MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

WORLD HEALTH ORGANIZATION

IDENTIFICATION OF MICROBIOLOGICAL COMPONENTS AND MEASUREMENT DEVELOPMENT AND TESTING OF METHODOLOGIES OF SPECIFIED CONTAMINANTS (AREA I)

IDENTIFICATION DE CONSTITUANTS MICROBIOLOGIQUES ET DE DOSAGE (MISE AU POINT ET ESSAI DE METHODES) DE CONTAMINANTS DONNES (DOMAINE DE RECHERCHE I)

Final Reports on Selected Microbiological Projects

Rapports finaux sur certains projets de nature microbiologique

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This volume is the eighty seventh of the Mediterranean Action Plan Technical Reports 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.

Ce volume constitue le quatre vingt septième 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.

PREFACE

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 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, exchange of information and assessment of the state of pollution and 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 inter-dependent 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.

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.

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.

Research and study topics included initially in the MED POL - Phase II were:

- development of sampling and analytical techniques for monitoring the sources and levels of pollutants. Testing and harmonization of these methods at the Mediterranean scale and their formulation as reference methods. Priority will be given to the substance listed in the annexes of the Protocol for the prevention of pollution of the Mediterranean Sea by dumping from ship and aircraft and the Protocol for the protection of the Mediterranean Sea against pollution from land-based sources (activity A);

- development of reporting formats required according to the Dumping, Emergency and Land-Based Sources Protocols (activity B);

- formulation of the scientific rationale for the environmental quality criteria to be used in the development of emission standards, standards of use or guidelines for substaces listed in annexes I and II of the Land-Based Sources Protocol in accordance with Articles 5, 6 and 7 of that Protocol (activity C);

- epidemiological studies related to the confirmation (or eventual revision) of the proposed environmental quality criteria (standards of use) for bathing waters, shellfish-growing waters and edible marine organisms (activity D);

- development of proposals for guidelines and criteria governing the application of the Land-Based Sources Protocol, as requested in Article 7 of that Protocol (activity E);

- research on oceanographic processes, with particular emphasis on surface circulation and vertical transport. Needed for the understanding of the distribution of pollutants through the Mediterranean and for the development of contingency plans for cases of emergency (activity F);

- research on the toxicity, persistence, bioaccumulation, carcinogenicity and mutagenicity of selected substances listed in annexes of the Land-Based Sources Protocol and the Dumping Protocol (activity G);

- research on eutrophication and concomitant plankton blooms. Needed to assess the feasibility of alleviating the consequences and damage from such recurring blooms (activity H);

- study of ecosystem modifications in areas influenced by pollutants, and in areas where ecosystem modifications are caused by large-scale coastal or inland engineering activity (activity I);

- effects of thermal discharges on marine and coastal ecosystems, including the study of associated effects (activity J);

- biogeochemical cycle of specific pollutants, particularly those relevant to human health (mercury, lead, survival of pathogens in the Mediterranean Sea, etc.) (activity K);

- study of pollutant-transfer processes (i) at river/sea and air/sea interface, (ii) by sedimentation and (iii) through the straits linking the Mediterranean with other seas (activity L);

The Contracting Parties at their 6th Ordinary Meeting (Athens, October 1989) agreed to:

(a) Re-orient the research activities within MED POL in order to generate information which will also be useful for the technical implementation of the LBS protocol in addition to supporting monitoring activities;

(b) replace as from 1990 research activities A-L by the following five new research areas:

Research area I - Characterization and measurement

This area will include projects which cover the characterization (identification of chemical or microbiological components) and measurement development and testing of methodologies of specified contaminants;

Research area II - Transport and dispersion

This area will include projects which aim at improving the understanding of the physical, chemical and biological mechanisms that transport potential pollutants from their sources to their ultimate repositories. Typical topics will be atmospheric transport and deposition, water movements and mixing, transport of contaminants by sedimentation and their incorporation in biogeochemical cycles. Priority will be given to the provision of quantitative information ultimately useful for modelling the system and contributing to regional assessments;

Research area III - Effects

This area will include projects relevant to the effects of selected contaminants, listed in Annexes I and II of the LBS and Dumping protocols, to marine organisms, communities and ecosystems or man and human populations. Priority will be given to effects and techniques providing information useful for establishing environmental quality criteria;

Research area IV - Fates/Environmental transformation

This area will include projects studying the fate of contaminants (including microorganisms) in the marine environment such as persistence or survival, degradation, transformation, bioaccumulation etc. but excluding transport and dispersion which is dealt in area II;

Research area V - Prevention and control

This area will include projects dealing with the determination of the factors affecting the efficiency of waste treatment and disposal methods under specific local conditions as well as the development of environmental quality criteria and common measures for pollution abatement;

(c) define target contaminants or toher variables at periodic intervals depending on the progress of implementation of the LBS protocol;

(d) select project proposals on the basis of their intrinsic scientific validity, their Mediterranean specificity, and encourage whenever possible bilateral and multilateral projects among Mediterranean countries from the north and the south of the basin.

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.

This eighty seventh volume of the MAP Technical Reports Series contains the final reports of six research projects completed with the framework of MED POL in Area I - "Identification of microbiological components and measurement development and testing of methodologies of specified contaminants".

PREFACE

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 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 continue 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.

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.

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'evnironnement, 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 Barcelona 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.

Les sujets de recherche et d'étude inclus initialement dans MED POL Phase II étaient les suivants:

- mise au point de techniques d'échantillonnage et d'analyse pour la surveillance des sources et des niveaux de pollution. Essai et harmonisation de ces méthodes à l'échelle

méditerranéenne, et formulation de méthodes de référence. Substances figurant sur les listes de priorité des protocoles sur les opérations d'immersion et sur la pollution d'origine tellurique (activité A);

- mise au point de la présentation type des rapports à soumettre en application des protocoles relatifs à l'immersion, à la pollution résultant de situations critiques et à la pollution d'origine tellurique, (activité B);

- élaboration des fondements scientifiques des critères de qualité de l'environnement qui serviront à définir des normes d'émission, des normes d'usage ou des directives concernant les substances énumérées dans les annexes I et II du protocole relatif à la pollution d'origine tellurique, conformément aux articles 5, 6 et 7 de ce protocole (activité C);

- études épidémiologiques relatives à la confirmation (ou révision éventuelle) des critères de la qualité de l'environnement (normes d'usage) proposés pour les eaux servant à la baignade, à la culture de coquillages et à l'élevage d'autres organismes marins comestibles (activité D);

- mise au point de projets de directives et de critères régissant l'application du protocole relatif à la pollution d'origine tellurique, conformément à l'article 7 de ce protocole (activité E);

- recherches sur les processus océaniques, et particulièrement sur la circulation en surface et les déplacements verticaux. Cette information est nécessaire à la connaissance de la répartition des polluants en Méditerranée et à la mise au point de plans pour parer aux situations critiques (activité F);

- recherches sur la toxicité, la persistance, la bioaccumulation et le caractère cancérigène et mutagène de certaines substances énumérées dans les annexes du protocole relatif à la pollution d'origine tellurique et du protocole relatif aux opérations d'immersion (activité G);

- recherches sur l'eutrophisation et les floraisons de plancton qui l'accompagnent. Cette information est nécessaire pour évaluer la possibilité de prévenir les effets et les dégâts causés par ces floraisons périodiques (activité H);

- étude des modifications de l'écosystème dans les zones soumises à l'influence des polluants et dans celles où ces modifications sont dues à d'importantes activités industrielles sur la côte ou à l'intérieur des terres (activité I);

- effets des pollutions thermiques sur les écosystèmes marins et côtiers, y compris l'étude des effets connexes (activité J);

- cycle biogéochimique de certains polluants intéressant particulièrement la santé (mercure, plomb, survie des organismes pathogènes dans la mer Méditerranée, etc.) (activité K);

- étude des processus de transfert des polluants (i) aux points de contact entre les cours d'eau et la mer et entre l'air et la mer, (ii) par sédimentation et (iii) à travers les détroits qui relient la Méditerranée aux mers voisines (activité L).

Les Parties contractantes au cours de leur sixième réunion ordinaire (Athènes, octobre 1989) ont convenu de:

(a) réorienter les activités de recherche menées dans le cadre du MED POL en sorte qu'elles engendrent des informations qui soient également utiles pour l'application technique du Protocole tellurique, en plus de l'appui apporté aux activités de surveillance continue;

(b) à compter de 1990, remplacer les activités A à L par les cinq nouveaux domaines de recherche ci-après:

Domaine de recherche I - Caractérisation et dosage

Ce domaine englobera des projets de recherche en matière de caractérisation (identification de constituants chimiques ou microbiologiques) et de dosage (mise au point et éssai de méthodes) de contaminants donnés;

Domaine de recherche II - Transfert et dispersion

Ce domaine englobera des projets visant à approfondir notre connaissance des mécanismes physiques, chimiques et biologiques qui véhiculent les polluants potentiels de leurs sources à leurs dépôts ultimes. Les sujets étudiés porteront notamment sur le transfert et le dépôt atmosphériques, les mouvements et le brassage des eaux, le transfert des contaminants par sédimentation et leur incorporation dans les cycles biogéochimiques. Priorité sera accordée à l'obtention de données quantitatives servant, en dernier ressort, à la modélisation des systèmes et à l'établissement des évaluations régionales;

Domaine de recherche III - Effets

Ce domaine englobera des projets relatifs aux effets de certains contaminants énumérés aux annexes I et II du Protocole tellurique et du Protocole relatif aux situations critiques: effets sur les organismes, les communautés et les écosystèmes marins, effets chez l'homme et parmi les populations humaines. Priorité sera accordée aux effets et techniques fournissant des données utiles pour établir les critères de qualité du milieu;

Domaine de recherche IV - Destinées/transformation dans l'environnement

Ce domaine englobera des projets portant sur l'étude de la destinée des polluants (micro-organismes y compris), dans le milieu marin, et notamment sur la persistance et la survie, la dégradation, la transformation et la bio-accumulation, etc., mais non sur le transfert et la dispersion qui sont traités dans le domaine II;

Domaine de recherche V - Prévention et lutte antipollution

Ce domaine englobera des projets traitant de la détermination des facteurs conditionnant l'efficacité des méthodes d'épuration et d'élimination des déchets sous des conditions locales spécifiques ainsi que de l'établissement de critères de qualité du milieu et de mesures communes de réduction de la pollution;

(c) définir des contaminants cibles ou d'autres variables à des intervalles périodiques en fonction de l'état de l'avancement de l'application du Protocole tellurique;

(d) choisir les propositions de projet sur la base de leur valeur scientifique intrinsèque, leur spécificité méditerranéenne et, chaque fois que possible, encourager les projets bilatéraux et multilatéraux entre les pays méditerranéens du nord et du sud du bassin.

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.

Ce quatre vingt septième volume de la Série des rapports techniques du PAM comprend six rapports finaux exécutés dans le cadre de la Phase II du MED POL, dans le Domaine de recherche I - "Identification de constituants microbiologiques et de dosage (mise au point et essai de méthodes) de contaminants donnés".

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COMPARISON OF METHODS FOR THE ISOLATION OF *SALMONELLA* FROM NATURAL WATERS

by

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1. INTRODUCTION

Sewage contains many microorganisms which may provoke infective diseases to humans. *Salmonella* infections appear to be one of the most typical examples of an enteric disease that is transmitted from animals to humans. More than 2,000 serovars of *Salmonella* have been described and all are considered to be potentially pathogenic for animals including man. Salmonellosis in humans can produce symptoms ranging in severity from intestinal disturbances to even death (Taylor *et al.*, 1984).

The transmission of *Salmonella* may occur both through food products, such as meat, dairy products, and egg by-products (Gustavsen & Breen, 1984; Holmberg *et al.*, 1984), and by contact between animals and humans directly through the faecal-oral route (Lyons *et al.*, 1980) or indirectly via faecally contaminated-waters (Johnston *et al.*, 1986).

The concentration of *Salmonella* which are discharged into sewage depends on the incidence of diseases in the population and on the number of pathogens excreted by infected persons (Pipes, 1982; Hejkal *et al.*, 1984). The number of salmonellas detected in the faeces of infected persons ranged between 10^5-10^7 colony forming units (CFU) per gram of faeces, although the amount excreted by the asymptomatic carriers is lower.

Classically, the level and extent of microbiological pollution of natural waters has been determined by the enumeration of microbial faecal indicators. Nevertheless, the development of new techniques, and the fact that there is no a direct relationship between the presence of these faecal indicators and the pathogenic microorganisms have led to the suggestion of the study of direct techniques to detect and enumerate pathogenic microorganisms in waters (Greenberg & Ongerth, 1966, Dutka, 1973: Dutka & Bell, Hood *et al.*, 1983).

The isolation and detection of *Salmonella* is a difficult multifactorial process (Hart *et al.*, 1982). For this reason, several authors have studied, designed and tested different detection and isolation methods for a long time. However a standard method for *Salmonella* detection and enumeration is still not available (Hart *et al.*, 1982), although several methods have been proposed in recent years by the Standard Methods for the Examination of Water and Wasterwater (Anon., 1980; 1985).

The problem to detect *Salmonella* in polluted natural waters is based on the high number of serotypes of this genus, which present different physiological and cultural characteristics and on the low numbers of these microorganisms in this environment. The last factor implies the use of several special concentration techniques, such as: (a) Ultrafiltration on fiber glass filters at acid pH (Rolland & Block, 1980a; (b) Membrane filtration on electronegative cellulosic filters (Kenner & Clark, 1974; Presnell & Andrews, 1976; Moriñigo

et al., 1986); (c) Use of absorbent pads and immersion into natural waters (Moore, 1952; Sears *et al.*, 1984); and (d) Centrifugation of the samples.

A large number of different methods have been proposed to detect these pathogens: (a) Phagetyping combined with chromatographic analyses (Hirsh & Martin, 1983); (b) Radiometric techniques based on the agglutination with specific H sera (Stewart *et al.*, 1980); (c) Enzyme immunoassays (EIA) (Minisch *et al.*, 1982; Aleixo *et al.*, 1984); (d) Use combined of enrichment techniques with selective broths, and then culture with specific antisera (Surdy & Haas, 1981); (e) Specific antibodies labelled with fluorescent dye (Early & Patterson, 1982); (f) Use of techniques of pre-enrichment, enrichment and isolation on selective media, together with incubation at restrictive temperatures (Rappaport *et al.*, 1956; Vassiliadis *et al.*, 1974; Moriñigo *et al.*, 1986).

Because of the ubiquitous nature of these microorganisms in several environments, the use of a powerful epidemiological tool is necessary to discriminate among the strains involved in an outbreak. Biochemical and serological methods for the primary identification and the antimicrobial susceptibility pattern are sufficient to confirm the epidemiological relationship between different isolates; however, there is an increasing need for more detailed discrimination or typing of *Salmonella* strains.

Outbreak strains have traditionally been traced by several different methods, including serotyping (Saxena *et al.*, 1983), biotyping and colicinetyping (Baker & Old, 1979; 1980; Ishiguro *et al.*, 1979), phage-typing (Thiel & Recla, 1984), antimicrobial susceptibility testing (Falbo *et al.*, 1982; Chugh & Suheir, 1983; Sojka *et al.*, 1986), and plasmids analysis (Bezanson *et al.*, 1983; Olsvik *et al.*, 1985). Several studies have demonstrated that plasmid profile analysis of *Salmonella* could be a reliable epidemiological tool for the differentiation of epidemic and non-epidemic strains from outbreaks (Taylor *et al.*, 1982; Brunner *et al.*, 1983; Farrar, 1983), or elucidate the epidemiology of these food-borne pathogens (Scheinbach & Hong, 1988). However, only a few reports of its application to environmental, food and animal isolates have been published (Riley *et al.*, 1983; Holmberg *et al.*, 1984; Nakamura *et al.*, 1986).

2. OBJECTIVES

In this research project, we have planned the following investigation objective: *Comparison of methods for the isolation and characterization of Salmonella from natural waters.*

This global objective has been studied from the following particular objectives:

(A) Evaluation of the selectivity and the recovery efficiency of eight enrichment broths and of eleven plating media, which are used to the detection of *Salmonella*. To study this objective, we have designed laboratory experiments and field experiences to test the selectivity, recovery capability and real efficiency of the media selected to detect and to enumerate *Salmonella* from polluted natural waters.

(B) Characterization and identification of the presumptive salmonellae isolated from contaminated-water, clinical and food sources, to compare the reliability of the typing methods, such as serotyping, phage-typing, antimicrobial susceptibility testing and plasmid profile analysis, as universal epidemiological markers.

3. MATERIALS AND METHODS

3.1 Evaluation of the Selectivity and the Recovery Efficiency of different Enrichment Broths and Plating Media Used for Detection of *Salmonella*

3.1.1 Laboratory experiments

3.1.1.1 Microorganisms

The types and sources of microorganisms used in this study are given in Table 1. *Salmonella* spp and *S. enteritidis* were isolated from seawater and clinical samples, respectively. All the cultures were stored at -20E C in milk broth supplemented with 20% glycerol and at 4E C in Tryptic Soy agar (TSA) (Difco Laboratories Inc., Detroit, MICH).

3.1.1.2 Media

Brain-Heart-Infusion agar and broth (BHIA, BHIB) (Difco) were used as control media of the growth of all the microorganisms tested.

The enrichment media used for the selectivity and enumeration comparative studies were the following: Tetrathionate Broth (TB) (Difco); Selenite Cystine Broth (SCB/36) (E. Merck, D-6100. Darmstadt 1, FGR); Selenite-F Broth (SFB/36) (Bonde, 1981), Selenite-F Broth supplemented with 10 µg per ml of sodium novobiocin (Sigma Chemical Co., St. Louis, MO) (NSFB/36) (Dutka, 1973); Rappaport-Vassiliadis Broth (RV/43) (Vassiliadis *et al.*, 1976), and this medium supplemented with 10 and 20 µg/ml of sodium novobiocin (NRV(10)/43 and NRV(20)/43, respectively) (Moriñigo *et al.*, 1983; 1984; 1986), and supplemented with 40 µg/ml of the same antibiotic (NRV(40)/43) (Alcaide *et al.*, 1982). All media were supplemented with 15% of agar-agar (Difco). The suffixes 36 and 43 indicate incubation at 36E C and 43E C, respectively.

The isolation media used for the selectivity and enumeration comparative studies were: Bismuth Sulphite (BS) agar (Difco); Brilliant Green (BG) agar (Difco); Brilliant Green-Phenol Red-Lactose-Sucrose (BPLS) agar (Merck); Eosin-Methylene Blue (EMB) agar (Difco); Hektoen Enteric (HE) agar (Difco); Salmonella-Shigella (SS) agar (Difco); Trypticase Soy-Brilliant Green (TSBG) agar (Moats & Kinner, 1976); Trypticase Soy-Xylose-Lysine (TSXL) agar (Moats & Kinner, 1976); Xylose-Lysine-Deoxycholate (XLD) agar (Difco) and two modifications of the TSBG and TSXL used by Moats (1978), that consisted in the addition of 10 µg/ml of sodium novobiocin (Sigma) (TSBGN and TSXLN, respectively).

3.1.1.3 Culture preparation

For the experiments of microbial qualitative growth, all the microorganisms were grown in Brain-Heart Infusion broth (BHIB) (Difco) for 24 h at 36E C. Incubated cultures of each of the microorganisms tested were centrifuged at 12,000 g for 20 min, washed in sterile 0.85% saline solution and their turbidities adjusted at 0.5 of the MacFarland scale with sterile phosphate buffered saline solution. The elapsed time required for these operations was similar for all the microorganisms and never up to 5 min.

A loopful of the microbial suspensions (ca. 1.5×10^5 cfu/ml) was streaked on each of the plating media. The plates were incubated at 36E C and the results were recorded at 24 and 48h.

3.1.1.4 Enumeration experiments

The spread plate technique was used for these experiments. Amounts of 0.1 ml of the different *Salmonella* serotype cultures, prepared as above mentioned were spread onto the surface of each of the eleven plating media tested. The plates inoculated were incubated at 36E C, and the colony counts were recorded after 72 h. The calculations for each experiment were carried out following the equation (1):

$$ry\ percentage\ ' \frac{number\ of\ colonies\ grown\ on\ selective\ medium}{number\ of\ colonies\ grown\ on\ BHIA}$$

Table 1

Types and sources of microorganisms used in the evaluation of the selectivity and recovery efficiency of different enrichment broths and plating media used for detection of *Salmonella*

MICROORGANSISMS	REFERENCE	SOURCE
YEAST		
Candida albicans	1349	Spanish Type Culture Collection
GRAM-POSITIVE		
Bacillus megaterium	44	Spanish Type Culture Collection
Micrococcus luteus	244	Spanish Type Culture Collection
Staphylococcus aureus	232	Spanish Type Culture Collection
GRAM-NEGATIVE		
Citrobacter freundii	401	Spanish Type Culture Collection
Enterobacter aerogenes	400	Spanish Type Culture Collection
Escherichia coli	102	Spanish Type Culture Collection
Klebsiella pneumoniae	143	Spanish Type Culture Collection
Proteus mirabilis	170	Spanish Type Culture Collection
Pseudomonas aeruginosa	108	Spanish Type Culture Collection
Salmonella spp	100	Isolated from seawater
Salmonella enteritidis	497	Clinical isolate
Salmonella paratyphi B	504	Spanish Type Culture Collection
Salmonella tuphi	409	Spanish Type Culture Collection
Salmonella typhimurium	159	Spanish Type Culture Collection
Serratia marcescens	137	Spanish Type Culture Collection

3.1.1.5 Induction of stress injuries on the microorganisms

The comparative enumeration experiments were also carried out using *Salmonella* cultures under stress conditions. The stress was induced by inoculation of the cells to Erlenmeyer with 250 ml of seawater filtered through 0.22 µm membrane filters (Millipore Ibérica, Madrid, Spain) and exposure for 24 h. The filtration treatment removed the biotic components of the seawater.

Double-agar layer (Anderson *et al.*, 1983) and direct plating on the selective media were used for the quantification of the stressed cells.

3.1.2 Field samples

3.1.2.1 River water samples

The water samples were collected at five stations, named VT-1, VT-2, VT-3, VT-4 and VT-5 (Figure 1), in the upper reaches of the Guadalhorce river (Malaga, Spain) near Villanueva del Trabuco, a village with approximately 6,000 inhabitants, Station VT-2 was in the village sewage effluent discharge. The distances between station VT-2 and VT-1, VT-3, VT-4 and VT-5 were 200, 500, 1,500 and 4,500 m, respectively.

The samples were collected in sterile amber glass bottles of 500 and 1,000 ml capacity, from about 10-20 cm below the water surface, against the water flow, avoiding still water and low flow areas: they were taken to the laboratory in refrigerated containers maintained at 4E C. Examination was carried out within 5 h of sampling. A total of 45 samples was examined.

3.1.2.2 Seawater samples

The sampling area was located in a marine coastal zone influenced by the Guadalhorce river mouth (Malaga-Spain). This river receives polluted discharges from industrial and domestic sources.

The seawater samples were collected at five sampling stations (A1 to A5) (Figure 2), one located at the centre and at a distance of 250 m, equally separated on a semi-circle centered on the river mouth. The stations were located in practice by taking cross bearings. These samples were taken in sterile amber glass bottles of 500 and 1000 ml capacity, from about 10-20 cm below the water surface. They were refrigerated at 4E C and processed within 5 h of sampling. A total of 36 samples were analyzed.

3.1.2.3 Media

The selective enrichment media and culture conditions used were the following: Buffered Peptone Water (BPW) for pre-enrichment (Edel & Kampelmacher, 1973); Rapapport-Vassiliadis (RV) broth incubated at 43E C for 48-72 h; RV broth supplemented with 10, 20 and 40 µg/ml of sodium novobiocin [NRV(10), NRV(20), and NRV(40)], respectively, incubated at 43E C for 48-72 h; Selenite Cystine (SC) broth incubated at 36EC and 43E C for 48-72 h (SC/36 and SC/43, respectively); Selenite F (SF) broth incubated at 36E and 43E C for 24-72 h (SF/36 and SF/43, respectively); and SF broth supplemented with 10 µg/ml of sodium novobiocin (Dutka, 1973) with incubated at 36E C and 43E C for 24-72 h [NSF(10)/36 and NSF(10)/43, respectively].

Xylose Lysine Deoxycholate agar (XLD) (Difco) and Trypticase Soy Brilliant Green agar (TSBG) were used as plating media.

3.1.2.4 Sample processing

The MPN technique (Anon. 1980) and buffered peptone water (BPW) as pre-enrichment medium were used in the protocol specified in Figure 3. Amounts of 100 and 10 ml of water were separately filtered through 0.45 µm pore size membrane filters (Millipore) (Fair & Morrison, 1967; Claudon *et al.*, 1971), and 1 ml of 1:10 sample dilutions was directly inoculated into BPW tubes. All tubes were incubated at 36E C for 18-24 h. From each BPW tube was inoculated to the selective media in the ratios of 1:100 for RV/43 and the supplemented media, and 1:10 for selenite media, according to Vassiliadis *et al.* (1978), Mulindwa & Pietzsche (1979) and van Schothorst & Renaud (1983). After incubation at 36E C or 43E C for 48-72 h, a loopful from each of the selective broth tubes was plated on XLD and TSBG agars. The transparent colonies with or without black centres (typical salmonella morphology) were tested by the standard biochemical procedures, according to the methods of Edwards & Ewing (1972) and Le Minor (1984).

Those isolates that matched the biochemical profile of genus *Salmonella* were later confirmed with API-20E System (API System); their serological characteristics were investigated by slide agglutination using commercial antisera (Difco), according to the Kauffmann-White scheme (Edwards & Ewing, 1972).

Isolates from every tube in which salmonellas were biochemically and serologically confirmed were regarded as positive. The MPN of salmonellas per 100 ml was obtained from probability tables (Anon. 1980).

3.1.2.5 Statistical analysis

The non-parametric test of Kendall's concordance coefficient was used to compare the different media and incubation conditions (Siegel, 1956). This method allowed us to estimate the agreement of the efficiency range of the media on different sampling days. Once this concordance was established, it was possible to determine the efficiency order of the media tested. The statistical test became parametric by analogy to the chi-square.

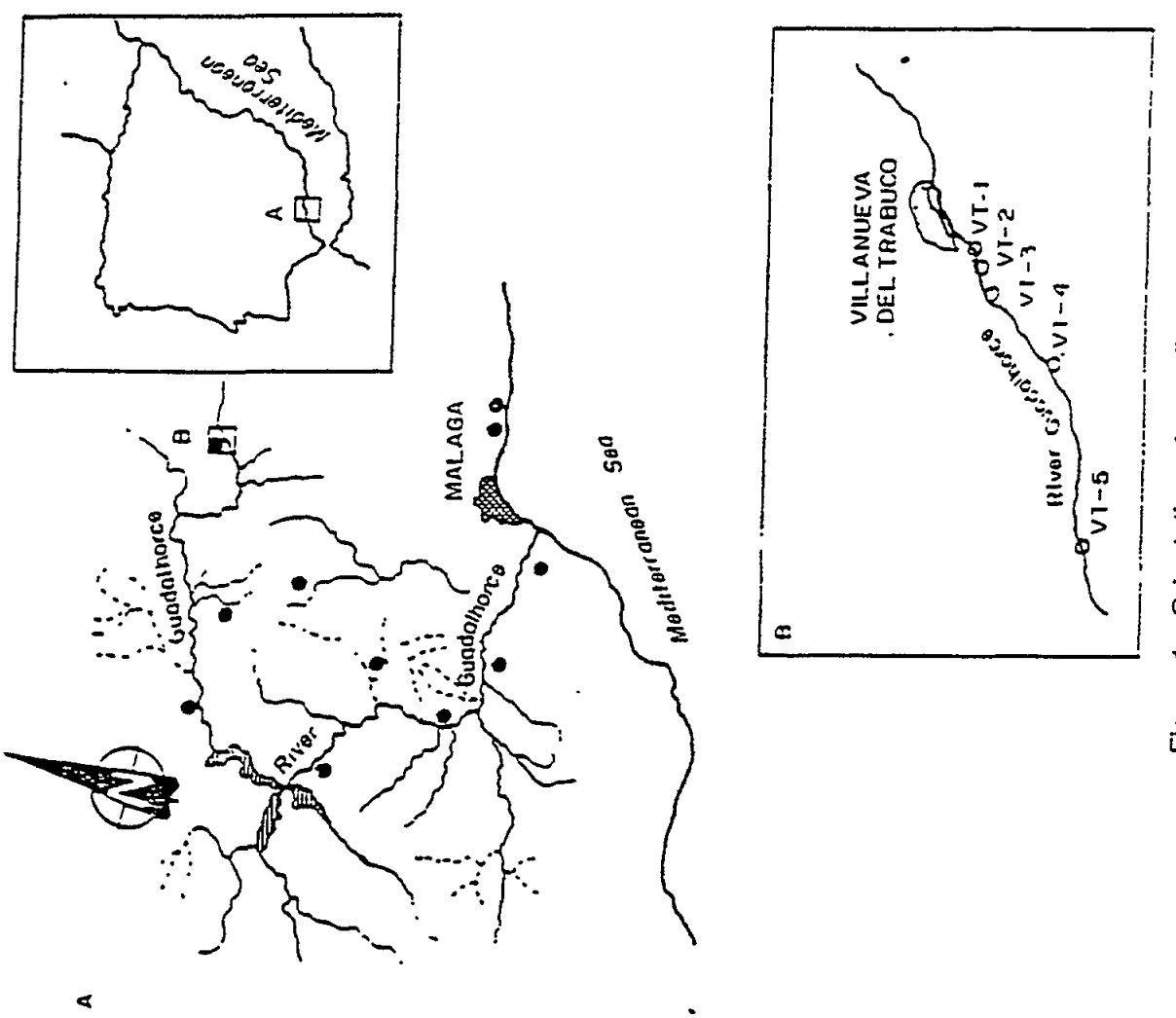

Figure 1. Orientation of sampling zone.
A. Course of the River Guadalhorce;
B. Distribution of the sample stations.

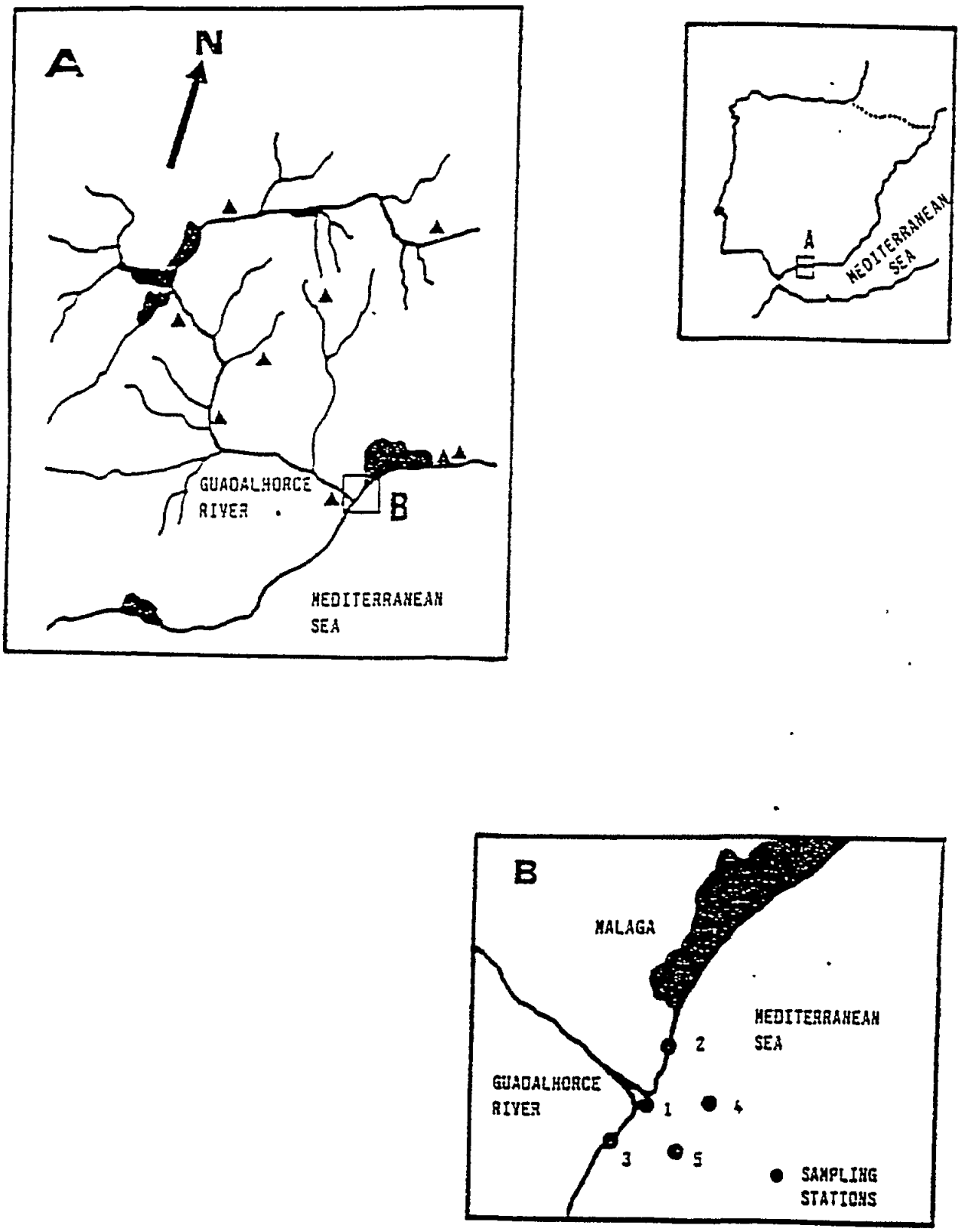

Figure 2. Sampling station situation

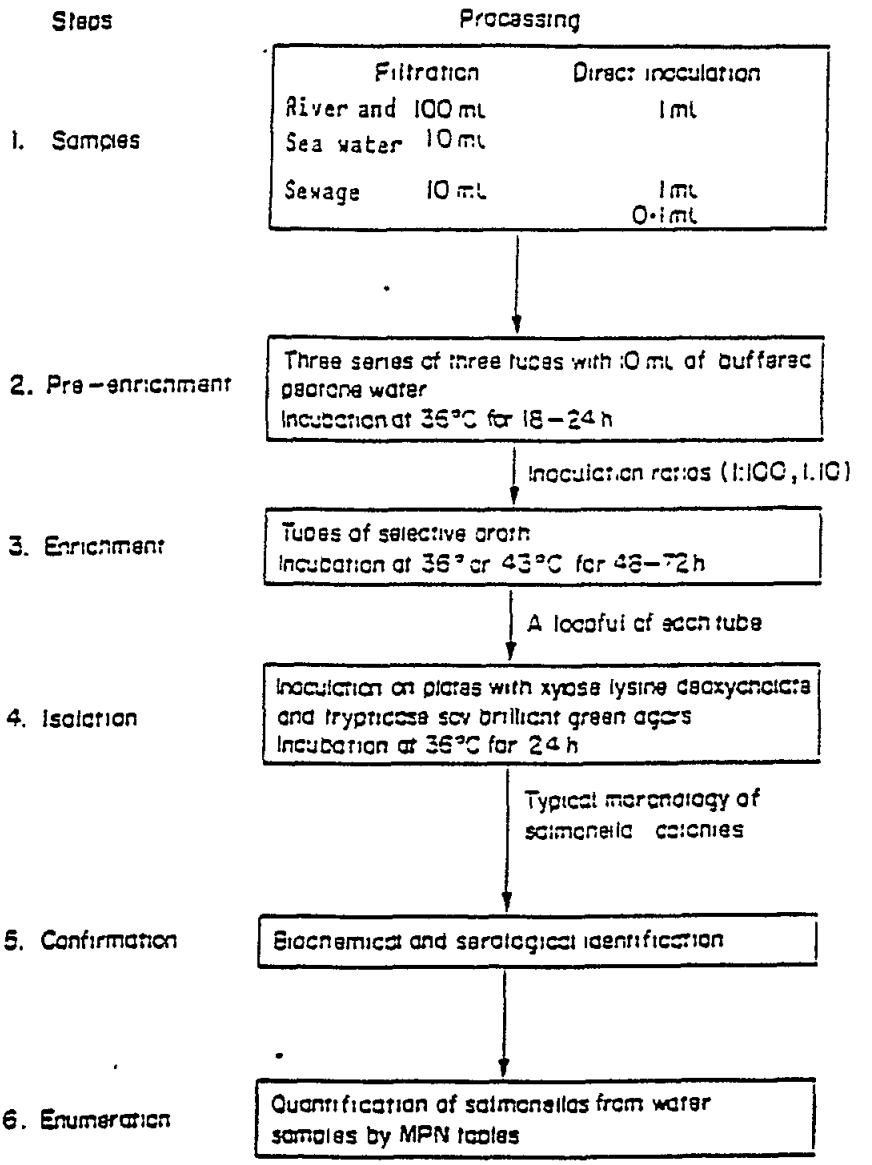

Figure 3. Sample processing scheme.

3.2 Characterization of *Salmonella* Isolated from Different Sources

3.2.1 Bacterial strains

Salmonella strains were divided into the following three groups according to source and epidemiological significance: (i) isolates from contaminated food (26 strains); (ii) isolates from different outbreaks in various locations of the provinces of Malaga and Madrid (Spain) (72 strains); and (iii) isolates from contaminated natural waters (fresh water and seawater) (72 strains). All the isolates were biochemically confirmed using the API 20E System (BioMerieux, Spain) and serologically characterized by the slide agglutination test with *Salmonella* polyvalent O and group antisera (Difco), as well as by the tube agglutination test with H antisera (Difco Laboratories, USA). All strains were sent to the National Reference Centre of *Salmonella* (Majadahonda, Madrid, Spain) for confirmation of serotyping.

3.2.2 Antimicrobial susceptibility testing

The disk diffusion method described by Barry & Thornsberry (1991) on Mueller-Hinton agar (Difco) was used to test the resistance of the isolates to the following antimicrobial agents (supplied by BioMerieux, Spain): Colistin (Cl), 10 µg; nalidixic acid (Na), 30 µg; gentamicin (Gm), 10 µg; streptomycin (Sm), 10 µg; kanamycin (Km), 30 µg; tetracycline (Tc), 30 µg; chloramphenicol (Cm), 30 µg; ampicillin (Ap), 10 µg; carbenicillin (Cb), 100 µg; cephalothin (Cf), 30 µg; trimethoprimsulphamethoxazole (SxT), 1.25 µg and 23.75 µg; tobramycin (Tm), 10 µg; and neomycin (Nm), 30 µg.

3.2.3 Phage-typing

Phagetypes were determined by means of 25 typing phages belonging to a new phage-typing scheme developed in our laboratory (Castro *et al.*, 1992). Phagetypes were established according to the patterns of reaction to the typing phages using a modified mnemonic index of the type described by Farmer (1970).

3.2.4 Plasmid profile

Plasmid DNA was extracted from lysed *Salmonella* isolates by a modification of the Kado & Liu (1981) technique (Toranzo *et al.*, 1983). Portions of DNA samples were electrophoresed through 0.7% agarose (type I) (Sigma) in Tris-acetate buffer (40 mM Tris, 2 mM disodium EDTA; adjusted to 7.9 with glacial acetic acid) at 90 V and 150 mA for 2 h with an horizontal apparatus. The gels were, then, stained with ethidium bromide solution 0.5 µg per ml and photographed through a UV transluminator (LKB) with Plus-X-Pan film and a 23 A Wratten filter. The approximate molecular mass of plasmids (in megadaltons, MDa) was determined by comparison with reference plasmids of known molecular mass, e.g., R40a (96 MDa) and plasmids from *Escherichia coli* V517 ranging from 35.8 to 1.4 MDa.

4. RESULTS

4.1 Evaluation of the Selectivity and the Recovery Efficiency of Enrichment Broths and Plating Media Used for Detection of *Salmonella*

4.1.1 Laboratory experiments

4.1.1.1 Enrichment media

Table 2 shows the results obtained from the qualitative growth experiments. All the enrichment broths selected, avoided the growth of strains of *Bacillus megaterium, Micrococcus luteus, Staphylococcus aureus* and *Candida albicans* tested. However, some of these media allow a slow growth of *Pseudomonas aeruginosa* and other strains of *Enterobacteriaceae (Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens* and *Proteus mirabilis).* On the other hand, SCB (at 36E C and 48 h of incubation) and SFB (at 36E C for 24 and 48 h of incubation) were the only media that allowed the growth of all serotypes of *Salmonella* tested. There are clear differences regarding to the growth respect to the temperature and time of incubation. The incubation at 43E C is more selective than 36E C, and in all cases a time of incubation for 48 h produces an increase of the microbial growth, compared to some strains which are unable to growth in the first 24 h. The strains of *Salmonella* tested, except *S. typhi*, need at least 48 h of incubation to exhibit moderate growth. The media NRV(20)/43 and NRV(40)/43 produce a slower growth of the strains of *Salmonella* than RV/43 and NRV(10)/43.

SFB/36 and NSFB/36 were the only media which recover *S. typhi*. Different experiments (non represented in this study) were carried out to check if the selective effect of the media incubated at 43E C was due to the high temperature or to the composition of the media. The results obtained showed that the temperature is an important selective agent, and in this way *S. marcescens, P. aeruginosa* and *C. freundii* showed an optimal growth in the RV/43 and their modifications incubated at 36E C, but at 43E C the growth of these strains was not observed (Table 2).

The mean recovery percentage of the *Salmonella* serotypes, except *S. typhi*, without stress treatment on each medium was calculated by equation (1) (see page 4). The average of six experiments for each medium is shown in Figure 4. In six media tested were obtained recovery percentages equal or higher than 90% (TB/43, SCB/36, SFB/36, NSFB/36, RV/43, NRV(10)/43). The addition of 20 and 40 µg/ml of antibiotic provokes lower recoveries than previous media (80% and 65%, respectively).

Table 3 summarizes the enumeration of stressed *Salmonella* on enrichment media. The double-agar layer was much more efficient than the direct plating assay for recovering the stressed salmonellas, with increase of the efficiency recovery ranged between 1.2 and 55.45% for SFB/36 and NRV(40)/43, respectively. The results obtained with the double-agar layer technique showed that the negative effect exerted by the novobiocin on the stressed *Salmonella* cells recovered with the modifications of RV/43 was lowered.

The statistical analysis of the selectivity and recovery of the media tested using the Kendall's coefficient are shown in Table 4. The best results for selectivity were obtained with SFB and SCB, while for recovery in the NSFB and SFB media were obtained that highest results.

Table 2

Qualitative growth of different microorganisms on the enrichment media at 24 and 48 h of incubation

MICROORGANISMS	TB		SCB		SFB		NSFB		RV	NRV(10)	NRV(20)	NRV(40)
	24	48	24	48	24	48	24	48	48	48	48	48
YEAST												
Candida albicans	-	-	-	-	-	-	-	-	-	-	-	-
GRAM-POSITIVE												
Bacillus-megaterium	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus luteus	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-
GRAM-NEGATIVE												
Citrobacter freundii	-	+	+	++	+	++	+	++	-	-	-	-
Enterobacter aerogenes	-	-	+	+	+	+	+	+	-	-	-	-
Escherichia coli	-	-	-	-	+	+	+	+	-	-	-	-
Klebsiella pneumoniae	-	-	++	+++	++	+++	++	++	-	-	-	-
Proteus mirabilis	++	+++	+	++	++	+++	++	++	+	-	-	-
Pseudomonas aeruginosa	-	-	+	+	+	++	+	+	-	-	-	-
Salmonella spp	-	-	+	+++	++	+++	++	++	++	++	++	++
Salmonella enteritidis	-	+	++	+++	++	+++	++	+++	++	++	++	++
Salmonella paratyphi B	-	+	++	+++	++	+++	++	++	++	++	++	++
Salmonella tuphi	-	-	-	+	++	+++	-	-	-	-	-	-
Salmonella typhimurium	-	+	-	+	++	+++	++	+++	++	++	++	++
Serratia marcescens	-	-	+	+	+	++	+	+	-	-	-	-

+++ Optimal growth ++ Moderate growth + Low growth - No growth ** Enrichment broths without growth at 24h
TB: Tetrathionate broth; SCB: Selenite Cystine broth; SFB: Selenite-F Broth; NSFB: Selenite-F broth supplemented with 10 µg/ml of sodium novobiocin; RV: Rappaport-Vassiliadis Broth; NRV(10): Rappaport-Vassiliadis Broth supplemented with 10 µg/ml of sodium novobiocin; NRV(20): Rappaport-Vassiliadis Broth supplemented with 20 µg/ml of sodium novobiocin; NRV(40): Rappaport-Vassiliadis Broth supplemented with 40 µg/ml of sodium novobiocin.

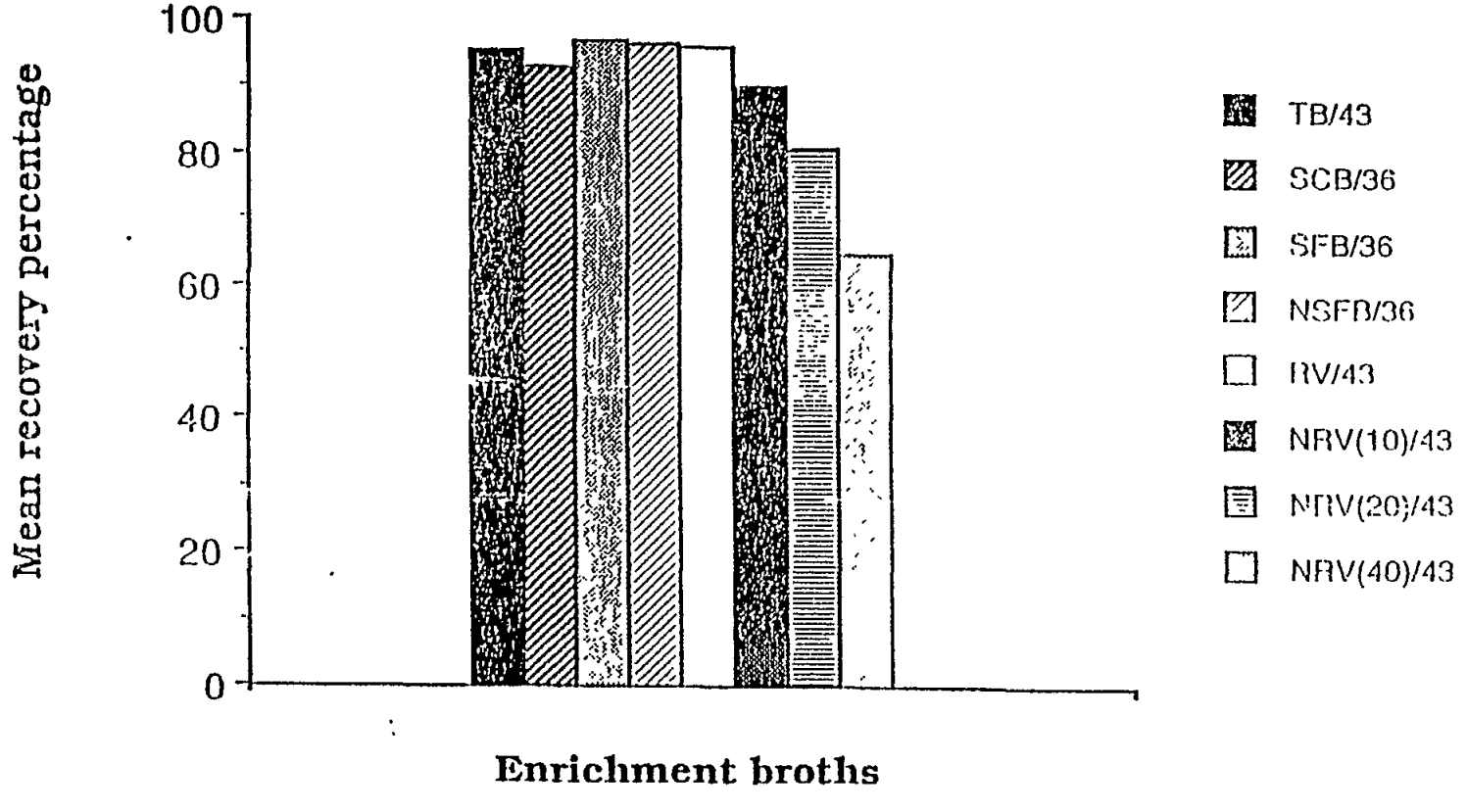

Figure 4. Mean recovery of different *Salmonella* serotypes on enrichment broths (Mean of six different experiments) (*Salmonella typhi* is not included)

Table 3

Recovery efficiency of the enrichment media for stressed *Salmonella* cells

MEDIA	MEAN RECOVERY PERCENTAGE* (± Standard deviation)	
	Direct technique	Double agar layer technique
TB/43	73.10 ± 18.31	97.00 ± 5.19
SCB/36	60.50 ± 13.43	91.00 ± 15.58
SFB/36	97.00 ± 14.30	99.00 ± 11.30
NSFB/36	96.80 ± 15.70	98.00 ± 10.70
RV/43	84.33 ± 13.65	97.53 ± 3.42
NRV(10)/43	78.44 ± 20.05	91.41 ± 15.24
NRV(20)/43	41.90 ± 21.34	87.36 ± 18.58
NRV(40)/43	26.25 ± 18.08	81.70 ± 21.13

* Average of the means calculated for each serotype (excluding *S. typhi*) from six experiments.

Table 4

Statistical analysis (Kendall's coefficient) of the selectivity and recovery of the enrichment media used

Characteristic		ENRICHMENT BROTHS							
		TB[1]	**SCB**[2]	**SFB**[2]	**NSFB**[2]	**RV**[1]	**NRV(10)**[1]	**NRV(20)**[1]	**NRV(40)**[1]
Selectivity									
Salmonellas growth at 24h		6[a]	2.5	1	2.5	6	6	6	6
Salmonellas growth at 48h		8	2	1	3	4.5	4.5	6.5	6.5
Inhibition of Gram positive microorganisms		4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Inhibition of other Gram negative microorganisms		5	6	7.5	7.5	4	2	2	2
	Rj	23.5	15	14	17.5	19	17	19	19
	Rank	8	2	1	4	6	3	6	6
Recovery									
Salmonellas without stress		4	5	1	2	3	6	7	8
Salmonellas stressed (direct plating)		5	6	1	2	3	4	7	8
Salmonellas stressed (double agar layer)		4	6	1	2	3	5	7	8
	Rj	13	17	3	6	9	15	21	24
	Rank	4	6	1	2	3	5	7	8
(Selectivity+Recovery)	Rank	6	3.5	1	2	5	3.5	7	8

[a] Ranking of the media depending on the order in the test considered

[1] Incubation at 43E C

[2] Incubation at 36E C

4.1.1.2 Isolation media

The results obtained from the qualitative growth experiments are shown in Table 5. In all of the eleven media tested the selected *Salmonella* serotypes did not exclusively growth, since an optimal growth of several microorganisms other than *Salmonella* was observed. All the serotypes of *Salmonella*, however, showed an optimal growth at 48 h of incubation on BG, BS, BPLS, HE and XLD agars. On the contrary, TSBGN and TSXLN media inhibited the development of any serotype of *Salmonella*. Although, the addition of novobiocin at concentration of 10 µg/ml to these media improved their selective capabilities.

EMB agar showed the lowest selectivity, because it allowed the growth of all microorganisms tested. On the contrary, HE agar had the highest selectivity, since this medium inhibited the growth of two enterobacteria microorganisms and *P. aeruginosa*, while other media such as BS, SS, TSBG and TSXL (with and without novobiocin) only inhibited the growth of *P. aeruginosa* and XLD to *Escherichia coli*.

The mean recovery percentage of the several *Salmonella* serotypes, except *S. typhi*, without stress treatment on each plating medium was calculated using the equation (1). The average of six experiments for each medium is represented in Fig. 5. Recoveries higher than 90% was obtained for all the media, except for SS agar, which only recovered 27% of the *Salmonella* cells.

The enumeration of *Salmonella* cells under stressed conditions in the plating media tested is summarized in Table 6. The double-agar layer was found to be more efficient for *Salmonella* recoveries than the direct plating assay. The stress injury exerted on the salmonella recovery of *Salmonella* provoked a decrease of about 25% in the media BS, BPLS, TSBG, TSBGN, TSXL and TSXLN. A 50-60% of reduction was achieved in BG, EMB and XLD agars; and a higher than 90% of decrease of the initial concentration was observed on HE and SS media. When the double-agar layer technique was used, the *Salmonella* recovery increased in all the media and only the SS agar presented a low recovery (13.54%). The highest recovery of stressed *Salmonella* cells was obtained on XLD agar with a percentage of up to 93%. XLD agar was the best plating medium for the recovery of *Salmonella* (Table 7), and TSBG, BPLS and BS presented an acceptable capabilities for the isolation of this microorganism from water samples.

4.1.2 Field samples

4.1.2.1 River water samples

Table 8 shows the numbers and percentages of *Salmonella* recovered from samples from each of the five sampling stations in seven enrichment media and at 36E and 43E C for SC, SF and NSF.

There was a close relationship between the *Salmonella* levels obtained by the different methods and also the closeness of the sample station to the pollution source.

Table 5

Qualitative growth of different microorganisms on the selective media at 48 h of incubation

MICROORGANISMS	**BG**	**BPLS**	**BS**	**EMB**	**HE**	**SS**	**TSBG**	**$TSBG_N$**	**TSXL**	**$TSXL_N$**	**XLD**
YEAST											
Candida albicans	-	-	-	+	-	-	-	-	-	-	-
GRAM-POSITIVE											
Bacillus-megaterium	-	++	-	+	-	-	-	-	-	-	-
Micrococcus luteus	-	+	-	+	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	+	-	-	-	-	-	-	-
GRAM-NEGATIVE											
Citrobacter freundii	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+++
Enterobacter aerogenes	+	++	+	++	-	+	+++	++	+++	+	-
Escherichia coli	+	+	+	++	-	+	+	+	+	+	+++
Klebsiella pneumoniae	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+++
Proteus mirabilis	++	+++	+++	+	+++	+++	+++	++	+++	++	+++
Pseudomonas aeruginosa	+	+	-	++	-	-	-	-	-	-	+
Salmonella spp	+++	+++	+++	++	+++	++	+++	++	+++	++	+++
Salmonella enteritidis	+++	+++	+++	++	+++	+++	+++	++	+++	++	+++
Salmonella paratyphi B	+++	+++	+++	++	+++	+++	+++	++	+++	++	+++
Salmonella tuphi	+++	+++	+++	+	+++	++	+	-	+	-	+++
Salmonella typhimurium	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+++
Serratia marcescens	++	+	++	++	+++	++	+	+	+	+	+++

+++ Optimal growth + Low growth - No growth

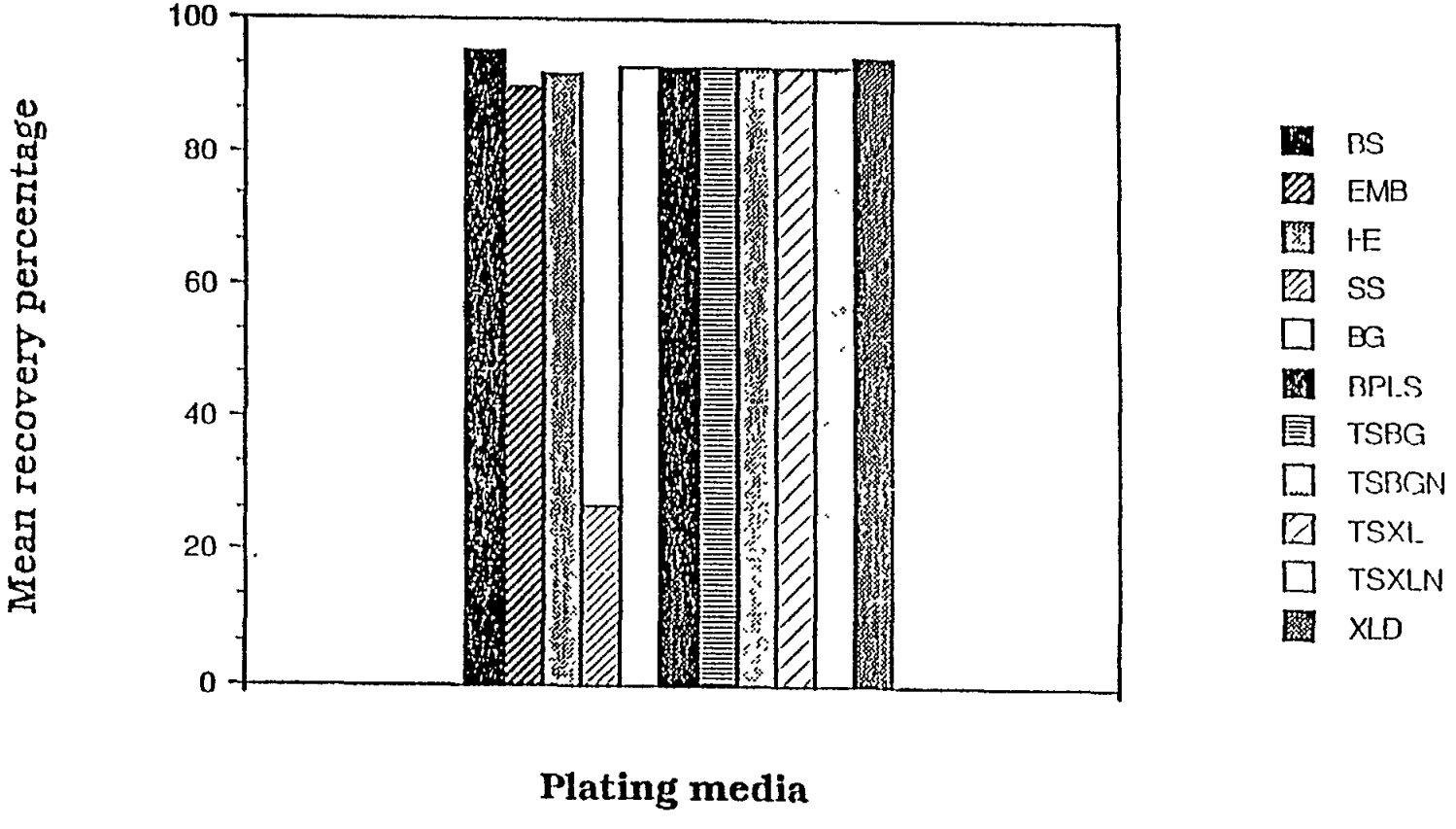

Figure 5. Mean recovery of different *Salmonella* serotypes on plating media Mean of six different experiments) (*Salmonella typhi* is not included)

Table 6

Recovery efficiency of the media for stressed *Salmonella* cells

MEDIA	**MEAN RECOVERY PERCENTAGE*** **(± Standard deviation)**	
	Direct technique	**Double agar layer technique**
Bismuth sulphite (BS)	74.90 ± 25.56	76.95 ± 22.64
Eosin-methylene blue (EMB)	42.93 ± 21.22	79.96 ± 14.94
Hektoen enteric (HE)	12.28 ± 0.58	74.42 ± 42.01
Salmonella-Shigella (SS)	0.25 ± 0.50	13.54 ± 12.52
Brilliant green (BG)	51.82 ± 46.02	76.19 ± 23.77
Brilliant green-phenol red-Lactose-Sucrose (BPLS)	78.36 ± 26.62	81.24 ± 16.78
Trypticase soy brilliant green (TSBG)	74.73 ± 11.41	83.32 ± 16.25
TSBG with 10 µg/ml sodium novobiocin ($TSBG_N$)	73.92 ± 26.03	85.34 ± 12.73
Trypticase soy xylose lysine (TSXL)	72.90 ± 12.53	84.15 ± 11.35
TSXL with 10 µg/ml sodium novobiocin ($TSXL_N$)	73.65 ± 20.15	83.39 ± 10.27
Xylose lysine deoxycholate (XLD)	48.73 ± 10.41	93.09 ± 9.40

* Average of the means calculated for each serotype (excluding *Salm. typhi*) from six experiments.

Table 7

Statistical analysis (Kendall's coefficient) of the selectivity and recovery of the plating media used

Characteristic	PLATING MEDIA										
	BG	**BPLS**	**BS**	**EMB**	**HE**	**SS**	**TSBG**	**$TSBG_N$**	**TSXL**	**$TSXL_N$**	**XLD**
Selectivity											
Salmonellas growth at 24h	6.5[a]	2.5	8	10	5	6.5	2.5	10	2.5	10	2.5
Salmonellas growth at 48h	3	3	3	9	3	8	6.5	10.5	6.5	10.5	3
Inhibition of Gram positive microorganisms	5	10	5	11	5	5	5	5	5	5	5
Inhibition of other Gram negative microorganisms	10	10	5	10	1	5	5	5	5	5	5
Rj	24.5	25.5	21	40	14	24.5	19	30.5	19	30.5	15.5
Rank	6.5	8	5	11	1	6.5	3.5	9.5	3.5	9.5	2
Recovery											
Salmonellas without stress	3.5	1	3.5	10	9	11	3.5	7	7	7	3.5
Salmonellas stressed (direct plating)	7	1	2	9	10	11	3	4	6	5	8
Salmonellas stressed (double agar layer)	9	6	8	7	10	11	5	2	3	4	1
Rj	19.5	8	13.5	26	29	33	11.5	13	16	16	12.5
Rank	8	1	5	9	10	11	2	4	6.5	6.5	3
(Selectivity+Recovery) Rank	8	3	4.5	11	6	10	2	7	4.5	9	1

[a] Ranking of the media depending on the order in the test considered.

Table 8

Concentrations (MPN) and percentage of recovery of salmonellas in samples of polluted river water using different enrichment media

Enrichment medium	Sampling station											
	VT-1		VT-2		VT-3		VT-4		VT-5		Total	
	MPN mean	Positive occurrence	MPN mean	Positive occurrence	MPN mean	Positive occurrence	MPN mean	Positive occurrence	MPN mean	Positive occurrence	No. of samples	% recovery
RV/43	1.61	3/7	150.7	10/10	93.13	9/9	55.88	8/9	3.77	9/10	45	86.66
NR10/43	1.55	2/7	0	8/10	93.03	8/9	55.86	8/9	3.13	7/10	45	73.33
NR10(10)*	1.61	3/7	150.0	10/10	93.68	9/9	56.01	8/9	3.76	9/10	45	86.66
NR10(20)*	1.61	3/7	0	9/10	93.13	9/9	55.86	8/9	3.76	9/10	45	84.44
SC/36	0.00	0/7	114.2	1/10	0.03	1/9	0.00	0/9	0.00	0/10	45	4.44
SC/43	0.04	1/7	5	7/10	1.44	2/9	1.81	4/9	0.07	2/10	45	35.55
SF/36	0.15	1/7	128.7	0/10	0.00	0/9	0.03	1/9	0.09	1/10	45	6.66
SF/43	0.00	0/7	1	7/10	2.88	2/9	0.17	3/9	0.36	4/10	45	35.55
NSF(10)/36	0.15	1/7	2.00	0/10	0.04	1/9	2.41	2/9	0.11	2/10	45	13.33
NSF(10)/43	0.00	0/7	6.53	6/10	2.84	4/9	2.91	6/9	0.69	6/10	45	50.00
			0.00									
			6.01									
			0.00									
			6.01									

* Modified RV/43 medium proposed by the authors.

RV/43, Rappaport-Vassiliadis medium; NR10/43, Rappaport-Vassiliadis medium supplemented with 40 µg/ml sodium novobiocin; NR10(10) and NR10(20), Rappaport-Vassiliadis medium supplemented with 10 and 20 µg/ml sodium novobiocin, respectively; SC/36 and SC/43, selenite cystine broth incubated at 36 and 43E C, respectively; SF/36 and SF/43, selenite F broth incubated at 36E and 43E C, respectively; NSF(10), selenite F broth supplemented with 10 µg/ml sodium novobiocin.

MPN, Most probable number.

The highest average percentage of salmonellas recovered, 86.66% of samples examined, was obtained with two media: RV/43 and NRV(10). The influence of the degree of water pollution clearly shows as, in those sampling stations without direct sewage discharge, the positive detection percentage was under 50%, while stations further from the pollution source (VT-4 and VT-5) show an increase in the detection percentage of *Salmonella* (about 90% of these media). On the other hand, SC and SF media incubated at 36E C showed the lowest average positive recovery percentages, all below 10%. Remarkably, *Salmonella* could not even be detected in the sewage outfall (station VT-2) with those media. Recovery percentages were increased by incubation at 43E C alone, or by the addition of sodium novobiocin. The latter gave recoveries up to a maximum of 50% (NSF).

There was a clear influence of the growth of competitive organisms on the selectivity of the *Salmonella* isolation media XLD and TSBG. The results suggest that SF medium alone is the least selective, whether or not it is supplemented by sodium novobiocin and at both 36E and 43E C; it allows a high proliferation of background microorganisms. On the other hand, both malachite green oxalate and sodium novobiocin media are highly selective, especially those that contain both inhibitory substances; by strongly inhibiting the competitive flora, growth of salmonellas is unrestricted.

Eight hundred and fifty-five presumptive *Salmonella* colonies were isolated from XLD and TSBG media. Of these isolates 90.52% (744) were confirmed as *Salmonella* spp; five predominant serotypes and other self-agglutinable serotypes were obtained (Table 9). Excepting SF, all the enrichment media had a high specificity, up to 90% confirmation. The SF medium, which had low specificity and selectivity, both with and without sodium novobiocin, was not adequated for enumeration purposes or for serotyping isolates of salmonellas from natural waters.

The Kendall's coefficient (w) results are shown in Table 10. They refer to both the counts and the recovery percentages in different media. On the basis of both total and partial analysis of results the most efficient enumeration media for *Salmonella* are those that contain malachite green, and also those derived from the media proposed by Rappaport *et al.* (1956). Incubation at 43E C and addition of sodium novobiocin significantly increased the recovery from the enrichment media.

4.1.2.2 Seawater samples

The results of detection and enumeration of *Salmonella* from different polluted seawater samples using seven enrichment media, are summarized in Table 11. The highest *Salmonella* counts and percentages of detection were obtained from RV broth, although the media NRV(10) and NRV(20) presented similar percentages of recovery. There was a close relationship between the recovery rate of these media and the pollution degree of the samples. *Salmonella* could not be isolated from all samples using selenite cisteine or selenite-F incubated at 36E C and 43E C.

The growth of competitive microorganisms in selenite-F medium at 36E C was high, but the incubation at 43E C or the addition of novobiocin to the medium, or both factors together, provoked a slight inhibition of the background flora. On the other hand, the RV medium and its modifications were highly selective, inhibiting the competitive flora sharply.

Table 9

Number of presumptive *Salmonella* isolations, confirmation percentages and *Salmonella* serotypes isolated in the enrichment media tested

Enrichment medium	No. of presumptive colonies	Confirmation percentage	*Salmonella* serotypes isolates
RV/43	424	93.87	*typhimurium* *blockley* *enteritidis* *london* *weltevreden*
NRV(10)	110	95.46	*typhimurium* *blockley* *weltevreden* *london*
NRV(20)	104	98.08	*blockley* *london* *typhimurium*
NRV(40)	98	94.90	*enteritidis* *blockley* *typhimurium*
SC/43	25	92.00	*blockley*
SF/43	49	55.11	*blockley*
NSF/43	45	57.78	*typhimurium* *blockley*

Table 10

Kendall's coefficient applied to bacterial counts and positive percentage recoveries of *Salmonella* in the different media selected

Enrichment medium	SAMPLING STATIONS						
	VT-1	VT-2	VT-3	VT-4	VT-5	Global	Positive detection
RV/43	29.0*	23.5	21.0	25.5	25.0	124.0	10.0
NRV(10)/43	29.0	23.0	19.0	21.5	27.0	119.5	10.0
NRV(20)/43	29.0	28.5	21.0	27.5	26.5	132.5	11.5
NRV(40)/43	35.0	36.0	32.5	27.5	40.0	171.0	18.5
SC/36	45.5	80.5	70.5	73.5	79.5	349.5	45.5
SC/43	42.5	60.5	67.5	64.0	74.5	309.0	31.5
SF/36	43.0	74.5	72.0	71.5	77.0	338.0	43.5
SF/43	45.5	62.5	64.5	66.0	67.0	305.5	34.0
NSF/36	41.5	84.0	69.0	67.5	75.0	337.0	39.5
NSF/43	45.5	67.5	58.0	50.5	58.5	280.0	31.0
Kendall's coefficient (w)	0.12+	0.63	0.73	0.64	0.58	0.51	0.85
Chi-square	7.40#	69.2	58.3	50.8	51.7	270.3	38.0

* Rj values + At $p<0.01$ # At $p<0.001$

Table 11

Number (MPN/100ml) and occurrence of *Salmonella* in samples of polluted seawater using different enrichment media

Enrichment broths	SAMPLING STATIONS										Total Percentage recovery
	A1		A2		A3		A4		A5		
	MPN[a] mean	Positive occurrence	MPN mean	Positive occurrence	MPN mean	Positive occurrence	MPN mean	Positive occurrence	MPN mean	Positive occurrence	
RV	31.1	6/6	1.7	5/6	0.4	2/6	0.2	2/6	0.3	3/6	50
NRV(10)	28	6/6	1.7	5/6	0.4	2/6	0.2	2/6	0.2	2/6	47.2
NRV(20)	24.8	6/6	1.6	5/6	0.4	2/6	0.2	2/6	0.2	2/6	47.2
NRV(40)	23	6/6	1.3	4/6	0.2	1/6	0.1	1/6	0.1	1/6	36.1
SF/43	0.7	1/6	0.7	1/6	0	0/6	0	0/6	0	0/6	5.5
NSF(10)/43	0.7	1/6	0.7	1/6	0	0/6	0	0/6	0	0/6	5.5

MPN, Most Probable Number/100 ml.
[a]Mean values from 6 different experiments.
In the SC/36, SC/43, SF/36, and NSF(10)/36 Salmonellae were not isolated from the samples tested.

Five hundred and seven presumptive colonies of *Salmonella* were isolated from the plating media. Of these isolates, 462 (91.12%) were confirmed as *Salmonella* spp (Table 12). The predominant serotypes isolated were *S. blockley, S. london, S. ohio, S. senftemberg, S. thompson, S. typhimurium* and *S. virchow* (Table 13). The media possessing selenite in their compositions showed low specificity and selectivity, both with and without sodium novobiocin. These were not useful for enumeration purposes or for serotyping isolates of *Salmonella* from seawater.

Kendall's coefficients of concordance applied to the recovery percentages in different media are shown in Table 14. The most efficient media for detecting *Salmonella* were those containing malachite green, with and without sodium novobiocin.

4.2 Characterization of *Salmonella* Isolated from Different Sources

Table 15 summarizes the serotype distribution and the number of phagetypes, antimicrobial patterns and plasmid profiles of the three groups of *Salmonella* isolates (food, outbreak and environmental strains). *Salmonella* serotypes most frequently isolated were *Salmonella enteritidis* (48.8%) and *S. typhimurium* (20%), although only three serotypes *S. enteritidis, S. typhimurium* and *S. infantis* were consistently isolated from all the sample groups.

Table 12

Percentages of confirmation of presumptive colonies of *Salmonella* isolated from different selective enrichment media

Enrichment medium	No. of presumptive colonies	Confirmation as *Salmonella*(%)
RV	148	97.9
NRV(10)	108	98.1
NRV(20)	98	97.9
NRV(40)	83	98.8
SF/43	25	44.0
NSF(10)/43	45	48.9

Table 13

Number of *Salmonella* serotypes isolated from seawater samples in different enrichment media

Serotypes	ENRICHMENT MEDIA						Total (%)
	RV	NRV(10)	NRV(20)	NRV(40)	SF/43	NSF(10)/43	
S. blockley	16	18	26	ND[a]	1	ND	61 (13.2)
S. braenderup	ND	ND	7	5	ND	ND	12 (2.6)
S. bovis-morbificans	4	ND	ND	ND	ND	ND	4 (0.8)
S. enteritidis	12	ND	ND	ND	ND	ND	12 (2.6)
S. infantis	1	6	7	5	ND	1	20 (4.3)
S. london	8	12	8	12	3	8	51 (11)
S. menden	ND	6	ND	ND	ND	ND	6 (1.3)
S. montevideo	ND	ND	7	ND	ND	ND	7 (1.5)
S. muenchen	4	ND	ND	ND	ND	ND	4 (0.8)
S. ohio	16	18	ND	12	ND	ND	46 (9.9)
S. oraniemburg	ND	ND	ND	5	ND	ND	5 (1.1)
S. postdam	12	ND	ND	ND	ND	ND	12 (2.6)
S. senftemberg	15	6	7	6	ND	ND	34 (7.3)
S. thompson	32	20	7	19	2	4	84 (18.2)
S. typhimurium	16	18	6	12	4	7	63 (13.6)
S. virchow	9	2	21	6	1	2	41 (8.9)
Total	145	106	96	82	11	22	462
Percentage	31.4	22.9	20.8	17.7	2.4	4.8	100

[a]ND: Note detected

Table 14

Statistical analysis using the Kendall's concordance coefficient of the positive percentage recovery of *Salmonella* in different selected media

Enrichment medium	Sampling stations					
	A1	A2	A3	A4	A5	Global
RV	11.5[a]	14.5	21	21.5	18	86.5
NRV(10)	15.5	14.5	21	21.5	21.5	94
NRV(20)	17.5	16.5	21	21.5	21.5	98
NRV(40)	15.5	21.5	21	23	24.5	105.5
SF/43	35.5	33.5	28	26.5	28	151.5
NSF(10)/43	35.5	33.5	28	26.5	28	151.5
Kendall's coefficient	0.38[*]	0.24[*]	0.04[**]	0.019[**]	0.045[**]	3.91[*]

[a] Rj values

[*] Significant values applying the chi-square test at $p<0.01$

[**] Significant values applying the chi-square test ar $p<0.001$

Food isolates presented a wide variety of phagetypes, antimicrobial patterns profiles, and no epidemiological relationship was observed among *Salmonella* isolates. Similar results were obtained with the environmental strains, which presented a high number of plasmidless strains. On the other hand, the isolates from outbreaks possessed common serotypes and phagetypes, but a great variety of plasmid content.

Several plasmid patterns were found in the 170 isolates. Many of them harboured large plasmids (from 82.8 to 110 MDa) that have generally been related to virulence properties (Popoff *et al.*, 1981; Nakamura *et al.*, 1985; Lujan *et al.*, 1990). The large plasmids were not used for pattern comparison of isolates because of their proven instability on subculture (Sansonetti *et al.*, 1981; Litwin *et al.*, 1991). Plasmids under 20 MDa were used for comparison of plasmid profiles. The 30 plasmid groups of the *Salmonella* isolates used for comparative purposes are included in Table 16. Patterns containing from one to five plasmids of low molecular mass were found in 72 isolates. The most frequently detected plasmid groups were D5 (two plasmids of 2.0 and 2.2 MDa) in 12.5% of strains with low molecular mass plasmids, and O3 (one 2.0 MDa plasmid) in 7.6% of the isolates tested. On the other hand, plasmids of 1.5, 2.0, 2.2 and 3.7 MDa were frequently present in the different pattern groups, corresponding to 30.6, 33.3, 25 and 19.4% of the strains, respectively.

The correlation between serotype and plasmid patterns is shown in Table 17. Only 5 serotypes, *S. enteritidis, S. typhimurium, S. virchow, S. blockley,* and *S. ohio* could be subdivided into 17, 13, 3, 5 and 2 plasmid groups, respectively. The other serotypes presented only one plasmid group. *S. typhi, S. thompson, S. weltevreden,* and the non-typable strains could not be included in any established plasmid group. On the other hand, all plasmid groups were limited to a single serotype, except groups O2, O3, O5, O6, O7, D2, D3, D5, D8 and D10.

Table 15

Serotype distribution and numbers of phagetypes, antimicrobial and plasmid profiles of the *Salmonella* isolates according to their origins

Source	Serotype	Number of serotypes	Number of		
			Phagetypes	Antimicrobial profiles	Plasmid profiles
Food	*S. enteritidis*	16	15	11	14
	S. virchow	3	3	3	3
	S. typhimurium	2	2	2	2
	S. typhi	2	2	1	1
	S. anatum	1	1	1	1
	S. infantis	1	1	1	1
	S. newport	1	1	1	1
	TOTAL	26	25	20	24
Outbreak no. 1	*S. enteritidis*	13	2	4	8
	S. virchow	1	1	1	1
Outbreak no. 2	*S. enteritidis*	19	6	10	11
	S. typhimurium	5	4	4	5
	S. virchow	1	1	1	1
	S. infantis	1	1	1	1
Outbreak no. 3	*S. typhimurium*	9	5	4	6
Outbreak no. 4	*S. enteritidis*	22	15	14	11
	S. typhimurium	1	1	1	1
	TOTAL	72	36	40	45
Environment	*S. typhimurium*	17	12	12	9
	S. enteritidis	13	6	9	3
	S. london	9	6	5	3
	S. blockley	7	5	4	6
	S. ohio	7	6	4	3
	S. weltevreden	3	3	3	2
	S. infantis	2	2	2	2
	S. thompson	2	2	2	1
	S. bovismorbificans	1	1	1	1
	Self-agglutinable	11	9	8	5
	TOTAL	72	52	50	35

Table 16

Plasmid patterns of 170 *Salmonella* strains from different sources

Patterns	Presence of *Salmonella* plasmid of size (Md)																Number of isolates
	0.7	1.5	2.0	2.2	2.4	2.6	3.7	5.2	1.2	1.8	4.4	4.1	1.0	3.1	4.8	3.5	
O1	+																3
O2		+															6
O3			+														7
O4				+													2
O5					+												4
O6						+											2
O7							+										4
O8								+									1
O9												+					1
D1		+								+							2
D2		+									+						2
D3		+		+													6
D4		+					+										1
D5			+	+													9
D6			+		+												2
D7			+			+											1
D8						+	+										2
D9							+				+						1
D10					+						+						4
D11												+					2
D12								+					+				1
T1		+		+									+				1
T2		+	+		+												1
T3							+					+		+			1
T4			+		+		+								+		1
T5							+			+							1
T6		+					+		+								1
T7						+				+							1
F1		+	+				+						+				1
F2		+					+					+		+			1
NP														+		+	1
																	98

NP: Plasmidless strains or strains with plasmids which were not grouped into a pattern.

Table 17

Correlation between serotype and plasmid pattern of *Salmonella* isolates from different sources

Serotype (n)	Number of isolates in plasmid groups																													NP[a]
	O1	O2	O3	O4	O5	O6	O7	O8	O9	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	T1	T2	T3	T4	T5	T6	T7	F1	F2	
S. enteritidis (83)		1	5	2	2	1	2					5		6		1	1	1	2	2	1		1			1		1			48
S. typhimurium (43)	3					1	1	1	1	2	1		1				1		2					1	1		1				17
S. virchow (5)					1		1							1																	1
S. infantis (4)														1																	3
S. blockley (7)		1	1												2														1	1	1
S. london (9)											1																				8
S. ohio (7)			4									1																			2
S. weltevreden (3)	1																														3
S. bovismorbificans (1)					1																										
S. anatum (1)																					1										
S. newport (1)																															
NT[b](11)																															

[a]NP: Strains not grouped into a pattern.
[b]NT: Untypable strains.

The relationship between the serotypes and the plasmid patterns according to the isolation source is given in Table 18. Only O5 plasmid group was common to the isolates from all three sources, although O3, O4, O7, D5, and D8 groups were detected from strains isolated from food and outbreaks (O3 and O4 in *S. enteritidis*, O7 and D5 in *S. virchow* and *S. enteritidis*, and D8 in *S. enteritidis* and *S. typhimurium*). On the other hand, only the O6 group was common to the strains from outbreak and environmental sources.

All the serotypes could be divided into several phagetype patterns, e.g. *S. enteritidis* into 37 phagetypes, of which 29 comprised one strain, 5 phagetypes with two strains, and 3 phagetypes with more than 5 strains (phagetype 84 with 9 strains, phagetype 56 with 13, and phagetype 115 with 20). The *S. typhimurium* isolates were divided among 20 phagetypes, 13 of which possessed one isolate, 3 phagetypes with two isolates, phagetypes 114 and 115 with 3 isolates, phagetype 122 with 4 isolates, and phagetype 61 with 5 isolates. *S. london* could be divided into 5 phagetypes, 3 of which had one isolate, and phagetypes 110 and 135 with three isolates each. The rest of the serotypes were divided into different phagetypes comprising one or two isolates.

Resistance to one or more of the antimicrobial agents tested was detected in 140 of the 170 *Salmonella* strains screened (82.3%). According to their origin, the antibiotic resistance percentages were 86.9% in food, 75% in outbreaks, and 87.5% in environmental isolates (Table 19). The frequencies of resistance to individual antibiotics are also given in Table 19. The resistance to streptomycin was the most commonly found (overall resistance of 48%), followed by nalidixic acid and tetracycline resistances (32.7 and 32.1%, respectively). On the other hand, only one strain was found to be gentamicin-resistant.

Table 18

Comparison of plasmid groups of various related *Salmonella* serotypes from different sources

Source and serotypes	Number of isolates tested	Number of strains	Plasmid group
Food			
S. enteritidis	16	1	O3, O4, O5, O8
		2	D5
		10	None[a]
S. typhimurium	2	1	O8, None
S. virchow	3	1	O7
S. infantis	1	1	None
Outbreaks			
S. enteritidis	54	1	T7,T5,02,04,06,D7, D9,T1,T2
		2	O7,D10,D11
		4	O3,D5
		26	None
S. typhimurium	15	1	O7,D2,D8,T3,T4
		2	D1,D10
		6	None
S. virchow	2	1	O5,None
S. infantis	1	1	D5
Environment			
S. enteritidis	13	1	D5
		12	None
S. typhimurium	17	1	O6,O9,D4,T6
		3	O1
		10	None
S. infantis	2	2	None

[a] None, None of the patterns described in Table 2.

Table 19

Distribution of antimicrobial resistance among the strains according to their isolation source

Antimicrobial agent[b]	Food (n=26)	Outbreaks				Environmental			Global %	Overall %
		#1 (n=14)	#2 (n=26)	#3 (n=9)	#4 (n=23)	Fresh-water (n=40)	Seawater (n=32)	Total (%)		
Sm	10(38.5)[a]	-	7(26.9)	9(100)	15(65.2)	27(67.5)	18(56.2)	62.5	43.1	48.0
Cm	5(19.2)	-	4(15.4)	3(33.3)	2(8.7)	3(7.5)	2(6.2)	6.9	12.5	12.9
Tc	7(26.9)	2(14.3)	8(30.8)	1(11.1)	7(30.4)	17(42.5)	16(50.0)	44.4	25.0	32.1
Na	10(38.5)	50(35.7)	9(34.6)	8(88.9)	11(47.8)	6(15.0)	4(12.5)	13.9	45.8	32.7
Cf	2(7.7)	-	2(7.7)	-	1(4.3)	-	-	-	4.2	4.0
Cb	1(3.8)	-	9(34.6)	1(11.1)	-	-	2(6.2)	2.8	13.9	6.8
Cl	2(7.7)	-	-	-	-	-	1(3.1)	1.4	-	3.0
SXT	-	-	-	-	-	3(7.5)	1(3.1)	5.5	-	1.8
Tm	-		1(3.8)	-	-	-	10(31.2)	14.8	1.4	5.4
Ap	-	-	9(34.6)	2(22.2)	-	-	1(3.1)	1.4	15.3	5.6
Gm	-	-	-	-	-	-	1(3.1)	1.4	-	0.5
Km	-	-	-	-	20(86.9)	-	3(9.4)	4.2	27.8	10.7
Nm	-	-	-	-	8(34.8)	-	3(9.4)	4.2	11.1	5.1
Susceptible	5(19.2)	8(57.1)	8(30.8)	-	-	7(17.5)	2(6.2)	12.5	22.2	18.8

[a] Number of isolates (percentages of resistance)
[b] Abbreviations: See text

It is interesting to note that the distribution of antimicrobial resistance of *Salmonella* isolates varied according to their origin. The environmental *Salmonella* strains differed sharply regarding their resistance pattern from the strains isolated from food and outbreaks. The strains isolated from water exhibited a higher resistance to streptomycin (62.5%), tetracycline (44.4%), trimethoprim-sulphamethoxazole (5.5%), and tobramycin (14.8%), but all of the strains were sensitive to cephalothin. *Salmonella* isolates from outbreaks presented a higher resistance to carbenicillin (13.9%), ampicillin (15.3%), kanamycin (27.8%), and neomycin (11.1%),although these isolates were shown to be susceptible to colistin.

The distribution of the resistance combinations detected among the serotypes isolated from the different sources is indicated in Table 20. The most frequent resistance pattern displayed by the *S. enteritidis* strains was the susceptibility to all the antimicrobial agents tested (19 strains), followed by resistance to nalidixic acid (8 strains). In the case of *S. typhimurium*, the resistance pattern most frequently obtained was the combination streptomycin-nalidixic acid (Sm-Na) with 6 strains. Among *S. ohio* and *S. london* isolates the predominant resistance was to streptomycin and tobramycin, respectively.

The distribution of the multiresistant patterns within the different serotypes is given in Table 21. None of these combinations was common to strains from all three sources, although resistance triplets, such as Sm-Cm-Tc and Sm-Cm-Na were detected in strains isolated from food and outbreaks, and the combination Sm-Tc-Na was distributed among isolates from environmental and clinical sources. The pattern Sm-Tc-Na was the most common in environmental strains, whereas in outbreaks the most common patterns were Sm-Km-Nm, Sm-Na-Km, and Sm-Cm-Tc-Cb-Ap.

Table 20

Relationship between serotypes and antimicrobial resistance to 0, 1 and 2 agents

Serotype	Resistance pattern	Number of isolates		
		Food	Outbreaks	Environmental
S. enteritidis	Susceptible	3	15	
	Na	3	5	
	Cl	1	-	
	Tc	2	1	
	Cf	1	1	
	Sm	1	-	
	Cm	-	1	
	Km	-	1	
	Sm-Na	1	-	
	Cb-Na	1	-	
	Tc-Na	-	3	
	Km-Na	-	2	
	Ap-Cb	-	1	
	Sm-Km	-	3	
	Nm-Km	-	-	
	Sm-Tc	-	-	
	Sm-Tm	-	-	

Serotype	Resistance pattern	Number of isolates		
		Food	Outbreaks	Environmental
S. typhimurium	Susceptible	-	-	2
	Sm	-	-	4
	Tc	-	-	1
	Tc-Na	1	-	-
	Sm-Cl	1	-	-
	Sm-Na	-	5	1
	Sm-Tc	-	-	4
	Sm-Tm	-	-	1
S. virchow	Susceptible	-	1	-
	Sm	1	-	-
	Cf	1	-	-
	Sm-Tc	-	1	-
S. newport	Na	1	-	-
S. infantis	Sm	-	1	-
	Tc	-	1	-
	Sm-Cm	1	-	1
S. typhi	Susceptible	2	-	-
S. london	Susceptible	-	-	2
	Sm	-	-	4
	Tc	-	-	1
	Sm-Tc	-	-	1
S. blockley	Susceptible	-	-	2
	Sm	-	-	2
	Tc	-	-	2
	Sm-Tc	-	-	1
S. weltevreden	Susceptible	-	-	1
	Na	-	-	1
S. thompson	Tc	-	-	1
	Sm-Tm	-	-	1
S. ohio	Tc	-	-	1
	Tm	-	-	4
S. bovismorbificans	Sm-Tc	-	-	1

Table 21

Distribution of the multiresistant patterns in the different serotypes

Antimicrobial profile	Food				Outbreaks				Environmental			
	Serotype	A	B	(MW)[a]	Serotype	A	B	(MW)	Serotype	A	B	(MW)
Sm-Cf-Tc	*S.virchow*		2	(2;2.2)								
Sm-Cm-Tc	*S.enteritidis*	1	1	(17.7)	*S.enteritidis*	2	1	(3.7)				
Sm-Cm-Na	*S.enteritidis*	1	2	(17.7;36)	*S.typhimurium*	1	2	(2.6;3.7)				
Sm-Cm-Na					*S.typhimurium*	1	0					
Sm-Cf-Na					*S.enteritidis*	1	1	(2.6)				
Sm-Tm-Tc									*S.ohio*	1	2	(1.5;2)
Sm-Tc-Na					*S.typhimurium*	1	0		*S.typhimurium*	1	0	
Sm-Tc-Na									*S.enteritidis*	2	0	
Sm-Na-Ap					*S.typhimurium*	1	1	(3.7)				
Sm-Cm-SXT									*S.weltevreden*	1	1	(85)
Sm-Km-Nm					*S.enteritidis*	2	1	(32.1)				
Sm-Km-Nm					*S.enteritidis*	1	1	(36)				
Sm-Cb-Ap					*S.enteritidis*	1	1	(56)				
Sm-Na-Km					*S.enteritidis*	1	2	(2;36)				
Sm-Na-Km					*S.enteritidis*	1	3	(1.8;2.3;16.6)				
Sm-Na-Km					*S.enteritidis*	1	5	(2;4.4;14.8;30;33.2)				
Cl-Na-Tc									*S.ohio*	1	1	(1.5)
Cl-Na-Ap					*S.enteritidis*	2	0	(2;36)				
Nm-Na-Km					*S.enteritidis*	1	2	(2;36)				
Nm-Tc-Km					*S.enteritidis*	1	2	(2;36)				
Na-Tc-Km					*S.enteritidis*	1	2					
Sm-Cm-Na-Tc	*S.enteritidis*	1	1	(36)								
Sm-Cm-Na-Tc	*S.anatum*	1	1	(2.5)								
Sm-Cm-Km-Nm									Self-agglutinable	1	1	(39.5)
Sm-Tc-Km-Na									*S.london*	1	3	(1.5;1.8;32.1)
Sm-Cm-Tc-SXT									*S.typhimurium*	1	2	1.5;3.7)
Sm-Km-Na-Nm									*S.typhimurium*	1	3	(1.2;15;3.7)
Sm-Km-Na-Tc					*S.enteritidis*	1	0					
Cf-Tc-Cb-Ap					*S.enteritidis*	1	0					
Tm-Na-Cb-Ap					*S.typhimurium*	1	4	(1.8;2.3;16.6;42.7)				
Tc-Na-Cb-Ap					*S.enteritidis*	1	1	(56)				
Sm-Km-Na-Nm-Tc					*S.enteritidis*	1	2	(12;56)				
Sm-Cm-Tc-Cb-Ap					*S.enteritidis*	1	3	(19.4;21;42)				
Sm-Cm-Tc-Cb-Ap					*S.enteritidis*	1	5	(2;4.4;14.8;30;33.2)				
Sm-Cm-Tc-Cb-Ap					*S.enteritidis*	1	2	(1.2;1.5)				
Sm-Km-Cb-Ap-Tc-Tm-SXT					*S.enteritidis*	1	3	(1.2;1.5;55)				
					S.enteritidis	1	4	(1.8;2.3;24;58)				
									S.typhi	1	3	(2.6;40.2;110)

[a] MW: Molecular weight in Md

5. DISCUSSION

5.1 Evaluation of the Selectivity and the Recovery Efficiency of Enrichment Broths and Plating Media Used for the Detection of *Salmonella*

5.1.1 Laboratory experiments

5.1.1.1 Enrichment media

There are some factors with an importance on the efficiency of the isolation of *Salmonella* from natural waters, such as the sampling method, culture media and the incubation conditions (Hart *et al.*, 1982).

The enrichment media tested in this study inhibited the growth of Gram-positive microorganisms, the selectivity degree for Gram-negative microorganisms varying in function to their chemical compositions. Thus, the media containing selenite are less inhibitory than other media, although growth of *S. typhi* was only detected on these media. Tetrathionate broth (TB/43) and enrichment media with brilliant green are more selectives, but TB and RV/43 allowed the development of *Proteus mirabilis*. These results are in agreement with those obtained by Rhodes *et al.* (1985). *P. mirabilis* and *Citrobacter freundii*, which may also developed in TB/43, are the main contaminant background flora of the selective *Salmonella* media, and they growth as salmonella-like S_2H positive colonies; and for this reason, they can modify the detection of *Salmonella* by formation of false-negative colonies. On the contrary, the media containing brilliant green in their composition produced a total inhibition of *C. freundii* and *P. mirabilis*, except RV/43, although the growth of *Proteus* was retarded until 48 h of incubation.

TB/43 and the media possessing brilliant green showed a slow growth of *Salmonella*, and inhibition of the *S. typhi* development. These results are concordant with those obtained by several authors (Harvey & Price, 1979; Muller, 1982; Moriñigo *et al.*, 1987). This serotype only showed growth in the media with selenite as inhibitory agent (Harvey & Price, 1979; Muller, 1982). However, the addition of novobiocin to media with selenite inhibits the recovery of *S. typhi*. The poor growth of *Salmonella* in TB/43 could be due to the potential toxicity of this medium on these microorganisms, this hypothesis agrees with the results obtained by Vassiliadis *et al.* (1974), Harvey & Price (1979), Bailey *et al.* (1981) and D'Aoust (1981).

All the media assayed showed percentages of recovery higher than 80% (Figure 4), except for RV/43 supplemented with 20 and 40 µg/ml of novobiocin. The restrictive temperature of 43E C did not affect the growth of salmonellas negatively.

The double-agar layer method improves the recoveries of *Salmonella* in all cases. This observation confirms the importance of pre-enrichment, because this pre-enrichment step allows the recovery of sublethal injured cells produced by the stress induced by the environment (Anderson 1983; Rhodes *et al.*, 1983; Moriñigo *et al.*, 1989). The addition of sodium novobiocin to the RV/43 medium increased its selectivity, although a reduction in the recovery of *Salmonella* was observed. Similar results were reported by Restaino *et al.* (1977). This effect may have been produced by the action of the antibiotic on the injured *Salmonella* cells and thereby reducing their growth.

From Table 4, it can also be concluded that SFB, NSFB, SCB, and RV/43 could be good enrichment media for the detection of *Salmonella* from water samples, although RV/43 does not recover *S. typhi*. However, it is necessary to carry out studies with all this enrichment

broths using samples of polluted natural waters to test the real efficiency of each one of them. This has been the objective of following investigations in this report.

5.1.1.2 Isolation media

The detection and enumeration of allochtonous microorganisms in an adverse environment, are more difficult when selective media are used (Bissonnette *et al.*, 1975; Anderson *et al.*, 1983; Moriñigo *et al.*, 1985). Since the stress induced by the environment affects the microbial growth, avoiding them to develop optimally on the selective media.

An optimal growth of all *Salmonella* serotypes was achieved after 48 h on the media BG, BPLS, BS, XLD and HE, although, these media allowed the development of organisms frequently belonging to the background flora, mainly *Citrobacter* and *Proteus* species, that grow as salmonella-like H_2S-positive colonies. Similar observations were also done by Taylor & Schelhart (1971), Moats & Kinner (1976) and Moats (1978).

The addition of sodium novobiocin to the TSBG and TSXL agars increased significantly their selectivity, although a total inhibition of *C. freundii* and *P. aeruginosa* was not achieved. According to the results obtained by Restaino *et al.* (1977), in these media, however, a reduction of the growth of *Salmonella* serotypes was observed, and a total inhibition of *S. typhi.*

The high recovery percentage of *Salmonella* obtained in the different selective media, except for SS agar, indicates that any of them can be used as recovery medium in standard conditions. These same conclusions were pointed out by Andrews *et al.* (1979), although combinations of several media yielded them highest recoveries. The addition of novobiocin did not affect the enumeration of *Salmonella* serotypes, except for *S. typhi*.

The recovery rate of stressed *Salmonella* cells is lower than in non-stressed conditions. However, the use of double-agar layer technique, with XLD agar, showed similar recovery percentages to those of non-stressed conditions. BPLS, TSBG and TSXL (the last media with and without novobiocin) are also acceptable for the quantification of stressed *Salmonella* cells using the double-agar layer technique. However, SS agar only recovers 13.5% of stressed salmonellas, which may be explained by an intrinsic toxicity of the medium, or by an increase of the selectivity when the cells were physiologically injured. BS agar is more efficient than BG agar when there are stress conditions, a conclusion which agrees with the results reported by Andrews *et al.* (1979), D'Aoust *et al.* (1980) and Alcaide *et al.* (1984) for food, polluted waters and shellfish samples, respectively. If there is no stress induced by the environment, however there are not significant differences in recovery rate between both media.

In short, XLD agar was the best plating medium for the recovery of *Salmonella* (Table 7). This conclusion is similar to the results obtained by Andrews *et al.* (1979) and by those of Rhodes & Quesnell (1986) who proposed the XLD agar as a substitute of SS agar for the official standard guidelines for food analysis. From Table 7 it can also be concluded that TSBG, BPLS and BS are acceptable in the isolation of *Salmonella* from environmental samples using the double-agar layer technique.

5.1.2 Field samples

5.1.2.1 Riverwater samples

Vassiliadis *et al.* (1976; 1978), Alcaide *et al.* (1982) and Moriñigo *et al.* (1983) obtained higher *Salmonella* recoveries from different sources in RV/43 and NRV(40). In Table 8, however, it can be seen that the highest percentages of *Salmonella* were detected with RV/43 and NRV(10). Although both media, NRV (40) and NRV (10), gave similar recovery rates, the later showed higher specificity and selectivity.

The pre-enrichment step using BPW (Grunnet, 1975) reduced the environmental stress. Two factors, the enrichment broth and the restrictive incubation temperature, may be responsible of the increase of environmental stress in the routine technique without pre-enrichment step (Harvey & Price, 1979). Furthermore, the pre-enrichment technique increases the *Salmonella* isolation efficiency (Edel & Kampelmacher, 1973; Thomason *et al.*, 1977; Vassiliadis *et al.*, 1978; Edgar & Soar, 1979; Fricker, 1984). On the other hand, it also avoids the absorption of malachite green oxalate by the membrane and deepens the colour, which may exert a negative effect. Both the growth of background microorganisms and the number of false-positive colonies in the isolation media, were reduced by the pre-enrichment technique (Fricker, 1984).

The best results, in both the recovery and enumeration of *Salmonella*, were obtained in the media containing malachite green, whether or not they were supplemented with sodium novobiocin, as this was less toxic than SC and SF media; this observation agrees with those reported by Vassiliadis *et al.* (1979). This lower toxicity is attributed by Rappaport & Konforti (1959) to the antitoxic effect of magnesium chloride and also to the presence of free amino acids which stimulate the growth of *Salmonella*. The antitoxicity power of the magnesium chloride is confirmed by Alcaide *et al.* (1984), who demonstrated a lowered selectivity of NRV (40) medium when magnesium chloride was replaced by magnesium sulfate.

The percentage of *Salmonella* spp. confirmed in SC broth is a very high (92%), which contrasts with its low isolation of presumptive colonies of *Salmonella* (25) because of the clear toxicity of the medium for both *Salmonella* and the competitive background microorganisms.

The selectivity of SF broth is much lower than that of modified Rappaport's medium, with or without the addition of sodium novobiocin, even though the antibiotic concentrations in these media are more than sufficient to reduce most of the competitive microorganisms that usually appear in RV/43 and SF without the antibiotic. Consequently, the excessive growth of competitive organisms in the SF broth may be due simply to the large inoculation volumes of the pre-enrichment process which vastly exceed the low selectivity (Vassiliadis *et al.*, 1979).

If the effects of incubation temperatures and the addition of sodium novobiocin in SC and SF broths are compared, it can be seen that, in all cases, the specific temperature of 43E C yielded a six- to nine-fold increase in the percentage of *Salmonella* detected. Similarly, sodium novobiocin gave a two-fold increase in recovery of *Salmonella* in SF broth, with an increased effect when incubated at 43E C.

Comparison studies of several modified Rappaport's broths show that in 23.07% of experiments RV/43, NRV(10) and NRV(20) detected higher concentrations than the NRV(40)

medium. The latter was better to the other media with lower sodium novobiocin concentrations, but only in the samples with high levels of faecal contamination (2.56%). This phenomenon may be due to the effect of high novobiocin concentration (40 µg/ml) on the growth of *Salmonella* in the isolation media. Thus, NRV(40) medium is not the most appropriated for the detection and enumeration of salmonellas in natural waters with low, or moderate, faecal pollution levels. However, with heavily polluted water samples *Salmonella* was optimally detected by the medium. These results agree with those obtained by Alcaide *et al.* (1983, 1986). The best medium for enumerating *Salmonella* in samples from moderately contaminated natural waters was the NRV(10) broth, because it recovered similar numbers of *Salmonella* than RV/43, and furthermore, an increase in the antibiotic concentrations decreased the number of competitive organisms, without apparently affecting the growth of *Salmonella*.

5.1.2.2 Seawater samples

Vassiliadis *et al.* (1978), Alcaide *et al.* (1982), Moriñigo *et al.* (1983; 1986), had obtained high *Salmonella* recoveries from different sources using RV broth and its modifications. The results of this study with seawater samples are in agreement with those observations.

The best recovery percentages and enumerations of *Salmonella* were obtained from RV broth and from this medium supplemented with 10, 20 and 40 µg/ml of sodium novobiocin (Table 2). These media possessed also a higher capability to inhibit competitive microorganisms than the media with selenite in their composition. These results agree with those obtained by Vassiliadis (1983), van Schothorst & Renaud (1983) and Xirouchaki *et al.* (1982). This inhibition may be due to a synergic effect of malachite green and sodium novobiocin which produces almost a total inhibition of competitive flora, although a scarce growth of lactose-negative microorganisms was detected in RV and NRV(10) in some seawater samples.

The media with selenite are not appropriated to isolate and enumerate *Salmonella* from seawater, because of the high toxicity of selenite cystine medium, both for *Salmonella* and the competitive flora, and by the low selectivity of selenite-F broth, with and without sodium novobiocin (Wilson *et al.*, 1975; Moriñigo *et al.*, 1986). The low selectivity of selenite-F medium with the large inoculation volumes of pre-enrichment broth (Vassiliadis *et al.*, 1979) provokes a high growth of competitive organisms.

When the effects of the incubation temperature on the growth of *Salmonella* in selenite-F medium is considered, it can be observed that in all cases, the best results were obtained at 43E C. These results are in accordance with those reported by other authors (Rappaport & Konforti, 1959; Harvey & Price, 1979; D'Aoust, 1981; D'Aoust *et al.*, 1980; 1982; Rhodes *et al.*, 1985), and probably may be attributed to the higher inhibition of the growth of competitive microorganisms at this restrictive temperature.

The NRV(40) broth showed a lower efficiency to enumerate salmonellae from seawater samples than the other media with malachite green. This finding is not consistent with the results obtained by Alcaide *et al.* (1982); 1984). This can be explained by the lower level of faecal pollution of the samples, because the polluted discharges of the river in the sampling area were diluted, and the NRV(40) broth is not the most appropriate medium for the detection and enumeration of *Salmonella* in natural waters with low or moderate levels of faecal pollution (Moriñigo *et al.*, 1986).

In this study, the toxicity observed for the selenite-cystine broth when it is used to enumerate and detect *Salmonella* was higher than that observed in our previous study with water samples from a polluted river (Moriñigo *et al.*, 1986). The explanation of this fact may be due to the higher stress induced to by the microorganisms in the marine environment.

In short, the best medium for *Salmonella* enumeration in seawater samples with low or moderate levels of faecal pollution was RV broth (Table 14), although the media NRV(10) and NRV(20) possessed similar performance characteristics, mainly when the faecal pollution degree was high.

5.2 Characterization of the Presumptive *Salmonella* Isolated from Different Sources

For an optimal bacteriological diagnosis and successful epidemiological tracing, the classification of species into smaller units is of great importance. Serological and biochemical typing of *Salmonella* isolates can only be regarded as first step in this respect. However, modern typing methods based on the analysis of genotypical characteristics may provide a useful tool for epidemiological studies.

This study shows that the incidence of serotypes, plasmids, drug resistance patterns and phagetypes was extremely high in *Salmonella* isolates from the three sources (Table 15). Similar data have been published previously (Terakado *et al.*, 1980; Holmberg *et al.*, 1984; Nakamura *et al.*, 1986).

We would expect that, in outbreaks in which there is a strong epidemiological association with a common exposure (food or water), the *Salmonella* isolates would prove to be the same regardless of the test used. However, the comparison of strains from four outbreaks with strains isolated from water and food brings to light the absence of a standard by which a definite classification of related or unrelated can be obtained (Table 15). Thus, isolates with the same serotype, phagetype, plasmid and drug-resistance profiles have different sources. On the other hand, two strains which have the same serotype may differ by the loss or gain of a plasmid or by any change in phage susceptibility (Threlfall *et al.*, 1978; Bezanson *et al.*, 1982).

Agarose gel electrophoresis of DNA from 170 *Salmonella* strains isolated from different sources revealed a heterogeneous plasmid population (Tables 16 and 17). These results are consistent with other studies of plasmids from strains of *Enterobacteriaceae* (Moller *et al.*, 1978) and from *Salmonella* (Taylor *et al.*, 1982). The plasmid analysis of bacteria has gained acceptance as a tool for identifying *Salmonella* isolates (Schaberg *et al.*, 1981). Different authors have reported the usefulness of plasmid profile analysis in the study of the epidemiology of infection by *Salmonella* (Brunner *et al.*, 1983; Riley *et al.*, 1983), as well as in the definition and identification of bacteria originating from the same clone (Orskov & Orskov, 1983). However, in the present study we were unable to establish strong evidence of the epidemiology of the strains by means of their plasmid profiles (Table 18). These findings contrast with previous reports (Taylor *et al.*, 1982; Olsvik *et al.*, 1985), whose authors were able, by means of plasmid profiles, to group *S. typhimurium* strains from common sources and to characterize the spread of such strains. Olsvik *et al.* (1985) established that the presence of a single plasmid cannot always be used to characterize a clone, and it may be necessary to carry out restriction endonuclease digests when strains contain plasmids of similar molecular weight.

In this study, we have compared the small plasmids (less than 20 MDa) present in *Salmonella* strains because these plasmids are prevalent in *Enterobacteriaceae* (Moller *et al.*, 1978; Jamieson *et al.*, 1979). Small plasmids under 5 MDa are found in both antibiotic-resistant and antibiotic-susceptible *Salmonella* strains, but the functions of several of these small plasmids are still unknown. Taylor *et al.* (1982) reported that the strains that were devoid of small plasmids contained one single high molecular weight plasmid, higher than 50 MDa for the drug-resistant strains, and over 20 MDa for the drug-sensitive strains. Such plasmids should be capable of self-transfer, since the transfer operon of the F plasmid comprises about 15 MDa of DNA (Willetts & Skurray, 1980). We have observed that, while most *Salmonella* strains susceptible to antibiotics were plasmidless (42.8%), a high number of these strains (25%) contained plasmids of both high and low molecular weight. In the case of drug-resistant strains, 52 did not harbour plasmids (37.1%), 32 contained only small plasmids (22.9%), and 23 strains possessed only large plasmids (16.4%), the remaining strains presented both small and large plasmids (23.6%). These strains may in fact harbour "plasmid aggregates" consisting of a transfer factor and small plasmids encoding drug resistance (Anderson & Lewis, 1965). Some small plasmids may be formed by dissociation of large plasmids present in the same host cell (Taylor *et al.*, 1982).

The spread of drug resistance in *Salmonella* is a relatively recent phenomenon. Current investigations show a sharp increase of antibiotic-resistance among *Salmonella* strains, yielding percentages higher than 80% (Chugh & Suheir, 1983; Sojka *et al.*, 1986). In this study, resistance to one or more antibiotics was recorded in 82.3% of the strains tested, demonstrating the epidemic spread and persistence of resistant clones of *Salmonella* in Spain.

Several authors have used the resistance typing of *Salmonella* strains for epidemiological purposes, mainly to characterize clones with a specific resistance (Anderson, 1975; Rowe & Threlfall, 1984). However, we have found that antimicrobial resistance testing was less specific in the identification of related isolates than other epidemiological markers. these findings agree with those obtained by other authors (Holmberg *et al.*, 1984).

Resistance to different antimicrobial agents has been reported to be mediated by plasmids. Thus, streptomycin-sulfonamides resistance in *Salmonella* is coded by a plasmid of approximately 5.5 MDa (Grinter & Barth, 1976; Araki *et al.*, 1987). Tetracycline resistance is mediated in *S. typhimurium* by a plasmid of 24 MDa (Olsvik *et al.*, 1985) or by one of 96 MDa (Nakamura *et al.*, 1986), and by two of 50 and 3 MDa in *S. dublin* (Nastasi *et al.*, 1987). Streptomycin-tetracycline resistances are coded by two plasmids of 71.1 and 2.5 MDa (Jayaratne *et al.*, 1987) or by one of 62 MDa (Nakamura *et al.*, 1986) or 140 MDa (Holmberg *et al.*, 1984). In addition, multiple drug-resistance patterns have been related to the presence of R-type plasmids linked to cryptic plasmids (Taylor *et al.*, 1982). In this study, resistance to 3 or more antimicrobial agents was observed in 43 strains, the most frequent resistance pattern being the profiles Sm-Km-Nm, Sm-Na-Km, and Sm-Cm-Tc-Cb-Ap with 3 isolates each (Table 21). The Sm-Km-Nm resistance pattern was observed in 2 strains of *S. enteritidis* with one plasmid of 32.1 MDa, and in one strain that harboured a 36 MDa plasmid. Sm-Na-Km profile was observed in 3 strains of *S. enteritidis* with varying plasmid content, one strain with 2 plasmids (2 and 36 MDa); one strain with 3 plasmids (1.8; 2.3 and 16.6 MDa); and the last with 5 plasmids (2; 4.4; 14.8; 30 and 33.2 MDa). Finally, the multiple resistance pattern Sm-Cm-Tc-Cb-Ap was recorded in 3 strains of *S. typhimurium* with 2, 3 and 4 plasmids each, with molecular weights of 1.2 and 1.5 MDa; 1.5 and 55 MDa; and 1.8; 2.3; 24 and 58 MDa, respectively.

Plasmids of similar molecular weights linked to antimicrobial resistance have been reported by other authors. Holmberg *et al.* (1984) described several resistance profiles associated with the presence of plasmids. Thus, the most common pattern Sm-Tc-Cb-Ap was observed in strains with 1 plasmid (140 MDa); with 2 plasmids (5.7 and 73 MDa); and with 3 plasmids (4; 5.5 and 87 MDa). In the present study, this resistance pattern seems to be linked to the presence of low molecular weight plasmids (from 1.2 to 1.8 MDa). Nakamura *et al.* (1986) described a plasmid-linked resistance to Sm-Cm-Su-Tc with 110 MDa. A similar plasmid has been obtained in our study in a multiple resistant strain of *S. typhimurium*, with resistance to Sm-Km-Cb-Ap-Tc-Tm-SXT, although this strain also harboured two additional plasmids of 2.6 and 40.2 MDa. It is worthwhile noting that resistance to SXT was only displayed by environmental strains and was always linked to the presence of large plasmids. Similar results have been obtained in other enterobacteria strains (Tschape *et al.*, 1986).

Phage-typing has been the selected method in the reference laboratory to differentiate among serovars. This technique is rapid and provides a reproducible and highly discriminatory method of subdivision (Threlfall & Frost, 1990). Phage-typing has been particularly useful in the epidemiological investigation of several salmonellosis outbreaks (Baker *et al.*, 1980; Chart *et al.*, 1989). In the present study, we used a phage-typing scheme developed in our laboratory (Castro *et al.*, 1992), which permits us to discriminate and group the serotypes of *Salmonella* with similar drug-resistance and plasmid profiles into different phagetypes. For isolates from outbreaks, mainly from outbreaks 1 and 2, phage-typing was the most efficient technique for establishing the fact that they were identical. However, in environmental strains, plasmid analysis may be preferable, and even the antimicrobial resistance could serve as a good marker of related strains in food-borne isolates. Certain plasmid profiles and phagetypes were seen repeatedly in the various outbreaks studied, which suggests that they are relatively stable markers.

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OCCURRENCE OF ENTERIC AND NON-ENTERIC INDICATORS IN SOUTHERN GREECE COASTAL WATER

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ABSTRACT

A survey of the occurrence of enteric and non enteric indicators in seawater was undertaken along the beaches of Southern Greece during the summer 1988. Total coliforms, *Faecal coliforms, Faecal streptococci, Staphylococcus aureus, Pseudomonas aeruginosa, Aeromonas hydrophila, Total fungi* and *Candida albicans* were detected in 78.5%, 71.6%, 86.8%, 6.8%, 12.4%, 5.6%, 89.4% and 3.7% of the 265 samples respectively. *Faecal streptococci* was recovered in 15.1% and 19.6% of the samples in which *Total coliforms* and *Faecal coliforms* and respectively were not recovered. In 1.1% of the samples *Staphylococcus aureus* was isolated and both *Total coliforms* and *Faecal coliforms* were absent. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* were recovered in 0.75% and 1.5% of the samples in which *Total coliforms* and *Faecal coliforms* respectively were not present. The corresponding numbers for *Total fungi* were 19.6% and 24.9%. *Candida albicans* was detected in 1.5% and 1.9% of the samples in which *Total coliforms* and *Faecal coliforms* were not detected.

The validity of the supplementary recommended indicators are discussed.

KEY WORDS-Occurrence; Indicators; Greece; Coastal water.

1. INTRODUCTION

Total coliforms (TC) and *Faecal coliforms* (FC) are the main organisms indicating the possibility of faecal contamination of recreational water. However epidemiological studies (Cabelli *et al.*, Seyfried *et al.*) indicate that enterococci could be best enteric indicator for assessing the quality of marine water. As many swimmers are affected by non-enteric symptoms (Mujeriego *et al.*) (symptoms from infected skin, ear, nose and throat), a number of supplementary indicators are proposed to be included in the study of the pollution of the coastal recreational waters to ensure proper seawater quality evaluation. We evaluated TC, FC and the following supplementary recommended indicators, *Faecal streptococci* (FS), *Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa), Aeromonas hydrophila (A. hydrophila), Total fungi* (TF) and *Candida albicans (C. albicans)* in order to find if they provided valid additional information on the sanitary quality of the seawater.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Water samples were collected during the summer of 1988 (1/6/88-30/9/88). Seawater was collected 1m in depth, 200-500 m from the shoreline sites (35 beaches) (Figure 1). Some areas along the coast are subject to highly polluted terrestrial effluents. Samples were collected in sterile bottles 20 cm under the surface, maintained at 4E C and processed within 6h. All samples were collected between 10.30 AM and 3.30 PM in order to sample the recreational waters during their peak utilization by recreationers. The sampling frequency was every 20 to 3 weeks time. Some beaches were sampled three times during the day (morning - noon - evening). Sampling in the morning took place before the arrival of swimmers, at noon when the greater number of swimmers was observed and at night when swimmers had left.

2.2 Microbial Analysis

All monitoring was performed with the following media, incubation temperature and techniques: for TC and FC Lauryl tryptose broth (double and single strength (Oxoid) incubated at 35±0.5E C for 48h with Most Probable Number (MPN) technique. TC and FC confirmation were performed according to the standard method techniques; for FS Slanetz and Bartley medium (Oxoid) incubated at 42±0.5E C for 48h with MF technique; FS were verified by catalase production, growth in brain heart infusion broth at 44.5E C within 48h and growth in bile broth medium after 72h at 35E C; for *P. aeruginosa* Centrimide agar (Oxoid) incubated at 42E C for 48h with MF technique. Typical and atypical colonies were verified by using Milk agar; for *S. aureus* m *Staphylococcus* medium (Difco) incubated at 35E C for 24h with MF technique. Three to five colonies were picked, gram stained (typical micro specic morphology) and confirmed with catalase, coagulase and DNAse tests; for *A. hydrophila* Glutaminate-Starch-Penicillin (GSP) agar modified by us (unpublished data) (elevation of pH at 8 and incorporation of ampicillin 10 mg/l to the original formula) incubated at 30E C for 48h with MF technique.

A. hydrophila were identified by API 20 NE system (La Balme les Grottes 38390 Montalieu-Vercieu, France) using the modification of the diluent proposed by Mac Donell *et al.* for estuarine samples. Total fungi and for *C. albicans* Cooke Rose Bengal agar (Difco Laboratories) incubated at 25E C for 5 days with MF technique. The identification of fungi and *C. albicans* was based on colonized morphology and gram stain.

3. RESULTS

Table 1 shows the percentage of samples with the number of organisms/100 ml of water. Of 265 samples of coastal water monitored during bathing season, TC and FC were detected in 78.5% and 71.6% respectively. FS was isolated from 86.8% of samples, *S. aureus* from 6.8% *P. aeruginosa* from 12.4%, *A. hydrophila* from 5.6% *Total fungi* from 89.4% and *C. albicans* for 3.7% of the monitored samples. In all the above enumerations even one cell per 100 ml of water was calculated. The number of TC, FC and FS exceeded 100/100 ml of water in 20 (7.5%), 19(7.1%) and 37(13.9%) respectively.

More than 100 *S. aureus* per 100 ml of water were found in 3(1.1%) of samples in which the number of TC and FC was also high.

Of the 265 samples of coastal water monitored 4(1.5%) contained >100 *P. aeruginosa* per 100 ml of water. In all four cases the number of TC and FC were low (3 samples 40 TC/100 ml and 30 FC/100 ml and 1 sample 60 TC/100 ml and 40 FC/100 ml of water).

More than 100 *A. hydrophila*/100 were found in 7(2.6%) out of 256 samples. In four of them TC and FC were present in high number as well (>100/100 ml).

No sample was found that had >100 ml *C. albicans* per 100 ml of water. The correlation between the presence of FS, *S. aureus, P. aeruginosa, A. hydrophila* and the absence of TC and FC is shown in Table 2.

Table 3 shows the correlation between numbers/100 ml of *S. aureus, A. hydrophila, P. aeruginosa, Total fungi* and *C. albicans* and numbers/100 ml of enteric indicators (TC, FC, FS).

From this table it is obvious that the concentration of the different examined indices do not correlate. Samples with low number of faecal microorganisms yielded high numbers of non-enteric microorganisms and vice versa.

In Table 4 we observed the fluctuations in the concentration of the organisms throughout the day. That is although the water was clear around noon when the number of swimmers reaches its peak, the concentration of microorganisms in the water in the morning or at night (and in some cases in both times) rose to a level which renders them inappropriate for swimming. So it seems that beach pollution can be attributed to a greater percent to pollution sources such as sewage from hotels, restaurants, and homes which are usually emptied at night or early in the morning when the activity of the inhabitants is more pronounced. In contrast the presence of swimmers does not worsen the degree of pollution of the seawater.

Polluted beaches are selfcleaned during the day but once pollution has exceeded a certain limit this ability is markedly reduced as in Alissos where the concentration of TC and FC exceeded 2400/100 ml of water.

FS was found in 15.1% and 19.6% of the samples in which TC and FC respectively were not recovered. In 3 samples (1.1%) *S. aureus* was isolated and both TC and FC were absent. *P. aeruginosa* was recovered without the presence of TC in two cases and without the presence of FC in four cases. *A. hydrophila* was present without the presence of FC in two cases. *Total fungi* was found in 19.6% and 24.9% of the samples in which TC and FC respectively were not recovered. *C. albicans* was found in 1.5% and 1.9% of the samples in which TC and FC respectively were not present.

4. DISCUSSION

The evaluation of the sanitary quality of seawater has dealt primarily with TC and FC associated with faecal pollution. The data presented here indicate that in a high number of samples (19.6%) FS were present (even in low density) and FC absent. Of 265 samples of coastal water monitored 230 (86.7%) were found polluted using as indicator FS while using FC as indicator the polluted samples were 190 (71.6%). So, if the medium employed for enumerating FS in seawater by the MF technique ensure maximum recovery, it may be not unrealistic this enteric indicator to replace FC in assessing seawater sanitary quality. The use

of enteric indicators only as guidelines for bathing beaches is questionable since they could not serve as indicators of different opportunistic pathogens which more commonly cause infections at sites other than the gastrointestinal tract.

It was considered that the more important pathogens causing infection through contact were *Staphylococci coagulase* positive, *Pseudomonas aeruginosa* and *fungi*. Coagulase positive strains are potential pathogens causing a wide range of infections. Favero *et al.* and Seyfried *et al.* claimed that the morbidity among swimmers are related well to staphylococcal count. *S. aureus* was found in 6.8% of the samples with relative high recovery rate (15 samples with 60 organisms/100 ml and 3 samples with more than 100/100 ml). This percentage is lower relating to other author's findings (Mujeriego *et al.*) probably because the beaches are located in scarcely populated areas and the number of bathers was small. As the main source of *S. aureus* in recreational water is the bathers, the percentage numbers would be higher in populated beaches. In 1.1% of samples *S. aureus* was present while TC and FC were absent.

P. aeruginosa is a potential pathogen which has been increasingly implicated in ear, throat and skin infection through bathing in contaminated waters. *P. aeruginosa* was isolated from 12.4% of samples. The main reasons for the low recovery rate of *P. aeruginosa* is its poor survival in seawater (Vasconcelos and Swartz 1976).

The correlation between the presence of *P. aeruginosa* with TC and FC was 100% and 97.1% respectively. This high correlation rates is in close agreement with those obtained by Yoshpe-Purer and Golderman in Israeli coastal water. *Total fungi* and *C. albicans* very important causative agents of contact diseases were found present in a considerable high number of samples in which FC and TC were absent (see Table 2).

Studies made by Araujo *et al.* demonstrated positive correlation between the presence of aeromonads and the faecal indicators that means that faecal indicators and the *A. hydrophila* had mainly the same origin (Araujo *et al.*). Our results agree closely with theirs in this field.

This study leads to the following conclusions.

a. It is valuable to include three sets of standards TC, FC and FS for assessing the quality of marine water.
b. *S. aureus* and *C. albicans* are advisable supplementary indicators at populated sea beaches.
c. *P. aeruginosa* and *A. hydrophila* are not useful supplementary indicators for monitoring the sanitary quality by the coastal water.

Table 1

Occurrence of organisms in Southern Greece coastal waters

Organisms	No. of beaches	No. of samples	No. of samples with the following no. of organisms/100 ml of water				
			<2	2-10	11-100	101-500	>500
Total coliforms	31	208(78.5%)*	0	89	70	20	29
Faecal coliforms	31		0	79	70	19	22
Faecal streptococci	34	190(71.6%)	21	84	88	37	-
S. aureus	13		0	0	15	3	-
P. aeruginosa	19	230(86.8%)	3	18	8	4	-
A. hydrophila	11		0	1	7	7	-
Total fungi	35	18(6.8%)	0	94	143	0	-
C. albicans	8	33(12.4%)	1	8	1	0	-
		15(5.6%)					
		237(89.4%)					
		10(3.7%)					

* No. in parentheses indicate the percentage of positive samples

Table 2

No. of samples with the supplementing assessed microorganisms where TC and FC were absent

Microorganisms	No. of samples		
	Total	Without TC	Without FC
Faecal streptococci	230	40(15.1%)*	52(19.6%)
S. aureus	18	3(1.1%)	3(1.1%)
P. aeruginosa	33	2(0.75%)	4(1.5%)
A. hydrophila	15	1(0.38%)	2(0.75%)
Total fungi	237	52(19.6%)	66(24.9%)
C. albicans	10	4(1.5%)	5(1.9%)

* No. in parentheses indicate the percentage of positive samples

Table 3

Correlation between numbers/100 ml of *S. aureus, A. hydrophila, P. aeruginosa,* Total fungi and *C. albicans* and the numbers/100 ml of enteric indicators (TC, FC, FS)

No of organisms/100 ml	No of samples with *S. aureus*	No of samples and No of organisms/ 100 ml of seawater								
		TC			FC			FS		
		#10	11-100	>100	$10	11-100	>100	#10	11-100	100
#10 11-100 >100	0 15 3	7 1	5 0	3 2	7 1	5 1	3 1	9 1	5 1	1 1
	No of samples with *A. hydrophila*									
#10 11-100 >100	1 7 7	10 3	04 1	03 3	11 3	04 1	02 3	12 1	05 6	00 0
	No of samples with *P. aeruginosa*									
#10 11-100 >100	21 9 4	112 3	43 1	64 0	112 3	44 1	63 0	114 2	84 1	21 1
	No of samples with T. fungi									
#10 11-100 >100	94 143 0	5576 0	2536 0	1431 0	5681 0	2637 0	1225 0	5570 0	2751 0	12220
	No of samples with *C. albicans*									
#10 11-100 >100	8 1 0	800 0	01 0	00 0	800 0	01 0	00 0	300 0	300 0	200 0

Table 4

Occurrence of organisms/100 ml of seawater during the same day

Region	Time of sampling	Organisms/100 of seawater							
		TC	FC	FS	*S. aureus*	*P. aeruginosa*	*A. hydrophila*	TF	*C. albicans*
ELEON	morning	460	150	9	0	3	0	15	0
	noon	0	0	0	0	0	0	8	0
	night	460	75	0	0	5	0	0	0
PSATHO-PYRGOS	morning	$240	2100	1	0	0	150	80	0
	noon	0	3	0	0	0	0	30	0
	night	11 9	0	1	0	0	0	20	0
AG. VASILIOS	morning	0	0	0	0	0	0	5	0
	noon	0	0	0	0	0	0	5	0
	night	0	0	3	0	0	0	2	0
ACTEON	morning	9	9	200	0	0	0	5	0
	noon	0	0	20	0	0	0	5	0
	night	460	460	100	0	0	0	2	0
RIO	morning	150	28	200	0	8	0	20	0
	noon	4	0	200	0	2	0	0	0
	night	28	21	80	0	0	0	0	0
ROITIKA	morning	0	0	0	0	0	0	15	0
	noon	0	0	0	0	0	0	30	0
	night	3	3	6	0	0	0	30	0
MONO-DENDRI	morning	15	15	3	0	0	2	10	0
	noon	0	0	0	0	0	0	20	10
	night	0	0	1	0	0	0	50	0
VRAH-NEIKA	morning	4	4	2	100	0	0	30	0
	noon	3	0	1	0	0	0	15	0
	night	0	0	1	0	0	0	30	0
ALISSOS Tarantella	morning	0	0	0	0	0	0	10	0
	noon	0	0	0	0	0	0	35	0
	night	9	9	0	0	0	0	70	0
ALISSOS Tarantella	morning	$240	$240	200	0	0	200	20	0
	noon	0	0	7	0	0	20	15	0
	night	$240 0 $240 0	$240 0 $240 0	9	0	0	5	50	0
ALIKES	morning	0	0	0	0	0	0	50	0
	noon	5	4	0	0	0	0	40	0
	night	0	0	0	0	0	0	30	0

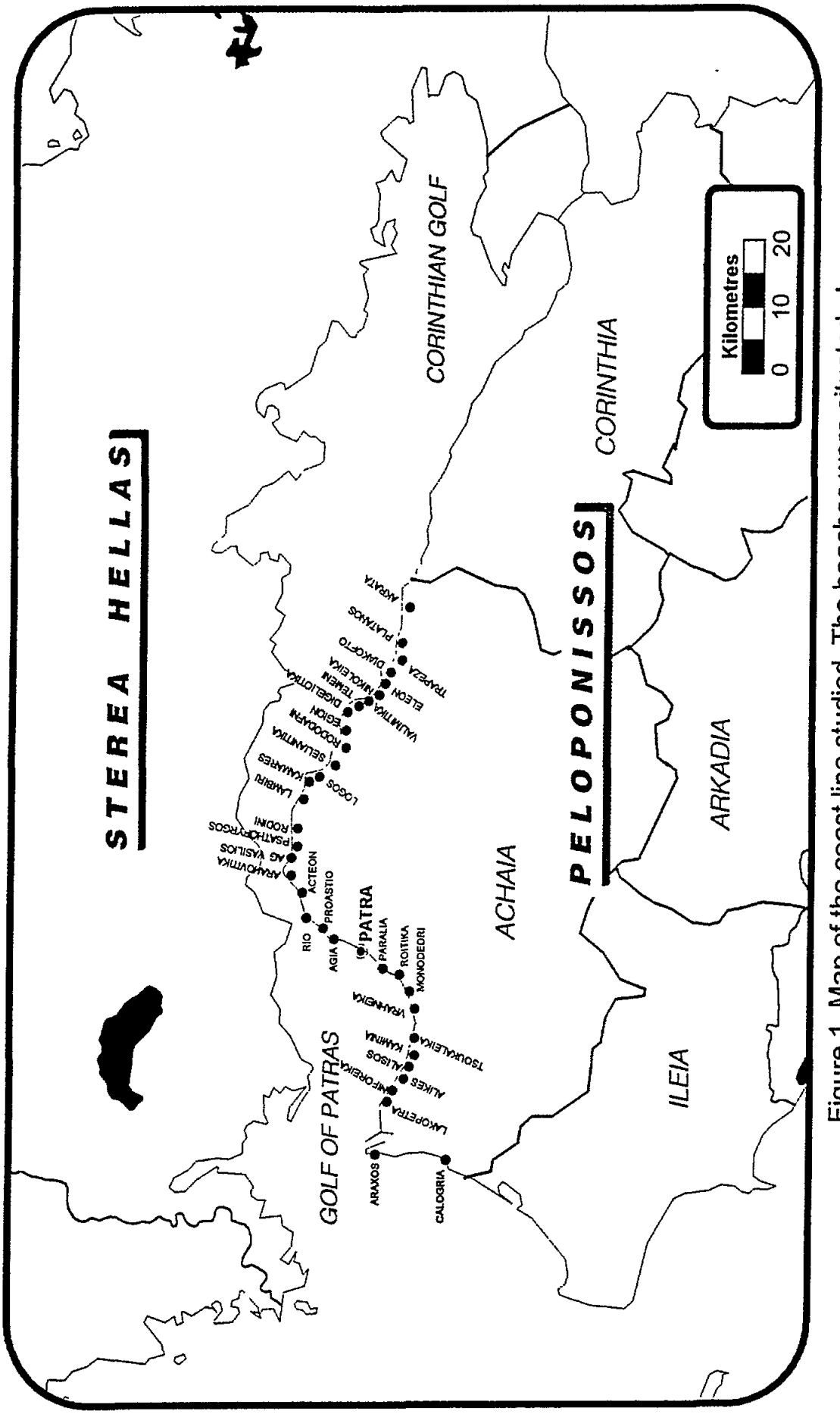

Figure 1. Map of the coast line studied. The beaches were situated along 90 km of Peloponissos coast in Mediterranean sea.

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MONITORING OF SELECTED BACTERIA AND FUNGI IN SAND AND SEAWATER ALONG THE TEL AVIV COAST

by

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1. INTRODUCTION

The detection and enumeration of indicator organisms in sand is of great importance in assessing presence of the bacterias, viruses, fungi and parasites (10). This quality criterion was used in assessing the risk of contracting different illnesses associated with the contact of humans with sand (6). Increased levels of bacteria in marine sediments create a potential health hazard for several months (3). The bacteria adsorbed to the estuarine sediment rich in organic content, survive, and act as a reservoir of pathogens which may be resuspended by any kind of turbulent activity and can pollute the seawater and sand (1, 24, 25). Therefore illnesses associated with eye, ear, skin, gastrointestinal disorders and respiratory diseases of bathers, can be predicted by testing bacterial indicators of seawater (14, 21, 23, 27). In addition to the standard bacterial indicators in the Standard Methods for the examination of water and wastewater, the 17th edition (8) *Campylobacter jejuni* (Cj) was proposed with caution as a standard indicator (7). Since recent studies on *Campylobacter* isolation from various aquatic habitats performed in the central area of Washington it has received considerable attention as a possible cause of bacterial enteritis in man (7). *Campylobacter* was also demonstrated in a river system (2) and in mud (18) and therefore was proposed as an additional bacterial parameter by many state authorities. The primary aim of this project was to assess the prevalence of Standard Bacterial Parameters (SBP) and Potential Pathogenic Bacteria (PPB) including fungi in sand. The study of the most effective method for the isolation of *Campylobacter jejuni* in sand and seawater and the comparison of a modified method with the method proposed in Standard Methods (8) was another aim. This study was performed in collaboration with the Dan Region Association of Towns (sewerage). It was supported by a research grant from the WHO Regional Office for Europe within the framework of the long-term Programme of Pollution Monitoring and Research in The Mediterranean sea (MED POL Phase II).

2. MATERIALS AND METHODS

2.1 Selection of sand and sea samples

Samples of sand and seawater were collected twice per week, on Mondays and Thursdays between 12 Noon and 1, during the summer of 1989. Five beaches in the Tel Aviv area, were chosen, some of them because of their proximity to the main sewage outflow. Technical advances and modernization in the treatment of sewage and the cease of all sewage outflow into the sea of Tel Aviv area, between 1990 and 1991 caused us to change our plan of studies. Five beaches were studied in 1990 and three beaches in 1991, twice weekly in each case: samples were taken alternatively, Monday/Tuesday and Tuesday/Friday twice a day, once

early in the morning and once at noon. The sampling was performed by the Dan Region Association of Towns (sewerage). (DRATs).

2.2 Sampling sites

Samples of sand were collected 2-3 meters in wards from the shoreline at 10 centimeters depth in the sand in 200 ml. sterile bottles. Seawater was taken in 200 ml sterile bottles 1-3 meters from the shoreline, in the sea, so that about one half of the collector body was covered by the water. Samples of sand and seawater for Monitoring *Campylobacter* were taken respectively in 200 ml and 500 ml sterile bottles.

2.3 Processing of the sand samples

20 grams of sand were weighed under aseptic conditions in a 200 ml sterile bottle and resuspended in 180 ml Phosphate Buffer Saline (PBS) ph 7.2, shaken lightly for one minute and allowed to stand for ten minutes for sedimentation.

2.4 Membrane filter (MF) procedure

The supernatant was filtered by the MF method (8) using Gelman membrane filter, diam 47 mm, mean pore diam 0.45 µm. The Gelman system of six filter-holding funnel assemblies was used.

2.5 Bacterial parameters

In the first stage (1989) a Faecal coliforms (FC) *Escherichia coli* (EC), Faecal streptococci (FS), *Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* (Sa) and Fungi were studied.

In the second and third stages (1990-1992), FC, EC, FS, Pa, Sa and Fungi were studied. The monitoring of *Campylobacter jejuni* in sand and seawater was introduced as a part of the Project only in 1991 and 1992. The bacterial Parameters for seawater monitoring of beaches in Tel Aviv area performed by DRATs were: Faecal coliform and *E. coli*.

2.6 Culture media

A. Faecal coliform: m-Fc base dehydrated media (Difco-0883-01-1).
The Most Probable Number (MPN): Lactose broth, double strength (8).

B. *Escherichia coli*: MUG media, described by Mates and Shaffer (20).

C. Faecal streptococci: m-Enterococcus (m-E) agar (Difco-0746-01-8) (8).

D. *Pseudomonas aeruginosa*: m-PA agar are not available in dehydrate form media was prepared according to the procedure described in Standard Methods.

E. *Staphylococcus aureus*: The S-4 media was prepared according to Morgenstern and Katznelson, 1982 (22).

2.7 Temperature and incubation time

FC: 44.5E C for 24 hours (on m-FC agar)
EC: 35.5E C (on MUG) 4 hours
FS: 41E ± 0.5E C for 48 hours (on m-E agar)
Pa: 42E ± 0.5E C for 48 hours (on m-PA agar)
Sa: 37E C for 24 hours (on S-4, egg yolk tellurite agar).

3. PROCEDURE FOR CAMPYLOBACTER CULTURE

3.1 Media and sand processing

Sand samples for *Campylobacter* isolation were taken in 200 ml bottle containing 100 ml Nutrient Broth (Difco-0003-01-6) with 5% lysed horse blood. 80 grams of sand were weighed in aseptic condition in 1 500 ml bottle containing 360 ml of PBS and shaken lightly, then left in rest for 10 minutes. The supernatant (400 ml) was filtered through MF (0.45 µm pore diam.) 400 ml of seawater from the same beach were filtered in parallel under the same conditions. The MF were then immersed in Broth Enrichment Medium (BEM) (26) for 48 hours at 42E C.

3.2 Enrichment medium for *Campylobacter jejuni* isolation

10 ml of BEM were transferred into a wide-mouth (57 mm) container. 500 ml of seawater or sand-supernatant were filtered and the filter immersed in the container, and incubated for 48 hours at 42E C. The MF were set up on selective media for Cj isolation.

3.3 Selective media for *Campylobacter jejuni* isolation

Three selective media were controlled: a-medium, b-medium and c-medium:

a-medium:

Nutrient agar 23.0 gr (rehydrate to 1000 ml, autoclave and cool to 50E C) add:

Lysed horse blood	25.0 ml
Supplement SR 84 (Oxoid)	1.0 vial
Supplement SR 117 (Oxoid)	1.0 vial
Supplement SR 125 (Oxoid)	0.94 ml.

b-medium (Modified CCDA-Preston) (19):

Blood free selective agar base	45.5 gr (Oxoid CM 739)
Cephoperazon	32.0 mgr
Distilled water 1000.0 ml	

c-medium (Butzler's medium) (20):

Blood agar base no. 2 (Oxoid CM 271)	40.0 gr
Human or sheep blood (whole)	7.0 ml %
Bacitracin	25.000 IU
Novobiotin	50.0 mgr
Cephalotin	15.0 mgr
Cycloheximide	10,000.0 IU
Distilled water 1,000.0 ml	

3.4 Temperature and incubation time

42E ± 0.5E C for 48 hours into a Jar in microaerophilic conditions by gas generating sachet (Oxoid BR-56).

3.5 Identification and confirmation

A. Carbol Fuchsin staining (1:10) for 5 minutes. Look for specific forms.
B. Sensitivity to Cephalotin (30 µm) = unsensitive.
C. Sensitivity to Naladixic acid (NAC) 30 µm = sensitive.
D. Oxidase test (by hydrogen peroxide) = the test should be negative.
E. Catalase test (by Catalase from bovine liver-BDH-no.39008) = the test should be positive.

4. PROCEDURE FOR ISOLATION OF CANDIDA ALBICANS

4.1 Media for Candida Albicans culture (MCA)

For 300 ml:

Glycine	3.0 gr
Maltose	9.0 gr
Sodium Sulphate	0.9 gr
Bismuth ammonium citrate	1.5 gr
Cloramphenicol	0.15 gr
Cycloheximide	0.45 gr
Distilled water 270.0 ml	

Heat to 50E C and then adjust first pH to 7.1 and then to pH 8.0 and add:

Agar	4.5 gr

Boil and cool to 50E C, add:

10 x Yeast nitrogen base	30.0 ml

mix and pour 3 ml liquid media in 6 cm diam Petri plates.

4.2 Culture technique using medium for Candida albicans (MCA)

100 ml of seawater or sand supernatant were filtered as showed before by MF method. The filter was seeded face up on the selective MCA media at 37E C for 48 hours. Brown colonies were picked up and transferred to Saboureaud selective media for reculture. In parallel a smear of the brown colonies and the Germ tube test for *Candida* were performed.

A. The smear was fixed and stained by Gram, and examined for chlamydospores, blastospores and hyphae.

B. The germ tube test (19). Material was taken from a brown colony and transferred to a tube containing 0.5 ml to 1 ml human inactivated serum. The tube was heated at 37E C for 2-4 hours and then examined microscopically (x40 Objective; x12 Ocular) for short lateral hyphal filaments (germ tubes).

5. RESULTS

The results of the first Phase studies performed in 1989 are summarized in table 1; fifty samples of sand and seawater were taken from 5 beaches in the Tel Aviv area. The bacterial density of PPB parameters established by the Geometric mean was not negligibly high and should be related for statistical purposes to 1 gram of sand. Faecal coliforms and *E. coli* counted in sand were proportionally less than in seawater. However isolation of *Pseudomonas aeruginosa* and other unidentified Pa strains was greater in sand than in seawater. Fungi, probably *Candida albicans* (Ca) were found in almost all samples of sand but none in seawater.

The results of the second Phase performed in 1990, including PPB and the preliminary data on the isolation of *Campylobacter jejuni* in 134 samples of sand and seawater in parts of six different beaches, using the selective a-medium (Materials and Methods), are summarized in Tables 2 and 3. The results of the isolation of PPB presented in Table 2 show a higher density of bacterial indicators and PPB in Tel Baruch and Sheraton beaches as compared to 1989 (Table 1). The Geometric mean of PPB in sand was always higher in seawater. The results summarized in Table 3 present the Geometric Mean (GM) of *Campylobacter* and *Candida* isolated in sand and seawater. *Campylobacter* show a relatively low level of isolation in sand and very low or absent in seawater. The GM of *Candida* was also very low compared to 1989.

Campylobacter jejuni occurrence in sand and seawater in three recreational beaches in the Tel Aviv area and their correlation with standard indicator bacteria was the objective of the third and last Phase of the study. Two selective media for Cj isolation were used: b-medium (like a-medium) and c-medium (Butzler's medium) (14) recommended in Standard Methods (8). The study was executed in 1991 (The data of monitoring, between January - April 1992 were not included in this paper) and the results are presented in Tables 4 and 5. The sampling of four beaches including a total of 127 samples of sand and 115 samples of seawater show a very low level of *Campylobacter* and PPB isolation of the Tel Aviv area (three of them were monitored in 1989 and 1990). 51 samples of sand (51/127) and 53 (53/115) of seawater were suspected for Cj. Only 7 of the sand samples and 5 of seawater were polluted with *Campylobacter jejuni* the remaining were polluted by Pa (14/50); Ec (36/50); *Candida* (41/50).

Table 1

Geometric mean of Bacterial density found in sand and seawater during study of five beaches in the Tel Aviv area of the Mediterranean sea between 01/05/89 and 01/10/89

Bacterial Parameter	No. of samples (24x2 weeks)	Geometric Mean distribution in 1 gram of sand /100 ml seawater									
		Country Club (Tuk 1)		Tel Baruch North		Tel Baruch South		Sheraton		Frishman (Bugrasow)	
		Sand	Sea Water	Sand	Sea Water	Sand	Sea Water	Sand	Sea Water	Sand	Sea Water
Fc. coli	42	16	24	7	26	31	35	24	21	11	32
E. coli	42	2	24	27	9	27	7	25	5	24	16
S. aureus	42	8	9	11	9	15	6	6	24	15	9
P. aeruginosa	40	19	10	14	11	18	15	3	3	11	8
Fc. Strept.	44	2	6	2	7	4	3	2	4	12	8

Fc. coli = Faecal coliforms
E. coli = Escherichia coli
S. aureus = Staphylococcus aureus
P. aeruginosa = Pseudomonas aeruginosa
Fc. Strpt. = Faecal streptococci.

Table 2

Size group percent distribution of Standard Bacterial Parameters in seawater
of five beaches in the Tel Aviv Area of Mediterranean sea during
a study performed by Dan Region Association of Towns (sewerage)

Beach	Bacterial Parameter	Size Group Distribution in 100 ml of seawater							
		<10	%	10-100	%	100-1000	%	<1000	%
Country Club North (Hatuk 1)	Faecal coliform	42	87.5	6	12.5	0	0.0	0	0.0
	Escherichia coli	48	100	0	0.0	0	0.0	0	0.0
Tel Baruch North	Faecal coliform	36	75.0	9	18.7	3	6.3	0	0.0
	Escherichia coli	43	89.6	5	10.4	0	0.0	0	0.0
Tel Baruch South	Faecal coliform	39	81.3	7	14.6	1	2.05	1	2.05
	Escherichia coli	44	91.7	3	6.3	0	0.0	1	2.0
Sheraton	Faecal coliform	29	60.4	13	27.1	6	12.5	0	0.0
	Escherichia coli	35	72.9	9	18.7	4	8.4	0	0.0
Frishman Bugrasow	Faecal coliform	26	53.1	16	32.6	6	12.3	1	2.0
	Escherichia coli	32	65.3	11	22.4	6	12.3	0	0.0

Frishman = Bugrasow;
Tel Baruch north = Tel Baruch 1;
Tel Baruch south = Tel Baruch 2.

Table 3

Geometric mean density of potentially pathogenic bacteria in sand and seawater (SW) in six recreational beaches of the Mediterranean sea in the Tel Aviv Area between 01/11/89 and 01/11/1990

Bacterial Parameter	No.* of samples	Geometric Mean Distribution in 1 gr sand/100 ml seawater											
		Hanehim		Hatuk		Tel-Baruch N.		Sheraton		Bugrasow		Ch. Clore	
		Sand	Sea water	Sand	Sea water	Sand	Sea water	Sand	Sea water	Sand	Sea water	Sand	Sea water
Fc. Coli	134	13	8	36	7	68	21	42	25	92	75	33	21
E. coli	101	12	8	7	11	42	14	19	20	21	11	22	10
S. aureus	134	31	19	67	9	28	15	96	6	24	6	81	9
P. aeruginosa	134	6	2	22	19	14	11	44	5	53	12	31	10
Fc. strept.	134	20	4	13	5	10	4	13	7	15	4	10	3
Fungi (Ca)	134	99	11	90	21	44	77	162	75	298	40	99	31

* This number includes samples taken twice from the same beach

Fc = Faecal coliforms; *E. coli = Escherichia coli; S. aureus = Staphylococcus aureus; P. aeruginosa = Pseudomonas aeruginosa;* Fc. Streptococcus = Faecal Streptococci; Ca = *Candida albicans*.

Table 4

Prevalence of *Campylobacter jejuni* and *Candida albicans* in sand and seawater in three recreational beaches in the Tel Aviv area of the Mediterranean sea between 04/07/90 and 31/11/90

Bacterial Parameter	Number of samples	Geometric Mean Distribution in 1 gr/100 ml seawater					
		Hanehim		Hatuk 1		Sheraton	
		Sand	Sea water	Sand	Sea water	Sand	Sea water
Campylobacter	34	13	2	18	6	20	3
Candida Albicans	34	24	2	13	2	30	2

Table 5

Size group percent distribution of Standard Bacterial Parameters in seawater of three beaches of Tel Aviv area of the Mediterranean sea between 01/05/91 to 30/10/91. Study performed by Dan Region association of Towns (sewerage) in collaboration with P.H. Central Laboratory

Beach	Bacterial Parameter	Size Group Distribution in 100 ml seawater							
		<10	%	10-100	%	100-1000	%	>1000	%
Hanehim	Faecal coliforms	17	35.4	17	35.4	12	25.0	2	4.2
	Escherichia coli	26	54.2	15	31.2	6	12.5	1	2.1
Hatuk 1 (Country Club North)	Faecal coliforms	42	87.5	6	12.5	0	0.0	0	0.0
	Escherichia coli	48	100	0	0.0	0	0.0	0	0.0
Sheraton	Faecal coliforms	29	60.4	13	27.1	6	12.5	0	0.0
	Escherichia coli	35	72.9	9	18.8	4	8.3	0	0.0
Bugrasow (Frishman)	Faecal coliforms	26	53.1	16	32.6	6	12.2	1	0.0
	Escherichia coli	32	65.3	11	22.5	6	12.2	0	0.0
Ch. Clore	Faecal coliforms	23	47.9	11	22.9	5	10.4	9	18.8
	Escherichia coli	30	62.5	6	12.5	8	16.7	4	8.3

Table 6

The effect of enrichment media on the recovery of *Campylobacter jejuni* from sand and seawater, cultured in a- and b- media, between 01/12/90 and 31/12/91

Source of sample	Total Number of Samples	Number of samples suspected for Cj (*)												
		Pre-enrichment in BEM culture						Non Pre-enrichment culture						Significance of pre-enrichment
		a-M		b-M		c-M		a-M		b-M		c-M		(p=)
Sea(**)	127	3	2.4%	9	7.1%	5***	-	2	1.6%	1	0.9%	5***	3.9%	p=0.09
Sand(**)	115	51	44.3%	52		7***	6.1%	52	-	49	-	7***	6.1%	p=0.1

a-M = a-medium;
b-M = b-medium;
c-M = c-medium;
p = T-student test coefficient.
(*): before the confirmation of Cj Centre;
(**): sampling was performed by Dan Region Association of Town (sewerage), The Ecological Department
(***): these isolates were confirmed as *Campylobacter*.

Table 7

Results of the prevalence of Standard Bacterial Parameters (SBP) and Potential Pathogenic Bacteria (PPB) in the sand and seawater of three beaches, monitored in 1991 in Tel Aviv between 01/12/90 and 31/12/91

Source of sample	Total No of Samples	Number of Positive Samples (1000 germs/100ml)												p=
		Number of Positive by SBP (*)						Number of Positive by PPB (**)						
		coliform (m-endo)		Fc/Ec (MUG)		Pa (m-Pa)		coliform (less)	Fc/Ec (m-Fc/ MUG)	FcS	Sa S4	Pa m-Pa	Ca MCA	
Sea (**)	127	9	7.8%	13	10.2%	0	0.0%	9	3	0	1	0	1	Sg*
Hatuk 1		0	0.0%	0	0.0%	0	0.0%	0	1	0	0	0	0	
Sheraton		7	5.5%	6	4.7%	0	0.0%	6	1	0	0	0	0	
Ch.Clore		2	1.6%	7	5.5%	0	0.0%	3	1	0	1	0	1	
Sand	115	12	10.4%	7	6.1%	5	4.3%	12	36	2	4	14	41	Sg
Hatuk 1		3	2.6%	1	0.9%	0	0.0%	2	5	0	2	4	11	
Sheraton		4	3.5%	2	1.7%	2	1.7%	4	12	1	0	3	9	%
Ch.Clore		5	4.4%	4	3.5%	3	2.6%	7	13	1	2	7	21	

m-Fc = Faecal coliforms; m-endo = coliforms; MUG = media for *Escherichia coli*;
FcS = Faecal Streptococci; Sa = *Staphylococcus aureus*; Pa = *Pseudomonas aeruginosa*;
S4 = media for Sa; m-Pa = media for Pa; Ca = *Candida albicans* = Monillia;
MCA = media for *Candida albicans*; (*): Statistically significant, p=<0.05, T-student test for statistical analysis;
(**): the sampling was performed by Dan Region Association of Town (sewerage), Ecological Department;
SBP = Standard Bacterial Parameter; PPB = Potentially Pathogenic Bacteria.

Table 8

Size Group percent distribution of Bacterial Parameters in seawater of three beaches in the Tel Aviv area of the Mediterranean sea study performed by Dan Region Association of Towns (sewages) between 01/05/91 and 30/10.91 in collaboration with the P.H. Laboratory

Beach	Bacterial Parameter	Size Group Distribution (in 100ml seawater)(*)							
		<10	%	10-100	%	100-1000	%	>1000>	%
Tuk 1	Faecal coliforms	42	87.5	6	12.5	0	0	0	0
	Escherichia coli	48	100	0	0	0	0	0	0
Sheraton	Faecal coliforms	27	55.1	19	38.8	3	6.1	0	0
	Escherichia coli	35	72.9	9	18.7	4	8.3	0	0
Ch.Clore	Faecal coliforms	23	47.9	11	22.9	5	10.4	9	18.7
	Escherichia coli	30	62.5	6	12.5	8	16.6	4	8.3

(*) Results received from the monitoring of Tel Aviv beaches performed in collaboration with Dan Region Association of Towns (Sewage) Ecological Department.

6. DISCUSSION

Standard Bacterial Parameters (SBP) for evaluation of the bacterial quality of seawater and sand are: total coliforms, Faecal coliforms and Faecal streptococci (10). We have considered that these SBP are insufficient to assess the quality of recreational beaches and therefore decided to monitor three additional parameters, two of which also Potentially Pathogenic Bacteria (PPB): *Staphylococcus aureus* (Sa), *Pseudomonas aeruginosa* (Pa) and pathogenic fungi: *Candida albicans* (Ca). We also decided to eliminate the monitoring of total coliforms considering the evaluation of Faecal coliforms and *Escherichia coli* sufficient as indicators of faecal contamination, i.e. enteric pathogens. The data received in these three years of monitoring, indicate differences between data obtained in 1989, 1990 and 1991 and in the correlation between SPB and PPB in sand and seawater. An interesting feature was found in 1989 (Table 1) compared to 1990 and 1991, where Faecal coliforms, *Escherichia coli* and Faecal streptococci were count less in sand than in seawater, results which contradict the findings of Paul La Liberte and Grimes (24). They demonstrated the extend of survival of *E. coli* in sediment because of the fine soil particles and high organic content. The sediment-bound indicator bacteria are suspended by the movement of the sea and the bacteria penetrate the sand. We attribute these specific results to the low turbulent activity of the sea in this specific year particularly in the summer., This phenomenon was not seen again in 1990 or 1991 (tables 3, 7). In addition we found a higher count of indicator bacteria in Tel Baruch and Sheraton beaches probably because of the vicinity of the main sewerage outflow in the north of Tel Aviv City. This phenomenon was seen again in 1990 (table 3). In 1991 the number of indicator bacteria counted in sand and in seawater dropped sharply (table 7). This was probably caused by the intensive work done by the Dan Region Association of Towns (DART). The results of the monitoring of PPB show an extended survival of *Pseudomonas aeruginosa* and other unidentified species as well as *Staphylococcus aureus* and *Candida* and other unidentified species of fungi, in higher number in sand than in seawater (table 1, table 3, table 7). The number of PPB counted in sand and in seawater also dropped dramatically at the end of 1990 and during 1991 (Table 7) as was also seen with the SBP. Nevertheless, the presence of these PPB in a real higher number in seawater explain the illnesses which can be contracted by non-swimmers in contact with the sand. This possibility was discussed by Fattal *et al.* 1986 (1) in a study on the association between morbidity and microbial quality of seawater and show a significant relationship between the SBP and PPB. The high density of PPB and enteric bacteria in sand found in the monitoring of Tel Aviv beaches support the study of Burton *et al.*, 1987 (4). He found that the level of pathogens was many times higher in sea-sediments than in seawater creating a potential health hazard for a long period of time. *Campylobacter* has received considerable attention in the cause of bacterial enteritis in humans, and considerable research was done on the isolation with food and water supply (3, 13, 15). The detection of *Campylobacter jejuni* (Cj) results from its ability to grow in the presence of different agents with lethal injury which are different in seawater or sand compared with faeces where the organism may be present in a large number (7). The first problem to solve was a method for enrichment and resuscitation of the bacteria from seawater or sand and the second to find the best media for cultivation and identification of Cj. Rogol *et al.*'s (7) enrichment and transport media, Butzler's cultivation media (5) and Preston's media (3) were used to study the prevalence of Cj in three beaches of the Tel Aviv area. The results presented in Table 4 show an isolation rate of between 13 to 20 bacteria in 1 gr of sand compared to 2 to 6 in 100 ml seawater (Table 4). The data presented in Table 6 reveal that *C. jejuni* isolation rate was higher in nutrient agar-lysed horse blood media (a-M) and in Preston's blood free selective agar media (b-M) than in Butzler's medium (c-M). The Cj isolation was higher in sand (up to 20/34) and the BEM pre-enrichment procedure (7) seem to increase the Cj isolation rate in sand and also in seawater. The isolation

of Cj from seawater was very low (6/34) and confirm the results of other studies (9, 16). The isolation rate of Candida albicans in sand was higher (up to 30/34) than in seawater (2/34). It is now a reality that *Campylobacter jejuni* can be a potential hazard factor for enteritis in humans and one of the reservoirs can be the sand (7). The results of a cooperative study with DART during 1991 (Tables 2, 5, 8) show that the Tel Aviv beaches were almost clean from PPB and also the Standard Bacterial Parameters were very low. This dramatic drop came after a long period of years in which untreated sewage was discharged along coastline of Tel Aviv beaches through many outfalls, the largest of these at Reeding Electric Power Station. The resulting pollution has long been a cause of concern and, in 1989 led the City Council of Tel Aviv to begin considering options for remedial actions. In the northern most beach of Tel Aviv, near the main outfall, a chlorinator was built for sewage and surface sewage streams coming from Hertzelia City. The Marina built in the last years in the North of Tel Aviv City also helped to reduce the number of PPB and SBP counted in the sea and sand of the Hanehim and Tuk (Country club) beaches. The Sheraton area had a relatively polluted beach, and was influenced by the Hayarkon river discharging into the sea. A sewer was projected to drain Hayarkon river and an engineering project was applied to permit seawater to flow into the Hayarkon river and to dilute the waste. The surface sewage was directed to the main outfall and drained in the main waste flow, treated and then discharged into the sea at 800 meters from the coastline. The three years monitoring of the Tel Aviv Mediterranean area provided many answers to some problems and requirements discussed at a Joint WHO/UNEP Consultation held in Athens from 22-26 September 1987 (21): (a) the prevalence of pathogenic fungi in bathing beaches, mainly in sand and a draft reference method for determination of *Candida albicans*; (b) data on fluctuation in the population density of the three Standard Bacterial Parameters previously mentioned as a result of various environmental factors; (c) the prevalence of *Campylobacter* in sand and seawater and the development of a standard method for the isolation of Cj. Data from this investigation gave information concerning the value of standard indicator bacteria and predict the implication of *Campylobacter* spp. found in seawater and sand for Public Health as a cause of enteritis in humans (7). More studies are needed for the standardization of the methods for cultivation and confirmation of Candidae and Campylobacters in sand and seawater.

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EVALUATION OF CCDA MEDIUM FOR RECOVERY OF CAMPYLOBACTER FROM MARINE WATERS

by

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1. INTRODUCTION

A great variety of broths and media containing different antibiotics and other selective substances have been used for the isolation of thermophilic campylobacters from clinical, veterinary, food and water samples (Patton *et al.* 1981; Bolton and Robertson 1982, Bolton *et al.* 1984, Rothenberg *et al.* 1984, Rogol *et al.* 1985, Merino *et al.* 1986, Gun-Munro *et al.* 1987, Burnens y Nicolet 1992, Stelzer *et al.* 1991).

Most detection methods for campylobacters in waters include an enrichment step using a rich basal medium, lysed blood, and a combination of antimicrobial agents. Subculture is done on a selective agar containing antibiotics and often lysed blood.

The requirement for blood in selective media can be a problem for some laboratories that do not normally use blood in their culture media. An important solution for these laboratories is the development of blood-free medium, selective media not containing blood and described by the literature (Bolton *et al.* 1984, Hutchinson and Bolton, 1983, Korhonen and Martikainen 1990). Blood-free media have been shown to be similar or more efficient than other selective media in detecting *C. jejuni* and *C. coli* in human and animal faeces (Goosens *et al.* 1986, Gun-Munro *et al.* 1987), and surface waters (Korhonen and Martikainen 1990).

We therefore compared two enrichment broths, modified Preston broth (PB) and campylobacter blood-free selective broth (CCDB), and two growth media, Preston agar (PA) and campylobacter blood-free selective agar (CCDA), for the isolation of thermophilic campylobacters from marine waters.

2. METHOD

2.1 Sampling area

Samples were taken from Perelló near a sewage discharge area.

Water samples were transported at 4E C from the sampling site to the laboratory. The period of time between collecting the samples and initiating water tests never exceeded 2 h.

2.2 Campylobacter isolation

2.2.1 Culture media

Modified Preston broth (PB): suspend 10 g beef extract, 10 g peptone, and 5 g sodium chloride in 950 ml distilled water. Sterilize 15 minutes at 121E C. Dissolve 5000 IU polymyxin B, 10 mg rifampicin, 10 mg trimethoprim lactate, and 100 mg actidione in 4 ml acetone-distilled water (1:1). Add this solution and 50 ml lysed defibrinated sheep blood to cooled medium. Distribute 100 ml portions in flasks.

Modified Preston agar (PA): suspend 10 g beef extract, 10 g peptone, 5 g sodium chloride and 15 g agar in 950 ml distilled water. Sterilize 15 minutes at 121E C. Dissolve 5000 IU polymixin B, 10 mg rifampicin, 10 mg trimethoprim lactate, and 100 mg actidione in 4 ml acetone-distilled water (1:1). add this solution and 50 ml lysed defibrinated sheep blood to the cooled medium. Mix well and pour into Petri dishes.

Campylobacter blood-free selective broth (CCDB): suspend 10 g beef extract, 10 g peptone, 5 g sodium chloride, 4 g bacteriological charcoal, 3 g casein hydrolysate, 1 g sodium desoxycholate, 0.25 g ferrous sulphate and 0.25 g sodium pyruvate in 1000 ml distilled water. Let soak 10 minutes. Mix to suspend dissolved material and autoclave at 121E C for 15 minutes. Cool to 50E C and aseptically and 32 mg cefoperazone dissolved in 4 ml distilled water. Mix well and pour into Petri dishes.

Campylobacter blood-free selective agar (CCDA): suspend 10 g beef extract, 10 g peptone, 5 g sodium chloride, 4 g bacteriological charcoal, 3 g casein hydrolysate, 1 g sodium desoxycholate, 0.25 g ferrous sulphate, 0.25 g sodium pyruvate and 15 g agar agar in 1000 ml distilled water. Let soak 10 minutes. Dissolve the ingredients by gentle boiling and autoclave at 121E C for 15 minutes. Cool to 50E C and aseptically add 32 mg cefoperazone dissolved in 4 ml distilled water. Mix well and pour into Petri dishes.

2.2.2 Controls

C. jejuni NCTC 11168, *C. coli* NCTC 12110 and *C. laridis* NCTC 11352.

2.2.3 Confirmation of the growth of campylobacters

Suspect colonies were stained by Gram method.

2.2.4 Procedure

Filter a volume of 100 ml of sample through 0.45 µm pore size membrane filters. After filtration the membranes are removed and introduced in flasks with 100 ml of PB. Use the same procedure to inoculate CCDB medium. Incubate enrichment broths at 37E C for 4 hours, before transfer to 42E C for 44 hours, under microaerobic conditions (5% O_2, 85% N_2 and 10% CO_2). Using a loop, inoculate incubated enrichment broths (PB and CCDB) on 2 plates of PA and CCDB, respectively. Incubate plates at 42E C for 48 hours under microaerobic conditions.

Pick at least 1 colony of every colonial type and test for Gram stain reaction (campylobacters are characteristically Gram negative and curved or spiral in shape).

Incubate also one stock culture of a *Campylobacter* strain from a type collection (e.g. NCTC).

3. RESULTS

There were no differences between media with blood (PB-PA) and media without blood (CCDB-CCDA). The total number of positive samples was the same for both culture procedures (Table 1). When campylobacters were found, they were mostly growing well, often swarming all over the plate, on both plating media.

Control strains of *C. jejuni, C. coli* and *C. laridis* showed good growth on both culture methods (table 2).

Results in the study indicate that growth of campylobacters are similar on both methods.

Table 1

Occurrence of *Campylobacter* in Perelló site

Sampling time	media[a]	
	PB - PA	CCDB - CCDA
14.10.91	-[b]	-
21.10.91	+	+
04.11.91	-	-
11.11.91	-	-
02.03.92	+	+
09.03.92	+	+
11.05.92	+	+
18.05.92	-	-

[a]: PB - PA, modified Preston broth and modified Preston agar.
CCDB - CCDA, *Campylobacter* blood-free enrichment broth and *Campylobacter* blood-free enrichment agar.

[b]: -, absence; +, presence.

Table 2

Growth of *Campylobacter* reference strains

Media	*C. jejuni*	*C. coli*	*C. laridis*
	NCTC 11168	NCTC 12110	NCTC 11352
PB - PA	+	+	+
CCDB - CCDA	+	+	+

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COMPARATIVE DISTRIBUTION OF MICROBIAL AND YEAST POPULATIONS IN SAND AND SEAWATER

by

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1. MATERIALS AND METHODS

1.1 Sampling

Water and sand samples were collected from two swimming areas: one free swimming area (site no. 1) and one organized beach, run by a private Organization (site no. 2). Site 2 is more popular with swimmers than site 1. Site 1 is located at Voula at a distance of approximate 1 km. from site 2, which is located at the east part of Glyfada. A total of 84 samples were taken from the two beaches during a 3 year study (1988-1990). Winter samples (October-May) were taken monthly and summer samples (May-October) at 15 day intervals. Each time the number of swimmers present on the beach and water temperature were recorded.

1.2 Bacterial Cultures

1.2.1 Water samples

For the detection of coliforms, faecal coliforms and enterococci in the water samples, the membrane filtration technique was employed, using Sartorius filters with porosity 0.45 µm. Coliform counts were detected on Membrane Lauryl Sulphate agar (OXOID MM615) after incubation at 37E C for 24 hours. Faecal coliforms were detected on m-FC agar (DIFCO 0677-01-1) after incubation in a water bath at 44E C for 24 hours. Enterococci colonies were detected on m-enterococcus agar (OXOID) after incubation at 37E C for 48 hours. For the investigation of *S. aureus* the MPN Technique was used. Positive 5X5X5 tubes of modified *m-staphylococcus* were subcultured on Vogel-Johnson agar. (Mavridou *et al.* 1990)*. Typical colonies were tested for coagulase and catalase tests and for anaerobic lactose fermentation.

For the isolation of yeasts and moulds 100 ml sample were filtered and incubated on S.D.A. at 25E C until the CFU's became visible and could be differentially counted. The yeast isolates were then counted and identified according to their capacity to assimilate and ferment carbohydrates. Moulds were identified macro- and microscopically.

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* Mavridou A., Papadakis J.A., Lambiri M., (1990). Isolation of *S. aureus* from Seawater Samples. C.I.S.M. Athens, 1990.

For the isolation of *Pitirosporum orbiculare*, 5ml of water were mixed in 25ml of B.H. I.B. with olive oil and incubated at 25E C for 48 hours. One ml was subcultured in S.D.A. with olive oil. The identification was carried out micro, and macroscopically and according to their capacity to assimilate carbohydrates.

1.2.2 Sand samples

For the isolation of coliforms, faecal coliforms and enterococci from sand samples, the pour plate technique was employed using the same culture media as with the water samples. For the *S. aureus* counts the MPN technique was applied as with the water samples.

For the isolation of yeasts and moulds from sand, quantities of 1-2 gm. were mixed in 25 ml of B.H.I.B. and incubated at 25E C for 48 hours. One ml was then subcultured in S.D.A. and carried out as with water samples. For the isolation of *Pitirosporum orbiculare* 1-2 gm of sand were mixed in 25 ml of B.H.I.B. with olive oil and carried out as above.

2. RESULTS

From the 84 samples taken during the study, 41 being collected from each site, half were winter and half summer samplings.

The water temperature range was 8-22E C during water (median value = 15E C) and 20-30E C during summer (median value = 23E C). Five and forty swimmers/30 m of beach were present at the sites 1 and 2 respectively, with maximum values 110 and 100/30 m of beach. During winter, swimmers were recorded once on each of the beaches. In summer, 18/21 days swimmers were present on beach no. 1 and 20/21 days on beach no. 2.

Median values of the bacterial counts in water and sand samples of both sites are presented in Table 1. The median values of non zero/above the detectable limit are also presented of yeasts and moulds isolated from sand and water samples. In Table 3 appear the number and percentage of sampling days, when yeasts and moulds were investigated from sand and water samples from each site, yeasts and moulds of strictly environmental or strictly human origin are analyzed separately in the same table.

3. STATISTICAL ANALYSIS

Statistical analysis of the results was carried out using a number of approaches.

3.1 Comparison between sites

Water and sand samples from site 2 had significantly higher bacterial counts than from site 1, with the exception of faecal coliforms in sand samples (Table 1). There were no significant differences in the yeasts and moulds findings,

with the exception of yeasts of human origin in water samples (table 3) which were significantly more common in site 2 than in site 1 (Binomial test, P-O 023).

3.2 Comparison of the results according to season

Water and sand samples of both sites contained significantly more *S. aureus* in summer than in winter samples (Table 4). The differences between summer and winter findings in the samples are the same when all data are compared and when only the non-zero/above limit ones are analyzed. There is no clear difference in the number of moulds and yeasts according to season (table 5). Yeasts of human origin present in water samples are slightly more in winter (P = 0.08) and slightly more in sand in summer (P = 0.07).

3.3 Correlation with water temperature

Taking the summer and winter data together, there is a significant correlation between water temperature and *S. aureus* counts in water and sand samples from both sites. However, this correlation doesn't appear when summer and winter data are analyzed separately (Table 6). The correlations of water temperature with the other microbial and mycological counts are not significant.

3.4 Correlation with the number of swimmers

A significant correlation appears between the number of swimmers present on the beach and the *S. aureus* counts in water and sand samples (table 7). This is clearer at site 2, which presents higher swimming frequency than at site 1. There is one clearly significant association between the number of swimmers and the mycological counts. This is between the number of swimmers and yeasts (mainly yeasts of human origin) in water samples of site 2. A weaker correlation appears between the number of swimmers and yeasts of human origin in sand samples of site 2 (table 8).

3.5 Correlation between micro organisms

The Sperman correlation test was applied between the different bacterial and mycological counts. Taking the summer and winter data together, a very strong correlation exists between coliforms and faecal coliforms in water samples (rs = 0.535, t40 = 4.00, P = 0.0003 for site 1 and rs = 0.870, t40 = 11.2, P = 0.00001 for site 2) and in sand samples (rs = 0.763, t40 = 7.47, P = 0.0001 for site 1 and rs = 0.653, t40 = 5.45, P = 0.0001 for site 2). There is a very strong correlation between faecal coliforms and enterococci in water samples (rs = 0.521, t40 = 3.56, P = 0.0001 for site 1 and rs = 0.583, t40 = 4.18 and P = 0.002 for site 2). There is not any good correlation between coliforms and enterococci nor between *S. aureus* counts and the enteric bacteria counts. There is a significant correlation between the *S. aureus* counts and the presence of yeasts of human origin in sand samples (P = 0.012).

4. CONCLUSIONS

From the data collected from seawater and sand and their statistical evaluation, it is obvious that the number of bathers may influence the bacteriological quality of seawater and sand. The two bathing areas investigated are located at the same coastal zone and their bacteriological quality, judged by faecal indicators, was very similar. *Staphylococcus aureus*, and the pathogenic fungi *Candida albicans* and *Pitirosporum orbiculare* seem to correlate with the number of bathers (tables 7 and 8) especially in site 2.

Table 1

	All days (n=42)		Days were non-zero/above lower limit*				COMPARISON	
	SITE 1	SITE 2	SITE 1		SITE 2			
ORGANISM	median	median	n	median	n	median	z	p
WATER								
Coliforms	11.0	21.5	35	22.0	38	22.5	2.41	0.016
Faecal coliforms	1.5	9.5	25	4.0	30	17.5	2.98	0.003
Enterococci	4.5	12.0	25	13.0	31	30.0	2.88	0.004
S. aureus	5.0	6.5	31	8.0	33	11.0	2.16	0.031
SAND								
Coliforms	10	10	13	130	18	255	1.76	0.08
Faecal coliforms	10	10	8	100	9	110	0.46	0.65
Enterococci	10	10	9	30	15	155	2.43	0.015
S. aureus	20.0	30.0	31	50	31	70	1.98	0.048

n = 36 (n = 42 otherwise)
Lower limit = 2 Staphylococci (water)
10 coliforms (sand),
Faecal coliforms (sand),
enterococci (sand)
20 staphylococci (sand)

* Wilcoxon's test.

Table 2

Species of yeasts and moulds isolated from water and sand samples

ORGANISM	WATER	SAND
YEASTS		
C. albicans*	+	+
C. Krusei	+	+
C. tropicalis	-	+
C. parapsilosis	+	-
C. puilliermondi	+	+
C. catenulata	+	-
C. curvata	+	-
C. rugosa	+++	+
C. maritima	+	-
C. zeylonoides	+	-
C. rugela	+	-
C. salmonicola	+	-
Candida sp.	+	+
T. cutuneum	+	-
T. glabrata	+	-
T. colliculosa	+	-
Toluropsis sp.	+++	-
Rhodotorula	+	+
Sacharomuces	+	-
Geotrichum	+	+
Trichosporum	+	-
Pichia	+	-
Criptococcus albidus	+	-
*Pitirosporum orbicular**	+	+
MOULDS		
		+
Penicillium	+	+
Altenaria	+	+
A. niger	+	
A. fumigatus	+	
A. glancus	+	+
Aspergillus	++	+
Fusarium	+	
Nigrospara	+	
Cephalosporium	+	
Epicoccum	+	
M. sterile	+	
Mucor	+	+
Rhizopus	+	
Chrysosporium	-	+
Helminthosporium	-	+
Cladosporium	+	

* Pathogenic for human

Table 3

Frequency of presence/absence of human and non-human yeasts

	SITE 1		SITE 2	
ORGANISM	n(days) 42	%	n(days) 42	%
YEASTS (WATER)				
Presence	29	69	33	79
Environmental	4	10	2	5
Human	14	33	23	55
YEASTS (SAND)				
Presence	11	26	10	24
Environmental	0	0	1	2
Human	7	17	6	14

Table 4

Values of microbial parameters during winter and summer periods

	OCTOBER-MAY (n=21)				JUNE-SEPTEMBER (n=21)				DIFFERENCE MANN-WHITNEY uTEST			
	Median				Median							
	Zero/below limit				Zero/below limit				All days		Non-zero/limit	
ORGANISM	n	%	All days	Above limit	n	%	All days	Above limit	z	p	z	p
SITE 1												
WATER												
Coliforms	3	14	12	18.5	4	19	10	22	0.49	0.62	0.99	0.32
Faecal coliforms	10	48	1	4	7	33	2	7	0.98	0.33	0.33	0.74
Enterococci*	8	40	3.5	9	3	19	8.5	30	1.52	0.13	0.71	0.48
S. aureus	9	43	2	2	2	10	8	13	3.84	0.0001	3.16	0.002
SAND												
Coliforms	14	67	10	130	15	71	10	825	0.08	0.94	1.86	0.063
Faecal coliforms	17	81	10	80	17	81	10	195	0.15	0.88	1.15	0.25
Enterococci*	14	70	10	20	13	81	10	30	0.75	0.45	0.00	1.00
S. aureus	11	52	20	20	0	0	50	50	4.51	0.0001	2.61	0.009
SITE 2												
WATER												
Coliforms	2	10	50	58	2	10	15	20	1.98	0.048	2.30	0.021
Faecal coliforms	3	14	14	17.5	9	43	2	17.5	2.17	0.030	0.93	0.35
Enterococci*	3	15	12	21	2	13	20	35	0.42	0.68	0.36	0.72
S. aureus	7	33	2	3	2	10	20	20	4.20	0.0001	4.16	0.0001
SAND												
Coliforms	11	52	10	255	13	62	10	305	0.31	0.76	0.89	0.37
Faecal coliforms	16	76	10	180	17	81	10	40	0.60	0.55	1.61	0.11
Enterococci*	14	70	10	10	7	44	25	170	1.85	0.064	1.20	0.23
S. aureus	11	52	20	20	0	0	80	80	4.67	0.0001	2.89	0.004

* n=20 October-May, n=16 June-September.

Table 5

Distribution of positive for fungi samples during summer and winter periods

	October-May (n=42)		June-September (n=42)		Difference x 1	P
	Present		Present			
	n	%	n	%		
YEASTS (WATER)						
Presence						
Human	33	79	29	69	0.55	0.46
	23	55	14	33	3.09	0.08
MOULDS (WATER)						
Presence/ (Environmental)	38	90	34	81	0.88	0.35
YEASTS (SAND)						
Presence	7	17	14	33	2.29	0.13
Human	3	7	10	24	3.28	0.07

Table 6

Temperature	ALL			SUMMER			WINTER		
	rs*	t19	p	rs	t19	p	rs	t19	p
SITE 1									
S. aureus-water	0.581	4.52	0.0001	0.275	1.25	0.23	0.123	0.54	0.59
S. aureus-sand	0.672	5.74	0.0001	0.087	0.38	0.71	0.186	0.83	0.42
SITE 2									
S. aureus-water	0.603	4.78	0.0001	0.235	1.05	0.31	0.056	0.25	0.81
S. aureus-sand	0.772	6.60	0.0001	0.216	0.96	0.50	0.204	0.91	0.38

* Sperman rank correlation.

Table 7

Correlation between the number of bathers and microbial counts

	SITE 1			SITE 2		
	rs*	t19	p	rs	t19	p
Temperature	0.236	1.06	0.30	0.024	0.11	0.92
WATER						
Coliforms	0.421	2.02	0.058	0.083	0.36	0.72
Faecal coliforms	0.377	1.77	0.09	0.235	1.06	0.30
Enterococci*	0.295	1.15	0.27	0.055	0.21	0.84
S. aureus	0.393	1.86	0.078	0.688	4.13	0.0001
SAND						
Coliforms	-0.202	-0.90	0.38	-0.063	-0.28	0.79
Faecal coliforms	-0.143	-0.63	0.54	-0.050	-0.22	0.83
Enterococci*	0.026	0.10	0.92	0.230	0.88	0.39

* Sperman rank correlation.

Table 8

Correlation between the number of bathers and fungi present

ORGANISM	SITE 1				SITE 2			
	Mean no. of swimmers		Mann-Whitney		Mean no. of swimmers		Mann-Whitney	
	Absent	Present	z	p	Absent	Present	z	p
WATER								
Yeasts (presence)	28.6	7.5	0.56	0.57	13.7	47.9	2.42	0.016
Yeasts (human)	16.4	6.5	0.13	0.89	26.5	50.9	1.87	0.061
Moulds (presence/ environmental)	27.5	9.3	1.91	0.056	33.5	38.6	0.30	0.76
SAND								
Yeasts (presence)	10.8	22.0	0.97	0.33	30.6	53.1	1.61	0.11
Yeasts (human	10.3	25.2	1.21	0.23	31.8	65.0	1.93	0.054

EVALUATION OF VIRAL CONTAMINATION OF SHELLFISH THROUGH ENUMERATION OF PHAGES INFECTING *BACTEROIDES FRAGILIS*

by

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1. INTRODUCTION

There is growing concern about the discharge of human enteric viruses into estuarine and marine environments, where they could present health hazards in areas that are used either for recreational purposes or from which shellfish are harvested for human consumption. Human enteric viruses are introduced into the sea by means of inflowing raw or partially treated sewage, polluted rivers and by offshore sludge dumping practices. Shellfish such as oysters, mussels and clams growing in polluted waters may become contaminated. Because these species are often eaten raw or lightly cooked, the risk of infection is high. Molluscan shellfish are well identified as vectors of some human enteric viruses such as hepatitis A virus (HAV) and small round structured gastroenteritis viruses (Norwalk and Snow Mountain Agents), and therefore consumption of raw or inadequately cooked contaminated shellfish is considered a health hazard (Du Pont, 1986; Wanke and Guerrant, 1990).

In order to minimize the health risk of shellfish consumption, the sanitary quality of shellfish should be guarantied. For it two measures should be implemented. First, the present regulations based on bacterial standards must be properly enforced. However present regulations have not been fully successful in preventing viral transmission through shellfish consumption (Gerba and Goyal, 1979; Gill *et al.*, 1983; Portnoy *et al.*, 1975). Second new regulations should be enacted. Present day advances in methodology of viral recovery and identification will yield further information on quality requirements of growing waters as well as requirements of the depuration process. This knowledge, plus the establishment of some quality controls for growing waters, depuration processes and marketable shellfish based on viral standards, perhaps by means of some indicator organism like bacteriophages (IAWPRC, 1991) will minimize the risk of viral infections through shellfish consumption.

Phages potentially to be used as indicators of viruses in shellfish and estuarine waters include F-specific coliphages (Havelaar and Hogeboom, 1984; IAWPRC, 1991) and bacteriophages infecting Bacteroides fragilis (Jofre *et al.*, 1986; Tartera and Jofre, 1987). Somatic coliphages which are the more abundant and easy to count had been discarded since they can replicate in environment under some conditions (Vaughn and Metcalf, 1974).

This study has been performed in order to gain some information about the potential usefulness of phages infecting *Bacteroides fragilis* as indicators of human viruses in shellfish. For it, the following studies were performed using mussels: methods to recover phages infecting *Bacteroides fragilis* and their numbers as compared to numbers of other phages, bacteria and enteroviruses in mussels with different levels of contamination.

2. MATERIALS AND METHODS

2.1 Mussels

Naturally occurring mussels (*Mytilus edulis*) were harvested in polluted areas in order to set the methodology. Naturally occurring mussels belonging to sampling sites 1, 2, 3, and 4 (see next paragraph) were harvested during a 7 month period, from the beginning of June to the beginning of December, at a constant depth, between 2 and 4 meters. An additional set of naturally occurring mussels were harvested in a sampling point located between sampling sites 1 and 2 (in this set there was not any distribution through the year). Thirty two samples of mussels were obtained from different markets in Barcelona.

Samples were always refrigerated until they were processed, which was within 24 hours of collection. The mussels were washed and scrubbed thoroughly in running tapwater, surface disinfected with 70% alcohol, and opened aseptically, before processing for the different microbial analysis.

2.2 Description of the area

Naturally occurring mussels were collected at four sampling sites along a coastline which has the influx of a small river that is heavily polluted as the single major source of pollution. The average microbial load is indicated in Figure 1. During the summer months, the flow of the river and its microbial load decrease and consequently the microbial pollution of the sea is also reduced. Reasons for the decrease in the microbial load are diverse, among them effects of temperature and solar radiation on selfpurification and treatment of part of the river water prior to discharge into the sea. The profile of the shoreline at the study area is straight and faces South East. Sampling site 1 is located 350 m South of the polluting focus. The other three sites are located 1000 m (site 2), 3500 m (site 3) and 5000 m (site 4) North of the river mouth. No tides occur in the area, as is the case in all the Mediterranean Sea. Water movement in the area has two components, namely weak currents which flow mostly North-South and swells most frequently from South to North. Salinity of the water ranged from an average 33% at sampling site 1 to an average 34.5% at sampling site 4. Water temperature during the study period ranged from 15E C to 23E C. Bacterial loads at the different sampling sites are indicated in Figure 2. In Figure 3 is shown the seasonal variation of levels of bacteria in seawater at the sampling site 1. Variations in the other sampling sites parallel that of sampling site 1.

A fifth site was located between sampling sites 1 and 2.

2.3 Recovery of bacteria from mussels

Firstly, mussel meat of a minimum of 30 mussels per sample was homogenized in a blender for 5 minutes. The homogenate was used to perform the bacterial analysis without further processing.

2.4 Recovery of phages from mussels

2.4.1 Extraction methods

Four methods were assayed.

Method 1 (beef extract - borate buffer; Lemaitre, 1990). 100 ml of 3% beef extract-borate buffer (0.1 M, pH 9.5) were added to 100 g of mussel meat. After homogenization, 300 ml of the buffer was added to the homogenate and the pH was adjusted to 9.5. The mixture was stirred for 15 minutes. Then the pH was adjusted to 7.2. Finally the homogenate was clarified by centrifugation at 3200 r.p.m. for 15 minutes at 4E C. Phages were enumerated from the supernatant and in a few cases also from the homogenate before clarification.

Method 2 (glycine buffer). 100 ml of glycine buffer (0.25 M, pH 10.5) were added to 100 g of mussel meat. After homogenization, 250 ml of buffer were added to the homogenate and the pH was adjusted to 7.2. Finally, the homogenate was clarified by centrifugation at 3500 r.p.m. for 15 minutes at 4E C. Phages were evaluated from the supernatant.

Method 3 (phosphate buffer). 100 ml of phosphate buffer (Na_2HPO_4, 7g; KH_2PO_4, 3g; NaCl, 5g; $MgSO_4$ 0.1 M, 10 ml) were added to 100 g of mussel meat. After homogenization, 250 ml of buffer were added to the homogenate and the pH was adjusted to 10.5. The mixture was stirred for 15 minutes. Then the pH was adjusted to 7.2. Finally, the homogenate was clarified by centrifugation at 3500 rpm for 15 minutes at 4E C. Phages were evaluated from the supernatant.

Method 4 (Method described for the recovery of enteroviruses by Sobsey, 1978). 100 g of homogenized mussel meat were mixed with 700 ml of distilled water. pH was adjusted to 4.5 and the conductivity to 2000 mg per liter of ClNa. After centrifugation at 3500 rpm for 15 minutes, the supernatant was discarded. The pellet was resuspended in 7 volumes of buffer-glycine-saline pH 7.5 and the final pH was adjusted to 7.5 and the conductivity to around 8000 mg/l. Finally the homogenate was clarified by centrifugation at 3500 rpm for 15 minutes. Phages were evaluated from the supernatant.

All samples of naturally occurring mussels as well as those of mussels purchased in markets were tested according to method 1.

2.5 Concentration of phages from the supernatants after extraction

Two methods were assayed to concentrate phages from the supernatants after extraction. Method one was ultrafiltration (Pellicon System) and method two was organic floculation as described by Katzenelson (1976). Briefly, beef extract was added to a final concentration of 3%. pH was then adjusted at 3.5. The mixture was gently shaken for 30 minutes at room temperature. During this period flocculation occurs. Then the mixture was centrifuged for 20 minutes at 4500 rpm. Finally the pellet was resuspended in 50 ml of PBS.

2.6 Methods for the recovery of enteric viruses

Two methods were used for the recovery of enteric viruses from mussels samples.

Method 1 (beef extract-borate buffer). 100 ml of 3% beef extract-borate buffer (0.1 M, pH 9.5) were added to 100 g of mussel meat. After homogenization, 300 ml of the buffer was added to the homogenate and the pH was adjusted to 9.5. The mixture was stirred for 15 minutes. Then the pH was adjusted to 7.2 Finally the homogenate was clarified by centrifugation at 3200 rpm for 15 minutes at 4E C. Enteroviruses present in this cleared suspension were concentrated by organic floculation as indicated above.

Method 2 (method described by Sobsey for enteroviruses, 1978). 100 g of homogenized mussel meat were mixed with 700 ml of distilled water. pH was adjusted to 4.5 and the conductivity to 2000 mg per liter of ClNa. After centrifugation at 3500 rmp for 15 minutes, the supernatant was discarded. The pellet was resuspended in 7 volumes of buffer-glycine-saline pH 7.5 and the final pH was adjusted to 7.5 and the conductivity to > 8000 mg/l. Finally the homogenate was clarified by centrifugation at 3500 rpm for 15 minutes. Phages were evaluated from the supernatant.

2.7 Water samples

Water samples were collected monthly over an 8 month period from the beginning of May to the beginning of December. Seawater samples were collected 4 or 5 m offshore at a depth of 30 cm at the same sites where mussels were harvested. Seawater samples and samples of the polluting water course were collected in sterile containers, placed at 4E C immediately after collection and processed within 12 hours of collection. Only samples to be analyzed for *B. fragilis* phages received treatment prior to being assayed. This was a decontamination by filtration through polyvinylidene difluoride (PVDF)(Millipore) membrane filters as described elsewhere (Tartera *et al.*, 1992).

2.8 Bacteriological analysis

Total and faecal coliforms numbers in mussels were determined according to the method proposed by WHO for the Mediterranean area (WHO and UNEP, 1987), briefly Most Probable Numbers of total coliforms were determined with lactose broth (WHO and UNEP, 1987), positive tubes of lactose broth were used to inoculate Brilliant Green - 2% bile salts (WHO and UNEP, 1987) that were then incubated at 44.5E C. Tubes showing growth after 24 hours were recorded as confirmed for the presence of faecal coliforms. Faecal streptococci concentration were determined with Enterococcus Agar (Difco) as recommended by Yoshpe-Purer for marine environments (Yoshpe-Purer, 1989). *Clostridium perfringens* in mussels was determined according to Handford (1974). Detection limits for the different microorganisms were 2 per gram. Total coliforms, faecal coliforms and faecal streptococci in water were determined according to Standard Methods (APHA, 1971). In seawater samples faecal streptococci were determined according to Yoshpe-Purer (1989). *Clostridium perfringens* in water were determined according to Handford (1974).

2.9 Bacteriophage analysis

Bacteroides fragilis strain HSP_4O, grown on BPRM (Tartera *et al.*, 1992) was used in the quantification of *B. fragilis* phages. *E. coli* HS (pF amp)R, grown on Tryptone Agar supplemented with streptomycin (15 mg/l) and ampicillin (15 mg/l) was used for the quantification of F-specific coliphages (Debartolomeis and Cabelli, 1991). *E. coli* CN13 (Armon *et al.*, 1988) grown on Nutrient Agar containing nalidixic acid (100 mg/l), was used for the quantification of somatic coliphages (*E. coli* strains were kindly provided by R. Armon). All phages were quantified by the double-agar-layer method. Presence/absence tests were performed by enrichment and subsequent testing by the spot test. To measure phages in water

a minimum volume of 10 ml was titrated. To measure phages in mussel homogenates 10x1ml (equivalent to 2.5 g) replicates were performed for each homogenate for F-specific coliphages and phages of *B. fragilis*, and 5x1ml plus 5x0.1 ml replicas for somatic coliphages (equivalent to 2.375 g of mussel flesh). Presence/absence test were performed using the equivalent of 10 g of mussel meat.

2.10 Viral analysis

Enteroviruses were quantified by plaque-formation by inoculating confluent BGM cell monolayers as described elsewhere (Bosch *et al.*, 1986).

3. RESULTS

3.1 Methods to recover phages from mussels meat

Four methods were tested for the recovery of bacteriophages from mussel meat. Results shown in Table 1 indicate that there are not significant differences among the performances of the different methods. Only two methods seem to perform worse than the others for a particular group of bacteriophages. These differences refer to the elution with glycine buffer which seems to perform poorly for male specific coliphages and the method described by Sobsey which seems to perform poorly for phages infecting *Bacteroides fragilis*.

Regarding the realization of a final clarification steep in the extraction of viruses from mussels meat, results in Table 2 show that clarification by centrifugation improves the extraction. Better results are observed either when viruses were titrated by the PFU method or when presence of viruses in decreasing volumes of homogenate was tested through presence/absence tests.

Two methods of concentration of extracted bacteriophages were tested. None of them, probably due to the high content of organic matter of the final cleared suspension, gave good results (Table 3). However, ultrafiltration performed clearly better than organic floculation. The main problem of using ultrafiltration is that membranes clog very fast.

3.2 Microbial levels of naturally occurring mussels

Tables 4, 5, 6 and 7 show the microbial levels of mussels collected at sampling sites 1, 2, 3 and 4 during the year.

Levels of faecal bacteria in mussels showed an obvious decrease from the most contaminated to the least contaminated sampling site. In mussels from all sampling sites, *Clostridium perfringens* was more abundant than faecal coliforms and faecal streptococci. Except in sampling site number 4, faecal streptococci overnumbered faecal coliforms. However the high number of samples under the limit of detection of sampling point 4 may be the reason. At the four sites levels of total coliforms, faecal coliforms and faecal streptococci in mussels paralleled their levels in water. Bacterial concentrations per 100 g in mussels were far greater than bacterial levels in 100 ml of water in the area of each sampling site, indicating the occurrence of bioaccumulation. The seasonal distribution of bacteria also shows a clear variation that parallels the distribution of bacteria in waters (Figure 4). These results show that mussels in the area and periods in which they were collected behaved as expected.

The levels of bacteriophages in mussels showed an obvious decrease from the most contaminated to the least contaminated sampling sites (Tables 4, 5, 6 and 7). Temporary variations were also observed in the levels of bacteriophages in mussels. Variations in the four sampling sites were comparable. Figure 5 shows such variation in mussels collected at sampling site 1. These variations paralleled those observed in bacterial levels in water and mussels. Comparison of geometric means indicates that somatic coliphages and phages infecting *Bacteroides fragilis* decreased approximately in the same proportion from site 1 to site 4. However, the magnitude of the decrease of male specific coliphages was significantly greater. The calculation of the ratios between the different pairs of phages applied sample to sample clearly demonstrates that relative concentrations of F specific coliphages decrease with the distance from the main polluting focus (Figure 6).

Unfortunately, the levels of enteroviruses detected in these sets of mussels were extremely low and no comparisons could be established. We can not explain the low levels found in the more contaminated sampling sites. The only reason we can think of is that the method of recovery was not efficient enough.

3.3 Microbial levels of mussels purchased in markets

Levels of bacteria in mussels purchased in markets (Table 9) are lower than levels of bacteria in mussels collected in the less contaminated sampling site (sampling site 4). 75% of samples fulfilled the WHO regulations with regard to the levels of faecal coliforms (0 to 2 faecal coliforms per gram of shellfish flesh), the rest had between 3 and 8 faecal coliforms per 100 g of mussels flesh, which do not fulfil the criteria (Helmer *et al.*, 1991).

No enteric viruses were detected in the samples analyzed. Regarding bacteriophages the results of mussels purchased in markets confirm the observations made in naturally occurring mussels. Levels of F-specific coliphages and phages infecting *Bacteroides fragilis* are very similar, as it happens in sampling site 4, whereas levels of somatic coliphages are higher.

3.4 Relationship between phage and enteroviruses concentration

In order to study the relationship between the concentrations of enteroviruses and phages infecting *B. fragilis* in mussel meat, 21 sets of mussels were collected at different sampling sites located between sampling points 1 and 2. The results in Table 10 show that phages infecting *Bacteroides fragilis* were isolated more frequently than enteroviruses, 90.5% versus 23% of positive isolations. Results shown in tables 4, 5, 6 and 7 as well as results of Table 9 clearly confirm this observation. Moreover in none of the samples were enteroviruses isolated and phages of *B. fragilis* not. Further the levels of phages were significantly higher than those of enteroviruses. Due to the high number of samples in which no enteroviruses were isolated, it is difficult to establish an actual ratio. Moreover, making the supposition more advantageous to enteroviruses, that is, considering that in all cases in which enteroviruses were not isolated the value was the highest possible below the detection limit of the method used, the ratio numbers of phages of *B. fragilis*: numbers of enteroviruses is 22.1.

Table 1

Efficiency of recovery of the different types of bacteriophages according to the methods used to elute bacteriophages from mussels

Phage	Range of phages in the samples (per 100 ml)	Extraction method			
		Method 1	Method 2	Method 3	Method 4
B. fragilis	< 45 to 6526	1*	1.2±1.1 (9/16)**	1.0±0.7 (6/13)	0.6±1.0 (2/6) 0.4±0.2 (0/9)**
Somatic coliphages	180 to $1.6x10^5$	1	1.2±0.3 (3/6)	1.3±1.1 (5/7)	Not done
Male-specific coliphages	< 45 to $8.7x10^4$	1	0.7±0.3 (0/6)	1.2±0.3 (5/7)	Not done

* Values of method 1 were always considered as 1 and values reported for the other methods are always the fraction between the real value and the value with method 1

** Numbers in brackets indicate the fraction of samples with higher results with the method indicated as compared to method 1. For example 9/16 means that from 16 different experiments in which we compared method 1 with method 2 in 9 occasions the value obtained with method 2 evernumbered values obtained with method 1. At the same time gives an indication of the number of tests performed with each method.

*** Values of seeded homogenates. All the other values are of natural samples

Table 2

Recovery of bacteriophages infecting *Bacteroides fragilis* either after extraction by method 1. Phages were counted before clarification of the homogenate treated to extract the bacteriophages (A) and after clarification by centrifugation (B)

Method of titration	Number of samples	Phages in	
		A	B
PFUs	6	1*	2.5 (± 0.9)
P/A*	24	P(18)/A(11)**	P(15)/A(9)

Table 3

Efficiencies of concentration of viruses extracted from mussel meat

Method	Number of samples	% recovery (± SD)
Ultrafiltration	8	44.1 (± 23.4)
Organic flocculation	8	16.1 (± 7.6)

Table 4

Microbial load of mussels collected in sampling site 1.
Values are given for 100 g of mussel meat

Microbial parameter samples	Number of samples	Geometric mean (SD)	Values		% positive samples
			Max	Min	
ENT*	11	-	-	< 20	0
BFB	11	$3.3x10^2$ (5.2)	$1.3x10^4$	45	100
SC	11	$4.6x10^4$ (70)	$1.8x10^5$	180	100
F+C	11	$5.6x10^3$ (117)	$7.3x10^4$	9	100
TC	11	$7.0x10^4$ ($9.7x10$)	$2.4x10^5$	$1.1x10^3$	100
FC	11	$4.9x10^3$ ($2.2x10$)	$2.4x10^5$	< 200	90
FS	11	$1.9x10^4$ ($1.8x10$)	$1.6x10^5$	< 200	100
CP	11	$8.1x10^4$ (446)	$6x10^5$	$6x10^3$	100

ENT = enteric viruses; BFB = bacteriophages of *Bacteroides fragilis*;
SC = somatic coliphages; F+C = male specific coliphages;
TC = total coliforms; FC = faecal coliforms;
FC = faecal streptococci; CP = Clostridium perfringens
* Enteroviruses were recovered by method 1.

Table 5

Microbial load of mussels collected in sampling site 2.
Values are given for 100 g of mussel meat

Microbial parameter samples	Number of samples	Geometric mean (SD)	Values		% positive samples
			Max	Min	
ENT*	9	-	25	20	11
BFB	8	83 (10)	$9.5x10^3$	< 9	87.5
SC	9	$1.3x10^4$ (19)	$1.1x10^5$	9	100
F+C	9	$3.9x10^2$ (20)	$2.9x10^4$	9	100
TC	9	$1.2x10^4$ (1.0x10)	$2.4x10^5$	800	100
FC	9	$1.0x10^3$ (7.1x10)	$9.2x10^4$	< 200	88
FS	9	$5.2x10^3$ (84)	$8.2x10^4$	< 200	88
CP	9	$8.3x10^3$ (1.9x10)	$1.5x10^5$	< 200	77

ENT = enteric viruses; BFB = bacteriophages of *Bacteroides fragilis*;
SC = somatic coliphages; F+C = male specific coliphages;
TC = total coliforms; FC = faecal coliforms;
FC = faecal streptococci; CP = Clostridium perfringens
* Enteroviruses were recovered by method 1.

Table 6

Microbial load of mussels collected in sampling site 3.
Values are given for 100 g of mussel meat

Microbial parameter samples	Number of samples	Geometric mean (SD)	Values		% positive samples
			Max	Min	
ENT*	8	-	-	< 20	0
BFB	7	75.8 (20.8)	$2.3x10^4$	9	100
SC	8	$1.4x10^4$ (52)	$2.3x10^5$	$1.8x10^3$	100
F+C	8	89.0 (17.3)	$8.7x10^3$	9	100
TC	9	$3.9x10^3$ (6.0x10)	$9.2x10^4$	200	100
FC	9	$8.7x10^2$ (5.5x10)	$1.4x10^4$	< 200	77
FS	9	218.0 (147.0)	$9.0x10^4$	< 200	66
CP	9	$1.3x10^3$ (8.7x10)	$9.0x10^4$	< 200	88

ENT = enteric viruses; BFB = bacteriophages of *Bacteroides fragilis*;
SC = somatic coliphages; F+C = male specific coliphages;
TC = total coliforms; FC = faecal coliforms;
FC = faecal streptococci; CP = Clostridium perfringens
* Enteroviruses were recovered by method 1.

Table 7

Microbial load of mussels collected in sampling site 4.
Values are given for 100 g of mussel meat

Microbial parameter samples	Number of samples	Geometric mean (SD)	Values		% positive samples
			Max	Min	
ENT*	8	-	-	< 20	0
BFB	8	23.0 (6.0)	$9.0x10^2$	< 9	87.5
SC	8	$1.1x10^3$ (15.0)	$4.0x10^3$	9	100
F+C	8	19.4 (8.9)	$4.6x10^3$	9	100
TC	8	$4.0x10^4$ (7.2x10)	$5.4x10^4$	< 200	87.5
FC	8	776.0 (602.0)	$7.9x10^3$	< 200	75
FS	8	575.0 (851.0)	$2.5x10^4$	< 200	50
CP	8	$5.5x10^3$ (1.9x10)	$1.0x10^5$	< 200	75

ENT = enteric viruses; BFB = bacteriophages of *Bacteroides fragilis*;
SC = somatic coliphages; F+C = male specific coliphages;
TC = total coliforms; FC = faecal coliforms;
FC = faecal streptococci; CP = Clostridium perfringens
* Enteroviruses were recovered by method 1.

Table 8

Bacterial load of mussels purchased in markets.
Values are given per 100 g of mussel meat

Microbial parameter samples	Number of samples	Geometric mean*	Values		% positive samples
			Max	Min	
TC	32	256 < < 378	$2.4x10^4$	< 20	90
FC	32	9.2 < < 47.9	800	< 20	43
FS	32	8.5 < < 330	$2x10^3$	< 200	33*
CP	32	48 < < 397	$3.6x10^3$	< 200	60

TC = total coliforms; FC = faecal coliforms;
FC = faecal streptococci; CP = Clostridium perfringens

* Because of the high number of samples with bacterial levels below the limit of detection of the method used, instead of calculating the geometric mean and the standard deviation, we calculated the minimum value considering as 0 the samples below the limit of detection, and the maximum value considering the samples below the limit of detection as the maximum possible below the limit of detection.

Table 9

Levels of viruses and bacteriophages in mussels purchased in markets.
Values are given in presence/absence in the indicated amounts of mussel meat

	ENT* 10g	BFB		SC		F+C	
		2g	10g	2g	10g	2g	10g
P**	0/32	4/28	13/28	11/28	17/28	5/28	13/28
% P***	0	14	46	39	60	17	46

ENT = enteric viruses; BFB = bacteriophages of *Bacteroides fragilis*;
SC = somatic coliphages; F+C = male specific coliphages

* Enterovirus were extracted by method 1 in 16 samples and according to method 2 in the other half

** Positive presences / number of samples tested

*** % of positive presences

Table 10

Microbial load of mussels collected in an additional sampling site located between sampling sites 1 and 2. Values are given for 100 g of mussel meat

Microbial parameter	Number of samples	Geometric mean (SD)	Values		% positive samples
			Max	Min	
ENT*	21	< 28.2	161	< 30	23
BFB	21	239.9 (42)	$4.0x10^3$	< 30	90.5
SC	6	$5.2x10^4$ (316)	$2.2x10^5$	9.5x10	100
F+C	6	257.0 (20)	700	90	100
TC	10	$4.0x10^4$ (47.8)	$4.9x10^5$	6.2x10	100
FC	10	$5.3x10^3$ (67.6)	$6.3x10^4$	< 100	91.6
FS	14	$1.1x10^4$ (95.4)	$1.1x10^5$	< 100	84.6

ENT = enteric viruses; BFB = bacteriophages of *Bacteroides fragilis*;
SC = somatic coliphages; F+C = male specific coliphages;
TC = total coliforms; FC = faecal coliforms;
FC = faecal streptococci; CP = Clostridium perfringens
* Enteroviruses were recovered by method 2.

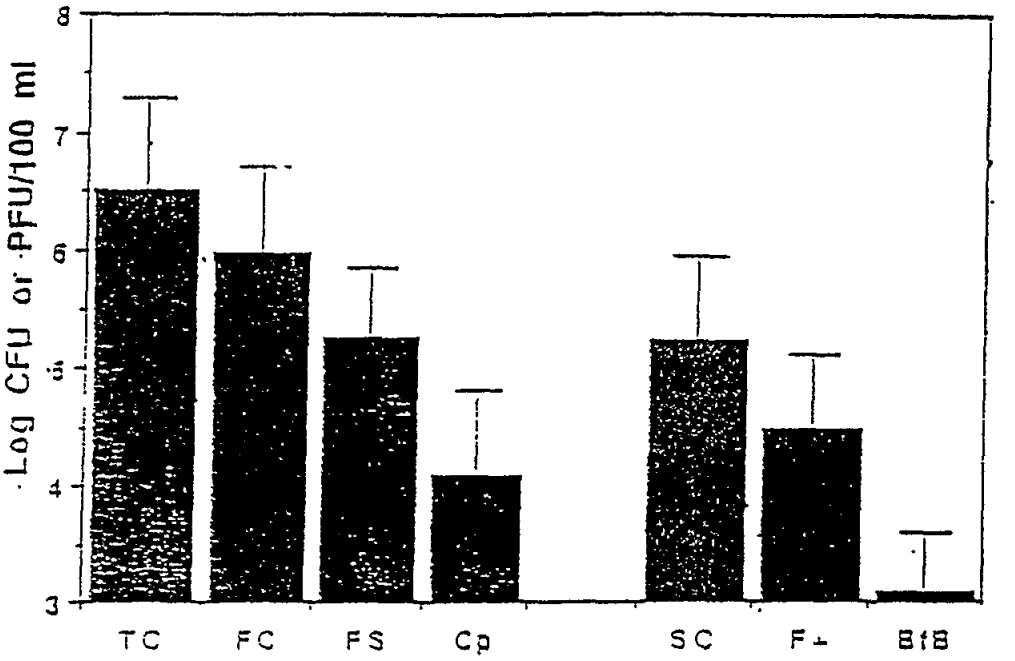

Figure 1. Levels of bacteria and bacteriophages in water from the polluting river.
TC: total coliforms; FC: faecal coliforms;
FS. faecal streptococci Cp: Clostridium perfringens;
SC: somatic coliphages; F+C: F-specific coliphages;
BFB. phages of *Bacteroides fragilis*

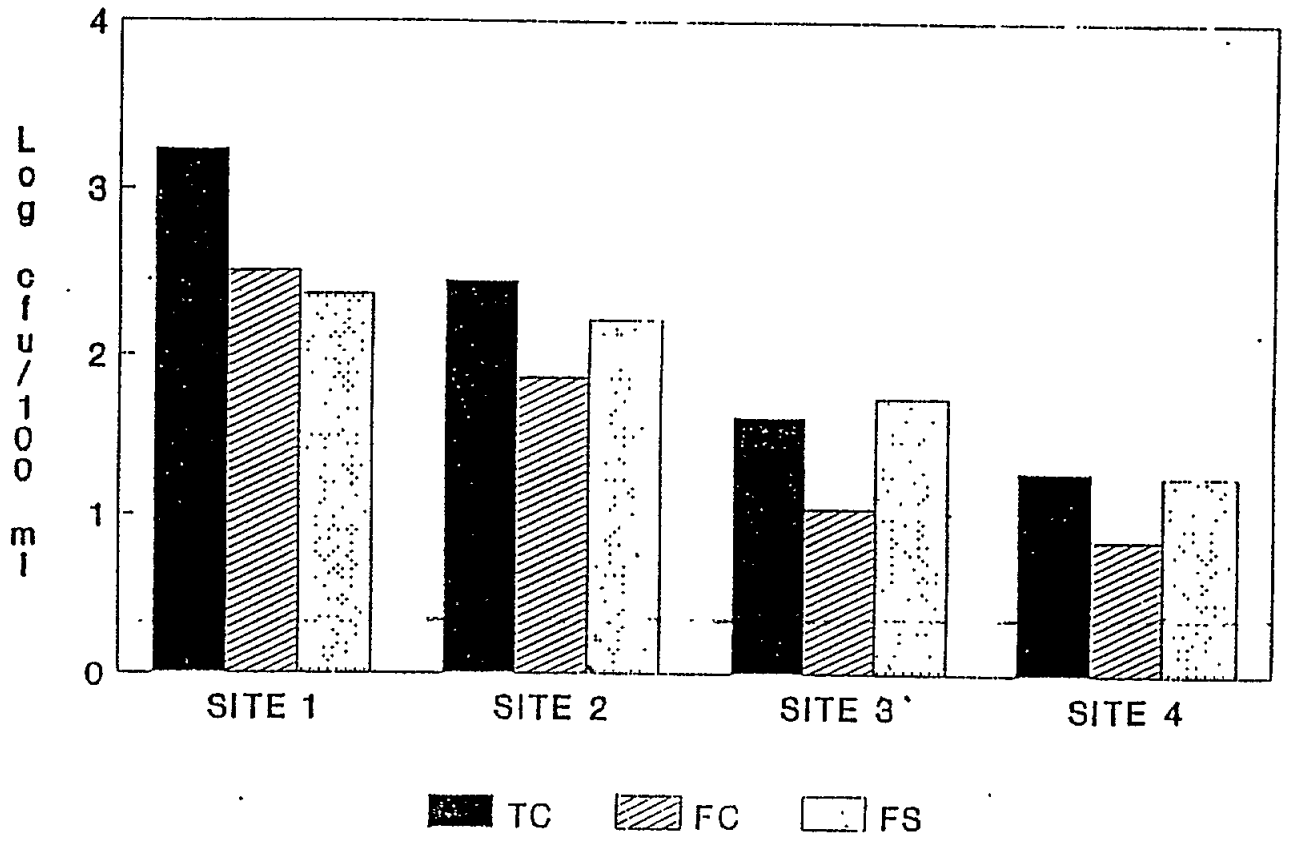

Figure 2. Levels of faecal bacteria in seawater overlaying the sites where mussels were collected.
TC: total coliforms; FC: faecal coliforms
FS: faecal streptococci

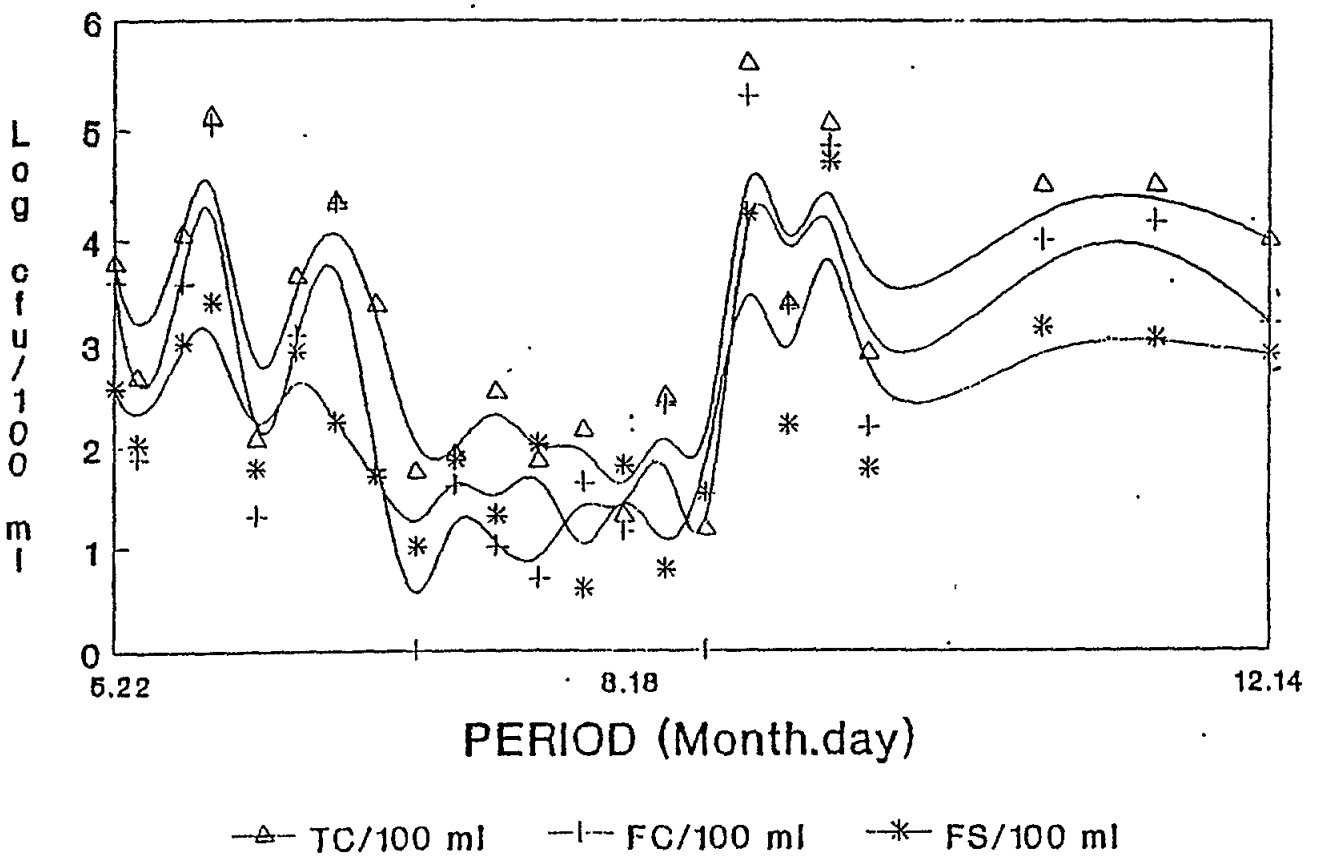

Figure 3. Seasonal variation of levels of faecal bacteria in seawater overlaying collecting site
Δ : total coliforms; + : faecal coliforms; * : faecal streptococci

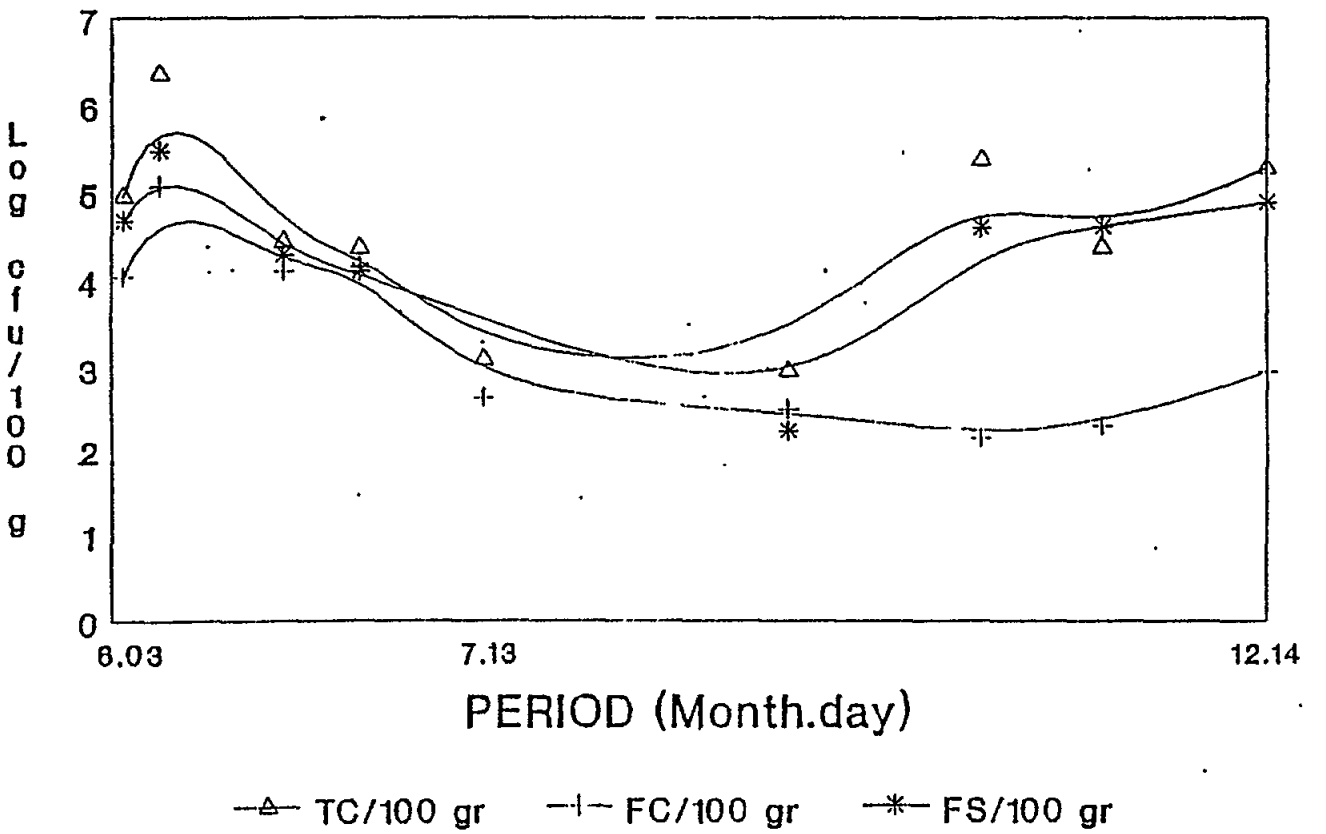

Figure 4. Seasonal variation of levels of faecal bacteria in mussels collected at site 1.
TC: total coliforms; FC: faecal coliforms; FS: faecal streptococci

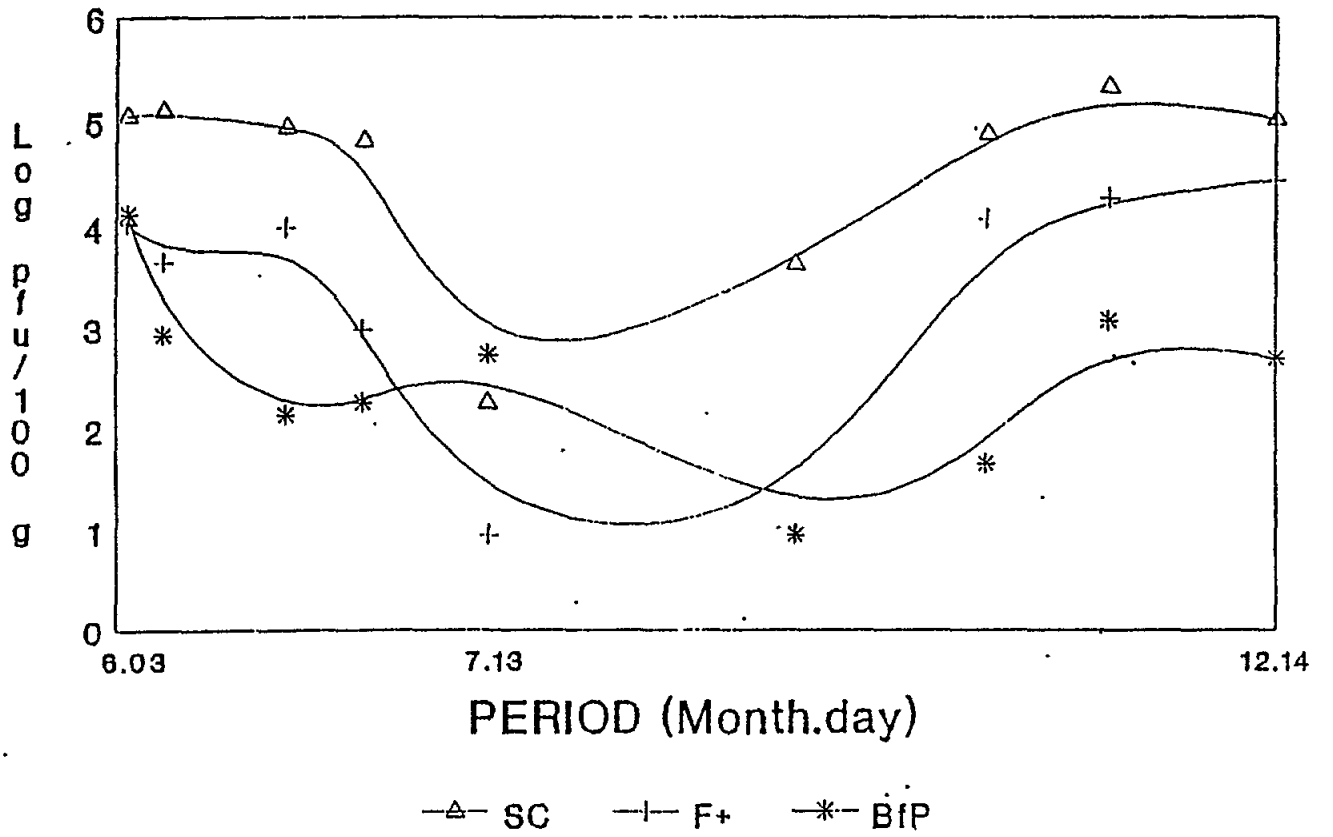

Figure 5. Seasonal variation of levels of bacteriophages in mussels collected at site 1.
Δ : somatic coliphages; +: F-specific coliphages; *: phages of *B. fragilis*

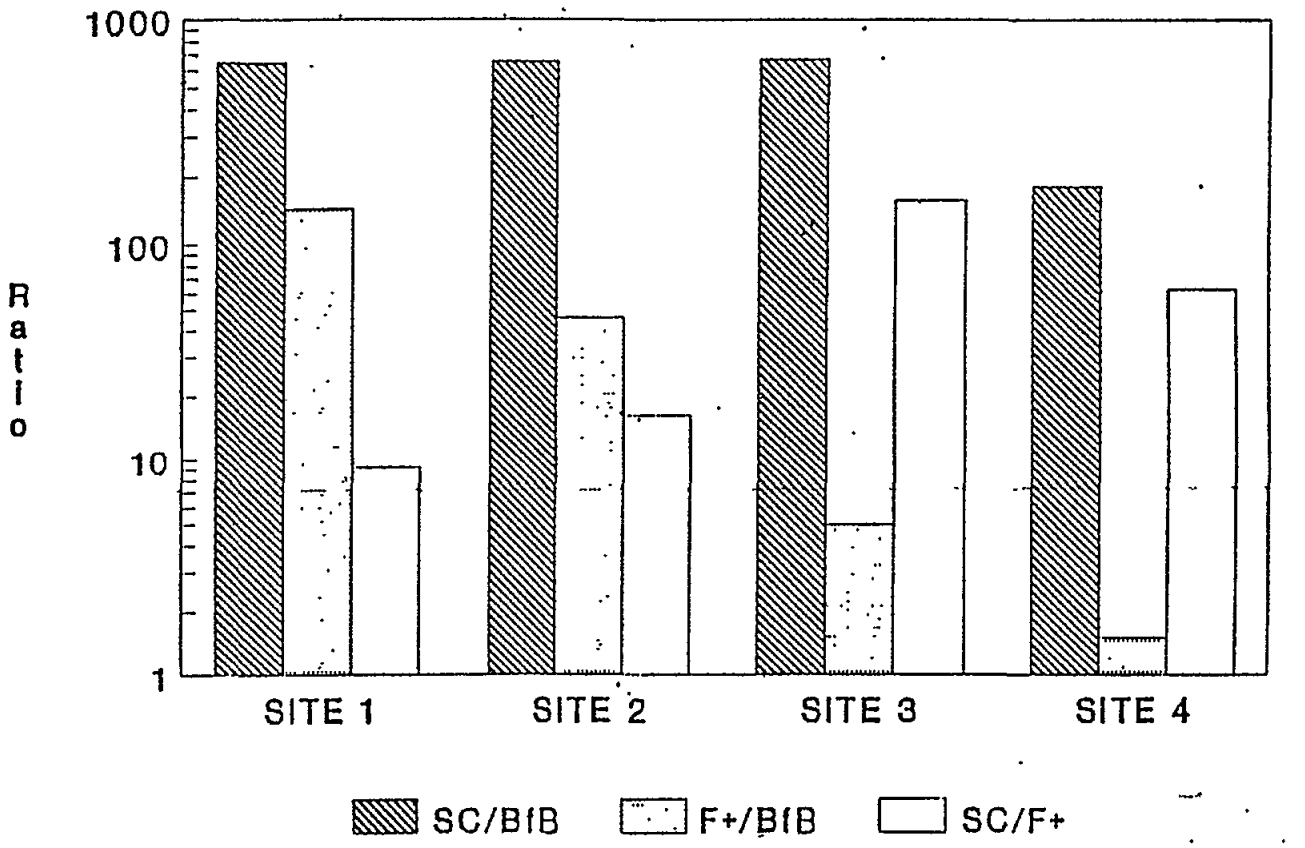

Figure 6. Ratios between (among) levels of bacteriophages in mussels collected at the four sampling sites.
(SC:BfP) somatic coliphages: phages of *B. fragilis*;
(F+:BfP) F-specific coliphages: phages of *B. fragilis*;
(SC:F+) somatic coliphages: F-specific coliphages

4. DISCUSSION AND CONCLUSIONS

Regarding the methodology for the recovery of bacteriophages infecting *Bacteroides fragilis*, results indicating the method of recovery does not influences significantly the recovery rates. These results are in agreement with previous results described for both somatic and F-specific coliphages (Brodisch *et al.*, 1986). According to results presented herein we will recommend either the method of borate-buffer plus beef extract or the method of phosphate buffer.

Regarding the presence of different groups of bacteriophages in mussels with different levels of faecal pollution we can draw two main conclusions. First conclusion refers to the relationship among numbers of present faecal bacterial indicators and bacteriophages and viruses. If we take in consideration the values of different microorganisms in the polluting fresh water and the values of the same microorganisms in mussels collected far away from the polluting focus, it is very clear that with the exception of *Clostridium perfringens*, ratios among phages infecting *Bacteroides fragilis* and somatic coliphages and bacteria favour bacteriophages. The difference between the geometric means of faecal coliforms and phages of *Bacteroides fragilis* in the polluting river is around 3 logs, whereas in any set of naturally occurring mussels studied is 1.5 logs. Behaviour of human enteric viruses seems to be similar to the one of bacteriophages, since previous results in sewage (Tartera *et al.*, 1989) show a difference of more than 5 logs, whereas some data shown here give an indication that the difference may be calculated as between 3 and 4 logs. This may be due to either different inactivation in seawater or to a different bioaccumulation. Further studies of inactivation and bioaccumulation need to be done. However data available strongly suggest that phages infecting *Bacteroides fragilis* behave very much like human enteric viruses regarding its accumulation in shellfish.

The second conclusion refers to relationships among different groups of bacteriophages. At the most polluted site the relative concentrations of the three phages are similar to the relative concentration in the polluted freshwater. Assuming that phages have not yet suffered inactivation, these data show that there are not significant differences in the bioaccumulation rates of the three groups of phages. However as we move further from this site, whereas the relative proportions among somatic coliphages and phages infecting *B. fragilis* do not change significantly, the ratios F-specific coliphages: somatic coliphages and F-specific coliphages: phages infecting *Bacteroides fragilis* clearly decrease. The same sort of information seems to be provided by the presence/absence tests performed with mussels purchased in markets, which are less faecally polluted than those collected in the less polluted sampling site. The decrease in the relative concentration of F+ coliphages in the least polluted sampling sites, most likely reveal differences in the survival rates of the three groups of phages, in the sense that somatic coliphages and phages infecting *B. fragilis* survive longer than F-specific coliphages do. We can conclude that in marine environments such as the one studied here, with salinities higher than 30% and relatively high water temperatures, F-specific coliphages are less useful than the other phages studied to index lasting faecal pollution (i.e. human enteric viruses). Since the usefulness of somatic coliphages for this purpose has been ruled out (Seeley and Primrose, 1980; Vaughn and Metcalf, 1974), and F-specific coliphages inactivate faster than the others, it is our contention that phages infecting *B. fragilis* have some advantages over other phages.

Although there is a requirement for further studies, the data presented here indicate that, using the available techniques, phages infecting *B. fragilis* are most abundant, more than 20 times, than human enteric viruses. A similar difference in concentrations was previously found in sewage (Tartera *et al.*, 1989).

The findings discussed here support further attention on phages infecting *Bacteroides fragilis* as candidates to surrogate indicators for human enteric viruses in shellfish.

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