

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Rome - Sampling station N.7 - Ostia											
6/5/88	37	14*	*	?	3	3	679	0	0	0	-
	42	464	*	?	9	9	560	0	0	0	-
1/6/88	37	3	33	1	1	1	2	1	50	1	1
	42	8	8	100	4	4	1	1	100	1	1
14/6/88	37	0	-	-	-	-	9*	8	89	4	4
	42	0	-	-	-	-	0	-	-	-	-
22/6/88	37	30*	8	27	4	4	12*,**	18**	?	3	3
	42	1	1	100	1	1	1*	1	100	1	1
9/7/88	37	9	5	56	5	5	10	3	30	3	2
	42	2	2	100	2	2	7	5	71	5	5
16/7/88	37	16	12	75	12	11	4	0	0	0	0
	42	13	8	61	8	8	7	2	29	2	2
2/8/88	37	3	2	67	2	2	4*	4	100	4	2
	42	7	5	71	5	5	10	4	40	4	4
$\bar{x}$	37	11					101				
	42	71					84				
Rome - Sampling station .8 - Ostia											
6/5/88	37	0	-	-	-	-	16	1	6	1	1
	42	2	1	50	1	1	42	0	0	0	-
1/6/88	37	1	1	100	1	1	1	0	0	0	00
	42	3	2	67	2	2	2	2	100	2	2
14/6/88	37	0	-	-	-	-	9*	0	0	0	0
	42	0	-	-	-	-	7	0	0	0	0
22/6/88	37	0	-	-	-	-	7	7	100	4	4
	42	0	-	-	-	-	2*	2	100	2	2
9/7/88	37	3	2	67	2	2	25	0	0	0	0
	42	0	-	-	-	-	1*	1	100	1	1
16/7/88	37	25**	34**	?	12	12	51	12	23	6	6
	42	65	2	3	2	2	80	0	0	0	0
2/8/88	37	2	1	50	1	1	13	4	31	4	0
	42	11	3	27	3	3	12	3	25	3	3
$\bar{x}$	37	4					17				
	42	12					21				

* background bacterial flora

** aesculin hydrolysis of colonies not identified as faecal streptococci on selective media

? bacteria different from faecal streptococci able to hydrolyse aesculin

Table 3

Results of the analyses of samples collected along the Adriatic coasts

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Ferrara - Sampling station N.1 - Lido di Volano											
10/5/88	37	88	2	2	2	0***	0	-	-	-	-
14/5/88	37	48	6	13	4	4	82	0	0	-	-
7/7/88	37	0	-	-	-	-	0	-	-	-	-
21/7/88	37	188	0	0	-	-	300	0	0	-	-
3/8/88	37	18	0	0	-	-	830	0	0	-	-
5/8/88	37	180	3	2	3	3	260	0	0	-	-
7/8/88	37	6	3	50	3	3	4	0	0	-	-
10/8/88	37	0	-	-	-	-	0	-	-	-	-
11/8/88	37	960	0	0	-	-	1200	0	0	-	-
17/8/88	37	20	0	0	-	-	160	0	0	-	-
19/8/88	37	184	10	5.4	4	4	500	30	6	4	0***
22/8/88	37	60	0	0	-	-	10	0	0	-	-
31/8/88	37	110	1	1	1	1	236	3	1.3	3	3
2/9/88	37	6	1	17	1	1	28	4	14	4	2***
6/9/88	37	18	0	0	-	-	50	1	2	1	0***
8/9/88	37	1	0	-	-	-	15	0	0	-	-
10/9/88	37	0	-	-	-	-	7	0	0	-	-
$\bar{x}$		111					217				
Ferrara - Sampling station N.2 - Spiggia Romea											
9/5/88	37	6	1	17	1	0***	0	-	-	-	-
14/5/88	37	5	2	40	2	2	15	0	0	-	-
7/7/88	37	2	0	0	-	-	32	0	0	-	-
21/7/88	37	220	0	0	-	-	287	1	0.3	1	0***
3/8/88	37	78	0	0	-	-	1100	0	0	-	-
5/8/88	37	32	0	0	-	-	24	0	0	-	-
7/8/88	37	0	-	-	-	-	0	-	-	-	-
10/8/88	37	0	-	-	-	-	2	0	0	-	-
11/8/88	37	160	0	0	-	-	240	1	0.4	1	1
17/8/88	37	50	0	0	-	-	110	0	0	-	-
19/8/88	37	158	4	3	4	4	256	8	3	4	0***
22/8/88	37	50	0	0	-	-	70	0	0	-	-
31/8/88	37	36	0	0	-	-	60	3	5	3	1(2***)
1/9/88	37	1	0	0	-	-	10	0	0	-	-
6/9/88	37	10	1	10	1	1	44	6	14	4	0***
8/9/88	37	8	1	13	1	1	8	1	13	1	0***
10/9/88	37	43	0	0	-	-	50	0	0	-	-
$\bar{x}$		48					136				

*** Colonies not confirmed because not grown

Table 3 continued

Date	Inc T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Ferrara - Sampling station N.3 - Lido di Volano e delle Nazioni											
9/5/88		0	-	-	-	-	0	-	-	-	-
14/5/88		9	0	0	-	-	13	0	0	-	-
7/7/88	37	11	1	9	1	0	152	0	0	-	-
21/7/88	37	200	0	0	-	-	320	0	0	-	-
3/8/88	37	490	0	0	-	-	1660	0	0	-	-
10/8/88	37	50	0	0	-	-	100	1		1	1
19/8/88	37	82	0	0	-	-	224	24		4	0
22/8/88	37	10	0	0	-	-	30	0	0	-	-
31/8/88	37	140	0	0	-	-	200	6		4	0***
2/9/88	37	5	0	0	-	-	8	0	0	-	-
6/9/88	37	50	0	0	-	-	100	1		1	0***
8/9/88	37	36	0	0	-	-	46	0	0	-	-
10/9/88	37	0	-	-	-	-	4	0	0	-	-
$\bar{x}$		83					220				
Ferrara - Sampling station N.4 - Lido delle Nazioni											
9/5/88	37	0	-	-	-	-	0	-	-	-	-
14/5/88	37	11	3	27	3	3	13	0	0	-	-
7/7/88	37	10	0	0	-	-	150	2	1.3	2	0***
21/7/88	37	90	0	0	-	-	160	0	0	-	-
8/8/88	37	90	0	0	-	-	1300	0	0	-	-
10/8/88	37	40	0	0	-	-	208	0	0	-	-
19/8/88	37	30	0	0	-	-	92	2	2	2	0
22/8/88	37	20	0	0	-	-	10	0	0	-	-
10/9/88	37	17	0	0	-	-	43	0	0	-	-
$\bar{x}$		34					220				
Ferrara - Sampling station N.4 - Lido delle Nazioni											
9/5/88	37	4	2	50	2	2	4	0	0	-	-
14/5/88	37	10	2	20	2	0***	4	0	0	-	-
7/7/88	37	0	-	-	-	-	120	0	0	-	-
21/7/88	37	240	2	0.8	2	1	410	0	0	-	-
8/8/88	37	990	0	0	-	-	1600	0	0	-	-
21/8/88	37	80	0	0	-	-	200	0	0	-	-
29/8/88	37	62	0	0	-	-	80	2	3	2	0
5/9/88	37	90	0	0	-	-	52	0	0	-	-
$\bar{x}$		185					309				

*** Colonies not confirmed because not grown

Table 3 continued

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Ferrara - Sampling station N.6 - Lido di Pomposa											
2/6/88	37	16	0	0	-	-	1	0	0	-	-
20/6/88	38	6	1	17	1	0***	3	0	0	-	-
8/7/88	37	21	1	5	1	0	180	0	0	-	-
22/7/88	37	85	0	0	-	-	172	0	0	-	-
1/8/88	37	830	0	0	-	-	1300	0	0	-	-
8/8/88	37	900	0	0	-	-	960	2	0.2	2	2
21/8/88	37	68	0	0	-	-	76	0	0	-	-
3/9/88	37	6	0	0	-	-	30	0	0	-	-
$\bar{x}$		241					540				
Ferrara - Sampling station N.7 - Lido degli Scacchi											
10/5/88	37	0	-	-	-	-	1	0	0	-	-
15/5/88	37	17	0	0	-	-	0	-	-	-	-
21/5/88	37	0	-	-	-	-	3	0	0	-	-
8/7/88	37	9	0	0	-	-	25	0	0	-	-
22/7/88	37	7	0	0	-	-	60	0	0	-	-
1/8/88	37	0	-	-	-	-	830	0	0	-	-
15/8/88	37	6	0	0	-	-	120	0	0	-	-
21/8/88	37	12	0	0	-	-	66	0	0	-	-
3/9/88	37	8	0	0	-	-	26	2	8	2	0***
$\bar{x}$		7					126				
Ferrara - Sampling station N.8 - Lido degli Scacci e Porto Garibaldi											
10/5/88	37	10	0	0	-	-	0	-	-	-	-
15/5/88	37	9	0	0	-	-	1	0	0	-	-
21/5/88	37	0	-	-	-	-	7	0	0	-	-
8/7/88	37	19	0	0	-	-	20	0	0	-	-
22/7/88	37	30	0	0	-	-	60	6	10	4	2
1/8/88	37	76	1	1.3	1	1	1160	1	0.03	1	1
8/8/88	37	46	0	0	-	-	96	0	0	-	-
15/8/88	37	28	0	0	-	-	106	0	0	-	-
21/8/88	37	110	0	0	-	-	140	4	3	4	0***
29/8/88	37	3	0	0	-	-	24	1	4	1	0***
$\bar{x}$	33						161				

*** Colonies not confirmed because not grown

Table 3 continued

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Ferrara - Sampling station N.9 - Porto Garibaldi											
6/6/88	37	0	-	-	-	-	0	-	-	-	-
20/6/88	37	3	0	0	-	-	3	0	0	-	-
8/7/88	37	4	0	0	-	-	5	0	0	-	-
22/7/88	37	10	0	0	-	-	16	0	0	-	-
1/8/88	37	660	10	1,5	4	2	990	3	0.3	3	1
8/8/88	37	860	2	0,2	2	2	1600	1	0.06	1	1
21/8/88	37	154	0	0	-	-	152	4	3	4	0***
3/9/88	37	21	0	0	-	-	52	1	2	1	0***
$\bar{x}$		214					552				
Ferrara - Sampling station N.10 - Lido degli Estensi											
6/6/88	37	2	0	0	-	-	0	-	-	-	-
20/6/88	37	0	-	-	-	-	3	0	0	-	-
8/7/88	37	6	1	17	1	1	10	0	0	-	-
22/7/88	37	42	0	0	-	-	68	1	1.4	1	0
1/8/88	37	9	0	0	-	-	60	0	0	-	-
8/8/88	37	14	0	0	-	-	960	0	0	-	-
15/8/88	37	288	0	0	-	-	560	2	0.3	2	0
21/8/88	37	9	0	0	-	-	40	2	5	2	0***
3/9/88	37	10	1	10	1	0	26	6	23	4	0(2/4***)
$\bar{x}$		42					186				
Ferrara - Sampling station N.11 - Lido degli Estensi											
1/6/88	37	6	0	0	-	-	0	-	-	-	-
6/6/88	37	5	1	20	1	1	3	0	0	-	-
20/6/88	37	1	0	0	0	-	0	-	-	-	-
27/6/88	37	3	0	0	-	-	53	0	0	-	-
8/7/88	37	2	0	0	-	-	48	1	2	1	1
22/7/88	37	18	0	0	-	-	32	0	0	-	-
1/8/88	37	4	0	0	-	-	330	0	0	-	-
8/8/88	37	160	0	0	-	-	1280	0	0	-	-
15/8/88	37	198	0	0	-	-	70	0	0	-	-
3/9/88	37	18	0	0	-	-	23	6	26	4	0(1/4***)
$\bar{x}$		42					184				

*** Colonies not confirmed because not grown

Table 3 continued

Date	Inc.T °C	N. of colonies on			N. of colonies		N. of colonies on			N. of colonies	
		MEA	EIA	(% EIA)	isolated from EIA	gram+ cat-	KF	EIA	(%EIA)	isolated from EIA	gram+ cat-
Ferrara - Sampling station N.12 - Lido degli Estensi e Lido di Spina											
5/6/88	37	22	0	0	-	-	1	0	0	-	-
11/6/88	37	21	1	5	1	1	25	0	0	-	-
18/6/88	37	0	-	-	-	-	0	-	-	-	-
25/6/88	37	2	1	50	1	1	2	0	0	-	-
7/7/88	37	1	0	0	-	-	30	0	0	-	-
21/7/88	37	9	0	0	-	-	26	0	0	-	-
1/8/88	37	6	0	0	-	-	30	1	3.3	1	0
8/8/88	37	140	0	0	-	-	440	1	0.2	1	1
21/8/88	37	8	0	0	-	-	80	0	0	-	-
4/9/88	37	2	0	0	-	-	25	1	4	1	0
$\bar{x}$		21					59				
Ferrara - Sampling station N.13 - Lido di Spina											
10/5/88	37	3	1	33	1	1	1	0	0	-	-
7/7/88	37	0	-	-	-	-	37	1		1	1
21/7/88	37	28	0	0	-	-	50	0	0	-	-
1/8/88	37	12	7	58	4	0	50	6	12	4	0
8/8/88	37	120	0	0	-	-	980	0	0	-	-
21/8/88	37	2	0	0	-	-	96	0	0	-	-
4/9/88	37	0	-	-	-	-	40	0	0	-	-
$\bar{x}$		24					179				
Ferrara - Sampling station N.14 - Zona di Bellocchio											
10/5/88	37	24	1	4	1	1	25	0	0	-	-
23/5/88	37	20	1	5	1	1	1	0	0	-	-
7/7/88	37	2	0	0	-	-	43	0	0	-	-
21/7/88	37	70	0	0	-	-	80	0	0	-	-
1/8/88	37	0	-	-	-	-	50	0	0	-	-
8/8/88	37	104	2	2	2	2	1600	0	0	-	-
15/8/88	37	2	0	0	-	-	92	0	0	-	-
21/8/88	37	35	0	-	-	-	10	2	20	2	0
4/9/88	37	2	0	-	-	-	46	1	2	1	0***
$\bar{x}$		29					216				

*** Colonies not confirmed because not grown

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