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 (1987-1990)

Rapports finaux sur certains projets de nature microbiologique (1987-1990)

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This volume is the sixtieth 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 soixantième 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 sixtieth volume of the MAP Technical Reports Series contains the final reports of five research projects completed within the framework of MED POL in Activity A between 1987 and 1990 - "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 soixantième volume de la Série des rapports techniques du PAM comprend cinq rapports finaux exécutés dans le cadre de la Phase II du MED POL, dans l'Activité A entre 1987 et 1990 - "Mise au point et essai des techniques d'échantillonnage et d'analyse pour la surveillance continue des polluants marins.

TABLE OF CONTENTS/TABLE DES MATIERES

Page

Studies on the occurrence of *staphylococcus aureus* and *pseudomonas aeruginosa* in coastal water in Israel

by Yona Yoshpe-Purer and Sicilia Golderman 1

Quantitative determination of *E. coli* from faecal coliforms in seawater

by A. Mates and N. Schaffer 17

Development of analytical techniques for monitoring the hygienic quality of shellfish

by L. Volterra 22

Investigation on neurotoxins in shellfish

by J.A. Papadakis 31

Evaluation de la spécificité et de la sensibilité d'une méthode fluorométrique de dénombrement d'*Escherichia coli* dans les échantillons d'eau et de sédiments marins par hydrolyse du 4-methylumbelliferyl-ß-D-glucuronide (MUG)

by M.J. Gauthier, J.J. Borrego and V.M. Torregrossa 43

STUDIES ON THE OCCURRENCE OF STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA IN COASTAL WATER IN ISRAEL

by

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1. INTRODUCTION

The bacterial quality of recreational waters is assessed by the same indicators employed for drinking water, namely total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS). These organisms mainly indicate the possibility of faecal contamination, hence the presence of enteric pathogens which are most important in drinking water. However, infections in recreational waters are not limited to enteric diseases but extend to the skin, ear, nose and throat, and many microorganisms that cause infection in these organs do not originate in the intestinal tract of warm-blooded animals. Stevenson (1) found that ailments of the upper respiratory tract represented about 50% of illnesses recorded in bathers, while gastro-intestinal disturbances were only about 20%. Mujeriego *et al.* (2) observed that the most frequent complaints recorded from bathers in marine coastal waters in Spain were skin diseases (morbidity rate of 2%) followed by ear and eye infections (1.5%), while intestinal infections were less than 1%. Foster *et al.* (3) maintain that the large majority of illnesses that are swimming related are transmitted by contact, not ingestion, however the problem of disease spread by contact has been relatively ignored.

Several bacterial species have been recommended as supplementary indicators for recreational waters - *Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and others. We selected to study the merit of *P. aeruginosa* and *S. aureus* as supplementary criteria for Mediterranean water, by assessing their occurrence in coastal water of our region and their rate of survival in seawater. These species were chosen for two reasons: a) they are both potential pathogens, b) they represent pollution from two different sources, *P. aeruginosa* originating in the environment and *S. aureus* in various organs of the body other than the alimentary tract.

Pseudomonas aeruginosa is a ubiquitous saprophite in many normal systems (4), widely distributed in soil, water, sewage, the mammalian gut and plants (5) which can proliferate in distilled water (6). The organism can be recovered from stools of about 10% of normal humans and consequently is frequently found in sewage, where their concentration may exceed 10^5 per 100 ml. (7). It is probably not a marine organism but can be recovered from seawater near outfalls and polluted river outlets (7). Numerous cases of folliculitis, dermatitis ear and urinary treat infections due to *P. aeruginosa*, that were acquired by bathing in contaminated water, are reported in the literature (8-15).

All strains of coagulase positive *Staphylococcus aureus* are potential pathogens causing a wide range of infection (16). They are found in nasal membranes, hair follicles, skin and perineun of warm-blooded animals (16), and their origin in bathing waters is undoubtedly human activity. They were found to be shed by bathers under all conditions of swimming (17) and were recommended as an index of pollution by bathers in swimming pools (17, 18), since they are more chlorine resistant than coliforms.

The relationship between *P. aeruginosa* and indicator organisms in aestuarine and coastal water was investigated in North America (19, 20), but we do not know whether these findings can be applied to the Mediterranean, where the climate is warmer. There is a dearth of information regarding the occurrence of *S. aureus* in marine water and the survival of both genera in this milieu. The present study intends to add some information on these subjects.

2. MATERIALS AND METHODS

2.1 Sampling

Samples were collected by sanitary inspectors in sterile bottles supplied by the laboratory and brought to the laboratory within 1-3 hours. Inoculation was performed as soon as possible, not later than 2 hours after arrival of samples. From 3 beaches, on which we collaborated with Dr. Fattal's group from the Hebrew University in Jerusalem in their epidemiological study, 49 samples were collected by them on Friday afternoons and Saturday mornings, when the bathing load was at its utmost. These samples were refrigerated and inoculated on Sunday morning.

2.2 Analysis

Analysis was performed by membrane filtration with the following media and incubation temperatures:

TC: m-Endo Agar LES incubated at 35 ± 0.5E C for 24h.
FC: m-Fc agar incubated at 44.5 ± 0.2E C for 24h.
P. aeruginosa (PA): m-PA-C medium (21) incubated at 42 ± 0.5E C for 24 and 48h.
S. aureus (SA): modified 4-S agar (22), where the basal medium contained 23.5 gr Plate Count Agar plus 50 gr NaCl per litre H_2O, incubated at 42 ± 0.5E C.

For TC and FC 10 and 50 ml of seawater were filtered and for *PA* and *SA* 100 ml or 50 ml.

2.3 Survival studies

The seawater employed in these experiments was sterilized by filtration through membrane filters (MF) of 0.45µ followed by 0.2µ porosity. Single strains of *P. aeruginosa* and *S. aureus* isolated from seawater were grown on nutrient agar slants at 35 ± 0.5E C for 24h. The slants were washed with 10 ml sterile phosphate buffer (pH7) and the bacteria were dispersed by shaking the tube in a vortex, diluted further in sterile phosphate buffer and added to 500 ml portions in brown one litre bottles, to give a final concentration of 10^3 bacteria per ml. For *P. aeruginosa* controls in sterile tap water were also inoculated. The bottles were kept on the bench at room temperature which was 8-12E C at night and 22-26E C in daytime. The salinity of the seawater was 34.7% and the pH was 8.11. The pH of the tap water was 7.40. Samples were withdrawn and inoculated as follows:

PA - Samples were withdrawn at 0 time, hourly intervals on the first day, followed by daily intervals. On the first two days, portions of 0.1 ml of two dilutions were spread on nutrient agar plates with a glass rod. On the following days when the number of viable bacteria declined and the inoculum had to be increased to 1, 10 and 20 ml it was filtered through MF which were placed on nutrient agar. All plates were incubated at 35 ± 0.5E C, the colonies were counted on the following day and the number of viable bacteria per 1 ml, was calculated.

SA - Experiments with the first two strains showed that the decline in the number of viable organisms during the first hours was negligible, therefore the following ones were sampled only at 0 time and daily intervals. Duplicate 0.1 ml portions of two dilutions were spread on nutrient agar, incubated at 35 ± 0.5E C. Parallel sets of samples were filtered through MF, one set was placed on 4-S agar incubated at 42 ± 0.5E C and the other was resuscitated on pads saturated with tryptic soy broth, incubated for 3 hours at 35E C then transferred to 4-S agar and incubated at 42E C. Colonies were counted after 24h and the number of viable organisms per 1 ml was calculated. When the size of inoculum had to be increased above 0.2 ml the plating on nutrient agar was omitted.

Selected 35 strains of *P. aeruginosa* were sent to Dr. Samra at the Asaf-Harofe hospital in Zrifin for serotyping.

3. RESULTS

During a period of two years (June 1983 to June 1985) 652 samples of coastal water were monitored for TC, FC, *PA* and *SA* (628 samples). The majority of samples were collected during the bathing season (May to October) when the sampling frequency was usually weekly and only 54 samples (8.3%) were collected during the winter. No seasonal variation was observed. In 1983 we monitored 323 samples from 31 beaches that were monitored routinely for FC in the framework of MED POL II (3 - 17 samples from each beach). From January 1984 till June 1985 we concentrated on 9 beaches, 3 from each of the most populated areas, namely Nathania, Tel Aviv and the Bat-Yam Rishon Lezion area. In these 280 samples FS were also monitored, but since we have our doubts regarding the validity of KF medium for monitoring FS in seawater, and therefore the accuracy of our results, the data will not be present here. The 49 samples for the 3 additional beaches (2 in Tel Aviv and one in Rishon Lezion) were collected during the bathing seasons of 1983 (37) and 1984 (12).

It was considered meaningless to calculate the geometric mean of the number of organisms in the various beaches since the number of *P. aeruginosa* and *S. aureus* in 55% and 39% of samples, respectively, was 0 or less than 2 (in samples where the amount of water collected did not suffice to filter 100 ml and we had to filter 50 ml) and in additional 6.5% and 5.3%, respectively, there was only one organism per 100 ml of water. Therefore the best scheme to present our data was to group the samples according to the number of each type of bacteria monitored, compare the percentage of samples that fell into each class and establish the relationship between the indicator organisms (TC & FC)_ and the species investigated by the number of samples from which they were recovered.

The results from each of the 9 beaches monitored for 2 years (35 - 47 samples) were analysed individually. The 211 samples from the other 22 beaches monitored in 1983 were grouped together and the 49 samples from the 3 beaches on which we cooperated with Dr. Fattal's group were analysed separately since they were monitored after 1-2 days of refrigeration.

The data for each organism monitored are presented in tables 1 - 4 and summarized in table 5. In order to determine whether the absence of indicator organisms also indicates absence of *P. aeruginosa* or *S. aureus*, the number of samples in which TC and FC were absent and *PA* and *SA* were present is summarized in table 6.

Of the 652 samples of water monitored only 15 (2.3%) contained more than 100 *PA* per 100 ml of water (table 3). In one of them there were 1000 and in 3 there was confluent growth on the filters with greenish-brown areas and the colonies could not be enumerated. Seven of these samples were from Tel Aviv beaches and the number of FC was high in 4 of them (600-2000 per 100 ml) and low in 3 samples (20-80 per 100 ml). The other 8 were single samples from various beaches in which the number of TC was low (12-70 per 100 ml) and FC were very few or absent.

The number of *SA* exceeded 100 per 100 ml of water in 27 samples (4.3%), ranging from 0 in some beaches to 12.8% in others (table 4). Fifteen of them were from the most crowded area in Tel Aviv, 11 from M42 and M43 and 4 from the two Tel Aviv beaches sampled by Dr. Fattal's group which are located in the same area. The highest number of *SA* recovered was 600 per 100 ml of water. The number of FC was elevated in 5 samples (140 to 10^3 per 100 ml) and low in 22 samples (2 to 100 per 100 ml). In 50 samples (8.6%) *SA* was isolated and FC were absent (table 6).

Survival rates of *PA* and *SA* in seawater at room temperature are presented in table 7 and figures 1 and 2. The data are based on the geometric mean of 5 strains of *PA* and 13 strains of *SA* and indicate that there is a vast difference in the survival rate of the two microorganisms during the first few days of exposure to seawater. While in *SA* about 84% of the bacteria were viable after 1 day and 63% after 2 days, dropping to 24% and 6% after 3 and 4 days, respectively, only 40% of *PA* survived after 1 day, 6% after 2 days and 1.2% after 4 days (table 7). There was considerable variation in the

resistance of individual strains of bacteria to the detrimental effect of seawater. In *PA* the survival rate after 2 days varied from 1.8-6% in 3 strains to 10-18% in the other two. However most bacteria that survived for 4 days (1-4%) persisted for 9 days and a few were found after 11 days. In *SA* the survival rate after 5 days varied from 0.2-3% in 8 strains, 12-18% in 4 strains and 50% in one strain. The latter persisted after 6 days dropping to 14.5% after 7 days and 0.03% in 9 days. In two strains 9 and 17 bacteria per ml were recovered after 10 days and 2-3 after 13 days.

Resuscitation was done only on *SA*. It had no beneficial effect during the first two days, a partial effect after 3-4 days and a pronounced effect after 5-8 days, in 5 out of 6 strains, increasing the number of colonies on the filters 4 to 22 fold.

The 35 strains of *P. aeruginosa* that were serotyped belonged to 15 different types, with H_{11}(6), H_{10}(5) and PS_{11}(5) predominating and 1-3 strains from other serotypes.

4. DISCUSSION

It was suggested that bacterial indicators designed to protect the water from enteric pathogens may, by themselves, not be adequate to insure the bacterial safety of recreational waters, where infection is liable to occur by contact rather than ingestion. In order to determine whether *P. aeruginosa* and *S. aureus* are valid supplementary indicators of pollution in the Mediterranean and their monitoring would add to the information gained by monitoring of coliforms, we assessed their occurrence in beaches of Central Israel during two years. As the number of winter samples was relatively small and no seasonal difference was observed in the occurrence of *PA* and *SA*, all samples were analysed together.

From 652 samples of coastal water monitored, TC and FC were detected in 91.6% and 82.1%, respectively. *PA* was isolated from 44.9% of samples including the ones with a single cell in 100 ml of water. In view of the fact that *PA* and coliforms often originate from the same source, namely sewage, the rate of recovery of *PA* is low compared to TC and FC. However, it is in good agreement with the 43.2% obtained by Robertson and Tobin (20) in Nova Scotia in an area where 97.2% of samples contained FC. In a less polluted area, with FC in 59.5% of samples they recovered *PA* from 2.4% and in polluted river water (100% with FC) *PA* was found in 78.6% of samples. The relatively low recovery rate of *PA* in evidently due to the poor survival of this species in seawater, as shown by our experiments.

The survival studies showed that the die-off rate of *PA* in seawater was quite pronounced, leaving 6% of viable cells after 2 days of exposure and 0.6% after 5 days (table 7). The few cells that survived this period seemed to adapt somehow to the marine environment and persisted there for 8 or 11 days, when experiments were terminated. It is possible that in the sea the bacteria may be slightly more resistant than in our laboratory experiments. Carson *et al.* (23) demonstrated that in *PA* strains isolated from distilled water, even one subculture on enriched medium at 37E C makes the bacteria more sensitive to disinfectants than naturally occurring strains. Although we employed *PA* strains that were isolated from seawater, they were subcultured on nutrient agar at 35E C before inoculation into the filtered seawater, which might have increased their sensitivity. However, since *PA* is not a marine bacterium (7) it can be assumed that its behaviour in the sea would follow the same pattern as in the laboratory. Vasconcelos and Swartz (24), who studied the survival of bacteria in seawater in a diffusion chamber *in situ*, also conclude that in seawater *PA* die-off more rapidly than *E. coli* and *S. aureus*. Cabelli *et al.* (19), who deducted that there was multiplication of *PA* in the water of their beaches, studied aestuarine and fresh water.

The number of *PA* exceeded that of TC and FC by 1-2 orders of magnitude in 5 samples (0.8%), in 6 samples (0.9%) *PA* were present (2-44 per 100 ml) and TC were absent and in 11 samples (1.7%) *PA* were present and FC were absent (table 6). It is interesting to note that 3 samples

from the last group were taken on the same day from 3 consecutive clean and uninhabited beaches, the number of *PA* in them was relatively high (280, 280 and 410 per 100 ml of water) and the number of TC was 70, 20 and 130 per 100 ml, respectively. The *PA* were all of the same serotype (H_{10}) and their origin was probably common, evidently not faecal. In one of these beaches the same serotype was isolated 2 weeks later (6 per 100 ml) possibly survivors of the previous contamination.

The data presented here indicate a 99% and 98% correlation between the presence of *PA* and TC and FC, respectively. Hence monitoring of *PA* in seawater will add little information to that gained by monitoring of coliforms about the sanitary quality of the beaches. This information, though sometimes interesting, does not seem to warrant the cost and effort involved. Nevertheless it may be a useful supplementary criterion for aestuarine, lake and river recreational waters (19, 25, 26), where it has a better chance of survival.

The main source of *S. aureus* in recreational waters is the bathers, who harbour the bacteria in their nasal membranes, on their skin and other parts of the body that come in contact with the water during bathing and swimming. We isolated them from numerous swimming pools, as did other workers (18,27). From the 628 samples of coastal water monitored we found *SA* in 60.7% of samples, including 33 samples (5.3%) with one cell per 100 ml, ranging from 49.5% in less populated beaches to 91% in beaches sampled during the highest bathing load. This percentage is quite high in view of the fact that some beaches are located in scarcely populated areas and others were sampled in the early morning hours, when the number of bathers was small.

SA was found in 4.6% of samples without TC and 8.0% of samples with no FC in 100 ml of water (reaching 11-14% in certain beaches). In additional 34 samples (5.4%) was the number of *SA* 4-10 fold higher than the few FC. This means that by monitoring only FC about 10-15% of samples contaminated with pathogenic bacteria would be considered clean. Favero (28) states that "water in swimming pools can quite readily pass tests for drinking water standards based on the absence of coliform bacteria and yet have a high density of staphylococci and streptococci and be associated with risk of acquiring illness. The same might be true for natural waters". Our findings confirm this statement for seawater.

In epidemiological studies on lake beaches Seifried *et al.* (29) found that morbidity among swimmers was related to staphylococcal counts, to faecal coliform levels and somewhat less to streptococcal counts. Total staphylococci were also related with eye and skin illnesses. They concluded that total staphilococci appeared to be more consistent indicators for predicting total morbidity rates among swimmers. Although correlation between coagulase positive staphylococci and morbidity was not established in the data analysed by Dr. Fattal's group of the Hebrew University in Jerusalem, the number of samples (45) was too small to draw any conclusion and further investigation in required. At this stage it seems to us that in populated beaches monitoring of *SA* as a supplementary indicator should be recommended.

The survival rate of *SA* in seawater was higher than that of *PA*. After 2 days of exposure 63.4% of cell were still viable (ranging from 29 and 34% in two strains to 94% in two others), however there was a sharp decline thereafter, falling to 24.3% after 3 days and 6.3% after 4 days, with only a few survivors on the following days (table 7 and figs 1 and 2). These findings were somewhat surprising. *SA* being salt tolerant and able to grow in media with 7.5% NaCl we expected them to survive longer in seawater. This led us to examine 13 strains, and although there was a difference in the resistance of the individual strains the trend was general. After 5 days the rate of survival was 0.2-3% in 8 strains, 12-18% in 4 strains and in one strain 49% survived after 6 days; however even in this strain there was less than 1 viable bacterium after 9 days. The detrimental factor in seawater is evidently not the salinity which was 34.7‰

Stress was not observed during the first two days of exposure, the colony counts being similar on nutrient agar, membrane filters placed directly on selective medium incubated at 42± 0.5E C and

resuscitation membranes. It was quite pronounced after 5-8 days, when resuscitation produced colony counts 4-22 times higher than on direct filters. As we never know how long the bacteria were exposed to the seawater before monitoring, resuscitation may be beneficial for monitoring *SA* in seawater and probably for chlorinated water as well.

In conclusion it can be said that while monitoring of seawater for *P. aeruginosa* may not be warranted, monitoring of *S. aureus* as a supplementary indicator in highly populated beaches is recommended.

Table 1

Distribution of Coastal-Water samples according
to the amount of total coliforms (TC) in them

Region	Sampling point	No. of samples	Number of TC per 100 ml of water			
			<2	2-10	11-100	>100
Nathania	M29 M30 M31	47 45 46	4 3 2	11 12 19	24 20 17	8 10 8
	total	138	4 (6.5%)	42 (30.4%)	61 (44.2%)	26 (18.8%)
Tel Aviv	M42 M43 M48	47 43 40	1 2 3	5 9 11	22 19 12	19 13 14
	total	130	6 (4.6%)	25 (19.2%)	53 (40.8%)	46 (35.4%)
Bat Yam Rishon-Lezion	M49 M52 M54	47 42 35	0 4 2	13 10 8	17 19 19	17 9 6
	total	124	6 (4.8%)	31 (25%)	55 (44.4%)	32 (25.8%)
Entire coast*	22 beaches	211	34 (16.1%)	60 (28.4%)	95 (45.0%)	22 (10.4%)
Tel Aviv and RL**	3 beaches	49	0 (0)	3 (6.1%)	24 (49.0%)	22 (44.9%)
All samples	34 beaches	652	55 (8.4%)	161 (24.7%)	288 (44.2%)	148 (22.7%

* The 22 beaches from about 10 km north of Nathania to south Rishon-Lezion that were monitored in the summer of 1983.

** The 3 beaches on which we cooperated with Dr. Fattal's group from the Hebrew University in Jerusalem, 2 of them in Tel Aviv and one in Rishon-Lezion

Table 2

Distribution of Coastal-Water samples according to the amount of faecal coliforms (FC) in them

Region	Sampling point	No. of samples	Number of FC per 100 ml of water			
			<2	2-10	11-100	>100
Nathania	M29	47	7	15	21	4
	M30	45	10	17	15	3
	M31	46	9	19	12	6
	total	138	26 (18.8%)	51 (37%)	48 (34.8%)	13 (9.4%)
Tel Aviv	M42	47	2	7	22	16
	M43	43	4	10	23	6
	M48	40	6	15	10	9
	total	130	12 (9.2%)	32 (24.6%)	55 (42.3%)	31 (23.8%)
Bat Yam and RL	M49	47	4	10	20	13
	M52	42	9	17	11	5
	M54	35	5	18	11	1
	total	124	18 (14.5%)	45 (36.3%)	42 (33.9%)	19 (15.3%)
Entire coats*	22 beaches	211	59 (28.0%)	83 (39.3%)	63 (29.9%)	6 (2.8%)
TA + RA**	3 beaches	49	1 (2.0%)	7 (14.3%)	27 (55.1%)	14 (28.6%)
All samples	34 beaches	652	116 (17.8%)	218 (33.4%)	235 (36.0%)	83 (12.7%)

* The 22 beaches from about 10 km north of Nathania to south Rishon-Lezion that were monitored in the summer of 1983.

** The 3 beaches on which we cooperated with Dr. Fattal's group from the Hebrew University in Jerusalem, 2 of them in Tel Aviv and one in Rishon-Lezion

Table 3

Distribution of Coastal-Water samples according
to the amount of *Pseudomonas aeruginosa (PA)* in them

Region	Sampling point	No. of samples	Number of *PA* per 100 ml of water			
			<2[1]	2-10	11-100	>100
Nathania	M29	47	34(7)	8	5	0
	M30	45	32(4)	7	6	0
	M31	46	32(1)	11	2	1
	total	138	98 (71.0%)	26 (18.8%)	13 (9.4%)	1 (0.8%)
Tel Aviv	M42	47	22(3)	20	2	3
	M43	43	29(6)	11	2	1
	M48	40	25(2)	10	2	3
	total	130	76 (58.5%)	41 (31.5%)	6 (4.6%)	7 (5.4%)
Bat Yam and RL	M49	47	22(3)	14	11	0
	M52	42	30(1)	6	6	0
	M54	35	29(2)	6	2	0
	total	124	81 (65.3%)	24 (19.4%)	19 (15.3%)	0
Entire coast	22 beaches	211	132(6) (62.6%)	54 (49.0%)	8 (16.3%)	2 (2.4%)
TA + RL**	3 beaches	49	15(7) (30.6%)	24 (49.0%)	8 (16.3%)	2 (4.1%)
All samples	34 beaches	652	402 (61.7%)	169 (25.9%)	66 (10.1%)	15 (2.3%)

(1) samples with one bacterium per 100 ml, altogether 42 samples (6.5%)

* The 22 beaches from about 10 km north of Nathania to south Rishon-Lezion that were monitored in the summer of 1983.

** The 3 beaches on which we cooperated with Dr. Fattal's group from the Hebrew University in Jerusalem, 2 of them in Tel Aviv and one in Rishon-Lezion

Table 4

Distribution of Coastal-Water samples according
to the amount of *Staphylococcus aureus (SA)* in them

Region	Sampling point	No. of samples	Number of *SA* per 100 ml of water			
			<2(1)	2-10	11-100	>100
Nathania	M29 M30 M31	44 43 44	18(3) 14(0) 21(3)	8 11 7	16 17 14	2 1 2
	total	131	53 (40.5%)	26 (19.8%)	47 (35.9%)	5 (3.8%)
Tel Aviv	M42 M43 M48	47 43 40	19(3) 15(1) 21(2)	8 11 7	14 12 10	6 5 2
	total	130	55 (42.3%)	26 (20.0%)	36 (27.7%)	13 (10.0%)
Bat Yam Rishon- Lezion	M49 M52 M54	47 42 35	21(3) 25(2) 18(3)	7 6 10	16 11 7	3 0 0
	total	124	64 (51.6%)	23 (18.5%)	34 (27.4%)	3 (2.4%)
Entire coast	22 beaches	198	100(9) (50.5%)	54 (27.3%)	42 (21.2%)	2 (1%)
TA* RL**	3 beaches	45	8(4) (17.8%)	10 (22.2%)	23 (51.3%)	4 (8.9%)
All samples	34 beaches	628	280 (44.6%)	139 (22.1%)	182 (29%)	27 (4.3%)

(1) samples with one bacterium per 100 ml, altogether 33 samples (5.3%)

* The 22 beaches from about 10 km north of Nathania to south Rishon-Lezion that were monitored in the summer of 1983.

** The 3 beaches on which we cooperated with Dr. Fattal's group from the Hebrew University in Jerusalem, 2 of them in Tel Aviv and one in Rishon-Lezion

<u>Table 5</u>

Occurrence of TC, FC, *PA* and *SA* in Coastal Water
(percent of samples that contained the stated Number of organisms

Region	No. of beaches	No. of samples	Organism	No. of organism per 100 ml of water			
				<2(1)	2-10	11-100	>100
Nathania	3	138	TC	6.5	30.4	44.2	18.8
		138	FC	18.8	37.0	34.8	9.4
		138	*PA*	71.0(8.7)	18.8	9.4	0.8
		131	*SA*	40.5(4.6)	19.8	35.9	3.8
Tel Aviv	3	130	TC	4.6	19.2	40.8	35.4
		130	FC	9.2	24.6	42.3	23.8
		130	*PA*	58.5(8.5)	31.5	4.6	5.4
		130	*SA*	42.3(4.6)	20.0	27.7	10.0
Bat Yam + Rishon-Lezion	3	124	TC	4.8	25.0	44.4	25.8
		124	FC	14.5	36.3	33.9	15.3
		124	*PA*	65.3(4.8)	19.4	15.3	0
		124	*SA*	51.6(6.5)	18.5	27.4	2.4
Entire coast*	22	211	TC	16.1	28.4	45.0	10.4
		211	FC	28.0	39.3	29.9	2.8
		211	*PA*	62.6(2.8)	25.6	9.5	2.4
		198	*SA*	50.5(4.3)	27.3	21.1	1.0
TA and RL**	3	49	TC	0	6.1	49.0	44.9
		49	FC	2.0	14.3	55.1	28.6
		49	*PA*	30.6(14.3)	49.0	16.3	4.1
		45	*SA*	17.8(8.9)	22.2	51.1	8.9
All samples	34	652	TC	8.4	24.7	44.2	22.7
		652	FC	17.8	33.4	36.0	12.7
		652	*PA*	61.7(6.5)	25.9	10.7	2.3
		628	*SA*	44.6(5.3)	22.1	29.0	4.3

(1) Percent of samples that contained one bacterium per 100 ml of water

* The 22 beaches from about 10 km north of Nathania to south Rishon-Lezion that were monitored in the summer of 1983.

** The 3 beaches on which we cooperated with Dr. Fattal's group from the Hebrew University in Jerusalem, 2 of them in Tel Aviv and one in Rishon-Lezion

Table 6

Number of samples with *PA* or *SA* where Coliforms were not recovered

Region	Sampling point	*P. aeruginosa*+			*S. aureus*+		
		No. of samples	TC^-	FC^-	No. of samples	TC^-	FC^-
Nathania	M29 M30 M31 9 others*	47 45 46 103	1 1 1 2	2 2 1 2	44 43 44 94	3 4 4 9	4(9%) 6(14%) 5(11.4%) 11(11.7%)
	total	241	5(2.1%)	7(2.9%)	225	20(8.3%)	26(11.6%)
Tel Aviv	M42 M43 M48 10 others*	47 43 40 56	0 0 0 1	0 1 0 1	47 43 40 56	1 2 1 1	3 5 3 3
	total	186	1(0.5%)	2(1.1%)	186	5(2.7%)	14(7.5%)
Bat Yam Rishon-Lezion	M47 M52 M54 5 others*	47 42 35 52	0 0 0 0	1 0 0 1	47 42 35 48	1 0 0 2	3 2 2 4
	total	176	0	2(1.1%)	172	3(1.7%)	11(6.4%)
TA + RL	3 beaches	49	0	0	45	0	0
All samples		652	6 (0.9%)	11 (1.7%)	628	27 (4.3%)	50 (8.0%)

* Out of the 22 beaches monitored in 1983

** The 3 beaches on which we cooperated with Dr. Fattal's group from the Hebrew University in Jerusalem, 2 of them in Tel Aviv and one in Rishon-Lezion

Table 7

Survival of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in seawater

Time	*P. aeruginosa* (5 str)			*S. aureus* (13 str)		
	Number of bacteria (geom. mean)	SD	% survival	Number of bacteria (geom. mean)	SD	% survival
0	2400	2.3		8200	2.0	
4h	1750	2.6	73.0			
24h	960	2.3	40.0	6920	1.9	84.4
30h	630	2.5	26.3			
2d	143	3.2	6.0	5200	1.9	63.4
3d	62	2.6	2.6	2000	3.0	24.3
4d	28	3.6	1.2	516	3.3	6.3
5d	14	6.4	0.6	236	4.5	2.9
6d	13	6.0	0.6	64	5.9	0.8
7d	13	6.0	0.6	8	15.0	0.1
8d	12	4.1	0.5	3	6.4	0.03

Figure 1

Survival of *S. aureus* and *P. aeruginosa* in seawater

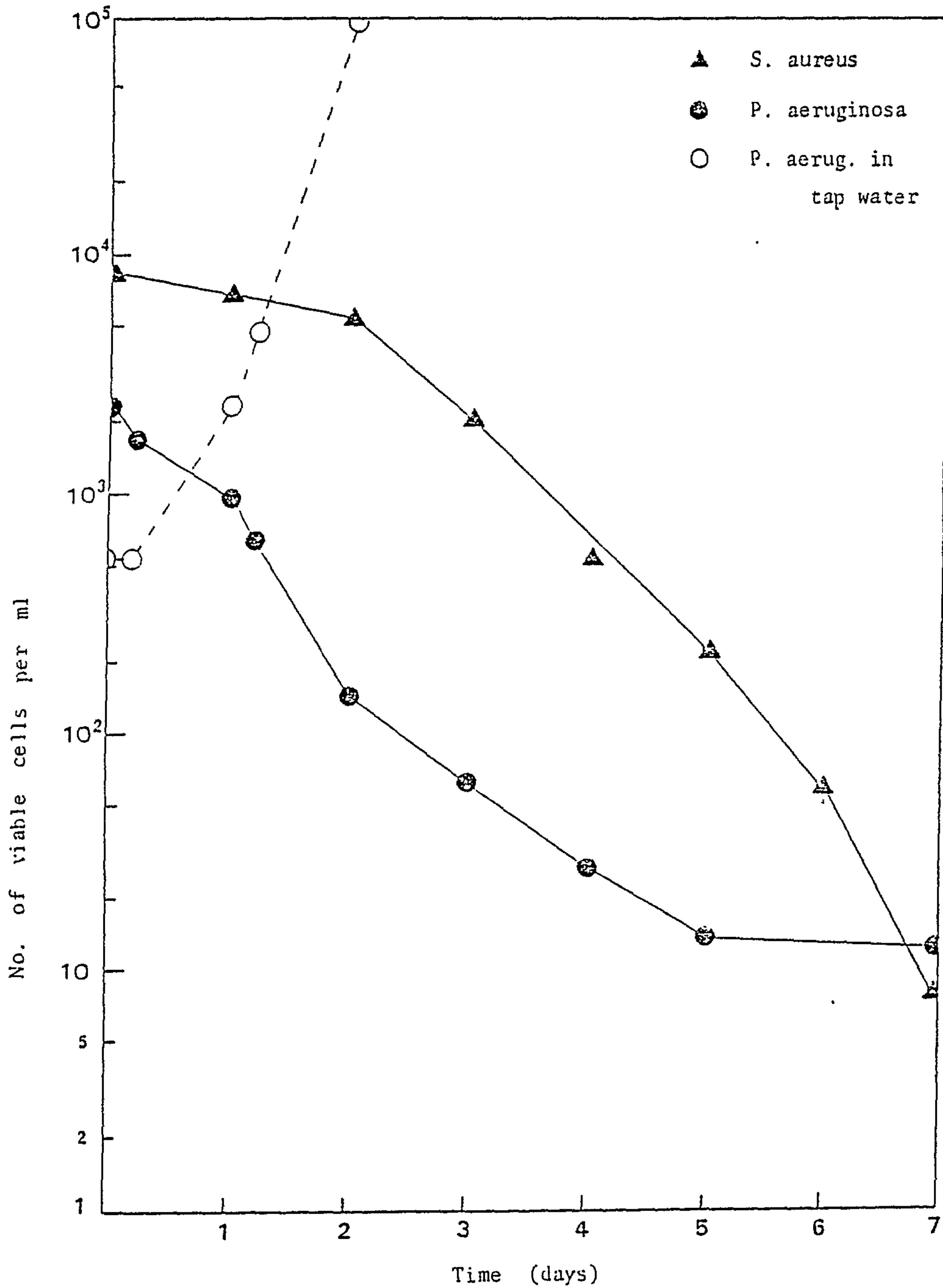
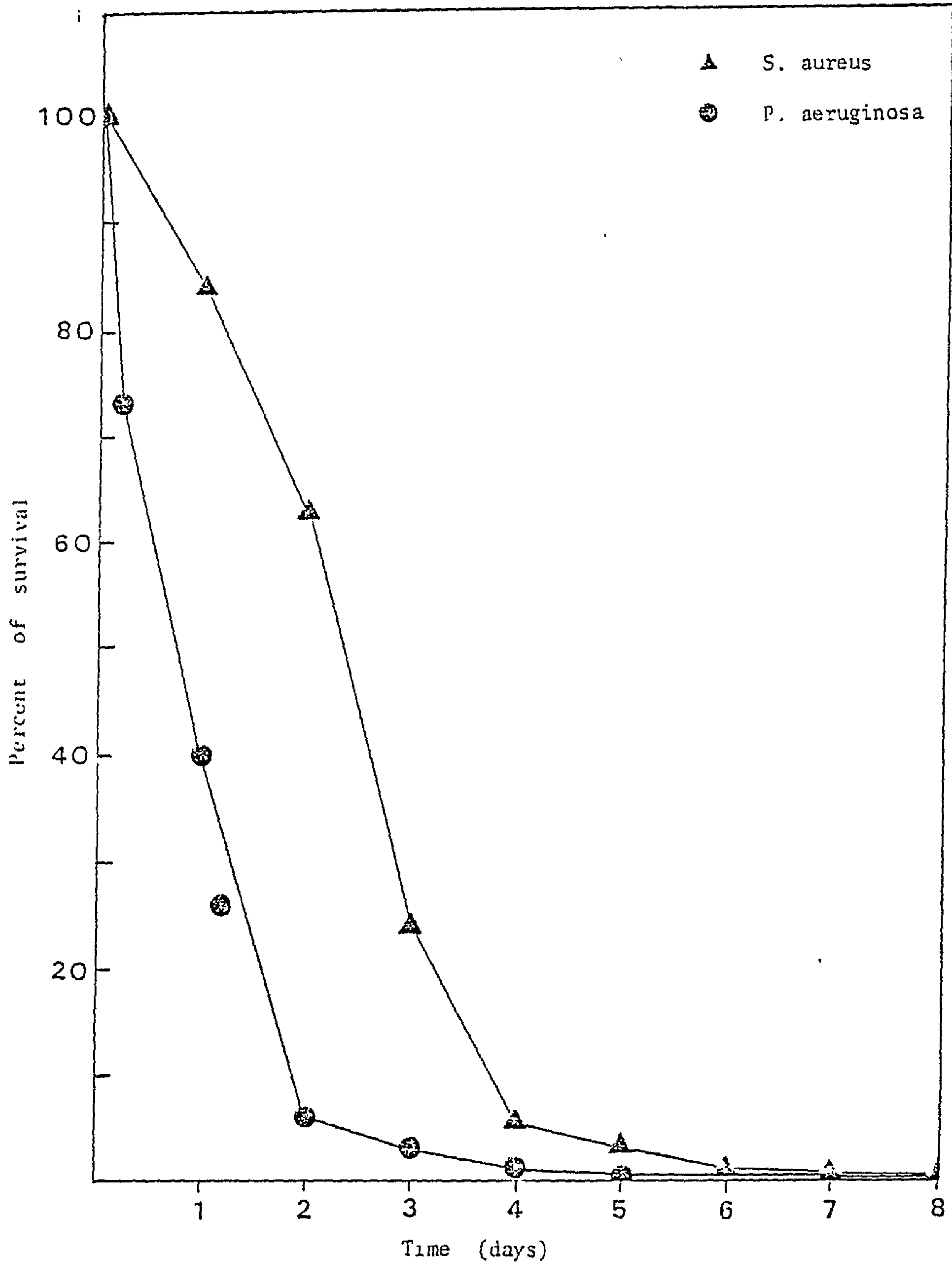


Figure 2

Survival of *S. aureus* and *P. aeruginosa* in seawater



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QUANTITATIVE DETERMINATION OF E. COLI FROM FAECAL COLIFORMS IN SEAWATER

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1. SUMMARY

A simple rapid method was developed for counting *E. coli* in seawater using the membrane filtration culture (MF) method. Following filtration, the membrane filters were incubated on mFC medium for 24 ± 2 hours at 44.5 ± 0.2E C for the determination of faecal coliforms. An *in situ* test for determination of *E. coli* was performed by transferring the membrane filters to nutrient agar containing 4-methylumbelliferyl-B-D-glucoronide, and incubating for 3 hours at 35E C, followed by detection of *E. coli* colonies by their fluorescence under longwave U.V. light.

Extensive biochemical confirmation tests on the isolates showed that all the fluorescent colonies (either lactose positive or negative) were *E. coli*. False negative reactions amounted to 8.4%.

2. INTRODUCTION

The discharge of sewage into the sea prompted the Public Health Authorities in various countries to look for means of indexing the quality of recreational water for bathing purposes (Committee of Water Quality Criteria, 1972; Flyn and Thislethwayte, 1965; Gammerson, 1979; MacKenzie and Livingston, 1983). Coliforms, faecal coliforms, *E. coli* and *Streptococcus faecalis* were all used as indicators of faecal pollution (Cabelli *et al.*, 1983). The U.S. Environmental Protection Agency established microbial standards for marine recreational waters based on faecal coliform counts (U.S. Environmental Protection Agency, 1976). This group of organisms, that are correlated with the possible transmission of enteric pathogens, are primarily *E. coli* and a thermo-tolerant type of *Klebsiella*.

In certain industrial effluents such as those discharged from paper mills and textile factories, the faecal coliforms isolated would mainly consist of a thermo-tolerant type of *Klebsiella* having no connection with a faecal source (Bauer, 1972; Capleans and Kanarek, 1984; Dufour and Cabelli, 1976). For this reason the counting of *E. coli* from faecal coliform counts would be a better indicator (Dufour and Cabelli, 1975) of faecal pollution of bathing beaches.

Feng and Hartman (1982) described a method for detection of *E. coli* based on the B-glucorodinase enzyme using a fluorgenic substrate of 4-methylumbelliferyl-B-D-glycoronide (MUG).

The purpose of our study was to develop a simple rapid method for the counting of *E. coli* on mFC medium following incubation at 44.5E C ± 0.2 for 22 hours based on the MUG reaction, thus enabling a better understanding of the source of the pollution.

3. MATERIALS AND METHODS

3.1 Sample areas

Marine water samples were collected from various sites along the Mediterranean Sea coast of Israel, during the summers of 1985 and 1986. The samples were collected from the shore at a water depth of 0.9m, placed in an ice box, transported to the laboratory within 3 hours and tested immediately.

3.2 MUG media

To 100 ml of sterile Nutrient Agar (DIFCO) at 46E C, 1 ml of a solution containing 10 mg/ml 4-methylumbelliferyl-B-D-glucoronide (Sigma) was added and mixed thoroughly. The medium was poured into 55 mm diameter plastic petri dishes, and stored at 4E C for up to 2 weeks.

3.3 Faecal coliform test (American Public Health Association, 1980)

Appropriate volumes of the water samples were filtered through a 47 mm diameter membrane filter having an average pore size of 0.45 um. The membranes were applied to the surface of plates containing (mFC) medium and incubated at 44.5 ± 0.2E C for 24 ± 2 hours. Blue colonies were counted as faecal coliforms while other types of colonies were considered as background.

3.4 MUG test

The blue colonies representing faecal coliforms were marked on the filter, which was then aseptically transferred to plates containing the MUG medium. Following incubation for 3 hours at 35E C, all the blue colonies lost their colour. The *E. coli* colonies were identified by their ability to hydrolyze the 4-methylumbelliferyl-B-D-glycoronide, producing a fluorogenic compound that was visible under longwave U.V. light.

3.5 Confirmation of colonies

Representative of fluorescent blue and non-blue colonies, as well as non-fluorescent blue colonies were transferred to MacConkey Agar and streaked for purity on Tryptic Soy Agar. The isolates were tested for growth on EC medium for 18 hours at 44.5 ± 0.2E C (American Health Association, 1980) and classified according to their species using the API 20 E test system (Analytas Product Inc., Plain View, N.T.).

4. RESULTS

One hundred and sixty-one (161) samples of seawater were tested for the presence of faecal coliforms and *E. coli*. The data presented in Table 1 indicate that in these seawater samples the number of *E. coli* counted by the MUG method was less than that of faecal coliforms. The spread of *E. coli* counts observed in the samples having more than 30 faecal coliforms illustrates even more the importance of counting the two groups of organisms separately.

The specificity of the test was examined by selecting colonies that were indicated as faecal coliforms (blue colonies) on mFC agar, either MUG positive or negative, as well as MUG positive non-faecal coliform colonies (non-blue colonies). The results which are given in Table 2 show that all the MUG positive colonies were *E. coli*. Among the *E. coli* isolated from the faecal coliforms and tested from these samples, 8.4% were MUG negative. *Klebsiella pneumoniae* was the predominant faecal coliform MUG negative organism that was isolated. Lactose-negative *E. coli* colonies were found in our seawater samples. These were not detected by the faecal coliform test, but positively identified by the MUG test.

Table 1

Faecal coliform and *E. coli* MUG positive counts in 100 ml seawater

Faecal coliform counts per 100ml seawater	*E. coli* MUG positive counts per 100 ml of seawater										Total number of samples
	0	1-15	6-10	11-20	21-30	31-50	51-100	101-150	151-200	200	
1-5	7	29									36
6-10	4	7	17								28
11-20		2	4	10							16
21-30		1	1	9	7						18
31-50	1	1	2	3	5	8					20
51-100				2	3	4	10				19
101-150				2		2	2	3			9
151-200				1				1			2
200							1		1	11	13
TOTAL	12	40	24	27	15	14	13	4	1	11	171

Table 2

Identification of faecal coliform organisms from seawater by the MUG test

	MUG+	MUG-
E. coli Lactose - positive	388	37
E. coli Lactose - negative	14	ND
Klebsiella pneumoniae		73
Enterobacter cloacae		4
Enterobacter sakazakii		1
Citrobacter sp.		2

5. DISCUSSION AND CONCLUSIONS

The use of faecal coliforms as indicators of faecal pollution of seawater, in preference to *E. coli*, is based on historical data and the relative convenience of the test, rather than on a purely scientific basis (Cabelli *et al.*, 1983). *E. coli* is the only member of the group found exclusively in the faeces of warm-blooded animals (Dufour, 1976), while other organisms can also be found in other types of pollution (Capleans and Kanarek, 1984). Various attempts have been made to introduce this species of bacteria as an indicator of faecal pollution.

The various methods were based primarily on the ability of the organism to produce acid from lactose, and indole from tryptophane at 44E C (Dufour and Cabelli, 1975; Dufour *et al.*, 1981). This approach excludes the non-lactose-fermenting *E. coli* that were found in a high proportion in some places, among them some strains causing diarrhoea (Sakazaki *et al.*, 1967). Furthermore, strains of non-type I *E. coli*, that are not indole producers, constituting about 10% of *E. coli* populations (Dufour *et al.,* 1981) would not be detected.

The use of microbial enzymes to detect indicator bacteria have been used in water microbiology (Dufour and Cabelli, 1975; Dufour *et al.,* 1981). Killan and Bullow (1976) showed that the production of B-glucoronidase is specific for *E. coli* in 97% of the strains tested by them. The only other Enterobacteriacea known to produce this enzyme were some strains of *Salmonella* and some *Shigella* species (Feng and Hartman, 1982).

The MUG test presented in this paper is simple and was found to be specific for *E. coli* that were either lactose positive or negative, and had no connection with indole production. As a result of the short incubation period and the release of the end-product of MUG that diffused into the surrounding medium, membrane filters containing up to 35 colonies per filter could be easily counted. The results obtained by this method give us an indication of both faecal coliform and *E. coli*, thus giving us a clue as to the source of the contamination of the beach. The test is easy, rapid, sensitive and specific, and also saves labour and material costs.

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DEVELOPMENT OF ANALYTICAL TECHNIQUES FOR MONITORING THE HYGIENIC QUALITY OF SHELLFISH

by

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1. INTRODUCTION

The aim of the project was to introduce in shellfish monitoring further microbiological parameters in addition to the classic and more used coliforms and enterococci.

At this purpose spore of clostridia, phages anti-*Escherichia coli, Aeromonas hydrophila, Pseudomonas* spp; and *Vibrion parahaemolyticus* have been examined, too. For these last three parameters it was necessary to define a methodology reliable for shellfish homogenate analysis.

Moreover, the concentration power of shellfish has been investigated examining specimens of mollusks, water and sediment contemporaneously collected in a same location. This was possible only for a limited number of the performed surveys.

2. MATERIALS AND METHODS

Methods, followed in the analyses, are summarized in Table 1.

Table 1

Methods followed for enumerating the selected microbiological parameters

Parameter		Methods and Media
Total and faecal coliforms (TC, FC)	MPN	MacConkey broth in the presumptive and confirmatory steps, as specified by WHO
Faecal streptococci (FS)	MPN	Azide Destrose Broth (36 ± 1 EC) with confirmation on Ethyl Violet Azide Dextrose Broth (36 ± 1 EC) (48+48 h)
Spore of Clostridia (s-Cl)	MPN	in standing tubes on TSN agar (46 EC 18-24h) after thermal inactivation of vegetative forms.
Phages anti-*E. coli* (Phages)	MPN	in Phage broth in the enrichment step (36 + 1 EC) followed by revealing on phage agar plates (36 ± 1 EC) (24+24 h) (1)
Aeromonas hydrophila (Ah)	MF	according to Cabelli (2)
Pseudomonas spp. (Ps-spp.)	MF	on Pseudosel agar at 20 EC (4-5 days) and on PSIA (3) at 30 EC (3-4 days) according Watkins (4)
Vibrio parahaemolyticus	MF	and on TCBS agar (36 ± 1 EC 24 h).

Samples of different shellfish species (*Mytilus galloprovincialis, Donax trunculus, Ensis siliqua minor, Chamela gallina*) have been collected in various mollusks beds located along the Thyrrenian coast (Figure 1): Gaeta Gulf (Loc. 1), Rio Martino (Loc. 2), Torvaianica (Loc. 3) and Capo d'Ischia (Loc. 4).

Figure 1 Location of the shellfish beds surveyed

While mussels derived only from Loc. 1, among the other stations (Loc. 2 and 3) more than one shellfish species could be captured (Tables 2 and 3) because of the presence of mixed banks.

Shellfish homogenates as well as sediment samples have been analyzed preparing a work dilution 1:10 with physiological buffered saline. Shellfish homogenates have been filtered by gauze in order to perform microbiological analyses by MF as specified in Table 1.

3. RESULTS

Results concerning all the analyses carried out on shellfish are reported in Table 2, while Table 3 refers data obtained on water, sediment and mollusks derived by a same bank. All these tables are divided in two sections: the first one on the left side referring to faecal indicators of pollution, the second one on the right side concerning the additional parameters more closely linked to general environmental conditions. Geometrical means and ratios among the faecal indicators of pollution have been calculated (Table 2). In the averages computing values <3 or <30/100 ml have been considered null.

From Table 2 mussels appear to be the most effective concentrators of all the researched microorganisms, including those of environmental interest such as *Pseudomonas* and *A. hydrophila*. Among the three kinds of clams living in the sediment: *Donax, Ensis* and lastly *Chamelea* result in decreasing order of microbial accumulation power, particularly looking at faecal coliforms.

Faecal streptococci once more appear not reliable faecal pollution indicators: as a general rule their number exceeds that of faecal coliforms varying between 1:6 to 1:80 from *Mytilus* to *Chamelea*. A number of halophilic bacteria and micrococci may grow in the so called "selective" media also because the incubation temperature fixed at 36 ± 1 EC has a limited selectivity. It has been suggested to increase it from 36 ± 1 EC to 42 EC.

A lack of fixed ratios among faecal indicators may be observed looking at the different mollusk species. Spores of clostridia, often postulated as possible alternative indicators of faecal contamination in shellfish, do not seem reliable. *D. trunculus* that may be recognized as a generalized bacterial concentrator, for instance, exhibits low titres of spores of sulphite reducers (Table 2).

Between clostridia and phages, phages appear the best substitutes or helpers of the actual microbial indicator of faecal pollution.

Among environmental bacteria, *V. parahaemolyticus* is widespread and easily accumulates inside shellfish. *Pseudomonas* spp. follow. *A. hydrophila* has a particular behavior: it has been not always revealed in shellfish homogenates also it sediments registered high titres of this microorganism. Optimization of culture methods and further observations will be necessary to ascertain this feature.

Out of the two methods followed to search for *Pseudomonas* and *V. parahaemolyticus* the use of PSIA (annex 1) instead of Pseudosel agar for the first group of bacteria gave the higher results (Table 3, number reported between brackets and marked by an asterisk), and the direct incubation of membranes on TCBS agar for vibrios resulted much more selective with respect to the specific species to be enumerated (Annex 2). However, also in this field additional confirmations will be necessary before to arrive at a conclusion.

Table 2

Results obtained on the different specimens of analyzed mollusks; shellfish derived by various location(loc)

Shellfish species		date	TC	FC	FS	s-Cl	Phages	Ah	Ps-spp.	Vp
Mytilus galloprovincialis	loc 1	3/12/87	24,000	31	24,000	40	460	16,000	36,000	4,000
		27/1/88	24,000	2,400	4,600	930	11,000	18,000	60,000	-
		23/2/88	24,000	7,920	7,920	15,180	10	0	660	1,320
		12/4/88	24,000	24,000	11,000	11,000	30	12,000	110,000	-
		24/5/88	24,000	230	24,000	30	24,000	28,000	7,200	-
		28/6/88	750	430	230	750	230	36,000	0	-
		X	13,470	1,058	6,131	409	256	3,893	3,227	2,298
		g	13 :	1 :	6 :	0.4 :	0.2			
Donax trunculus	loc 3	13/1/88	2,400	430	4,600	30	240,000	0	460,000	-
	loc 4	22/2/88	24,000	24,000	24,000	273	12,200	8,000	29,000	2,000
	loc 3	19/4/88	2,400	110	24,000	30	24,000	0	10,000	5,400
	loc 2	21/4/88	4,600	4,400	24,000	30	10,000	2,000	30,000	60,000
	loc 3	14/7/88	24,000	11,000	4,600	30	24,000	740,000	800	50,000
		X	6,867	2,228	12,395	6	27,887			
		g	3 :	1 :	6 :	0.003 :	13			
Ensis siliqua	loc 2	25/3/88	2,400	11,000	24,000	24,000	30	0	12,400	10,000
	loc 2	21/4/88	2,400	40	4,600	30	90,000	0	10,000	10,000
	loc 2	16/6/88	11,000	150	4,600	40	40	22,000	42,000	-
	loc 2	27/7/88	11,000	430	1,500	230	1,500	0	0	6,000
		X	5,138	410	5,254	122	271	12	1,511	8,434
		g	12 :	1 :	13 :	0.3:	0.7			
Chamelea gallina	loc 2	25/3/88	2,400	150	24,000	930	30	0	34,000	60,000
	loc 2	14/6/88	11,000	150	4,600	150	230	0	0	-
	loc 2	27/7/88	11,000	230	24,000	40	11,000	0	4,000	2,000
		X	6,622	173	13,838	177	136	0	514	10,954
		g	38 :	1 :	80 :	1 :	0.8			

Table 3

Water, sediment and shellfish analyses

Sample type	date	TC	FC	FS	s-Cl	Phages	Ah	Ps-spp.	Vp
Water (Shellfish Gaeta)	3/12/87	1,100	27	93	3	3	220	80	10
	27/1/88	240	43	210	3	4	40	600	-
	23/2/88	3	-	3	4	3	0	0	0
	12/4/88	4	4	3	3	3	0	400	400
	24/5/88	93	3	3	3	21	40	280	10
	28/6/88	75	43	9	3	3	20	50	0
Rio Martino Water	25/3/88	150	9	3	23	3	1,240	100	30
Sediment		93	90	30	430	30	16,000	4,000	0
E. siliqua		2,400	11,000	24,000	24,000	30	0	12,400	10,000
C. gallina		2,400	150	24,000	930	30	0	34,000	60,000
Water	24/6/88	23	3	31	4	15	440	120	-
Sediment		230	40	4,600	90	2,400	0	0	(2,000)*
E. siliqua		11,000	150	4,600	40	40	22,000	42,000	-
C. gallina		11,000	150	4,600	150	230	0	0	-
Water	27/7/88	7	3	28	3	3	20	20	-
Sediment		4,600	90	40	150	30	0	0	(2,000)*
E. siliqua		11,000	430	1,500	230	1,500	0	0	(6,000)*
C. gallina		11,000	230	24,000	40	11,000	0	4,000	-
Torvaianica Water	13/1/88	3	3	6	3	4	10	0	-
Sediment		30	30	70	30	230	0	2,000	-
D. trunculus		2,400	430	4,600	30	240,000	0	460,000	-
Water	14/7/88	43	4	7	3	2,400	50	50	-
Sediment		230	90	30	30	24,000	6,000	0	(4,000)*
C. gallina		24,000	11,000	4,600	30	24,000	740,000	800	-

Table 4

Concentration power typical of sediments and shellfish.
The results are reported as magnitude order on the base of the mean values

Sample type	TC	FC	FS	s-Cl	Phages	Ah	Ps-spp.	Vp
Gaeta Water	10^2	10^1	10^0	10^0	10^0	10^1	10^2	10^1
Mussels	10^4	10^3	10^4	10^2	10^2	10^3	10^3	10^3
Rio Martino Water	10^1	10^0	10^1	10^0	10^0	10^2	10^2	10^1
Sediment	10^2	10^2	10^2	10^2	10^1	10^1	10^1	0
E. siliqua	10^4	10^3	10^4	10^3	10^1	10^1	10^4	10^4
C. gallina	10^4	10^2	10^5	10^2	10^2	0	10^2	10^5
Torvainanica Water	10^0	10^0	0	0	10^2	10^1	10^0	
Sediment	10^1	10^1	10^0	10^0	10^4	10^2	10^1	
D. trunculus	10^4	10^3	10^3	10^0	10^6	10^3	10^4	

Table 3 shows the accumulation power typical of sediments and mollusks: sediments generally reach microbial titres 10-100 times higher than those of the overlaying waters, and, as a general rule, shellfish living in the sea bottom accumulate 10-100 times the bacterial population present in the surrounding sediments. This relation that looks clear enough for bacteria indicators of faecal pollution is less evident for environmental microbial flora (Table 4).

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ANNEX 1

PSIA = Pseudomonas Selective Isolation Agar

Soybean Casein Digest Agar (SCD) (DIFCO) as basal medium.
To 990 ml of SCD add 2ml of a crystal violet solution (0.1% in distilled water). Autoclave and, when medium has reached the temperature of 50 EC add 7 ml of nitrofurantoidine (0,5% in N,N-dimethyl formal*mmide).

Pseudomonas colonies grown on this medium at 30 EC after 3-4 days of incubation. Cream to brown colonies are enumerable as Pseudomonas spp.

ANNEX 2

Method for enumeration of *V. parahaemolyticus* on TCBS agar by MF.

0.1, 1, 10 ml of a shellfish homogenate previously filtered by sterile cotton gauze, have been filtered on a membrane with mesh of 0.,45 u pore diameter. Membranes have been poured on petri dishes containing TCBS agar (Oxoid CM 333/10625). The plates have been incubated at 36 + 1 EC for 24 h.

Colonies 2-4 mm in diameter, yellow-brown in colour or uncoloured with green center, able to induce a yellow pigmentation in the growth medium, have been enumerated as *V. parahaemolyticus*.

A significative number of colonies have been isolated, cloned and subjected to the miniaturized API systems in order to confirm the presumptive counts.

INVESTIGATION ON NEUROTOXINS IN SHELLFISH

by

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1. INTRODUCTION

The investigation started in June, 1985, after a small delay, as we had some difficulties in getting the saxitoxin vials in order, which Dr. J.E. Gilchrist of the Food and Drug Administration, U.S.A. had kindly mailed for us. It was completed on 23 January 1989.

For our investigation we harvested mussels from 3 growing areas, located in Elefsis bay. It can be seen from the attached drawing that the bay accepts a number of terrestrial effluents, the majority of which come from the industrial zones of Aspropyrgos and Elefsis. The shellfish growing area at Loutropyrgos is rather near the Elefsis industrial zone.

At Nea Peramos there are 2 shellfish growing areas from which we collected molluscs. This area may be considered as more clean than Loutropyrgos, as it is far from industrial zone but in the vicinity of a small urban zone with 2 small sewage outfalls.

The third shellfish growing area is located almost opposite Nea Peramos (see Figure 1) on the side of Salamis island, and it is a marine area in which there is not any recognized terrestrial source of pollution.

For reasons of comparison, a small number of mussels were taken from the central market of Athens between June 1986 to July 1987.

Water temperature was always recorded during sampling time. All other parameters were determined after transportation of the samples to the laboratory.

Seawater samples were collected from shellfish growing areas (4 sampling points) at a depth of 15 cm (approximately) below the surface and were transported within 3-4 hours to the laboratory, together with the mussels harvested from the same areas. The molluscs were collected by aseptic techniques and transported in sterilized glass jars. During transportation, they were kept in an ice-box and examined within 24 hours of collection.

In all, 140 samples of mussels and 128 samples of seawater were examined. All samples from the growing areas were collected with a boat. The total number of samples examined was not as great as originally envisaged, because of difficulties met on some sampling days, (rough seas, rain, strong winds, etc.).

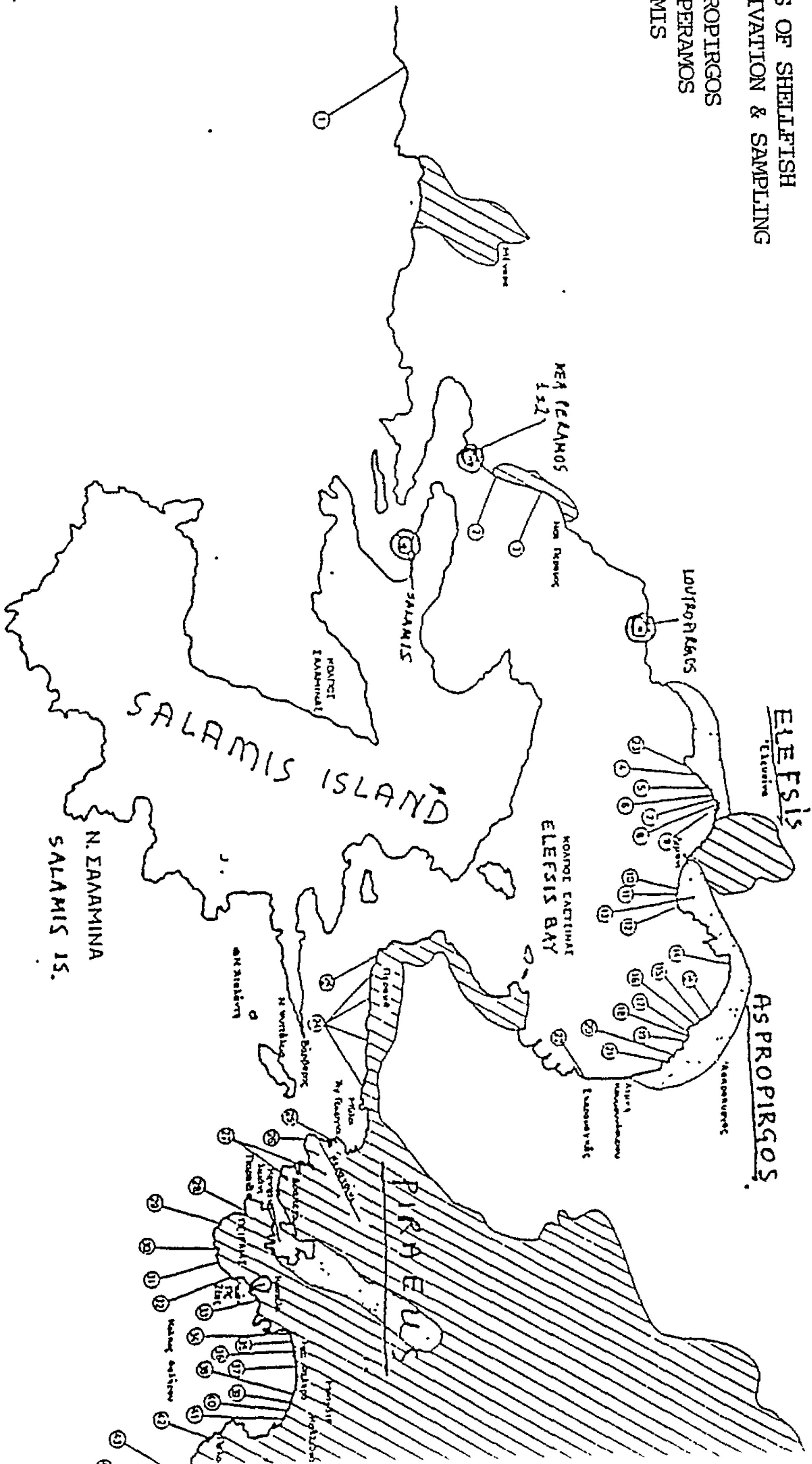
The following parameters were determined:

a) Seawater samples: pH, temperature, coliform count/100 ml., *E. coli* count/100 ml., Enterococci count/100 ml., Chlorophylls a, b, and c, absence or presence/count of *Gymnodinium* and *Gonyaulax*.

b) Shellfish: Coliform count/100 gr., *E. coli* count/100 gr., Enterococci count/100 gr., *Salmonella* in 25 gr. and quantification of Saxitoxin/μg/100 gr. of flesh.

The following techniques were used:

Figure 1



a) Seawater: For coliform and *E. coli* counts, the MPN technique with 5 tubes per dilution. For Enterococci, M.F. technique and the selective Enterococcus agar by Slanetz and Bartley (Merck). For Chlorophylls, the technique described in Standard Methods for the Examination of Water and Waste Water, 15th Ed. 1980 and measurement of O.D. in 3 wavelengths (630, 645, and 663 nm) for c,b, and a chlorophylls respectively. Phytoplankton was also examined according to the technique described by Utermohl (1931), using an inverted microscope. For both chlorophylls and phytoplankton determinations, invaluable help, as well as some confirmation of our results, was given by Mrs. Ignatiadou of the Dimocritos Institute.

b) Shellfish: For coliform and *E. coli* counts, the MPN technique with 5 tubes per dilution according to the WHO/UNEP (1983)* methodology. For Enterococci, the pour plate technique using Bacto-KF streptococcus agar (Difco).

For *Salmonella*, detection after a pre-enrichment step in buffered peptone, enrichment in Rappaport-Vassiliadis medium and also in Selenite broth and subculture on selective agar plates. For Saxitoxin quantification, a standard toxin was provided by Dr. J.E. Gilchrist from the U.S.A. Food and Drug Administration Department and dilutions of this standard were used to inoculate mice, according to the technique described in "Official Methods of Analysis" of the Association of Official Analytical Chemists, 13th Edition., 1980, pp 298-299. The mice used were kindly provided by the Pasteur Institute of Athens in which there is a special animal breeding unit.

2. RESULTS

2.1 General remarks

The medians and ranges of all parameters determined during the investigation are listed in Table 1. The same number of samples were examined from each shellfish-growing area, and repeated all season. However, during the hot season (June - September), a greater number of samples were taken than was the case for the other 3 periods.

From Table 1 it is obvious that the temperature (median and range) of seawater was almost identical in all shellfish growing areas. The densities of faecal indicator organisms (coliforms, *E. coli*, enterococci), if judged by the medians, were also the same. But if the ranges are considered, higher upper values were noted in all 3 indicators only in Loutropyrgos.

The medians of indicator organisms in mussels from the 4 growing areas were almost the same, but much higher medians were found in the market mussels. Moreover all samples of mussels from the growing areas had *E. coli* counts below the standards laid down in the Greek regulations (500/100 gr.), though in the market mussels 1 out of 12 showed a much higher count (i.e. 2,400).

Salmonella was not found in any of the samples examined. It should be noted that selenite broth was used for *S. typhi* isolation and Rappaport-Vassiliadis medium for the other salmonellas.

Chlorophyll-a density (mg/m^3) in seawater was the same (median and range) in the two sampling points of Nea Peramos but somewhat lower in Salamina and substantially higher in Loutropyrgos growing area. Considering the two sampling points of Nea Peramos as one, from the fluctuation of the mean values of chlorophyl-a per month (figure 2) it is obvious that there is a tendency of 2 peaks, one in April and a smaller in October-November.

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* Reference methods for marine pollution studies No. 5 Rev. 1, modified i.e. La.T.B. at 37 EC/48h, differentiation at 44 EC with Lactose-Tryptone-Lauryl Sulfate Broth (gas + indole) (Papadakis & Mavridou 1987, J. Epidemiology and Infection, 99,155).

Table 1

Descriptive statistics - All samples Median and Range (in brackets).

Sampling points	No. of samples	Seawater					Mussels			Salm. in 25g	P.S.P* μg/100gr
		Temp. EC	Colif. /100ml	*E. coli* /100ml	Enteroc. /100ml	Chl A mg/m^3	Colif. /100gr	*E. coli* /100gr	Enteroc. /100gr		
Loutropyrgos	33	19.0 (9-28)	<20 (-790**)	<20 (-490)	2 (-500)	1.76 (0-7.20)	230 (50-5350)	20 (-230)	800 ($-2x10^5$)	No	B.D.L.***
Nea Peramos 1	32	19.5 (10-28)	<20 (-50)	<20 (-50)	<1 (-130)	1.08 (0-6.72)	230 (-24000)	<20 (-460)	400 ($-1.5x10^6$)	No	B.D.L.
Nea Peramos 2	32	19.5 (10-28.5)	<20 (-50)	<20 (-20)	<1 (-8)	1.15 (0-6.80)	230 (20-3500)	<20 (-130)	200 ($-3.2x10^5$)	No	B.D.L.
Salamina	31	20.0 (9-29)	<20 (-1100)	<20 (-20)	<1 (-335)	0.81 (0-4.08)	230 (-24000)	<20 (-170)	200 ($-1.6x10^6$)	No	B.D.L.
Athens Market	12	-	-	-	-	-	1950 (230-24000)	50 (2400)	7200 ($200-8.6x10^6$)	No	B.D.L.

* Paralytic shellfish poisoning toxin (Saxitoxins)

** In cases where the minimum value is "<1", "<20", etc., only the maximum is given

*** Below detectable level of the method (32-55μg/100 gr.)

From a total of 128 samples of seawater from shellfish culturing areas only 12 and 18 samples showed the presence of *Gonyaulax* and *Gymnodinium* respectively. There was not any substantial difference in the frequency of presence or absence of these toxin producing organisms among the four sampling points. However, *Gonyaulax* was generally found in low numbers, up to 160 cells per liter, though *Gymnodinium*, when found, was almost always in higher numbers, up to 5,000 cells/L.

Finally, paralytic shellfish poisoning toxin (P.S.P.-table 1) in mussels (including market samples) was always below detectable level of the method.

2.2 Statistical evaluation

Comparing the percentages of samples with unmeasurable low concentrations of indicator organisms in seawater from the four sampling points (table 2), it is apparent that the differences were statistically highly significant ($P<0,0001$ for coliforms and enterococci $p=0.008$ for *E. coli*). Seawater samples from Loutropyrgos sampling point, differed significantly from the samples of the other points because Loutropyrgos had the smallest rate of samples with unmeasurable low concentrations of all indicator organisms. Seawater samples from the remaining areas showed almost the same rates with the exception of enterococci in the Salamina samples. The difference for enterococci in Salamina and the other three points was near to significant ($X^2_2 = 4,99$, $p=0,08$).

The percentages of mussels from the four harvesting areas (table 2) showed differences which were insignificant for Enterococci ($p=0.43$) and near to significant for *E. coli* ($p=0.08$). Mussels from Loutropyrgos had small rates of unmeasurable low concentration samples. On the contrary, the difference of rates of unmeasurable values between mussels from all harvesting areas and the market samples was significant ($p=0,027$) for enterococci and near to significant for *E. coli* ($p=0.09$). The market mussels had the smallest percentages of samples with unmeasurable low concentration of indicator organisms.

The comparison between samples with measurable concentrations of indicator organisms is given in Table 3. It can be seen that there was no significant difference among the concentrations of indicator organisms both in seawater and in mussels from different sampling points. However the comparison between all mussels from growing areas and the samples from the market showed a statistically highly significant difference ($p<0,0001$) for coliforms but insignificant for *E. coli* and enterococci. The market mussels had very high coliform median (1,950) though the corresponding median in all other mussels was almost 8 times lower (230). The *E. coli* median in both categories of mussels was the same and the median of enterococci in market samples was more than 3 times greater than in all harvested samples, but the difference is insignificant ($p=0.28$).

The correlations between measurements of the parameters studied are listed in table 4. Seawater temperature appeared to have a negative correlation with coliform levels ($p=0.051$) and a weaker one with *E. coli* only in mussels.

The coliform densities in seawater appeared to have a positive correlation with *E. coli* and enterococci of seawater ($p=0.001$) and a weaker correlation ($p=0.06$) with chlorophyl-a. Moreover coliform levels in seawater correlated also with coliform and *E. coli* densities in mussels ($p=0.005$ & 0,002). There was also a lower degree of correlation of seawater coliform levels with enterococci ($p=0.011$).

The densities of *E. coli* in seawater appeared to have a positive correlation with enterococci in seawater ($p=0.001$) and also with *E. coli* in mussels ($p=0.002$). A weaker correlation between *E. coli* in seawater and enterococci ($p=0.054$) and coliform levels ($p=0.08$) in mussels was noted. All the above correlations were positive.

Table 2

Percentage (%) of samples with Unmeasurably Low Concentration
(i.e. "<1", "<20", etc.)

Sampling Point	No. of samples	Seawater			Mussels		
		Colif.	*E. coli*	Entero	Colif.	*E. coli*	Entero
Salamina	31	94	97	58	3	71	42
Loutropyrgos	33	52	76	21	0	39	24
Nea Peramos 1	32	91	94	81	3	53	38
Nea Peramos 2	32	94	97	78	0	59	41
All seawater samples	128	82	91	59	2	54	38
Athens market	12	-	-	-	0	25	0
Differences between 4 sea sampling points	X^2_3	28.2	11.8	31.0	-	6.74	2.78
	P	<0.0001	0.008	<0.0001	-	0.08	0.43
Differences between market and all seawater samples	X^2_1	-	-	-	-	2.96	4.90
	P	-	-	-	-	0.09	0.027

Table 3

Comparisons between samples with measurable concentrations

Sampling point	Seawater						Mussels					
	Colif.		*E. coli*		Entero		Colif.		*E. coli*		Entero	
	n	median	n	median	n	median	n	median	n	median	n	median
Salamina	2	560	1	20	13	2	30	230	9	20	18	3250
Loutropyrgos	16	50	8	20	26	3.5	33	230	20	50	25	2000
Nea Peramos 1	3	50	2	50	6	4	31	230	15	50	20	1500
Nea Peramos 2	2	35	1	20	7	1	32	230	13	20	19	2000
All samples	23	50	12	20	52	3	126	230	57	50	82	2000
Athens market	-	-	-	-	-	-	12	1950	9	50	12	7200
Differences between 4 sea sampling points[1]	X^2_3	0.42	2.90		3.81		4.65		1.87		0.30	
	p	0.94	0.41		0.28		0.20		0.60		0.96	
Differences between market and all samples[2]	p	-	-		-		<0.0001		0.11		0.28	

(1) Kruskal-Wallis test
(2) Mann-Whitney test

Table 4

Correlations between measurements
(Spearman rank correlation coefficient r_s)

A. Sea sampling points (combined)

	Seawater				Mussels		
	Colif.	*E. coli*	Entero	Chl A	Colif.	*E. coli*	Entero
Temp.	0.11 (0.23)	0.07 (0.40)	-0.14 (0.11)	-0.11 (0.22)	-0.17 (0.051)	-0.15 (0.09)	0.05 (0.57)
Colif.		0.55 (0.001)	0.34 (0.001)	0.17 (0.06)	0.25 (0.005)	0.27 (0.002)	0.22 (0.011)
E. coli			0.32 (0.001)	0.11 (0.22)	0.16 (0.08)	0.28 (0.002)	0.17 (0.054)
Entero				0.05 (0.55)	0.20 (0.022)	0.21 (0.019)	0.22 (0.014)
Chl A					0.35 (0.001)	0.23 (0.009)	-0.09 (0.31)

Figure in brackets is level of significance P

B. Between Measurements In Mussels : Upper Triangle - Athens Market; Lower Triangle - All sea samples

	Colif.	*E. coli*	Entero
Colif.	-	0.52 (0.08)	-0.01 (0.97)
E. coli	0.43 (0.001)	-	0.45 (0.14)
Entero	0.11 (0.20)	0.16 (0.07)	-

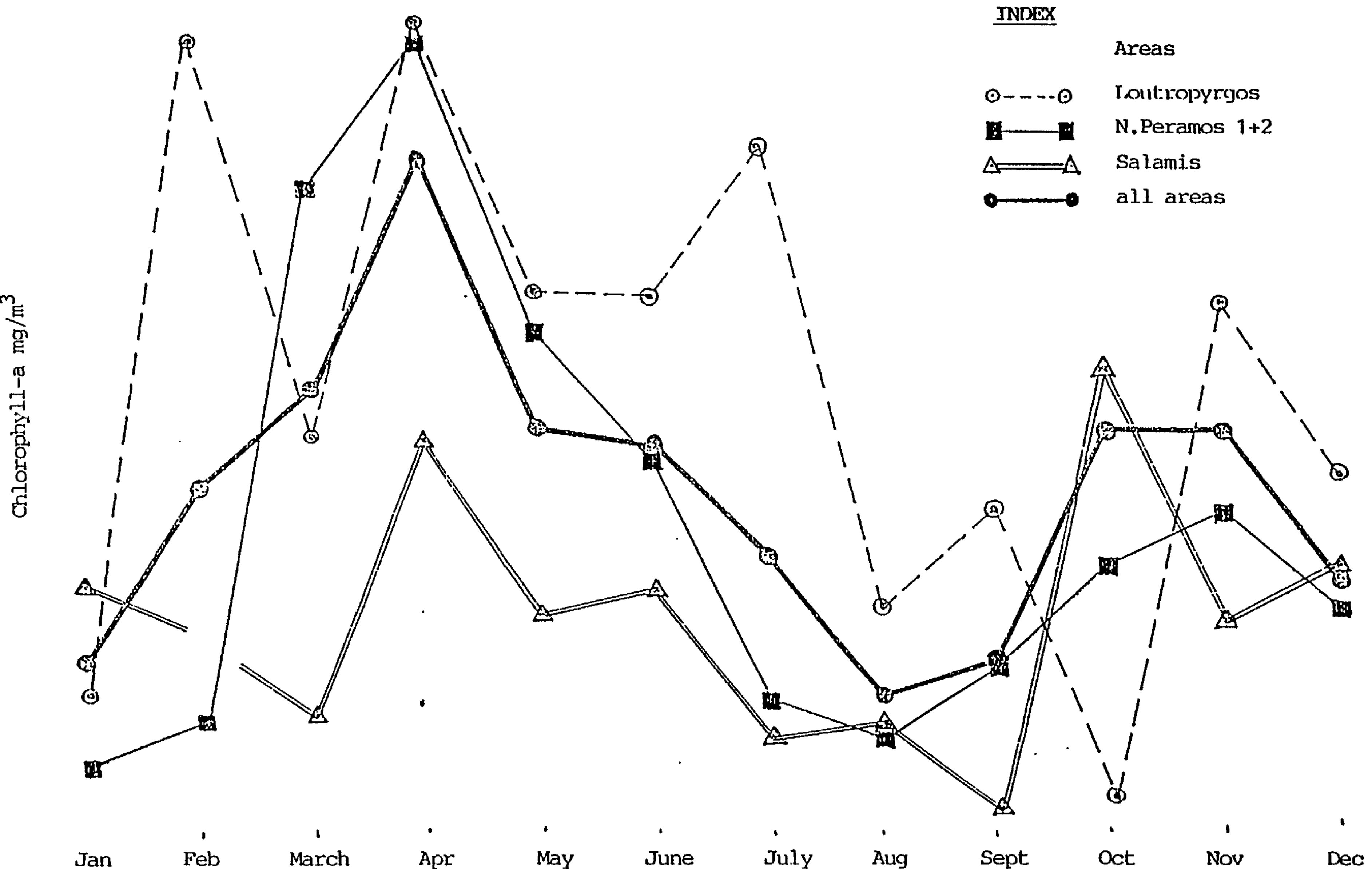

Figure 2 - Fluctuation of Chlorophyll-a in shellfish growing areas of Elefsis Bay

Chlorophyll-a densities in seawater appeared to correlate positively with coliforms and *E. coli* in mussels (p=0,054 and 0.08 respectively). Finally from the parameters in seawater, chlorophyl-a densities appeared to have a positive correlation with coliforms (p=0.001) and *E. coli* (p=0.009) in mussels.

It can also be seen from table 4 that in mussels coliform and *E. coli* counts were positively correlated (p=0.001) and possibly enterococci and *E. coli* (p=0.07). These correlations are seen less clearly for samples from the market because the sample size was small.

Statistical evaluation of chlorophyl-a values from the four sampling points gave significant differences (Kruskal-Wallis test $X^2_3 = 8.34$, p=0.04). If Loutropyrgos samples are omitted and the differences between the other 3 areas re-tested, then there are not significant differences ($X^2_2 = 1.30$, p=0.52). It is obvious that Loutropyrgos appeared to have a higher level of chlorophyl-a than the others.

The most important organism, *Gonyaulax*, was found in seawater only in January, July, August and September with a peak in August (figure 3). Both months corresponded to the lower densities of chlorophyl-a mean values.

Gymnodinium breve was found more often than *Gonyaulax* (figure 3) and it was not possible to observe a clear seasonal distribution. The number of observations was rather small for definite conclusions and statistical evaluations.

2.3 Conclusions

The object of this research was the examination of shellfish growing in seawater in Elefsis bay (a part of the greater Saronikos gulf) for the presence of neurotoxins during cold and hot seasons in relation to bacteriological and trophic grading of seawater.

Neurotoxin levels in mussels from the four growing areas were always below the detectable concentrations of the method used. Thus there was not any possibility for comparison between neurotoxin levels and the other parameters examined. However, the important toxin producing organism *Gonyaulax* was found in very low numbers (up to 160 cells/L) and only during the two periods of the lowest chlorophyll-a densities (January and around August-figure 3). It appears as though the presence of substantial numbers of other phytoplankton species do not favour the growth of *Gonyaulax*. In the contrary, *Gymnodinium breve* was found 8 months per year and even during the high peak of chlorophyll-a levels. Although chlorophyll densities depend on daily and previous solar radiation, and on some other parameters, it can be accepted for comparison in nearby areas. All four investigated areas had been exposed to the same radiation, and the same meteorological conditions. However, the degree of pollution and eutrophication was rather different in Loutropyrgos, which is closer to a number of terrestrial sources of pollution (figure 1). The Salamis sampling point is far from known pollution sources. These facts are in agreement with the observation that chlorophyll-a levels in Loutropyrgos were much higher than in the other three sampling points (difference statistically significant, p=0.04). The higher degree of pollution of Loutropyrgos is also indicated by the highest rate of samples with measurable concentrations for all indicator organisms (table 3) in seawater, and the lowest percentages of "unmeasurable" samples (p<0.0001 - table 2). Nevertheless, the values of indicator organisms in mussels did not exhibit statistically significant differences between sampling points with one exception, the *E. coli* values in mussels of Loutropyrgos. However, there was a difference between all harvested mussels and the market samples (table 2) which was near to significant for *E. coli* (p=0.09) and significant for enterococci (p=0.027) concerning the samples with unmeasurable concentrations. There was also a highly significant difference (p<0.0001) in coliform counts (measurable concentrations) between these two groups of mussels.

From the different correlations between measurements (table 4), coliform, *E. coli* and enterococci counts in seawater appeared to correlate well with each other (p=0.001). There was

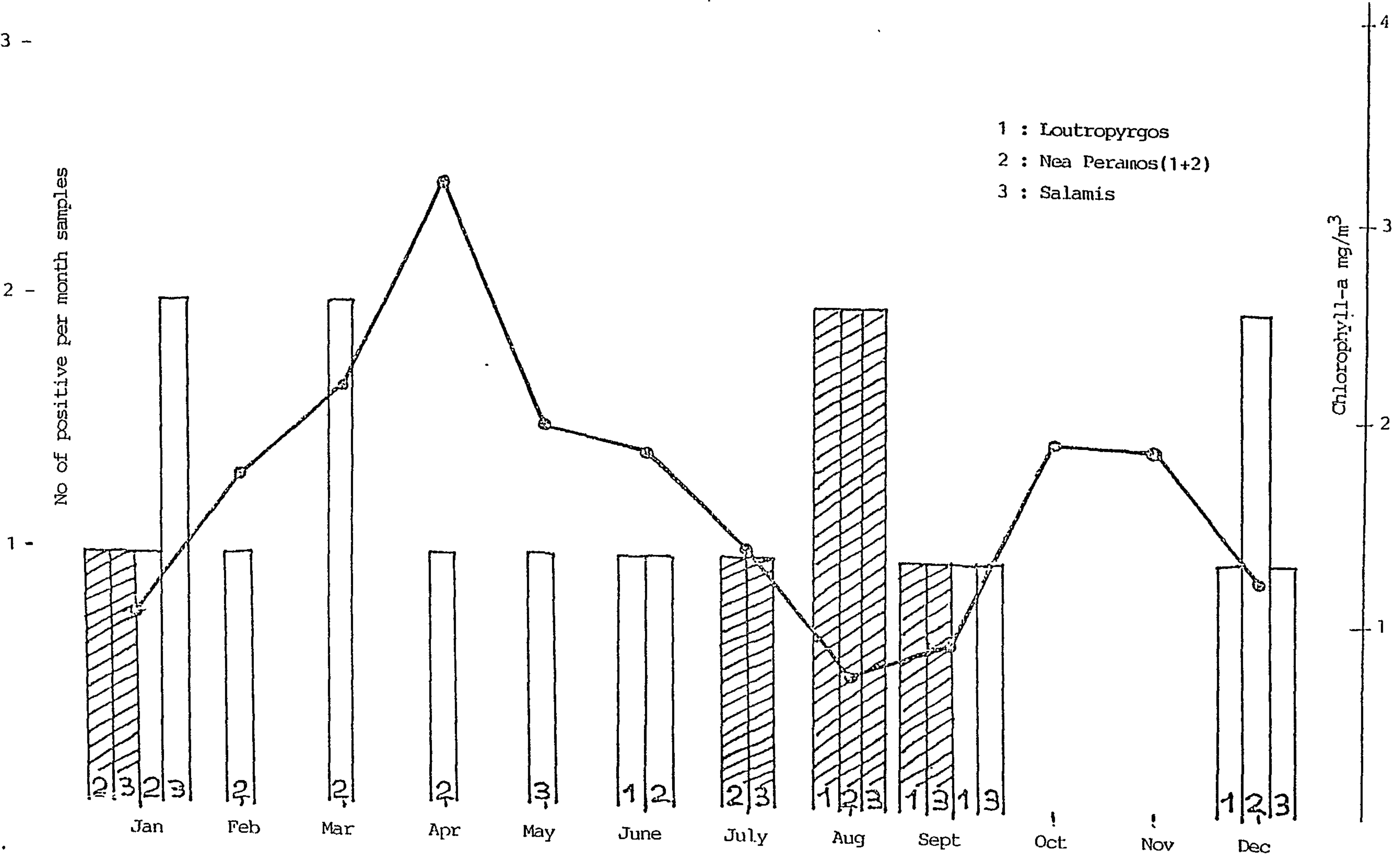

Figure 3 - Distribution per month of positive samples for *Gonyaulax* [hatched] and *Gymnodinium* [open] from 3 shellfish-growing areas of Elefsis Bay. (In comparison with Chlorophyll-a densities)

also a good correlation of seawater coliforms with coliforms and *E. coli* in mussels (table 4) and of *E. coli* with the same organism in mussels. The correlation of enterococci of seawater with the other indicator organisms in mussels appeared to be of lower degree and *vice versa*. An interesting fact is that chlorophyll-a also correlated positively with coliforms and *E. coli* in shellfish, but not with enterococci.

Finally, in mussels, it seems that there is a good correlation between coliform and *E. coli* densities ($p=0.001$) but not between enterococci and, especially, the coliforms.

EVALUATION DE LA SPECIFICITE ET DE LA SENSIBILITE D'UNE METHODE FLUOROMETRIQUE DE DENOMBREMENT D'Escherichia coli

DANS LES ECHANTILLONS D'EAU ET DE SEDIMENTS MARINS PAR HYDROLYSE DU 4-METHYLUMBELLIFERYL-ß-D-GLUCURONIDE (MUG)

by

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1. INTRODUCTION

La contamination des eaux marines par les bactéries telluriques d'origine fécale est traditionnellement évaluée par le dénombrement des représentants du groupe des coliformes totaux (= CT), définis par la Commission Internationale ISO comme l'ensemble des bacilles à Gram négatif, non sporulés, oxydase -, aérobies ou anaérobies facultatifs, capables de se multiplier en présence de sels biliaires ou d'autres agents de surface ayant des propriétés équivalentes, et capables de fermenter le lactose avec production d'acide et de gaz en 48 heures à une température de 35 à 37E C (+ 0.5E C).

La preuve a cependant été faite récemment que ce groupe des CT compte diverses espèces psychrotrophes non fécales (Leclerc et coll., 1983). Il semble donc préférable de rechercher plus spécifiquement la présence des coliformes fécaux (CF), définis par les mêmes propriétés que les CT avec une restriction en ce qui concerne la température d'incubation: ces germes, thermophiles, sont capables de fermenter le lactose avec production d'acide et de gaz à 44.5E C (+ 0.5E C).

Les CF comprennent un certain nombre d'espèces appartenant à la Famille des *Enterobacteriaceae: Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, Enterobacter aerogenes, Citrobacter freundii, Levinea malonatica, Levinea amalonatica* (Leclerc et coll., 1983). Dans certains types d'eaux usées cependant, une importante part des CF isolés sont représentés par des *Klebsiella* et des *Enterobacter* thermo-tolérantes, sans relation avec les matières fécales (Capleans et Kanarek, 1984). Pour cette raison, la numération spécifique de l'espèce *E. coli* a été recommandée à de nombreuses reprises pour le contrôle du degré de contamination bactérienne d'origine fécale des eaux naturelles et des aliments (Dufour et Cabelli, 1975; EPA, 1976; APHA, 1985; FDA, 1985).

Du point de vue pratique, la numération de cette bactérie, que ce soit dans les eaux ou les aliments, est habituellement effectuée de deux manières: par la méthode de filtration sur membranes et culture à 44.5E C sur milieux sélectifs solides (gélose de Chapman au TTC et au Tergitol, milieu mFC, milieu d'Endo, milieu EMB, etc.) et par la technique du MPN (Most Probable Number) en milieu liquide, qui implique un test présomptif suivi d'un test de confirmation (ALPHA, 1985). Ces techniques, toutes deux fondées sur l'acidification du milieu et la production de gaz dues à la fermentation du lactose, sont coûteuses et longues à développer; elles ne fournissent des résultats fiables qu'après 24 à 72 heures, parfois même 96 heures d'incubation.

En outre, ces méthodes de détection d'*E. coli* peuvent être inadéquates dans certains conditions, particulièrement lorsque le milieu dans lequel cette bactérie est recherchée est agressif pour elle, comme les eaux douces chlorées ou les eaux marines (Lin, 1973; Ray et Speck, 1973; Olson, 1978; Dutka et coll., 1979; Evans et coll., 1981). Il a été montré que les cellules d'*E. coli* qui ont subi un stress dans ces conditions, sont hautement sensibles aux fortes températures d'incubation (Rose et coll., 1975; Meadows et coll., 1980) et aux milieux sélectifs contenant certains agents tensio-actifs relativement toxiques (comme le désoxycholate de sodium) (Maxcy, 1973; Ray et Speck, 1973).

En outre, la méthode du MPN prête parfois à certaines interférences bactériennes (Geldreich et coll., 1972; Hussong et coll., 1981) et peut fournir des résultats faussement négatifs (absence de production de gaz en présence de coliformes confirmés) aux stades des tests présomptifs ou des tests confirmatifs (Evans et coll., 1981). D'autres facteurs ont aussi contribué à diminuer l'efficacité et la signification des méthodes de détection d'*E. coli*: production synergique de gaz à partir du lactose par des bactéries non coliformes (Greer et Nyhan, 1928), présence de souches d'*E. coli* incapables de fermenter le lactose et anaérogènes (Anderson et coll., 1980), existence de bactéries non coliformes fermentant le lactose (Hussong et coll., 1980; Hussong et coll., 1981).

L'utilisation de certaines activités enzymatiques spécifiques pour détecter les espèces indicatrices représente une alternative intéressante par rapport aux techniques culturales habituelles. C'est le cas pour la recherche d'*E. coli* grâce à l'examen de l'activité ß-glucuronidase. Cette enzyme, la ß-D-glucuronide glucuronohydrolase (3.2.1.31), catalyse l'hydrolyse des ß-glucuronides et le transfert des radicaux glucuronyl sur les alcools (Kushinski et coll., 1967). Elle est très spécifique de l'espèce *E. coli* approximativement 97% des souches de cette espèce examinées par Kilian et BÜlow (1976) la produisaient, alors que les autres entérobactéries en étaient privées à l'exception de certaines souches de *Salmonella* (Le Minor, 1979; Feng et Hartman, 1982), *Shigella* et *Yersinia* (Kilian et BÜlow, 1976; Feng et Hartman, 1982). Un nouveau test a donc été développé pour la détection rapide d'*E. coli* (24 heures), fondée sur la propriété que présente la ß-glucuronidase de cliver un substrat fluorogène, le 4-methylumbelliferyl-ß-D-glucuronide (MUG) en 4-methylumbelliferone fluorescente sous lumière ultraviolette à grande longueur d'onde. Ce test peut être réalisé en milieu liquide (MPN) (Dahlen et Linde, 1973; Kilian et BÜlow, 1976; Hansen et Yourassowsky, 1984) ou sur milieu solide par numération des colonies fluorescentes après culture sur gélose additionnée de MUG. Il a été proposé pour la recherche d'*E. coli* dans les eaux naturelles, les organismes et les aliments (Feng et Hartman, 1982; Robinson, 1984; Alvarez, 1984; Moberg, 1985; Perez et coll., 1986; Rippey et coll., 1987; Mates, 1987; Berg et Fiksdal, 1988). Il permet la reconnaissance d'*E. coli* parmi les CF, sans nécessité de tests confirmatifs, après 3 heures à 24 heures d'incubation pour l'examen des eaux (Berg et Fiksdal, 1988) et 48 heures pour les coquillages (Rippey et coll., 1987).

Un examen détaillé des données de la littérature consacrée à ce thème, montre cependant une certaine hétérogénéité dans les techniques mises en oeuvre pour tester la méthode au MUG (milieux de culture spécifiques très variés). Il est également difficile de comparer les résultats publiés car ils ont été réalisés à partir d'échantillons très divers: eaux douces, eaux potables, eaux de mer, aliments, coquillages, etc. Par ailleurs, on ne possède que peu de données sur la validité de la méthode pour la détection d'*E. coli* dans des échantillons d'origine marine (eaux, sédiments). La présente étude a donc été entreprise pour tester cette validité, pour des échantillons d'eaux de mer

et de sédiments provenant de trois zones méditerranéennes différentes, à l'aide de milieux et de réactifs rigoureusement identiques et suivant le même protocole expérimental. La standardisation de la méthode a permis de tester statistiquement sa sensibilité et sa spécificité selon la provenance des échantillons analysés et le manipulateur effectuant les tests.

2. MATERIELS ET METHODES

2.1 Echantillons

Trois zones de prélèvements d'échantillons ont été choisies le long du littoral méditerranéen, au voisinage des villes de Malaga (Espagne), Nice (France) et Palerme (Sicile, Italie). Dans chacune de ces trois zones, les échantillons ont été prélevés aux environs d'un rejet d'eaux usées en mer, selon le schéma suivant:

- une station dans la zone de mixage des eaux usées en mer (point de rejet) (A) (eau très contaminée);

- une station à environ 500 mètres de ce point de rejet. A ce niveau, deux échantillons ont été prélevés: eau (peu polluée) (B) et sédiments (C). La position exacte de ces points de prélèvements est présentée dans les Figures 1, 2 et 3.

Dans les trois zones sélectionnées, les échantillons ont été prélevés au cours des mois d'Avril à Juin 1988 (température de l'eau de mer: 18E C à 20E C environ), à une seule reprise. Ils ont été systématiquement transportés aux laboratoires respectifs dans des récipients réfrigérés (4E C) et analysés dans les deux heures qui ont suivi leur prélèvement.

2.2 Milieux de culture

Deux milieux sélectifs ont été utilisés pour dénombrer et isoler les souches d'*E. coli*:

- Milieu mFC Agar (Difco)
- Milieu Tergitol-7 Agar (Difco) additionné de 0,04 g/100 ml de chlorure de triphényl tétrazolium (TTC) (milieu Chapman-TTC).

Le test au MUG a été réalisé sur milieu Nutrient Agar (NA) (Difco), préparé de la manière suivante: à 100 ml de NA stérile ramené à 45E C, a été additionné 1 ml d'une solution aqueuse contenant 10 mg/ml de réactif MUG (milieu NA+MUG). Ce réactif provenait des Etablissements SIGMA. Après mélange à chaud (46E C), le milieu NA+MUG a été réparti en boîtes de Pétri plastiques (diamètre 55 mm). Ces boîtes ont été conservées au froid (4E C) et généralement utilisées pendant une période de 7 jours; aucune différence dans les résultats et l'intensité de la fluorescence n'a cependant été observée pendant les trois semaines qui ont suivi la préparation du milieu NA+MUG.

Le but de ce travail étant l'étude comparative de la valeur du test MUG effectué dans trois environnements différents (zones marines et laboratoires différents), il était nécessaire de standardiser rigoureusement tous les autres facteurs, et en particulier les milieux et réactifs employés, et les protocoles expérimentaux. Les trois laboratoires concernés ont donc utilisé des milieux (mFC Agar, Tergitol-7 Agar, Nutrient Agar) et des réactifs (TTC, MUG) provenant de lots uniques, acquis par le Laboratoire de Nice, homogénéisés et répartis en trois fractions, l'une utilisée sur place et les autres adressées aux Laboratoires de Malaga et de Palerme.

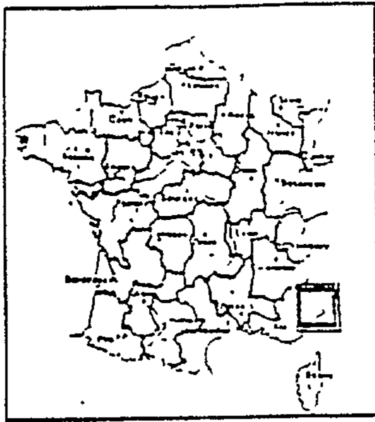


Fig. 1 - Localisation des stations de prélèvement des échantillons d'eau et de sédiment sur le littoral marin de Nice (France)

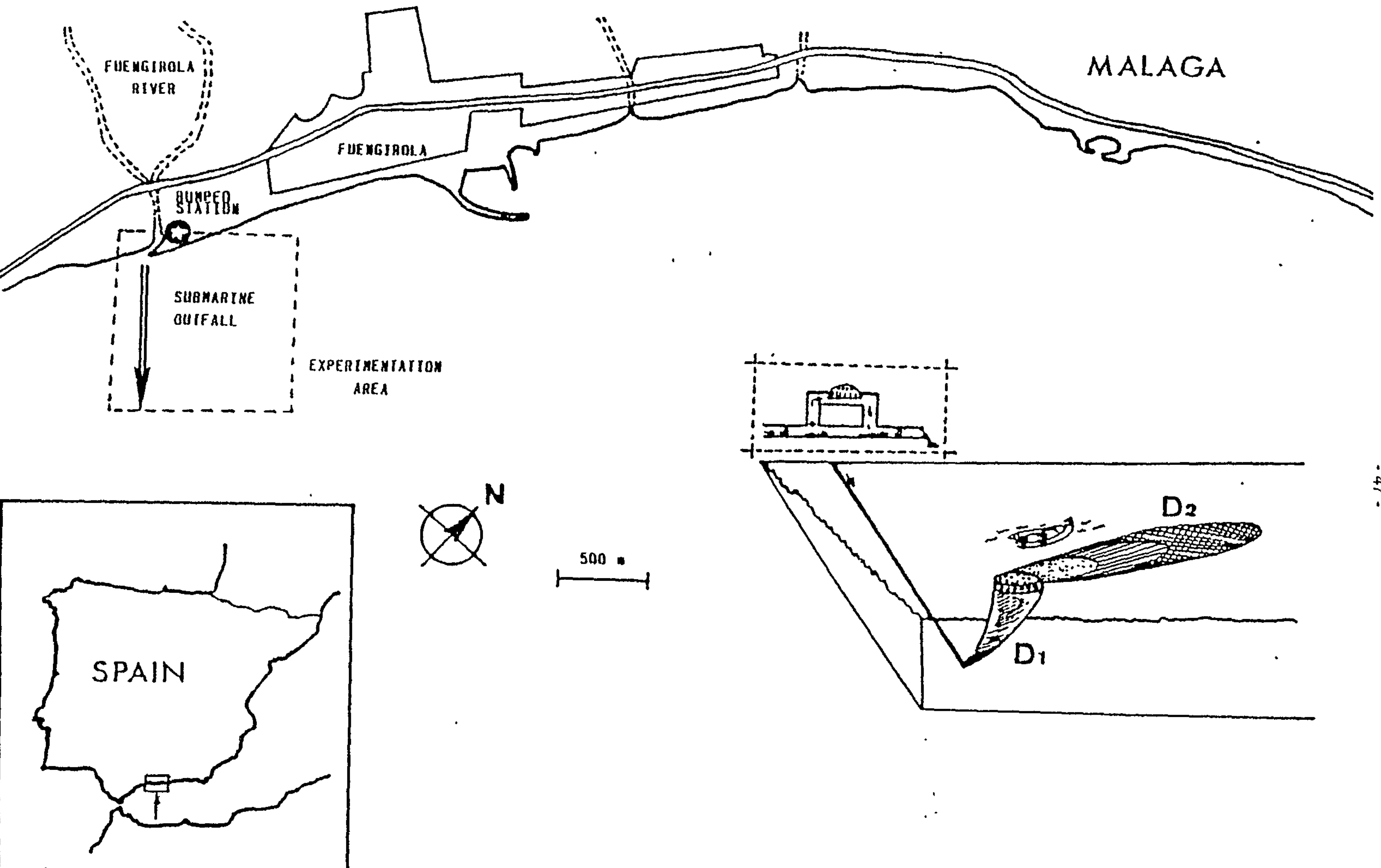
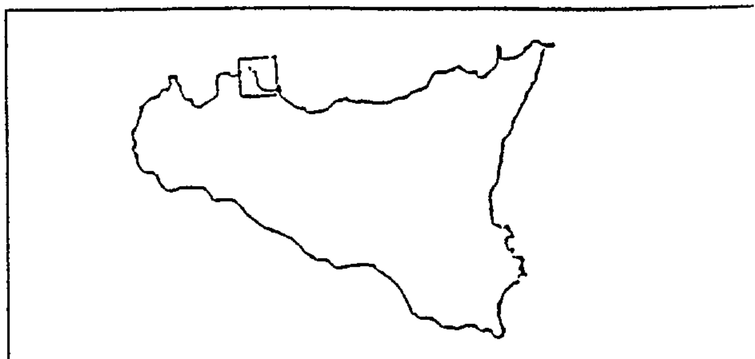
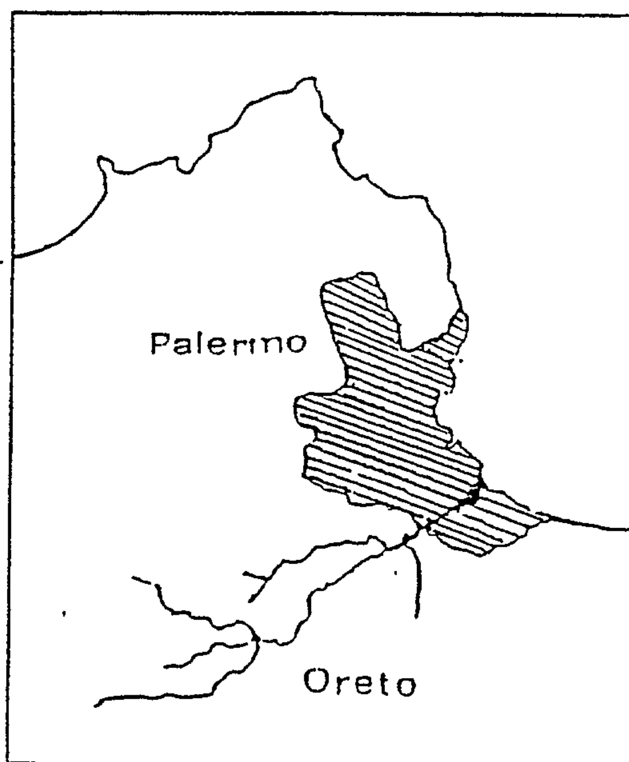
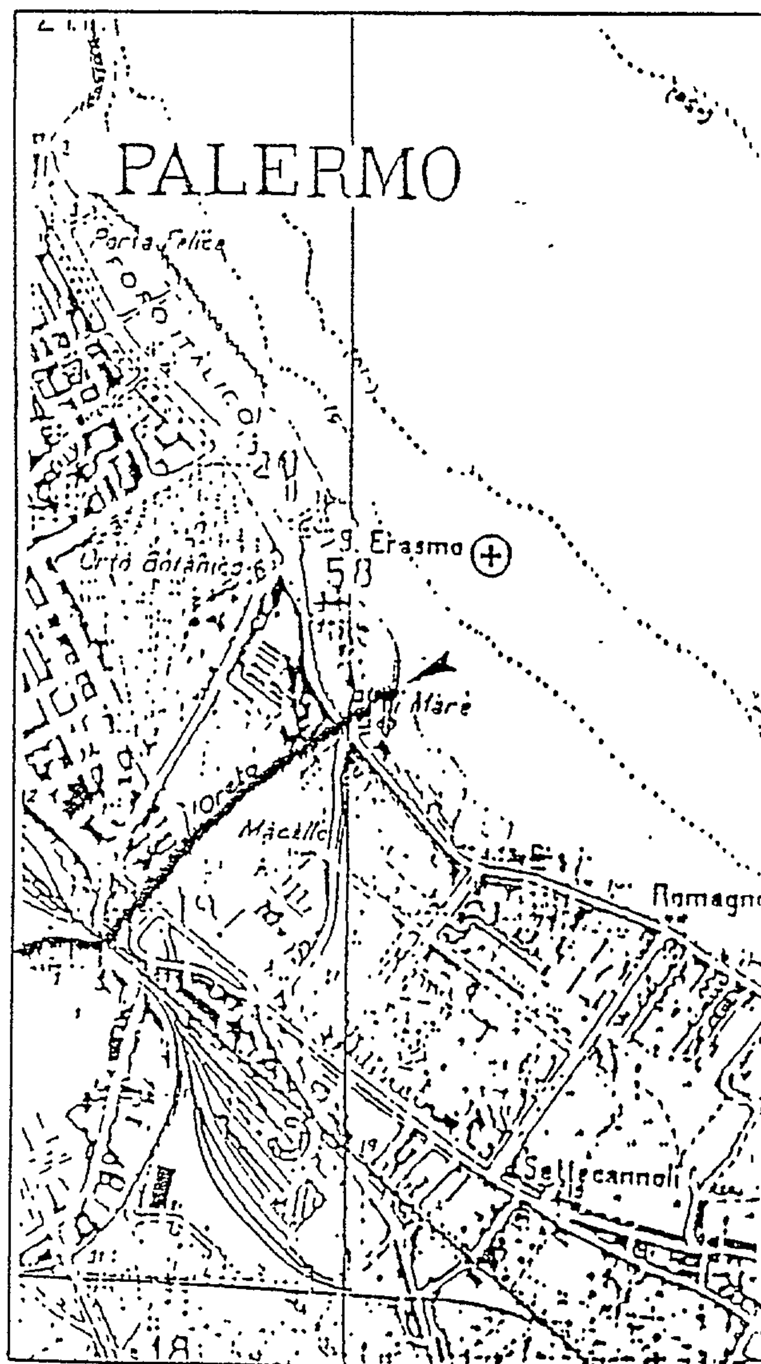


Fig. 2 - Localisation des stations de prélevement des échantillons d'eau et de sédiment sur le littoral marin de Malaga (Espagne)







Eau de mer très polluée, échantillon prélevé à l'embouchure de l'Oreto, près du rivage.

Eau de mer modérément polluée, échantillon prélevé à 500 m. au large de l'embouchure de l'Oreto.

Sédiment

Fig. 3 - Localisation des stations de prélèvement des échantillons d'eau et de sédiment sur le littoral marin de Palerme (Sicile, France)

2.3 Analyses bactériologiques

2.3.1 Méthodes de numération

Les échantillons d'eau ont été analysés sans traitement préalable. Les sédiments ont été suspendus en tampon phosphate salin (PBS) dilué au 1/10e (APHA, 1985); après homogénéisation et décantation rapide, le surnageant a été traité comme les échantillons d'eau.

La numération des *E. coli*, les test MUG et l'isolement des souches pour identification taxonomique ont été réalisés par la technique de filtration sur membranes (Millipore, diamètre 47 mm; pores de 0,45 micron). Des volumes appropriés d'eau et de sédiments ont été filtrés sur ces membranes: celles-ci ont ensuite été déposées à la surface des milieux sélectifs mFC et Chapman -TCC (les deux milieux pour chaque échantillon), plus incubées à 44.5E C (± 0.5E C) pendant 24 heures (± 2 à 3 heures). La numération des colonies considérées comme typiques de l'espèce *E. coli* a été effectuée selon les critères suivants:

- sur milieu mFC: colonies bleues plus ou moins foncées, à contour régulier ou légèrement ondulé;

- sur milieu Chapman-TTC: colonies orangées entourées d'un halo jaune pâle. Seules les membranes portant 20 à 30 colonies ont été utilisées pour les tests au MUG.

2.3.2 Tests au MUG

Pour les deux milieux sélectifs, les colonies typiques décrites ci-dessus ont été marquées sur les filtres. Ceux-ci ont ensuite été transférés aseptiquement dans des boîtes de Pétri contenant le milieu NA-MUG, qui ont alors été incubées pendant 3 heures à 36E C (± 1E C). Les membranes ont été examinées sous lumière ultraviolette (254 à 305 nm) pour la détection des colonies ayant hydrolysé le MUG (fluorescentes = colonies MUG +) parmi celles qui n'ont pas modifié ce réactif (non fluorescentes = colonies MUG -).

2.3.3 Isolement des souches MUG + et MUG - et tests d'identification

Pour chaque échantillon (A, B et C), quatre ensembles de souches ont été isolées sur milieux mFC et Chapman-TTC (environ 40 souches pour chaque type):

- souches à colonies typiques d'*E. coli* et à réaction MUG+;
- souches à colonies typiques d'*E. coli* et à réaction MUG-;
- souches à colonies non typiques d'*E. coli*, à réaction MUG+;
- souches à colonies non typiques d'*E. coli*, à réaction MUG-.

Chaque souche a d'abord été cultivée sur le milieu spécifique d'origine (mFC ou Chapman-TTC), puis transférée sur milieu Trypticase Soy Agar (Difco). Toutes les souches isolées ont fait l'objet d'une identification taxonomique à l'aide des tests suivants:

- coloration de Gram,
- cytochrome oxydase (coloration bleue en présence de NNN'N'-tétraméthyl paraphénylènediamine),
- catalase (dégagement d'oxygène en présence de H_2O_2 à 10 vol.),
- test d'oxydation-fermentation du glucose,
- tests des galeries API 20E (Analytical Products Inc.).

2.3.4 Tests de stabilité de l'activité ß-glucuronidase chez E. coli en survie dans l'eau de mer

Il a été récemment montré que les activités enzymatiques d'*E. coli* peuvent être modifiées pendant sa survie dans l'eau de mer (Munro et coll., 1987): certaines augmentent (phosphatase alcaline, certaines estérases, lipases), alors que d'autres diminuent rapidement (ß-galactosidase par exemple). Il semblait donc nécessaire d'examiner la stabilité de l'activité de la ß-glucuronidase en fonction du temps de contact des cellules avec l'eau de mer, de manière à déceler une variation possible de cette propriété chez *E. coli* en milieu marin.

Ce test a été réalisé avec quatre souches d'*E. coli*:

- trois souches sauvages:
  . H10407 (toxinogène LT ST, CFAI) (Evans et coll., 1975);
  . LT+ (toxinogène LT, isolée d'un malade au Bengladesh);
  . M (toxinogène, isolée au laboratoire);

- une souche de laboratoire (K12 C600).

Ces quatre souches développent une réaction MUG positive très nette.

Les bactéries ont été cultivées dans un bouillon nutritif (Nutrient Broth, Difco) pendant à 7 heures à 37E C. Les cellules ont été rincées trois fois en eau de mer stérile par centrifugation (10000 rpm, 4E C, 20 minutes), puis suspendues en eau de mer filtrée (membranes à pores de 0,22 micron), en flacons erlenmeyer de 500 ml contenant 200 ml d'eau de mer, jusqu'à une densité d'environ 2.10^6 cellules par ml. La survie de ces cellules a été suivie pendant 15 jours, à l'obscurité et à 23E C (± 3E C), par dénombrement des unités formant colonies (UFC) sur milieu NA puis NA+MUG (colonies MUG+ et MUG-), selon la technique de filtration sur membrane décrite ci-dessus.

Le test complet a été réalisé à deux reprises.

2.3.5 Analyse statistique des résultats

Un test de variance a été utilisé pour comparer les résultats obtenus dans les trois laboratoires, selon la méthode décrite par Sokal et Rohlf (1980). Les calculs ont été effectués à l'aide d'un programme Statview, sur micro-ordinateur Apple Macintosh Plus.

4. RESULTATS

L'ensemble de cette étude a porté sur 2600 souches (Malaga: 792; Nice: 960; Palerme: 848), isolées à partir des trois types d'échantillons (A, B, C) et sur les deux milieux spécifiques (mFC, Chapman-TTC).

Les résultats des tests de contingence (Chi^2) montrent que, quels que soient la région considérée et le type d'échantillon, il existe une liaison hautement significative entre l'aspect (typique ou non typique d'*E. coli*) des colonies sur les deux milieux utilisés et la réaction MUG (+ ou -): les colonies typiques étaient très significativement liées à la réaction MUG+ ($p < 0.00001$) (Tableau 1).

Les proportions des colonies MUG+ et MUG-, parmi les colonies typiques ou non typiques d'*E. coli* isolées sur mFC ou sur Chapman-TTC, dans les trois laboratoires associés, sont présentés dans le Tableau 2. Celui-ci fournit également les résultats des tests d'identification effectués sur l'ensemble des souches isolées au cours du travail.

Sur les 798 souches à colonies typiques MUG+ isolées dans les trois laboratoires (396 sur mFC, 402 sur Chapman-TTC), seules 35 souches (soit 4,3% du total, ou 5,5% des souches venant du mFC et 3,2% de celles isolées du Chapman-TTC) n'étaient pas identifiées à l'espèce *E. coli* (résultats faussement positifs): elles appartenaient aux espèces suivantes (selon le système de classification taxonomique API):

- *Salmonella spp.*
- *Shigella spp.*
- *Citrobacter spp.*
- *Citrobacter freundii*
- *Yersinia enterocolitica*
- *Enterobacter agglomerans*
- *Klebsiella pneumoniae*
- *Klebsiella oxytoca*

D'autre part, 14,9% des souches à colonies typiques d'*E. coli* mais à réaction MUG- (724 au total, dont 373 provenaient du milieu mFC et 351 du milieu Chapman-TTC) étaient identifiées à cette espèce. Ces réactions faussement négatives étaient beaucoup plus fréquentes parmi les souches isolées du milieu Chapman-TTC (21,3%) que parmi celles qui provenaient du milieu mFC (8,8%). Parmi ces souches à colonies typiques MUG-, et en dehors des *E. coli* confirmés, les espèces dominantes étaient différentes selon la zone considérée:

MALAGA :	*Citrobacter freundii*	(23,5%)
	Klebsiella pneumoniae	(14,8%)
PALERME:	*Citrobacter freundii*	(24,3%)
	Enterobacter cloacae	(19,6%)
	Klebsiella oxytoca	(14,5%)
	Klebsiella pneumoniae	(10,9%)
NICE:	*Klebsiella pneumoniae*	(75,8%)
	Enterobacter cloacae	(8,7%)
	Citrobacter spp.	(4,6%)

Par ailleurs 87,7% des colonies non typiques à réaction MUG+ étaient identifiées à *E. coli* (91,9% pour les souches isolées sur milieu mFC, 83,2% pour celles isolées sur milieu Chapman-TTC). La fréquence de ces souches d'*E. coli* MUG+ à colonies atypiques était cependant très différente selon le laboratoire où étaient effectués les tests. On notera en particulier qu'aucune de ces souches n'a été détectée dans l'eau de mer (polluée ou non) à Malaga, alors qu'elles étaient très fréquentes à Nice et moyennement fréquentes à Palerme dans tous les échantillons.

Enfin, dans le groupe "témoin" composé des souches à colonies atypiques et à réaction MUG-, on notait la dominance des espèces possédant une oxydase (84,1%, toutes provenances confondues). Parmi les souches oxydase-, les espèces les plus fréquentes étaient: *Enterobacter cloacae* (3,8%), *Citrobacter freundii* (3,2%) et *Klebsiella pneumoniae* (1,9%). On notera dans ce groupe l'isolement de cinq souches d'*E. coli*, qui cumulaient donc les deux caractères atypiques (aspect inhabituel sur les milieux de culture spécifiques et réaction MUG-).

L'analyse de variance effectuée sur l'ensemble des souches (Tableau 3) confirme le haut niveau de signification de l'identification d'*E. coli* par la réaction au MUG(MUG+) et l'aspect macroscopique des colonies (colonies typiques sur milieux sélectifs) ($p < 0,0001$).

Tableau 1

Résultats des tests de contingence (Chi ^{2}) effectués sur les colonies typiques et atypiques isolées sur les deux milieux sélectifs, par rapport à leur réaction MUG+ ou MUG- respectivement

		mFC				Chapman-TTC			
Région	Echantillon	Typiques		Atypiques		Typiques		Atypiques	
		C.C.[a]	N.S.[b]	C.C.	N.S.	C.C.	N.S.	C.C.	N.S.
Nice	A[d]	0,65	1 E-4	0,69	1 E-4	0,65	1 E-4	0,64	1.E-4
	B	0,66	1 E-4	0,66	1 E-4	0,69	1 E-4	0,61	1.E-4
	C	0,68	1 E-4	0,68	1 E-4	0,69	1 E-4	0,61	1.E-4
Palerme	A	0,64	1 E-4	0,69	1 E-4	0,63	1 E-4	0,69	1.E-4
	B	0,66	1 E-4	5	1 E-4	0,60	1 E-4	0,68	1.E-4
	C	0,67	1 E-4	0,71	1 E-4	0,65	1 E-4	0,67	1.E-4
				0,59					
Malaga	A	0,66	1 E-4	0,71	1 E-4	-[c]	-	0,49	1.E-4
	B	0,62	1 E-4	-	-	-	-	0,56	1.E-4
	C	0,64	1 E-4	0,66	1 E-4	0,67	1 E-4	0,73	1.E-4

(a) Coefficient de contingence;
(b) Niveau de signification;
(c) Aucune donnée analysée;
(d) A: eau de mer très polluée; B: eau de mer peu polluée; C: sédiment

Table 2

Résultats des tests au MUG effectués sur les souches isolées à Malaga, Nice et Palerme, à partir d'échantillons d'eau très polluée ou peu polluée et de sédiments, sur milieux mFC et Chapman-TTC. Ce tableau présente également l'identification de ces souches selon le système API

mFC – Colonies typiques

mFC	MUG+ Espèces	F	I	E	MUG- Espèces	F	I	E
	E. coli	37(92,5)	51(94,4)	45(100)	E. coli	4(10)	6(11,5)	6(13,3)
Eau	Salmonella spp.	1 (2,5)	-	-	K. pneumoniae	29(72,5)	7(13,4)	15(33,3)
de	Shigella spp	2 (5)	-	-	K. oxytoca	1(2,5)	9(17,3)	6(13,3)
mer	C. freundii	-	3(5,5)	-	K. ozaenae	-	1(1,9)	3(6,6)
très					Enterobacter spp	1(2,5)	-	-
polluée					E. sakazakii	1(2,5)	2(3,8)	3(6,6)
					E. cloacae	4(10)	12(2,3)	3(6,6)
					Citrobacter spp.	-	2(3,8)	-
A					C. freundii	-	6(11,5)	9(20)
					C. diversus	-	2(3,9)	-
					Serratia spp	-	1(1,9)	-
					S. liquefaciens	-	2(3,8)	-
					Klebsiella spp	-	2(3,8)	-
Total		40(100)	54(100)	45(100)		40(100)	52(100)	45(100)
	E. coli	37(92,5)	42(95,5)	39(86,6)	E. coli	2(5)	3(6,7)	3(7,7)
Eau	Salmonella spp	1 (2,5)	-	-	K. pneumoniae	32(80)	5(11,6)	3(7,7)
de	Shigella spp	2 (5)	-	-	E. cloacae	3(7,5)	6(13,9)	-
mer	E. agglomerans	-	1(2,3)	-	Enterobacter spp	1(2,5)	-	-
peu	Y. enterocolitica	-	1(2,3)	-	Citrobacter spp	2 (5)	2(4,6)	-
polluée	C. freundii	-	-	6(13,3)	C. freundii	-	15(34,9)	15(38,4)
					K. oxytoca	-	9(20,9)	3(7,7)
					Klebsiella spp.	-	2(4,6)	-
B					E. agglomerans	-	1(2,3)	3(7,7)
					Kluyvera spp	-	-	3(7,7)
					E. sakazakii	-	-	6(15,3)
					K. ozaenae	-	-	3(7,7)
Total		40(100)	44(100)	45(100)		40(100)	43(100)	39(100)
	E. coli	39(97,5)	39(97,5)	45(93,7)	E. coli	2(5)	3(7,5)	4(11,7)
	Shigella spp	1(2,5)	-	-	K. pneumoniae	28(70)	3(7,5)	8(23,5)
	C. freundii	-	1(2,5)	3(6,3)	E. cloacae	4(10)	8(20)	6(17,5)
					E. spp 1	1(2,5)	-	-
Sédiment					Citrobacter spp.	4(10)	3(7,5)	-
					E. sakazakii	1(2,5)	-	-
					C. freundii	-	17(42,5)	12(35,2)
C					Y. enterocolitica	-	1(2,5)	-
					E. agglomerans	-	2(5)	2(58)
					K. oxytoca	-	3(7,5)	2(5,8)
Total		40(100)	40(100)	48(100)		40(100)	40(100)	34(100)

mFC – Colonies atypiques

mFC	MUG+ Espèces	F	I	E	MUG- Espèces	F	I	E
A	E. coli	38(95)	22(95,6)	9(100)	Shigella spp	1(2,5)	-	-
	Salmonella spp.	1 (2,5)	-		C. violaceum	1(2,5)	-	-
	Shigella spp	1 (2,5)	-	-	K. pneumoniae	1(2,5)	-	1(2,5)
	K. pneumoniae	-	1(4,4)	-	V. metchnikovii	1(2,5)	-	-
					Kluyvera sp.	1(2,5)	-	-
					Y. enterocolitica	-	1(2,5)	-
					E. cloacae	-	2(5)	9(22,5)
					K. oxytoca	-	1(2,5)	1(2,5)
					C. freundii	-	-	3(7,5)
					Y. frederiksenii	-	-	1(2,5)
					E. aerogenes	-	-	1(2,5)
					OXIDASE +	35(87,5)	28(70)	24(60)
Total		40(100)	23(100)	9(100)		40(100)	40(100)	40(100)
B	E. coli	35(87,5)	6(100)	0	Cedecea spp	1(2,5)	-	-
	Shigella spp	4 (10)	-	-	K. pneumoniae	2(5)	-	1(2,5)
	Salmonella spp	1 (2,5)	-	-	V. metchnikovii	1(2,5)	-	-
					K. oxytoca	-	1(2,5)	4(10)
					C. freundii	-	1(2,5)	1(2,5)
					E. cloacae	-	-	1(2,5)
					E. aerogenes	-	-	1(2,5)
					E. agglomerans	-	-	3(7,5)
					K. ozaenae	-	-	1(2,5)
					OXIDASE +	36(90)	38(95)	28(70)
Total		40(100)	6(100)	0		40(100)	40(100)	40(100)
C	E. coli	37(92,5)	4(57,1)	9(100)	S. odorifera	1(2,5)	-	1(2,5)
	Shigella spp	3(7,5)	-	-	Cedecea spp.	1(2,5)	-	-
	C. freundii	-	3(42,9)	-	C. freundii	-	5(12,5)	3(7,5)
					E. cloacae	-	2(5)	4(10)
					K. oxytoca	-	-	1(2,5)
					E. coli	-	-	2(5)
					E. amnigenus	-	-	1(2,5)
					OXIDASE +	38(95)	33(85,5)	28(70)
Total		40(100)	7(100)	9(100)		40(100)	40(100)	40(100)

Chapman-TTC – Colonies typiques

Chapman-TTC	MUG+ Espèces	F	I	E	MUG- Espèces	F	I	E
	E. coli	36(90)	40(100)	51(100)	E. coli	2(5)	8(20)	20(55,5)
Eau	Shigella spp	4 (10)	-	-	K. pneumoniae	34(85)	7(17,5)	4(11,1)
de								
mer					Citrobacter spp.	1(2,5)	1(25)	-
très					E. sakazakii	1(2,5)	-	-
polluée					E. cloacae	2(5)	10(25)	-
					K. oxytoca	-	7(17,5)	-
					C. freundii	-	6(15)	-
A					C. diversus	-	1(2,5)	-
					S. odorifera	-	-	8(22,2)
					E. agglomerans	-	-	4(11,1)
Total		40(100)	40(100)	51(100)		40(100)	40(100)	36(100)
	E. coli	37(92,5)	37(92,5)	54(100)	E. coli	2(5)	7(17,5)	15(42,9)
Eau	Shigella spp	3 (7,5)	-	-	K. pneumoniae	36(90)	5(12,5)	-
de	C. freundii	-	1(2,5)	-	E. cloacae	1(2,5)	6(15)	5(14,3)
mer	K. pneumoniae	-	1(2,5)	-	Enterobacter spp	1(2,5)	-	-
peu	K. oxytoca	-	1(2,5)	-	E. agglomerans	-	6(15)	-
polluée					E. aerogenes	-	1(2,5)	-
					C. freundii	-	8(20)	10(28,2)
					K. ozaenae	-	1(2,5)	-
B					K. oxytoca	-	3(7,5)	5(14,3)
					Y. enterocolitica	-	1(2,5)	-
					S. odorifera	-	1(2,5)	-
					S. liquefaciens	-	1(25)	
Total		40(100)	40(100)	54(100)		40(100)	40(100)	35(100)
	E. coli	39(97,5)	38(95)	57(100)	E. coli	1(2,5)	4(10)	16(40)
	Shigella spp.	1(2,5)	-	-	K. pneumoniae	23(57,5)	1(2,5)	4(10)
	K. pneumoniae	-	1(2,5)	-	K. oxytoca	2(5)	6(15)	4(10)
	Citrobacter spp	-	1(2,5)	-	Enterobacter spp	2(5)	1(2,5)	-
Sédiment					E. cloacae	7(17,5)	8(20)	8(20)
					E. sakazakii	1(2,5)	-	-
					Citrobacter spp.	4(10)	4(10)	-
C					C. freundii	-	12(30)	8(20)
					E. agglomerans	-	2(5)	-
					Klebsiella spp	-	1(2,5)	-
					OXIDASE +	-	1(2,5)	-
Total		40(100)	40(100)	57(100)		40(100)	40(100)	40(100)

Chapman-TTC – Colonies atypiques

Chapman-TTC	MUG+ Espèces	F	I	E	MUG- Espèces	F	I	E
A	E. coli	33(82,5)	39(97,5)	0	Kluyvera spp	1(2,5)	-	-
	Salmonella spp	1 (2,5)	-	-	Shigella spp	1(2,5)	-	-
	Shigella spp.	6 (15)	-	-	K. pneumoniae	1(2,5)	-	6(15)
	K. oxytoca	-	1(2,5)	-	S. marcescens	1(2,5)	-	-
					V. metchnikovii	2(5)	-	-
					E. coli	-	1(2,5)	1(2,5)
					E. cloacae	-	3(7,5)	1(2,5)
					Yersinia spp	-	1(2,5)	-
					Citrobacter spp	-	2(5)	-
					C. freundii	-	2(5)	-
					K. oxytoca	-	-	2(5)
					OXIDASE +	34(85)	31(77,5)	30(75)
Total		40(100)	40(100)	0		40(100)	40(100)	40(100)
B	E. coli	30(75)	19(90,4)	0	V. metchnikovii	2(5)	-	-
	Salmonella spp	2 (5)	-	-	Shigella spp	1(2,5)	-	-
	Shigella spp	8 (20)	-	-	E. aerogenes	-	1(2,7)	-
	E. agglomerans	-	2(9,5)	-	C. freundii	-	3(8,3)	2(5)
					S. liquefaciens	-	1(2,7)	-
					K. oxytoca	-	1(2,7)	-
					S. odorifera	-	-	1(2,5)
					S. marcescens	-	-	1(2,5)
					E. cloacae	-	-	2(5)
					E. sakazakii	-	-	1(2,5)
					OXIDASE +	37(92,5)	30(83,3)	33(82,5)
Total		40(100)	21(100)	0		40(100)	36(100)	40(100)
C	E. coli	30(75)	5(83,3)	5(100)	C. freundii	-	2(5)	1(2,5)
	Shigella spp	10(25)	-	-	K. oxytoca	-	2(5)	-
	C. freundii	-	1(16,6)	-	K. pneumoniae	-	-	2(5)
					E. cloacae	-	-	3(7,5)
					E. coli	-	-	1(2,5)
					C. hermannii	-	-	1(2,5)
					OXIDASE +	40(100)	36(90)	32(80)
Total		40(100)	6(100)	5(100)		40(100)	40(100)	40(100)

Les tableaux 4, 5, 6, 7, 8, 9 et 10 présentent les données calculées pour évaluer plus précisément la spécificité et la sensibilité de la méthode MUG pour la reconnaissance et la numération d'*E. coli* dans les divers échantillons analysés (A, B ou C), selon le lieu de prélèvement et l'étude (3 zones) et le milieu de culture utilisé (2 milieux).

Si l'on évalue la spécificité de la technique sur la simple proportion des colonies typiques d'*E. coli* qui donnent une réaction MUG+, il est clair qu'elle est très élevée, cette proportion n'étant jamais inférieure à 91%. Ces résultats sont en accord avec les données publiées antérieurement par Kilian et Bülow (1976), Feng et Hartman (1982), Perez et coll. (1986) et Mates (1987), qui ont rapporté des pourcentages d'identification d'*E. coli* analogues: 97%, 96%, 93,5% et 91,6%, respectivement). La variation résiduelle de cette spécificité entre 90 et 100% est du même ordre pour nos résultats et pour ces données bibliographiques.

La même conclusion peut être tirée en considérant le pourcentage des *E. coli* confirmés dans chacun des quatre groupes des souches: typiques MUG+, typiques MUG-, atypiques MUG+ et atypiques MUG-, selon le milieu utilisé (Tableau 5). Le taux de résultats faussement négatifs est beaucoup plus élevé à partir du Chapman-TTC (17,5 à 25,8% moyenne générale: 21,3%) qu'à partir du milieu mFC (6,5 à 11,6% moyenne générale: 8,8%).

Si l'on utilise comme critère de spécificité le pourcentage des résultats réellement négatifs (souches non *E. coli* MUG-) par rapport à l'ensemble des résultats réellement négatifs et faussement positifs (souches non *E. coli* MUG- et souches non *E. coli* MUG+) (Tableaux 6, 7, 8, 9 et 10), on constate également que la spécificité de la méthode MUG est très élevée, beaucoup plus que celle de la méthode traditionnelle (numération sur milieu spécifique sans MUG) (Tableau 10). L'examen de ces résultats montre cependant qu'elle varie selon le milieu utilisé pour la culture des CF et qu'elle est moins importante lorsque cette culture est faite sur milieu Chapman-TTC). Sur ce point, il existait une différence très significative entre les résultats fournis par les trois laboratoires: ceux qui ont été obtenus à Nice (Sp = 90%) étaient nettement moins bons que ceux de Malaga ou de Palerme (Sp = 97,7 et 96%, respectivement).

La sensibilité de la méthode pour distinguer les *E. coli* par le test au MUG était élevée (> 90%) et relativement constante dans le cas de l'emploi du milieu mFC, ceci dans les trois laboratoires et pour les trois types d'échantillons analysés. Par contre, lorsque les CF étaient cultivés sur milieu Chapman-TTC, ces indices étaient beaucoup plus variables selon le laboratoire: 69,8 à 78,4% à Malaga, 88,7 à 91,5% à Palerme et 97,1 à 98,5% à Nice.

Il est par ailleurs très intéressant de considérer l'évolution du nombre de colonies MUG+ sur les membranes en fonction de la densité totale des colonies (Tableau 11). Ce test a été effectué à Malaga, sur 192 membranes ayant servi à la numération d'échantillons A, B ou C. Les résultats montrent que la visualisation "*in situ*" de la fluorescence des colonies MUG+ dépend beaucoup du nombre total des colonies portées par la membrane. Approximativement 40% des filtres étudiés portaient un nombre "optimal" de colonies de CF (soit entre 20 et 60 colonies typiques), alors que plus de 45% d'entre eux ne portaient moins de 20, et 15% plus de 60.

En ce qui concerne l'étude de la stabilité de l'activité ß-glucuronidase chez *E. coli* en survie dans l'eau de mer carencée en matières nutritives, aucune modification n'a été observée dans le pouvoir de clivage du MUG par les cellules des quatre souches testées, après 15 jours de contact avec l'eau de mer. Toutes les cellules qui ont conservé le pouvoir de cultiver sur milieu NA ont également conservé leur caractère MUG+ (Tableau 12).

Tableau 3

Résultats de l'analyse de variance en bloc complet à 4 facteurs pour la mise en évidence des corrélations entre la provenance de souches (3 zones d'études et 3 types d'échantillons A, B, C) la réaction MUG (+ ou -) et l'aspect des colonies des souches testées sur les deux milieux sélectifs (typiques d'*E. coli* ou atypiques)

Source	dl	Ó^{2}	Carré moyen	Test-F	P
Echantillon (A)	2	154.694	77.347	685	509
Milieu de culture (B)	1	5	5	4.427E-3	.9472
AB	2	34.75	17.375	.154	.8578
MUG (C)	1	1120.222	1120.222	9.92	.0028
AC	2	36.028	18.014	.16	.853
BC	1	46.722	46.722	.414	.5231
ABC	2	13.194	6.597	.058	.9433
Colonie (D)	1	2592	2592	22.952	1.DE-4
AD	2	14.083	7.042	.062	.9396
BD	1	6.722	6.722	.06	.8083
ABD	2	90.028	45.014	.399	.6735
CD	1	2403.556	2403.556	21.283	1.DE-4
ACD	2	70.861	35.431	.314	.7322
BCD	1	2.722	2.722	.024	.8773
ABCD	2	11.028	5.514	.049	.9524
Erreur	48	5420.667	112.931		

Tableau 4

Pourcentages de souches identifiées à *E. coli* dans les quatres groupes de souches à colonies typiques ou atypiques de cette espèce et à réaction MUG positive ou négative (souches mFC et Chapman-TTC confondues)

Prélèvements	Laboratoires (ou sites)	*E. coli* à colonies typiques MUG+ (a)	*E. coli* à colonies typiques MUG- (b)	*E. coli* à colonies atypiques MUG+ (c)	*E. coli* à colonies atypiques MUG- (d)
Eau de mer	Malaga	100	32	100	1.25
très polluée	Nice	91.25	7.5	88.75	0
(A)	Palerme	96.8	15.2	96.8	1.25
Eau de mer	Malaga	93.9	24.3	(e)	0
peu polluée	Nice	92.5	5	81.25	0
(B)	Palerme	94	12	92.6	0
	Malaga	97.1	27	100	4.2
Sédiment	Nice	97.5	3.75	83.75	0
(C)	Palerme	96.25	8.75	82.10	0

(a) Vrais positifs sur aspect des colonies et réaction MUG
(b) Faux négatifs sur réaction MUG
(c) Faux négatifs sur aspect des colonies
(d) Faux négatifs sur aspect des colonies et réaction MUG
(e) Aucune souche de ce type n'a pu être testée dans le prélèvement correspondant

Tableau 5

Pourcentages de souches d'*E. coli* dans les quatre groupes de souches à colonies typiques ou atypiques de cette espèce et à réunion MUG positive ou négative selon le type d'échantillon et la nature du milieu de culture utilisé (provenances confondues)

		E. coli à colonies typiques MUG+	*E. coli* à colonies typiques MUG-	*E. coli* à colonies atypiques MUG+	*E. coli* à colonies atypiques MUG-
A	mFC	95,6	11,6	95,8	-
	Chapman-TTC	96,9	25,8	90	1,6
B	mFC	91,4	6,5	89,1	-
	Chapman-TTC	95,5	20,8	80,3	-
C	mFC	96	7,9	89,2	1,6
	Chapman-TTC	97,8	17,5	78,4	0,8
A+ B+ C	mFC	96,7	8,8	91,9	0,55
	Chapman-TTC	97,7	21,3	83,8	0,87

Tableau 6

Indices de sensibilité et de spécificité de la méthode au MUG pour la numération d'*E. coli*, calculés selon la nature du milieu de culture utilisé ou celle de l'échantillon analysé, le laboratoire (ou site géographique) choisi et la méthode de numération utilisée (traditionnelle ou MUG)

Milieu de culture	Echantillon analysé (a)	Laboratoire	Méthode de numération	Indice de sensibilité (Se) (b)	Indice de spécificité (Sp) (c)
mFC	A	Nice	T(d)	51,9	51,8
			MUG(e)	94,9	93,8
		Palerme	T	72,1	45,5
			MUG	92,4	95,5
		Malaga	T	85	50,6
			MUG	90	100
	B	Nice	T	52,7	52,3
			MUG	97,3	90,7
		Palerme	T	88,3	48,8
			MUG	94,1	97,6
		Malaga	T	100	48,8
			MUG	92,9	92,7
	C	Nice	T	52,6	52,4
			MUG	97,4	90,7
		Palerme	T	91,4	53,1
			MUG	93,5	95,1
		Malaga	T	81,7	53,5
			MUG	90	95,8
TTC	A	Nice	T	53,5	52,8
			MUG	97,2	87,6
		Palerme	T	54,5	55,5
			MUG	90,8	98,6
		Malaga	T	98,6	70,9
			MUG	70,8	100
	B	Nice	T	56,5	54,9
			MUG	97,1	85,7
		Palerme	T	69,8	51,3
			MUG	88,9	93,2
		Malaga	T	100	66,7
			MUG	78,3	100
	C	Nice	T	57,1	55,5
			MUG	98,6	87,8
		Palerme	T	87,5	51,3
			MUG	91,5	96,3
		Malaga	T	92,4	61,9
			MUG	78,5	100

(a) : A = eau très polluée; B = eau peu polluée; C = sédiment

(b) : $Se = \frac{E.\ coli\ MUG+}{(E.\ coli\ MUG+) + (E.\ coli\ MUG-)}$

(c) : $Sp = \frac{Non\ E.\ coli\ MUG-}{(Non\ E.\ coli\ MUG-) + (Non\ E.\ coli\ MUG+)}$

(d) : T = technique traditionnelle (milieu sans réactif MUG)

(e) : MUG = technique au MUG

Tableau 7

Indices de sensibilité et de spécificité de la méthode au MUG selon
le milieu de culture employé, le laboratoire (ou site géographique choisi et
la méthode de numération utilisée. Cumulation des données obtenues pour les trois
types d'échantillons analysés (eau très polluée, eau peu polluée, sédiment)

Milieu de culture	Laboratoire	Méthode de numération	Indice de Sensibilité (Se) (a)	Indice de Spécificité (Sp) (b)
mFC	Nice	T (c)	52,4	52,2
		MUG (d)	96,5	93,2
	Palerme	T	81,8	49
		MUG	93,2	96,1
	Malaga	T	87,6	50,9
		MUG	90,7	96,1
Chapman-TTC	Nice	T	55,7	54,4
		MUG	97,6	87
	Palerme	T	67,3	57,7
		MUG	89,9	96
	Malaga	T	96,8	66,3
		MUG	75,9	100

(a) : Se = *E. coli* MUG+ / ((*E. coli* MUG+) + (*E. coli* MUG-))

(b) : Sp = Non *E. coli* MUG- / ((Non *E. coli* MUG-) + (Non *E. coli* MUG+))

(c) : T = technique traditionnelle (milieu sans réactif MUG)
(d) : MUG = technique au MUG

Tableau 8

Indices de sensibilité et de spécificité de la méthode au MUG selon le site géographique étudié (Nice, Palerme, Malaga) et la méthode de numération choisie (traditionnelle ou au MUG). Cumulation des données obtenues pour les trois types d'échantillons A, B, C) et les deux milieux de culture (mFC, Chapman-TTC)

Laboratoire	Méthode de numération	Indice de Sensibilité (Se) (a)	Indice de Spécificité (Sp) (b)
Nice	T (c)	54	53,4
	MUG (d)	97	90
Palerme	T	74,1	50,7
	MUG	91,4	96
Malaga	T	92,9	65,9
	MUG	82,2	97,7

(a) : Se = $\frac{E.\ coli\ MUG+}{(E.\ coli\ MUG+) + (E.\ coli\ MUG-)}$

(b) : Sp = $\frac{Non\ E.\ coli\ MUG-}{(Non\ E.\ coli\ MUG-) + (Non\ E.\ coli\ MUG+)}$

(c) : T = technique traditionnelle (milieu sans réactif MUG)
(d) : MUG = technique au MUG

Tableau 9

Indices de sensibilité et de spécificité de la méthode au MUG par rapport à la méthode traditionnelle, calculés en fonction de la nature du milieu de culture utilisé (mFC, Chapman-TTC). Cumulation des données obtenues pour les trois types d'échantillons (A, B, C) et dans les trois laboratoires (Nice, Palerme, Malaga)

Méthode de numération	Milieu de culture	Indice de Sensibilité (Se) (a)	Indice de Spécificité (Sp) (b)
T (c)	mFC	71,5	50,7
	Chapman-TTC	73,8	56,9
MUG (d)	mFC	93,8	95,1
	Chapman-TTC	87,6	93,5

(a) : Se = $\frac{E.\ coli\ MUG+}{(E.\ coli\ MUG+) + (E.\ coli\ MUG-)}$

(b) : Sp = $\frac{Non\ E.\ coli\ MUG-}{(Non\ E.\ coli\ MUG-) + (Non\ E.\ coli\ MUG+)}$

(c) : T = technique traditionnelle (milieu sans réactif MUG)
(d) : MUG = technique au MUG

Tableau 10

Indices de sensibilité et de spécificité de la méthode au MUG par rapport à la méthode traditionnelle, toutes données confondues (3 types d'échantillons, 3 laboratoires, 2 milieux de culture)

Méthode de numération	Indice de Sensibilité (Se) (a)	Indice de Spécificité (Sp) (b)
T (c)	72,7	56,8
MUG (d)	90,6	94,2

(a) : $Se = \frac{E.\ coli\ MUG+}{(E.\ coli\ MUG+) + (E.\ coli\ MUG-)}$

(b) : $Sp = \frac{Non\ E.\ coli\ MUG-}{(Non\ E.\ coli\ MUG-) + (Non\ E.\ coli\ MUG+)}$

(c) : T = techni que traditionnelle (milieu sans réactif MUG)
(d) : MUG = technique au MUG

Tableau 11

Proportion du nombre de colonies MUG+ selon le nombre total de colonies présentes sur les membranes (192 membranes testées)

Nombre colonies/ filtre	Nombre de colonies MUG+							%
	0	1-10	11-20	21-30	31-40	41-50	>50	
1-10	2,6	20,5						23,1
11-20		17,9	5,1					23
21-30		2,6	2,6					5,2
31-40		2,6	12,8	5				20,4
41-50		2,6		2,6				5,2
51-60			2,6		5,1			7,7
>60				2,6	5,1	2,6	5,1	15,4
Total	2,6	46,2	23,1	10,2	10,2	2,6	5,1	100

Tableau 12

Evolution du nombre des cellules cultivables et de la réaction au MUG de celles-ci pour quatre souches d'*E. coli* mises en survie dans l'eau de mer filtrée carencée en matières nutritives

Temps de contact avec l'eau de mer (jours)	Tests	Souches d'*E. coli*			
		H 10407	LT+	M	K12
0	CFU/ml	$2,4.10^6$ ($9,4.10^4$)[a]	$4,5.10^6$ ($2,6.10^5$)	$1,7.10^6$ ($7,4.10^4$)	$4,3.10^6$ ($1,1.10^5$)
	%MUG+	100	100	100	100
2	CFU/ml	$7,3.10^4$ ($2,4.10^3$)	$6,3.10^4$ ($3,2.10^3$)	$9,6.10^4$ ($3,2.10^2$)	3.10^3 ($2,2.10^2$)
	%MUG+	100	100	100	100
5	CFU/ml	$3,9.10^3$ (3.10^2)	$6,4.10^3$ (450)	$9,8.10^2$ (35)	60 (40)
	%MUG+	100	100	100	100
7	CFU/ml	68 (44)	40 (22)	25 (12)	2 (0,3)
	%MUG+	100	100	100	100
10	CFU/ml	68 (44)	40 (22)	25 (12)	2 (0,3)
	%MUG+	100	100	100	100
15	CFU/ml	25 (5)	12 (3)	7 (1,5)	<1 -
	%MUG+	100	100	100	-

a: Déviation standard, calculée sur deux expériences à partir de dénombrements effectués en triplicate.

5. DISCUSSION - CONCLUSION

La méthode MUG, associée à la technique de filtration sur membrane et à la culture des CF sur milieu sélectif peut effectivement représenter une alternative intéressante et fiable aux méthodes habituelles utilisées pour le dénombrement spécifique d'*E. coli* dans les échantillons d'eau ou de sédiments marins, lesquels nécessitent des tests confirmatifs biochimiques et/ou sérologiques longs et coûteux.

La spécificité et la sensibilité de cette technique, telles qu'elles apparaissent dans cette étude, sont élevées, ce qui confirme nettement sa validité: dans la presque totalité des situations, quels que soient la nature et le degré de contamination de l'échantillon (eau polluée ou non, sédiment), le site choisi, ou les conditions dans lesquelles sont réalisées les numérations (nature du milieu de culture, technique utilisée), la méthode MUG était toujours plus spécifique et plus sensible que la méthode traditionnelle pour le dénombrement d'*E. coli*. On doit cependant moduler cette conclusion car le pourcentage des résultats faussement négatifs (RFN) (souches d'*E. coli* à réaction MUG-) était relativement élevé dans certains cas, et la spécificité de la méthode variait assez largement selon le lieu de prélèvement et le milieu utilisé pour la culture des CF. Ainsi, on a vu que le nombre des RFN était parfois élevé (5 à 32%, selon la zone étudiée). En appliquant ce calcul au milieu de culture, on constate que le pourcentage des RFN était beaucoup plus important pour le groupe des souches isolées sur milieu Chapman-TTC. De ce point de vue, la différence entre l'ensemble des souches issues du milieu mFC et celui des souches isolées sur milieu Chapman-TTC était maximale pour le laboratoire de Malaga: les RFN atteignaient 13,3% à 17,6% (selon l'échantillon analysé) pour les colonies isolées du mFC et 40% à 55,8% pour celles qui provenaient du milieu Chapman-TTC. Ceci montre que la fiabilité du test MUG pour la détection spécifique d'*E. coli* sur les membranes utilisées pour la colimétrie, dépend fondamentalement du pouvoir de différenciation macroscopique des colonies de CF sur le milieu sélectif utilisé. Il semble clair que, de ce point de vue, le milieu de Chapman-TTC est moins fiable que le milieu mFC, et qu'il n'est donc pas adéquat pour une utilisation conjointe avec la méthode MUG pour la numération sélective d'*E. coli* dans des échantillons marins. Sur le plan pratique, les personnes qui ont effectué les isolements dans les trois laboratoires associés à cette étude s'accordent pour reconnaître que l'identification des colonies de CF sur le milieu de Chapman-TTC est plus délicate et laisse une plus grande part à l'interprétation subjective, ce qui explique certainement les différences de "spécificité" de la méthode MUG dans les trois laboratoires telles qu'elles ressortent des calculs présentés ci-dessus.

Par ailleurs, la présence de ß-glucuronidase paraît effectivement très spécifique d'*E. coli*, compte tenu du très faible pourcentage des résultats faussement positifs (RFP) (souches MUG+ non identifiées à *E. coli*) (4,3% des 798 souches MUG+ testées). Ces RFP étaient attribuables à des bactéries appartenant toutes à la famille des *Enterobacteriaceae*, et réparties dans les genres *Salmonella, Shigella, Citrobacter, Yersinia, Enterobacter* et *Klebsiella*. On remarquera cependant une plus grande diversité spécifique dans ce groupe de bactéries non *E. coli*-MUG+ pour les échantillons étudiés à Palerme et à Malaga. Par contre, seules les eaux prélevées à Nice et analysées sur milieu mFC contenaient des *Salmonella* (espèces non déterminées). Ces résultats sont globalement en accord avec ceux qui ont été publiés par Rippey et coll. (1987), concernant les CF isolés de coquillages marins: parmi ces bactéries, une activité ß-glucuronidase (réaction MUG+) a été décelée, en dehors d'*E. coli*, chez diverses espèces de *Shigella, Yersinia* et *Salmonella*, ainsi que des staphylocoques et des streptocoques. Kilian et Bülow (1976), Le Minor (1979) et Feng et Hartman (1982) ont également détecté cette activité chez *Shigella* et *Salmonella*. Robinson (1984) et Moberg (1985) ne l'ont par contre observée que chez des staphylocoques.

Certaines entérobactéries appartenant aux genres *Klebsiella*, *Citrobacter* et *Enterobacter* sont reconnues comme pouvant être confondues avec *E. coli* par la morphologie et la coloration de leurs colonies sur les milieux sélectifs (dont les milieux mFC et Chapman-TTC). Selon les résultats obtenus au cours de cette étude, il semble qu'elles puissent être distinguées d'*E. coli* par le test au MUG après culture sur milieu mFC. Parmi les 769 souches à colonies typiques d'*E. coli* isolées sur ce milieu dans les trois laboratoires, 396 (51,5%) étaient MUG-positives, et les 373 souches MUG-négatives appartenaient aux espèces suivantes: *K. pneumoniae* (34,8%), *K. oxytoca* (8,8%), *K.* spp. (1%), *K. ozaenae* (1,87%), *C. freundii* (19,8%), *C. diversus* (0,53%), *C.* spp. (3,48%), *E. cloacae* (12,3%), *E. agglomerans* (2,1%), *E. sakazakii* (3,48%), *E.* spp. (0,8%) et *Y. enterocolitica* (0,26%). Sur le milieu Chapman-TTC, la composition en espèces des souches typiques MUG-négatives était la suivante: *K. pneumoniae* (33,3%), *K. oxytoca* (7,7%), *K. ozaenae* (0,28%), *K.* spp.

(0,28%), *C. freundii* (12,5%), *C. diversus* (0,28%), *C.* spp. (0,28%), *E. cloacae* (13,4%), *E. agglomerans* (3,4%), *E. sakazakii* (0,56%), *E. aerogenes* (0,28%), *E.* spp. (1,1%), *S. liquefaciens* (0,28%), *S. odorifera* (0,28%) et *Y. enterocolitica* (0,28%). Ces résultats sont voisins de ceux présentés par Mates (1987) dans son rapport MED POL concernant la différenciation d'*E. coli* parmi les CF dans l'eau de mer par la méthode MUG, tout au moins en ce qui concerne les espèces dominantes (*K. pneumoniae, C. freundii, E. cloacae* et *E. sakazakii*). Notre étude montre que les espèces à réaction MUG négative dont les colonies peuvent être confondues avec celles d'*E, coli* sur les milieux sélectifs sont en fait relativement nombreuses, bien qu'en très petit nombre. Précisions à ce propos que cette discussion n'est valable que dans le système taxonomique API, utilisé par Mates et pour cette étude. Quelques tests effectués à l'aide d'autres systèmes (PASCO Difco par exemple) ont montré qu'il pourrait en être différemment si les tests d'identification des souches isolées étaient réalisés avec d'autres techniques et selon d'autres critères de détermination taxonomique.

En conclusion, il semble donc que le test au MUG soit plus sensible et plus spécifique, donc plus fiable, que la méthode traditionnelle, et qu'il soit donc recommandable pour le dénombrement en routine d'*E. coli* dans les échantillons marins, sans que les test confirmatifs soient nécessaires. On ne peut cependant cacher le fait qu'il est assez coûteux: 1 gramme de réactif MUG coûte environ 10.000 Francs Français, et permet la préparation de 10 litres de milieu NA-MUG, donc 2000 boîtes de Pétri pour le test (5 FF / boîte). On doit en outre le coupler à l'utilisation du milieu mFC et tenir compte du fait que la densité des colonies sur les membranes influence la précision du test: il ne devrait être réalisé qu'avec les membranes portant moins de 30 colonies.

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