MEDITERRANEAN ACTION PLAN
MED POL

UNITED NATIONS ENVIRONMENT PROGRAMME

WORLD HEALTH ORGANIZATION

BIOGEOCHEMICAL CYCLES OF SPECIFIC POLLUTANTS (ACTIVITY K)

CYCLES BIOGENOCHIMIQUES DE POLLUANTS SPECIFIQUES (ACTIVITE K)

Final reports on project on survival of pathogenic organisms in seawater

Rapports finaux sur le projet sur la survie des microorganismes pathogènes dans l'eau de mer

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

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

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

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

GENERAL INTRODUCTION

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

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

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

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

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

MED POL - Phase I (1976-1980)

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

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

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

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

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

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

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

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

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

MED POL - Phase II (1981-1990)

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

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

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

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

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

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

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

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

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

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

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

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

This fifty-fifth volume of the MAP Technical Reports Series contains the final report of a research project completed within the framework of MED POL Phase II in Activity K "Biogeochemical cycles of pollutants: survival of pathogens". Final reports on other projects will appear in future issues of the series.

INTRODUCTION GENERALE

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

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

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

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

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

MED POL - Phase I (1976-1980)

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

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

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

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

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

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

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

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

MED POL-Phase II (1981-1990)

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

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

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

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

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

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

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

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

La surveillance continue des polluants affectant le milieu marin de la Méditerranée ainsi que la recherche menée à leur sujet répondent en premier lieu aux prescriptions immédiates et à long terme de la Convention de Barcelone et des protocoles y relatifs, mais elles tiennent

également compte des facteurs requis pour la compréhension des relations existant entre le développement socio-économique de la région et la pollution de la mer Méditerranée.

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

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

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

Ce cinquante-cinquième volume de la Série des rapports techniques du PAM comprend le rapport final sur un projet de recherche terminé dans le cadre de la Phase II du MED POL, dans l'Activité K "Cycles biogéochimiques de polluants spécifiques: survie des Pathogènes". Les rapports finaux sur d'autres projets figureront dans les prochaines publications de la série.

SURVIVAL OF PATHOGENIC MICROORGANISMS IN SEAWATER

by

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1. INTRODUCTION

1.1 Pollution of the marine environment

Industrial progress and population increase of littoral zones, usually produce a series of consequences which negatively affect the environment. Coastal contamination is of particular interest, because it is the result of waste discharges from both industrial and domestic sources.

The polluting agents are of a physical, chemical or biological nature. Temperature, inorganic ions, acids and alkalis, organic matter, toxic and radioactive substances and diverse groups of microorganisms, are the most important. The different types of pollution are defined according to the contaminating agents, their causes and their effects. However, the World Health Organization (WHO, 1977) define the pollution of marine waters as follows:

"Man's introduction into the sea or estuary, either directly or indirectly, oil substances or energies which may have noxious effects. They may damage biological resources, health-hazards to human, be obstacles to marine activities, such as fishing, or cause the deterioration in the quality of sea water considered both for general use and as a recreative medium"

A very important aspect of pollution, which affects human health, is the presence of pathogenic microorganisms in sewage. These microorganisms belong to the following bacteria genera, of which *Salmonella, Shigella, Yersinia, Escherichia, Vibrio, Leptospira, Brucella, Staphylococcus and Pseudomonas,* are the most representative; viruses, such as enteroviruses, rotaviruses, hepatitis B virus; and pathogenic yeasts and protozoa, such as *Candida albicans, Entomoeba histolytica and Giardia lamblia.* These pathogens can cause infections through direct contact or through the consumption of contaminated marine organisms.

This supposition leads to a series of hypotheses which relate the presence of faecal matter in the water to the incidence of illnesses transmitted through water routes in the population (Pipes, 1982a):

(a) A direct and proportional relationship between the amounts of faecal material and the density of the faecal bacterial indicators has been established.

(b) In domestic sewage, the ratio between the densities of a determined faecal microorganism indicator and of that a specific pathogenic microorganism is more or less constant, depending on the incidence of illness present in the population.

(c) There is a direct quantitative relationship between the concentration of pathogenic microorganisms in the water and the incidence of the illness suffered in the human communities. The microbiological pollution produced by the sewage is studied by analysing determined microorganisms or substances whose densities are quantitatively related to potential risks to human health, and which are named indicators of faecal pollution.

To evaluate the quality of natural water, from a microbiological point of view, the microbial indicator should fulfil the following characteristics (Oliveri, 1982):

(1) The microbial indicator must be associated, in a constant and exclusive way, to the source of pollution which carries the pathogenic microorganisms. That indicator organism is detected in contaminated waters wherever the pathogens are present.

(2) The microorganism indicator concentration must always be superior to those of the pathogenic microorganisms.

(3) They must show a greater resistance to both the environmental conditions and sewage treatment processes than the pathogenic microorganisms.

(4) Isolation, enumeration and identification methods of the microbial indicators must be simple, rapid and economical.

Although the criteria for the selection and acceptance of the indicators seem easy, none of the microorganisms or groups of microorganisms considered fulfil all of them. The intended use of the water is also an important factor in the evaluation and consideration of the indicator system chosen.

Classically, the total coliforms, faecal coliforms, faecal streptococci, clostridia, sulphite-reducers and coliphages have been used as indicator organisms. In the last few years *Bifidobacterium bifidum, Bacteroides* spp. and *Lactobacillus* spp. have also been proposed as indicators (Cabelli *et al*, 1976; Bonde, 1977).

The two principal ways in which illness is transmitted when the causal aetiological agent is an allochtonous pathogenic microorganism in the marine environment are:

(a) The ingestion of water or contaminated marine products, which mainly produce gastrointestinal symptoms (Shuval, 1975; Suess, 1977).

(b) The contact of the skin or mucous membrane with the polluted water, causing illnesses known as washing infections. The most significant microorganisms responsible are: *Pseudomonas aeruginosa, Klebsiella* spp., *Staphylococcus aureus* and *Candida albicans.*

Some of the above mentioned microorganisms can grow and multiply in natural waters, although sewage is their principal or primary source. Others are discharged by bathers themselves, and do not have such a strict relationship with the microorganism indicators of faecal pollution (Dutka, 1973; Cabelli, 1978; Gauthier, 1980; Pipes, 1982a).

To choose the criteria involved in deciding the quality of natural waters, epidemiological studies are required, which must allow the evaluation of sanitary risks derived from the use of, or contact with them (Cabelli *et al*., 1974; WHO, 1977). In this way, the cause-effect relationship between the polluted environment and the potential risk of illness may be established (Moore, 1971; Geldreich, 1975).

Unfortunately, these epidemiological studies are scarce, difficult to carry out and occasionally inconclusive. Because of this, and in order to obtain an approximation to the problem, the relationships between the indicator and the pathogen microorganisms associated with the potential risks must be studied, as must other additional factors such as the volume of water consumed by the bather and the actual time he was in contact with the water. Other factors which favour the transmission of the infection from beaches are high temperatures, strong radiation or the incidence of the pathogen in the community (Cabelli, 1977; WHO, 1977); (Pipes, 1982b).

1.2. Survival of allochtonous microorganisms in seawater

The microorganisms contained in sewage are dispersed by the turbulence diffusion where they are discharged into the sea. The adaptable capacity of a microbial cell may be overcome on its contact with the hostile environment, producing physiological damage which may be sublethal or so intensive that it causes the death of the cell. This physiological damage or stress exerted by the marine environment on the allochtonous bacteria can be studied by observing either the bacteria structural disorganization or its inability to carry out a determined metabolic function when grown in a selective medium. However, these stressed cells can be developed in cultural media which do not contain inhibitory substances.

The importance of the study of the physiological damage to pathogenic cells is based on the non-detection of these cells in the performance of standard microbiological tests, which are based on the examination of selective cultural media (Hoadley, 1981).

A large variety of environmental conditions or factors exist which can kill, damage or simply cause the disappearance of microorganisms from the environment. These include physical, chemical and biological processes.

1.2.1 Physical dispersion

The coarse particulate matter contained in sewage has a tendency to settle rapidly in sea water fixing microorganisms which are adsorptioned onto it. Although this process of sedimentation plays an important role in the vicinity of waste discharge points (Mitchell & Chamberlain, 1975; Geldreich, 1978), it does not seem to be an essential factor in microbial disappearance considering the high bacterial concentration that is detected in these waters. On the other hand, the fine particles undergo a diffusion process whereby they transport a large quantity of microorganisms with them (Gauthier, 1980; Mujeriego *et al*, 1980; Borrego, 1982).

1.2.2 Salinity

The saline concentration of seawater oscillates from 3.3% to 3.8% while the most favourable habitat for allochtonus microorganisms requires a salinity level of close to 0.9%. This significant difference in salt concentration anticipates sea water's ability to inactivate those microorganisms. This may be achieved through osmotic shock or through the specific toxicity of the ions (Carlucci & Pramer, 1960a).

1.2.3 Sunlight

Several authors (Gameson & Gould, 1975; Kapuscinski & Mitchell, 1981; Fujioka & Narikawa, 1982) have proposed the importance of sunlight as an agent which inactivates the microorganisms discharged into the sea. They emphasize the importance of visible light compared with ultraviolet light which is not as powerful because of its weak penetration through water layers. Gameson & Gould (1975) establish the existence of a direct relationship between the degree of insolation and the bacterial inactivation, as the highest levels of bacterial inactivity in seawater coincide with daylight hours when the sunlight is strongest, and with those seasons of the year which are the brightest.

1.2.4 Temperature

It is commonly known that the increase of cellular metabolism is dependent on temperature. The accelerated activity of the microorganisms caused by this increase can produce a greater inactivation of the toxic factors in the water (Aubert & Aubert, 1969; Vasconcelos & Swartz, 1976).

Temperature may also have the additional indirect effect of stimulating predation phenomena and antibiosis (Vasconcelos & Swartz, 1976; Verstrate & Voets, 1976; Anderson *et al*, 1983).

1.2.5 Metallic ions

Due to the specific toxicity of some of the metallic ions to numerous organisms, they can exert a negative effect on the sewage flora in even relatively low concentrations (Jones, 1971, Jones & Cobet, 1975). Because of their ability to inactivate enzymatic systems, it seems that these ions, or complexes of heavy metals, form part of the seawater purifying processes.

1.2.6 Lack of nutrients

Seawater is a oligotrophic substrate as a nutritive source, with a low level of nutrients, organic matter being a limiting factor in the growth of polluting microorganisms (Savage & Hanes, 1971; Sinclair & Alexander, 1984). The presence of nutrients in the water stimulates bacterial growth, while intervening in the partial compensation of the bactericidal effects of other negative factors.

1.2.7 Predation

1.2.7.1 - Bacteriophages

Sewage contains high levels of bacterial viruses of different microorganisms which are also detected in seawater where this water is discharged. Phages are strict parasites and, usually they kill bacterial cells producing lysis plaques when the optimum conditions necessary for the growth of these bacteria occur. Because of these conditions, it is difficult to specify the degree of influence that the phages exert on the process of seawater purification (Borrego, 1982).

1.2.7.2 - *Bdellovibrio bacteriovorus*

This bacterial species is widely distributed in soil and freshwater, seawater and sewage. However, it is difficult to determine its true ecological role, as its specific action on microbial cells is an important characteristic of the bacteria (Starr & Seidler, 1971).

1.2.7.3 - Myxobacteria

These bacteria have the ability to either hydrolyze insoluble molecules or to lysis bacterial cell and use them as substrate. The purifying of these bacteria is well established. They act mainly on dead bacterial cell (Verstrate & Voets, 1976).

1.2.7.4 - Protozoa

Protozoa play an efficient part, directly or indirectly, in the purification process, eliminating the organic matter and also the bacteria in the environment (Mitchell, 1971; McCambridge & McMeeckin, 1980, 1981; Mallory *et al*, 1983). Bacteria play a significant role in the nutrition of the protozoa, principally, as growth factors, although, according to Dive's hypothesis (1973), there is a bacterial nutritive value rank for the macropredators, and for example, Enterobacteria are nutritionally valuable for the ciliates, but only slightly, if at all, for other protozoa.

1.2.8 Autochthonous flora

Marine bacteria are generally better adapted than allochtonous microorganisms to the concentrations of nutrients found in this environment (Sinclair & Alexander, 1984). Thus, a competitive phenomena for nutrients may occur in areas where the incidence of autochthonous flora is significant.

Several authors have proposed the importance of antibiotic substances in the water as inactivating agents of polluting microorganisms in the marine environment (Aubert & Aubert, 1969, Paoletti, 1970). Different microorganisms show the ability to synthesize antibiotic substances, such as *Actinomyces* spp., *Streptomyces* spp. *Bacillus* spp. and some seaweeds. A negative effect of these antibiotic substances on sewage flora has been observed in laboratory experiences, but its role "*in situ*" is of little importance as the production of antibiotics is conditioned by the growth of the microorganisms in very rich media, conditions which are only produced with difficulty in the sea (Mitchell, 1971).

1.3 Objectives

The main objectives of the present study were the following:

a - "*in situ*" survival studies of the indicator and pathogenic microorganisms using Pearson's technique.

b - Survival studies in the laboratory using pathogenic microorganisms, both isolated from natural waters and reference strains, with the objective to establishing the influence rate of the different marine self-purifying factors on the microorganisms.

c - Survival studies using diffusion chambers submerged in natural marine waters.

2. MATERIAL AND METHODS

2.1 Sampling zone

The studies of dispersion and survival "in situ" of microorganisms were carried out in a marine zone influenced by the discharges of a submarine outfall sited in Fuengirola (Malaga, Spain). It is at a depth of 30 m, and is situated 1000m offshore (Figure 1). The sampling techniques have been specified in the Progress Report of this Project.

2.2 Microorganisms

To perform the different experiences, we used the strains specified in Table 1.

2.3 Media

2.3.1 Control and enrichment media

2.3.1.1 - Tripticase-soy-broth (Difco)

This broth was supplemented with 0.3% yeast extract and 0.5% dextrose when used as inoculum and test medium for all the strains.

2.3.1.2 - Peptone buffered water (Edel & Kampelmacher, 1973)

It was used for pre-enrichment of salmonellas from natural water samples

Composition (grains per litre of distilled water)

Peptone	10.0
Sodium chloride	5.0
Na_2HPO_4 $12H_2O$	9.0
KH_2PO_4	1.5

pH 7.2

2.3.1.3 - Modified Rappaport-Vassiliadis broth (NR10(10)/43) (Moriñigo *et al*, 1986).

Composition (grams)

Solution A :

Tryptone	5.0
Sodium chloride	8.0

KH_2PO_4	1.6
Distilled water	1000 ml

Solution B:

Magnesium chloride	400.0
Distilled water	1000 ml

Solution C:

Malachite green oxalate	0.4
Distilled water	1000 ml

Solutions A,B and C were mixed in the following proportions: 100, 10 and 1, respectively. The mix was supplemented with 10 μg per ml of sodium movobiocin.

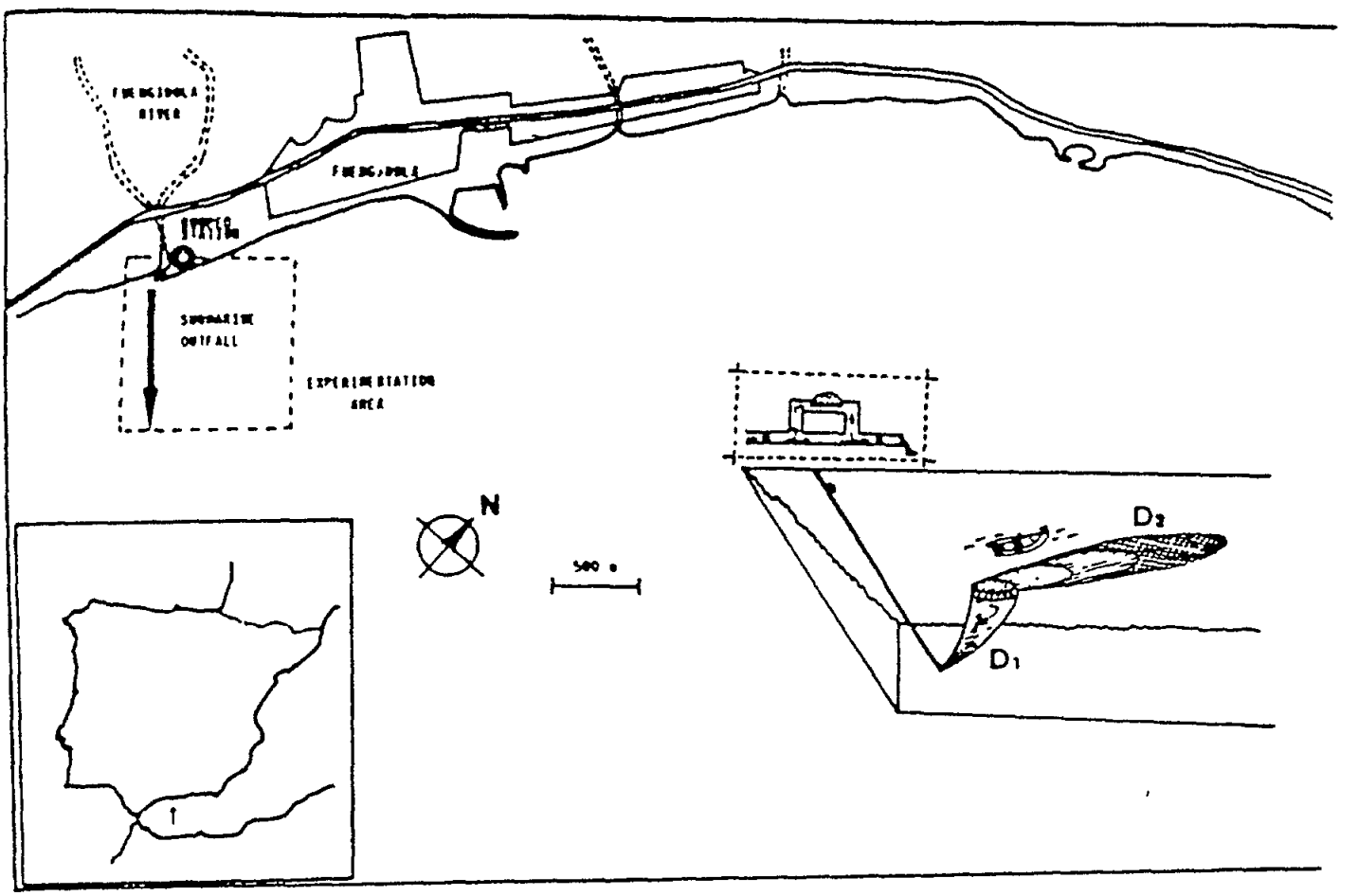


Figure 1

Location of the experimentation area of
the Fuengirola submarine outfall

Table 1

Strains used in the experiments

Microorganisms		Source of the strains	Utilization
Pseudomonas aeruginosa	110	Spanish Type Culture Collection	Diffusion Chamber and Laboratory
Pseudomonas aeruginosa	J-75	Isolated from sea water	Diffusion Chambers
Escherichia coli	381CO	Spanish Type Culture Collection	Bacteriophages Assays
Escherichia coli	25922	American Type Culture Collection	Diffusion Chambers and Laboratory
Escherichia coli	6	Isolated from sea water	Diffusion Chambers
Escherichia coli	17	Isolated from sea water	Diffusion Chambers
Salmonella thompson	41F	Isolated from sea water	Diffusion Chambers and Laboratory
Salmonella paratyphi	554	Spanish Type Culture Collection	Diffusion Chambers and Laboratory
Staphylococcus aureus	29213	American Type Culture Collection	Diffusion Chambers and Laboratory
Staphylococcus aureus	15	Isolated from sea water	Diffusion Chambers
Streptococcus faecalis	19933	American Type Culture Collection	Diffusion Chambers and Laboratory
Streptococcus faecalis	21	Isolated from sea water	Diffusion Chambers
Streptococcus faecium	2	Isolated from sea water	Diffusion Chambers
Candida albicans	1394	Spanish Type Culture Collection	Diffusion Chambers and Laboratory
Candida albicans	1	Isolated from sea water	Diffusion Chambers

2.3.1.4 - Luria broth

This medium was employed both for *E. coli* growth and bacteriophages enrichment.

Composition (grams per litre of distilled water)

Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0
pH = 7.2	

2.3.2 Enumeration media

2.3.2.1 - The following media were used in the filtration membrane technique.

(1) m Endo MF broth (Difco)

This was used for the quantitative recovery of total coliforms, supplemented with 1.2% of agar-agar (Difco).

(2) m FC base broth (Difco).

This was employed for the quantitative analysis of faecal coliforms. It was also supplemented with 1.2% of agar-agar.

(3) m Enterococcus agar (Merch)

This medium was used for the selective enumeration of faecal streptococci present in the samples.

(4) m PA-E agar (de Vicente *et al.*, 1986)

This medium is highly selective in the detection and enumeration of *Pseudomonas aeruginosa* from *seawaters*.

Composition (grams per litre of distilled water).

Yeast extract	1.0
Sodium chloride	5.0
HCl-Lysine	5.0
Xylose	2.5
Ammonium ferric citrate	1.0
Sodium thiosulfate	5.0
Magnesium sulfate $7H_20$	3.04
Phenol red	0.08
Agar-agar	15.0
pH = 7.2	

The ingredients were dissolved by autoclaving and cooling at 55-60EC. Before spreading the medium onto the Petri dishes, a 12 mg/l of kanamycin sulfate (Sigma) and 37 mg/l of nalidixic acid (Sigma) were added.

(5) BFR-O agar (Florido, 1985)

This medium was used in the enumeration of staphylococci and particularly *S. aureus,* from water samples.

Composition (grams per litre of distilled water)

Yeast extract	3.0
Tryptone	6.0
Beef extract	1.5
D-mannitol	20.0
Sodium chloride	100.0
Glycine	11.0
Sodium pyruvate	10.0
Potassium thiocyanate	25.0
Phenol red	0.025
Sodium azide	0.049
Agar	15.0
pH = 7.2	

(6) m-CA agar (Buck & Bubucis, 1978)

Medium used for the selective recovery of pathogenic yeast, especially *Candida albicans,* from soil and water samples.

Composition (grams per litre of distilled water).

Glycine	10.0
Maltose	30.0
Bismut ammonium citrate	5.0
Sodium sulphite	3.0
Cloramphenicol	0.5
Actidione	1.5
Agar	15.0
pH = 7.0	

The ingredients were dissolved by boiling at 100EC. After cooling at 45EC, the medium was supplemented with 10% of yeast nitrogen base (Difco), and the completed medium was sterilized by membrane filtration (0.45µm pore size).

2.3.2.2 The following media were used in the standard plate count (S.P.C.).

(1) Endo agar (Difco)

Selective and differential medium used for coliforms group plate counts.

(2) KF base agar (Merck)

This medium was used for detection and enumeration of faecal streptococci.

(3) Cetrimide base agar (Difco)

This was employed for the selective enumeration of *Pseudomonas aeruginosa*.

(4) Mannitol salt agar (Difco)

This medium is recommended for the selective isolation and enumeration of staphylococci.

(5) Xylose lysine deoxycholate agar (Pronadisa)

This differential medium is used in the isolation of salmonellae.

(6) CA agar

Specified in item 2.3.2.1 - 6.

(7) Trypticase soy agar (T.S.A.) (Difco)

An unselective medium employed in the recovery of different stressed cells.

2.3.2.3 - Other counting media used were as follows:

(1) Tryptose sulphite cycloserine (T.S.C.) (Harmon & Kantter, 1971)

This medium was used in combination with the tube poured technique (Bonde, 1977)) for the quantification of clostridia sulphite reducers.

Composition (in grams)

Tryptose	6.3
Yeast extract	2.1
Casitone	2.1
Ferric citrate	0.42
Sodium bisulphite	0.42
Agar	8.4
Distilled water	350 ml
pH = 7.6	

This medium was sterilized at 121EC for 15 minutes, and cooled at 45EC in a thermostatic bath. Before use, the medium was supplemented with 15.2 ml of a buffered solution of cycloserine (Sigma), previously sterilized by membrane filtration (0.22µm).

Composition (in grams)

K_2HPO_4	0.87
KH_2PO_4	0.048
D-cycloserine	0.5
Distilled water	350 ml
pH = 8.0	

(2) Luria agar

The composition was similar to Luria broth, but supplemented with 1.2% of agar-agar (Difco). Luria agar was employed as bottom layer agar in the double layer technique (Adams, 1959) for detection and enumeration of bacteriophages.

A modification of the medium which consists in the addition of 0.7%, and the suppression of yeast extract is called soft nutrient agar. It was used as top layer agar in the double layer technique (Adams, 1959).

(3) Phage assay broth (P.A.B.) (Kott, 1966).

Used as the nutritive medium for detecting bacteriophages in the More Probable Number technique.

Composition (Grams per litre of distilled water).

Beef extract	3.0
Meat peptone	5.0
Sodium chloride	5.0
Magnesium sulphate	0.2
Manganese sulphate	0.05

The medium was supplemented with 0.15 g/l of magnesium chloride before its use.

2.3.3 Solutions and reagents

2.3.3.1 - Phosphate buffered solution (APHA, 1985).

This solution was used as diluent and also for washing the membrane filtration equipment.

Composition (in grams)

Solution I :	KH2PO4	3.4
	Distilled water	100 ml
	pH = 7.2	
Solution II:	MgCl	8.1
	Distilled water	100 ml
	pH = 7.2	

To prepare the phosphate buffered solution, 1.25 ml of solution I and 5 ml of solution II were mixed with 1000 ml of distilled water.

2.3.3.2 - Phosphate buffered gelatine solution

Used for the dilutions and resuspensions of centrifuged cultures in the diffusion chambers experiments and also for the "in vitro" survival experiments.

Composition (in grams per litre of distilled water).

Gelatine	2.0
Na_2HPO_4	7.25
NaH_2PO_4	3.7
pH = 7.2	

2.3.3.3 - Physiological saline solution

This was prepared by adding 8.59 of sodium chloride to 100 ml of distilled water. This solution is used in the "in vitro" survival experiments.

2.3.3.4 - Artificial seawater (Sieburth, 1979)

This medium was used to simulate the effects which the chemical composition of sea water exert on the different microorganisms in laboratory experiments.

Composition (in grams).

Sodium chloride	21.50
Potassium chloride	0.61
Magnesium chloride	9.74
Distilled water	1000 ml

The ingredients were dissolved and sterilized by autoclaving at 121EC for 15 min. The following ingredients were added later:

Na_2SO_4	3.45
$NaHCO_3$	0.17
Na_2HPO_4	0.14

2.4 Detection and recovery of microorganisms

2.4.1 Membrane filtration technique

The principal objective of these methods consist in the concentration of the microbial cells present in a water sample. For this reason, the sample is passed through a membrane filter with a diameter which is smaller than that of the bacteria. Afterwards the filtered membrane is cultivated in the appropriate media for the detection and selective enumeration of the determined microorganisms. In the present work, we used the Standard Methods for the Examination of Water and Wastewater guidelines (1985).

The media and incubation conditions are the following.

2.4.1.1 - Total coliforms: m-Endo MF agar (2.3.2.1-1) at 36EC for 24 h. The characteristics of the colonies growth in this medium are a pink or deep red colour with a metallic bright green at the centre, or over all the colony. Counts of 20-80 colonies per petri dishes are considered as optimum.

2.4.1.2 - Faecal coliforms: m-FC MF agar (2.3.2.1-2) at 44.5EC in thermostatic bath for 24 h. Typical faecal coliforms colonies are characterized by their blue colour. In agreement with Presswood & Strong (1978) rosolic acid is suppressed, because the acid can negatively effect the counting. Optimum recount is established between 20-60 colonies per membrane.

2.4.1.3 - Faecal streptococci: m-Enterococcus agar (2.3.2.1-3) at 36EC for 48 h. To recover the stressed streptococci, the incubation period is delayed to 72 h (Lin, 1977). The membrane plates that contain between 20-100 typical streptococci colonies (pink to red colour and 0.5-2 mm diameter size) are used as optimum for the recount and concentrations calculations.

2.4.1.4 - *Pseudomonas aeruginosa*: m-PA-E agar (2.3.2.1-4) at 36EC for 48 h. The typical colonies show a brown-grey to black colour, and in most cases, the presence of diffusible pigment. Optimum recount of 20-80 colonies per membrane are established (de Vicente *et al,* 1986).

2.4.1.5 - *Staphylococcus aureus*: BFR-O agar (2.3.2.1-5) at 36EC for 48-72 h. The typical colonies in the medium are differentiated by their yellow colour and also by the yellow halo around the colony. The minimum and maximum limits of recount are 20-80 colonies per membrane.

2.4.1.6 - *Candida albicans*: m-CA agar (2.3.2.1-6) at 36EC for 48 h. The membrane filter has a 1.2µm pore size. Typical characteristics of these colonies are their dark-brown colour and mucous aspect. For the concentration calculations recounts of between 20=80 colonies per membrane dishes are used.

2.4.2 Spread plate technique

The plates were inoculated with a volume of less than 0.5 ml of the analysis sample, and the inoculum spread by use of a Drigalski and Conradi rod. This technique was employed for direct recount of the different microorganisms, changing in each case, the selective medium.

2.4.2.1 - Selective media:

- Coliform counts: Endo agar (2.3.2.2-1). The typical coliform colonies ferment the lactose that the medium contains and show a red colour. Incubation conditions are 36E C for 24h.

- Faecal streptococci counts: KF agar (2.3.2.2-2), where the typical colonies present a pink or red colour. Incubation conditions are 36E C for 48h.

- *Pseudomonas aeruginosa* counts: Cetrimide base agar (2.3.2.2-3). Its growth in this medium produces a pigmented colonies, whose colours vary between blue, green-blue, yellow-green or are colourless. Incubation conditions are 36E C for 48h.

- *Staphylococcus aureus* counts: Mannitol salt agar (2.3.2.2-4). These species growth giving yellow colonies that present a halo around them. Incubation conditions are 36E C for 36h.

- *Salmonella* counts: The xylose-lysine-deoxycholate agar (2.3.2.2-5) was used for the direct recount of salmonellas. This microorganism differs from the others because it produces colourless colonies with a black centre in XLD plates. Incubation at 36EC for 24h.

- *Candida albicans* counts: This count is performed on m-CA agar plates, where the typical colonies, of a drak-brown colour, appear after incubation at 36E C for 48h.

2.4.2.2 - Non-selective media

The detection of sublethally injured microorganisms may be affected by the use of selective media due to the sensibility of these stressed cells to inhibitory agents present in the selective media. To avoid this problem, all the samples were cultured in tryticase soy agar plates. These plates were incubated at 36EC for 24 h.

2.4.2.3 - Double layer agar count

Culture on non-selective media does not allow the differentiation of the microorganisms present in the water samples. Therefore, to detect the damaged cells of a particular microorganism, the double layer agar (non-selective and selective media) culture was used. The method consists in inoculating the sample onto tryticase soy agar plates and incubating at room temperature (20-24EC) for 3h. After this period of time the second layer of the selective medium maintained at 45EC, is added to the agar plates. The incubation conditions are the same as those mentioned above for the particular selective medium.

2.4.3 Depth inoculation in agar tube technique

This method was used for the investigation and count of clostridium sulphite reducer spores. Therefore, a water sample, previously heated at 80EC for 10 min, is required for the vegetative cell's death. Cellular revivification processes are carried out in tryptose sulphite cycloserine agar (2.3.2.2-1) using the depth inoculation in agar tube technique, consists in the addition of a determined volume of the water sample to a tube containing the melted medium by means of a sterilized pipette. The sample is homogeneously distributed from the bottom to the surface of the tube. Inoculated tubes are incubated into Gas-Pak jars, with CO_2 and H_2 atmosphere. Clostridium sulphite reducer colonies are shown in the medium with a black colour from the formation of a FeS precipitate.

2.4.4 Detection of bacteriophages using the Most Probable Number (Kott, 1966)

With this method a low concentration of phages in the sample can be detected. It was used for seawater whith a low faecal pollution level.

Three series of tubes, each one with 10 ml of phage assay broth (2.3.2.3-4), the first of them at doubled-concentration, were inoculated with 10, 1 and 0.1 ml, respectively, of the water sample. Afterwards, 0.1 ml of a logarithmic growth *E. coli* culture was added to each tube. Before incubation at 36EC for 16-18 h, all the tubes were shaken gently to improve the virus-host adsorption.

After the incubation period a loop of each incubated tube was transferred to Petri dishes inoculated with the host bacterial culture. These plates were incubated at 36EC for 6 h. The tubes that contain specific bacteriophages produce a lysis area over the plate surface, that is recorded by a positive test. With the positive results, the MPN of phages per 100 ml of sample was calculated for mean by a Poisson probability table.

2.4.5. Direct count of phages using the double agar layer technique

This technique is appropriate when the water sample possesses a titre of 10 plaques forming unit per 100 ml and it consists in adding 1 ml of the sample and 0.2 ml of logarithmic growth culture of the bacterial host to tubes contain soft agar melted at 45EC. After a brief homogenization, the tubes are poured onto Luria agar plates, following the technique described by Adams (1959) and modified by Borrego (1982). When the top layer of the plates are solidified, they are incubated at 36EC for 12-18 h in an inverted position.

2.4.6 Detection and quantification of salmonellae from natural waters

The method is an adaptation of the multiple tube technique and the protocol is the following:

Aliquots of 100 ml and 10 ml of the sample are filtered through membrane filter of 0.45 µm pore size, and are afterwards put into tubes with 10 ml of buffered peptone water (2.3.1.2) Aliquots of 1 ml of the sample are inoculated directly into the tubes with the above mentioned medium in total, three series, of three tubes, each containing the pre-enrichment medium, are used. They are incubated at 36EC for 24 h.

A 1:100 portion of each incubated pre-enrichment tube is transferred to another tube with the selective enrichment broth (NR 10/43), which is incubated at 43EC for 48-72 h. A loopful of each incubated tube is streaked onto XLD plates, which are also incubated at 36EC for 24 h.

Typical salmonella colonies grown on XLD plates are identified as *Salmonella* spp by biochemical characteristic profiles (Le Minor, 1984) and serotyping of the isolates (Edwards & Ewing, 1972). All the isolates that are coincident with Salmonella spp. profile are confirmed by API 20-e System-test.

2.5 Disappearance of the microorganisms in the marine environment

2.5.1 Methods

To studying microbial disappearance in sea water, experiments on sewage evolution in the marine surface from the Fuengirola (outfall) discharge were made, using Pearson's technique (1956). This technique consisted in simulating the sewage dispersion by the employing semi-submerged floats from the superficial effluent outfall.

The sampling was carried out at time periods: O (superficial effluent discharge), 7, 15, 30 and 60 min of drift of from the moment of discharge of the sewage outflow in the sea surface. For this sampling, a Zodiak Mark IOP with a Johnson motor of 15 CY was used.

All the samples were collected from the surface of the sea in 500 or 1000 ml sterilized amber bottles of 500 and 1000 ml. After sampling, the samples were refrigerated in darkness at 4EC in isothermic containers in which they were carried to the laboratory. Samples were processed in the laboratory within 6 h of collection.

In these experiments, we investigated the following microbial parameters:

a) Indicator microorganisms: Total coliforms, faecal coliforms, faecal streptococci, sulphite reducer clostridia and coliphages.

b) Pathogenic microorganisms: *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* spp and *Candida albicans.*

2.5.2 Mathematical calculations

2.5.2.1 - Initial dilution

This term refers to the decrease of in microbial concentration the sewage produced when it is mixed with seawater the discharge point and the surface effluent outfall.

The initial dilution was obtained as follows:

a) Sewage/superficial effluent outfall microbial concentrations calculations (Borrego *et al,* 1983a), or b) using the Cerderwall's equation:

$$D_1 = 0.54\ F\ (0.38\ Y/DF + 0.68)^{5/3}$$

where D_1 is the initial dilution; Y is the outfall depth (in metres); D is the diffuser diameter and F is the Froude's number (1), when Y in the velocity of the sewage:

$$F = Y/(0.27\ D)^{1/2} \quad (1)$$

2.5.2.2 - Superficial dispersion

The microbial dispersion on the marine surface is determined both by dilution processes and by microbial inactivation phenomena. For the calculation of the disappearance process, several mathematics models have been proposed, but the decrease logarithmic model has been used in this study, has been to prove as the most useful:

$$C = C_o \times 10^{-t/T90} \quad (2)$$

where C_o and C are the microbial concentrations in the time 0 and t, respectively; T90 is the characteristic constant that indicates the time that decreases the 90% of the microbial concentration.

The equation (2) is transformed to a lineal expression, where the T90 parameter is easily calculated:

$$\log C = \log C_o - t/T90 \quad (3)$$

The logarithmic concentration of each microorganism in each sample is plotted against the drift time, allowing us to make the lineal adjustments by square mean method.

2.6 Survival of experiments using diffusion chambers

2.6.1 Diffusion chambers design

The diffusion chambers employed in the microbial survival experiences in the sea were modelled in our Department, using the McFeters & Stuart (1972) and Fliermans & Gordons (1977) chambers as patterns.

Polycarbonate membranes of 90 mm diameter and 0.2 µm pore size (Nucleopore) were used to prevent the contamination of the test culture (Figure 2). Two hipodermic needles were fixed in the top of the chambers for the inoculation of the bacterial inoculum and the extraction of the analysis sample.

The needles were closed by means of paraffin lacres to prevent seawater passing into the chambers. The chambers were autoclaved at 121EC for 125 min, except the membranes sterilized by UV irradiation. The assembly of each piece was carried out aseptically.

2.6.2 Preparation of the microbial suspensions

Selected pure microorganism cultures were inoculated in trypticase soy broth supplemented with 0.3% of yeast extract and 0.5% of dextrose and then incubated at 36EC for 20-24 h.

The cells of the culture were concentrated, centrifuged at 3000x g for 20 min, and later purified by two cycles of resuspension-centrifugation in gelatine buffer. The last pellet was re-suspended in sterilized seawater.

This resuspension was diluted in sterilized seawater to obtain a final titre of 1×10^5 or 1×10^7 colony forming units per milimeter depending on the microorganism.

2.6.3 Inoculation of the microbial culture

The chambers filled with sterilized seawater were inoculated with 1 ml of the microbial suspensions using a sterilized syringe. Then, they were carried to the sampling zone, situated near the sports Club harbour (El Candado, Málaga, Spain). The time between inoculation and the immersion in to the sea was never more than 45 min. All the chambers were submerged at 1.5 m depth.

2.6.4 Sampling

Immediately after the culture inoculation into the chambers, the suspension was homogenized by an air repeated pump with a sterilized syringe, and the first sample obtained. Subsequently, samples were obtained at time intervals of 4, 24 and 48h, repeating the initial process. The samples obtained were carried to the laboratory in sterilized haemolysis vials.

2.6.5 Enumeration

The quantitative analysis of the different microorganisms was carried out using the spread plate technique, using parallel selective media and tripticase soy agar. The selective media used in these experiments have been described in section 2.4.2.1.

Processing of the samples was performed by consecutive dilutions in gelatine buffer and three plates of each media (selective and non-selective) of each dilution were inoculated. After dissemination of the inoculum, all the plates were incubated as previously mentioned. After this period of time the recount of growth colonies of the different microorganisms was carried out.

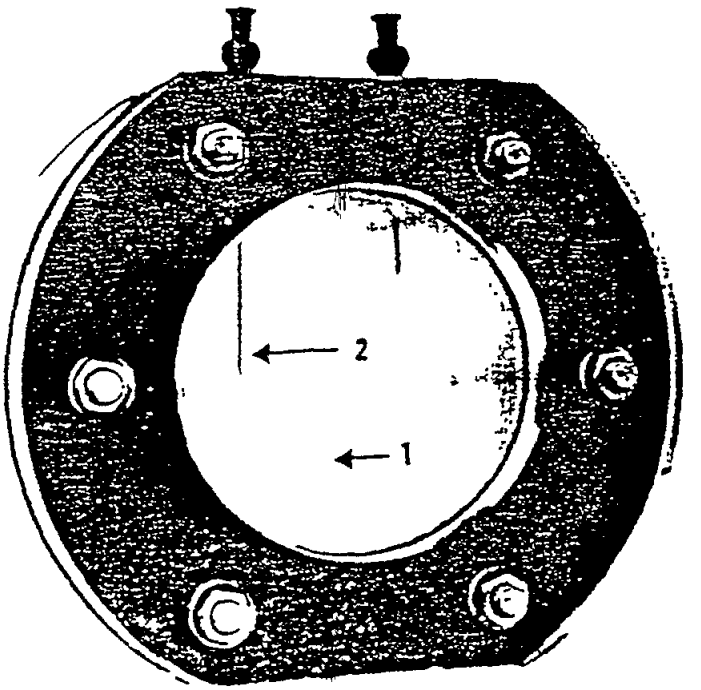


Figure 2: Scheme of the Diffusion Chamber

(1) *Polycarbonate membrane*
(2) Hypodermic needle

The colony counts comprising between 30 to 300 per plate (APHA, 1985), and the final microbial concentration were the only ones of significance for recount. The percentages of surviving and injured cells at specified times were obtained by the following equations:

$$\text{Survival cell percentage at time} = \frac{\text{count on non-selective medium at time}}{\text{count on non-selective medium at 0 time}} \times 100$$

$$\text{Injured cell percentage at time} = \left(1 - \frac{\text{selective medium recount at time}}{\text{non-selective medium recount at time}} \times 100\right)$$

2.7 Survival of microorganisms in laboratory experiments

To study the influence that the different environmental factors exert on the selected allochtonous microorganisms (indicators and pathogens), we designed experiments in which the different microbial suspensions undergo exposure to the factors cited under controlled laboratory conditions.

2.7.1 Cellular suspensions preparation

The methodology applied was similar to that described in section 2.6.2 and, the last re-suspensions were performed in gelatin buffer.

From the purified suspensions consecutive dilutions in gelatine buffer tubes were made to obtain concentrations of 1×10^5 and 1×10^7 c.f.u./ml, depending on the different microorganisms. Only 1 ml of this dilution was inoculated into a 250 ml capacity Erlenmeyer frasks containing 100 ml of the test medium.

2.7.2 Test solutions: factors studied

Five test solutions were investigated to study the effect of the different agents of seawater on the allochtonous microorganisms. A physiological saline solution (0.85%) was employed as control solution.

All the test solutions were kept in darkness and at a constant temperature of 18ºC, except an artificial seawater solution that exposed to constant sunlight (spectrum radiation 380-760 nm).

The different test solutions and their influence on the microorganisms are presented in Table 2.

2.7.3 Methodology

The different microbial suspensions were sampled at 0, 1, 3, 7 and 14 days from the beginning of the experiment.

The enumeration of different microorganisms was carried out by the spread plate technique on selective and non-selective media, from decimal dilutions of the test suspensions. All the tests were performed on triplicate-plates for each dilution and medium.

Table 2

Different test solutions and their negative effects on the microorganisms

Test solutions	Negative effects observed
Unpolluted crude seawater	Global microorganism inactivation
Artificial seawater	Chemical composition (especially salinity)
Filtered seawater	Influence of filtrable factors (toxins, antibiotic, bacteriophages, etc.)
Artificial seawater supplemented with 1% of sewage	Sewage effect
Illuminated artificial seawater	Visible light effect

3. RESULTS

3.1 Experiments on microbial disappearance in the marine environment

In this section the results obtained from studies of the evolution of pathogenic and indicator microorganism concentrations are presented. These were obtained from experiences which traced the sewage discharged by the submarine outfall situated in Fuengirola (Malaga, Spain).

In these studies the disappearance process is considered globally, including the decrease of the microbial concentrations produced both by the sewage dilution in the receptor environment, as by the inactivation or biological death of the microorganisms.

3.1.1 Initial dilution calculations

Initial dilution is calculated both by applying the formula developed by Cederwall (1963) and by experiments, the ratio between the microbial concentrations in the sewage pumping station and in the marine surface from the outfall effluent (Borrego *et al,* 1983a). Initial dilution results obtained in this study are specified in table 3.

The values of initial dilution calculated experimentally can be integrated into three groups; values inferior to 100 for non-bacterial microorganisms, coliphages and *C. albicans*, values from 100-200, for the indicator bacteria such as faecal coliforms, faecal streptococci, sulphite reducer clostridia and bacterial pathogens; and values superior to 200 obtained from total coliforms.

3.1.2 The calculation of horizontal dispersion

The T90 parameter is used to calculate both the dilution suffered by contaminating microorganisms on the surface of the sea and the process of "in situ" microbial inactivation.

In the Figures 3-7 the lineal adjustment of the inactivating dynamics of the indicator microorganisms depending on the immersion time in the evolution zone of the waters affected by the discharges of the submarine outfall are represented. In all cases, the significance levels of the correlation coefficients obtained are higher than to 99.9%.

The T90 values calculated by the exponential decrease equation with the values of T90 for each experiment are presented in table 4. Although differences in the T90 of each microorganism exist, they are not very significant. Coliphages have the highest T90 value while the sulphite reducer clostridia have the lowest.

The inactivation curves of the pathogenic microorganisms are shown in Figures 8-11, where significance levels of the correlation coefficients similar to those of the indicator microorganisms are seen (99.5%).

In Table 5 the partial and global values of the parameter T90 are reflected for each pathogenic microorganism studied. The values obtained by *Salmonella* spp. (8 min) and *P. aeruginosa* (11.3 min) are very similar to those obtained by the indicator microorganisms, while the T90 values of *S. aureus* (30.5 min) and *C. albicans* (22.7 min) are highest of the microorganisms studied.

3.2. Experiments on microbial survival in the sea, using diffusion chambers

The use of diffusion chambers permits the direct study of the influence of the sea on the survival of microorganisms. In this section we present the results obtained from inactivation in the marine environment using diffusion chambers. The samples analysis has been carried out using the methodology indicated in section 6 of the Material and Methods, employing both selective and non-selective media in order to determine the proportions of surviving and damaged cells.

Table 6 shows the percentage of injured cells for each microorganism tested at the various time intervals sampled. *E.. coli,* faecal streptococci, *P. aeruginosa* and *Salmonella* spp. present a 7% of cell survivors. This percentage is greater (8%) in the case of *C. albicans*, with *S.aureus* being the microorganism which presents the lowest rate of survival (6%) at the end of the time studied.

The largest proportion of damaged cells, on completion of the experiments, is found in the pathogenic microorganism, *S. aureus*, and the lowest percentage corresponds to *P. aeruginosa*. In this case, no similar or parallel behaviour is observed between *E. coli*, faecal streptococci, *P. aeruginosa* and *Salmonella* spp. In general, the proportion of injured cells increases, in all cases, the longer the microorganism is submerged in the sea.

In figures 12-29, the inactivation kinetics of the different microbial strains studied in selective and non-selective media are shown, as well as the representation of the average values of the strains of each microorganism.

The graphic values of T90 have been calculated from the representation of global kinetics. The values obtained are shown in table 7.

The greatest value of T90 observed on the non-selective medium (TSA) corresponds to the indicator *E. coli* (47.2 h), with *C. albicans*, with a T90 of 24 h, being the microorganism which shows the fastest inactivation kinetic. In the selective media, *S. aureus* is the microorganism with the least T90 value (3.2 h) and *C. albicans* shows the slowest kinetic inactivity (>48 h).

The kinetic inactivations of all the microorganisms tested on selective media is faster than that observed on tripticase soy agar (TSA), with the exception of *C. albicans* mentioned earlier.

3.3 Survival experiments in the laboratory

To evaluate the different factors present in the sea waters which influence the inactivation of the microorganisms studied, a series of experiments using different conditions and media were designed in the laboratory. The materials and methods used in these studies are specified in epigraph 7 of the Materials and Methods chapter of the present report. As in the diffusion

Table 3

Determination of initial dilution (D_1) of waste waters discharged from Fuengirola submarine outfall

Experiment	Theoretical	Value of initial dilution								
		Experimental								
		T.C.	F.C.	F.S.	SrC	Ph.	P.a.	S.a.	C.a.	S.sp.
1	75	153	20	94	100	81	159	106	128	-
2	75	366	96	206	-	29	69	384	188	13
3	75	196	2	108	62	9	70	145	31	258
4	75	138	388	61	246	208	149	54	15	48
Mean value	75	213	126	117	135	86	112	163	90	106

T.C.: Total Coliforms

F.C.: Faecal Coliforms

F.S.: Faecal Streptococci

SrC : Sulphitereducers-clostridia

Ph. : Coliphages

P.a. : *Pseudomonas aeruginosa*

S.a. : *Staphylococcus aureus*

C.a. : *Candida albicans*

S. sp.: *Salmonella sp.*

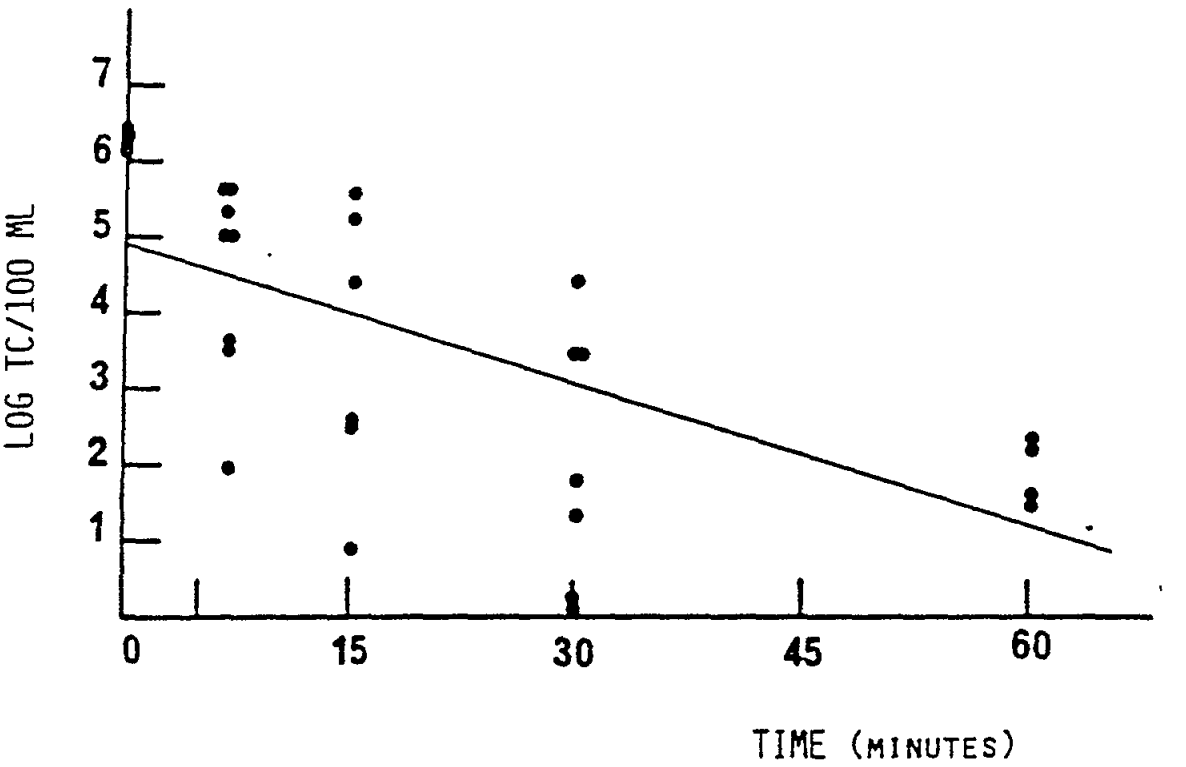


Figure 3
Disappearance kinetic of Total Coliforms in the evolution zone of the Fuengirola submarine outfall

$y = 4.9056 - 0.0165\ x$; $r = 0.5835$; $p<0.001$

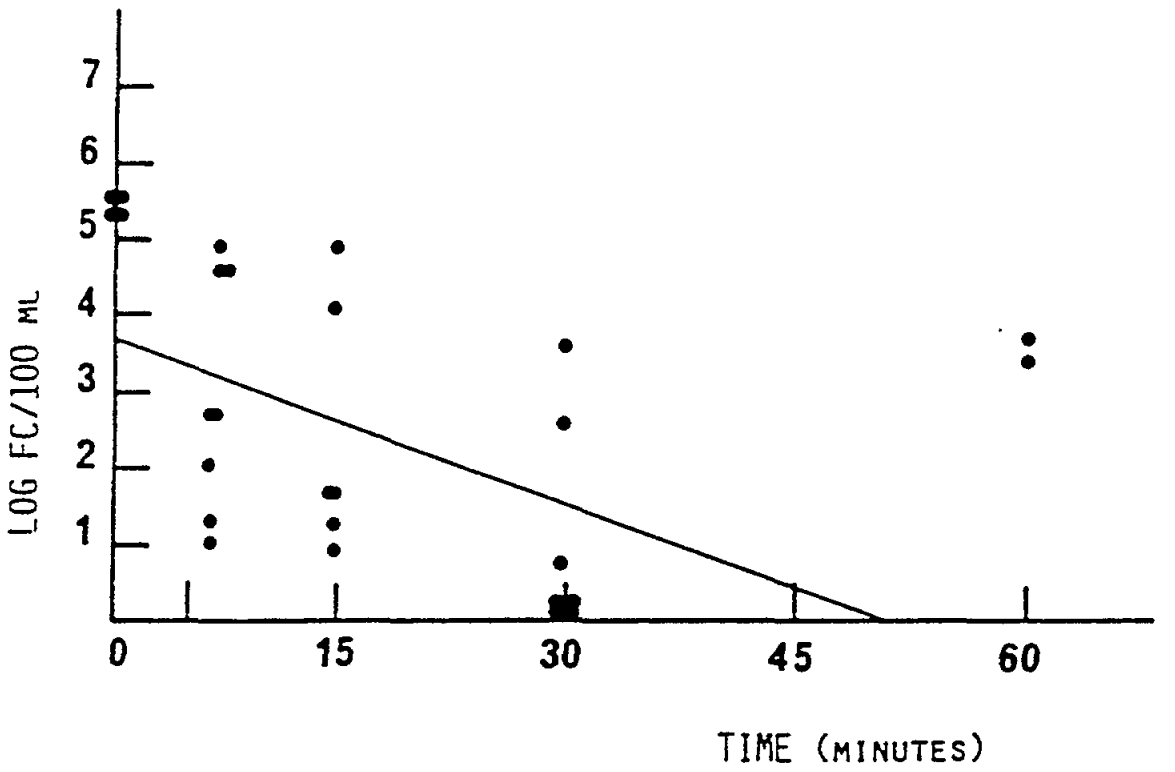


Figure 4

Disappearance kinetic of Faecal Coliforms in the evolution zone of the Fuengirola submarine outfall

$y = 3.7049 - 0.0775\ x$; $r = 0.6362$; $p<0.001$

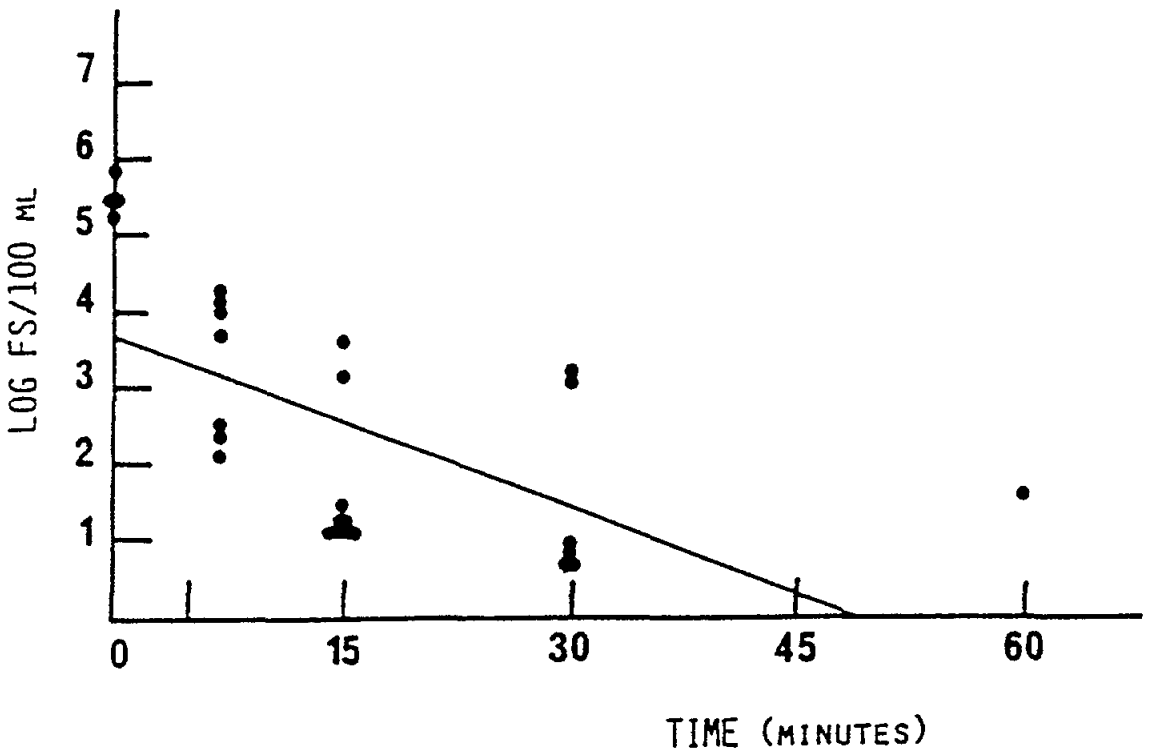


Figure 5

Disappearance kinetic of Faecal Streptococci in the evolution zone of Fuengirola submarine outfall

$y = 3.7057 - 0.0753\ x$; $r = 0.7227$; $p<0.001$

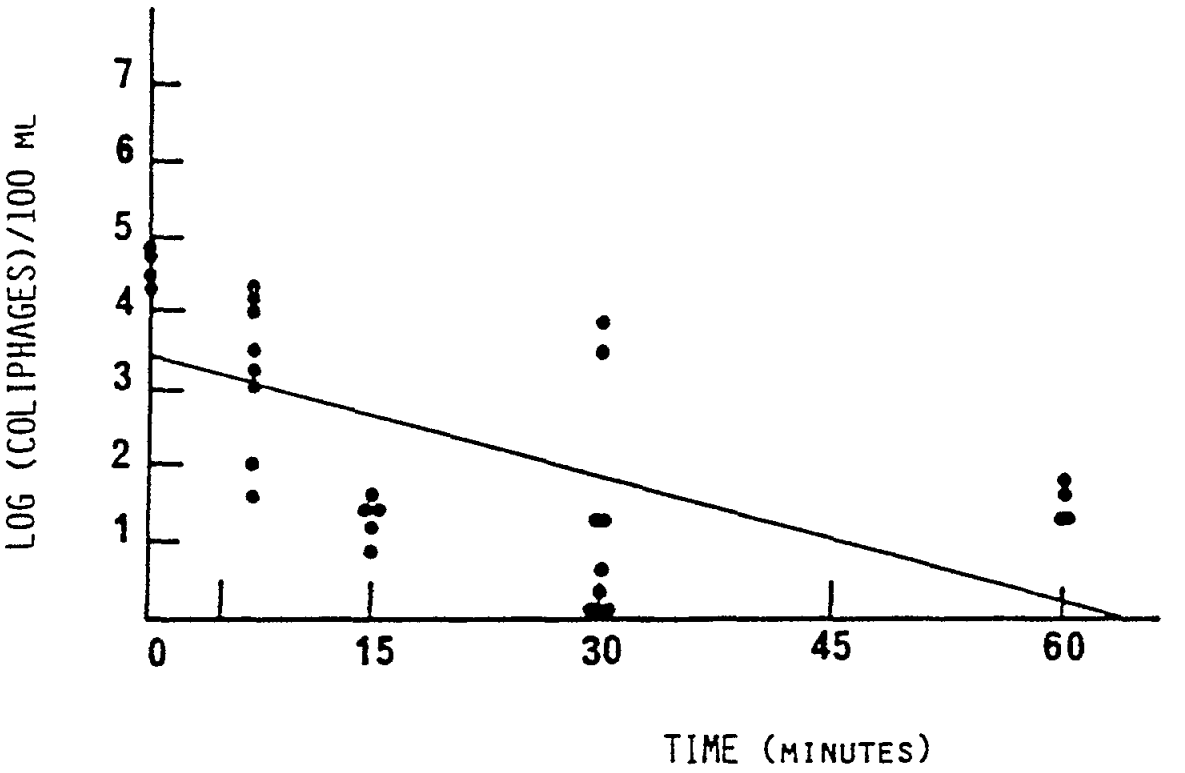


Figure 6

Disappearance kinetic of Coliphages in the evolution zone of the Fuengirola submarine outfall

$y = 3.4083 - 0.0520\ x$; $r = 0.5593$; $p<0.001$

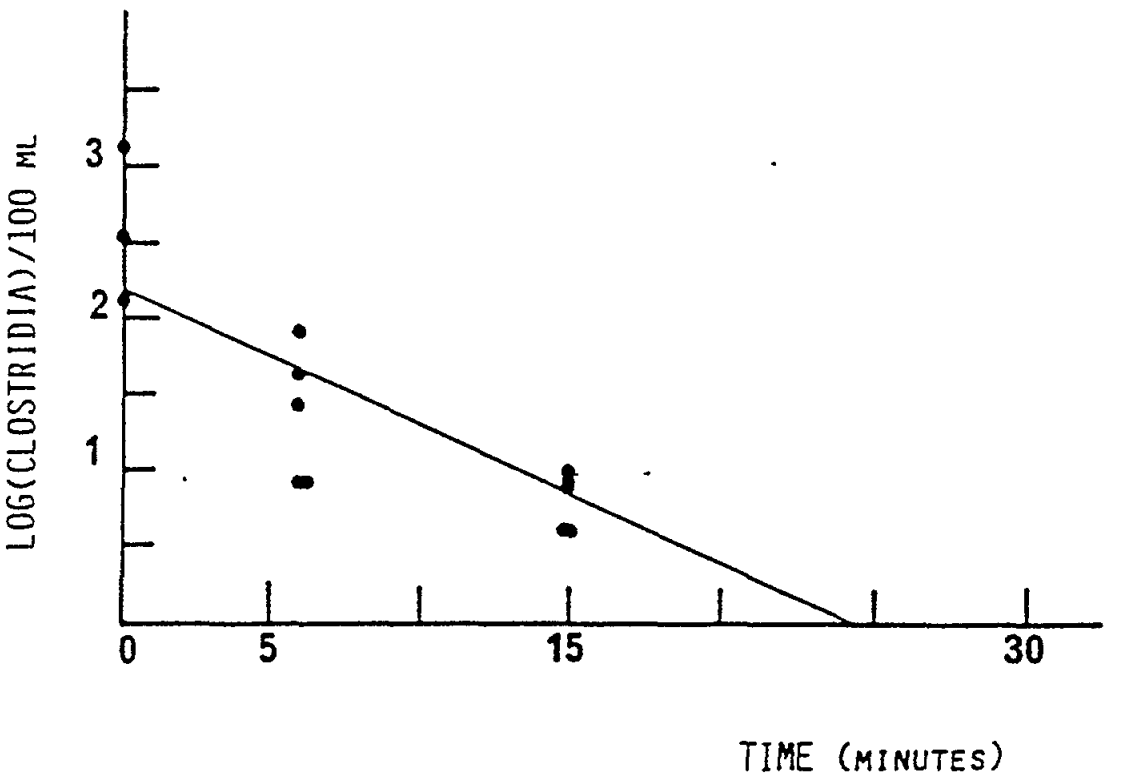


Figure 7

Disappearance kinetic of sulphitereducers-clostridia
in the evolution zone of Fuengirola submarine outfall

$y = 2.1518 - 0.0866\ x$; $r = 0.8091$; $p<0.001$

chamber experiments, selective and non-selective media were used for the recovery of the microorganisms tested.

In figures 30-71 the results of the survival curves of the strains studied are represented, under diverse conditions. Tables 8-14 show the percentages of damaged and surviving cells for each test solution used.

The inactivation kinetic obtained by the indicator microorganism *E. coli* is represented in figures 30-35. *E. coli* showed appreciable inactivation in the control test solution (0.85% saline solution). The percentage of cells which survived 14 days was over 100% (165%), which denotes a process of recuperation rather than one of inactivation of the suspended cells.

On the other hand, the percentage of damaged cells diminished during the experimental period.

Seawater produced a sharp decrease of the initial concentration of microorganisms which reached five orders of magnitude (Figure 31). This decrease is lessened when the water was previously filtered.

To verify the effects of other conditions, in which a determining factor is modified, suspensions in artificial seawater were used (Figure 33). This medium did not affect the survival of *E. coli* in any major way, although a decrease of the highest population, obtained in saline solution, was observed.

The influence on the survival of *E. coli* shown by determined exogenous factors of the environment, which are carried by the sewage discharged, were studied in suspensions of artificial seawater supplemented with 1% of domestic sewage.

Table 4

T90 values of indicator microorganisms in each experiment

	Partial T90[a]				
Parameter	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Global T90[a]
T.C.	14.20	15.72	6.56	6.02	16.3
F.C.	11.50	12.30	6.21	7.62	12.9
F.S.	8.81	24.33	7.17	8.20	13.3
SrC	-	11.50	10.25	9.28	11.5
	9.20	22.47	7.70	8.38	18.1

T.C. : Total Coliforms
F.C. : Faecal Coliforms
F.S. : Faecal Streptococci
SrC : Sulphitereducers-Clostridia
Ph. : Coliphages
[a] expressed in minutes

Table 5

T90 values of pathogenic microorganisms to each experiment

	Partial T90[a]				
Parameter	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Global T90[a]
S.a.	14.90	33.80	17.06	66.65	30.4
P.a.	6.75	14.90	3.61	15.38	11.3
C.a.	-	34.90	7.53	7.14	22.7
S.sp.	6.15	12.50	9.79	9.79	8.0

S.a. : *Staphylococcus aureus*
P.a. : Pseudomonas aeruginosa
C.a : *Candida albicans*
S. sp. : *Salmonella spp*
[a] expressed in minutes

Table 6

Percentages of injured and survival cells from each experiment during the experimentation time

Microorganisms	Percentage of injured cells			Percentage of injured cells		
	Sampling time (h)			Sampling time (h)		
	4	24	48	4	24	48
E. coli	29.51	52.53	82.39	59.28	22.57	7.75
Streptococcus faecalis	34.47	81.31	93.91	87.63	21.29	7.31
P. aeruginosa	45.59	57.27	54.16	33.74	34.05	7.43
C. albicans	95.41	37.79	79.45	73.50	8.60	8.00
S. aureus	81.05	96.50	96.21	38.51	21.18	5.97
Salmonella spp	70.90	76.32	78.43	104.00	32.88	6.84

Table 7

Grafic T90 values obtained from each microorganism
in the experiments with diffusion chambers

Microorganisms	Grafic T90 Values (h)	
	TSA	Selective medium
E. coli	47.2	39.2
Streptococcus faecalis	36.8	15.2
P. aeruginosa	40.8	28.0
C. albicans	24.0	48.0
S. aureus	33.6	3.2
Salmonella sp	42.6	25.2

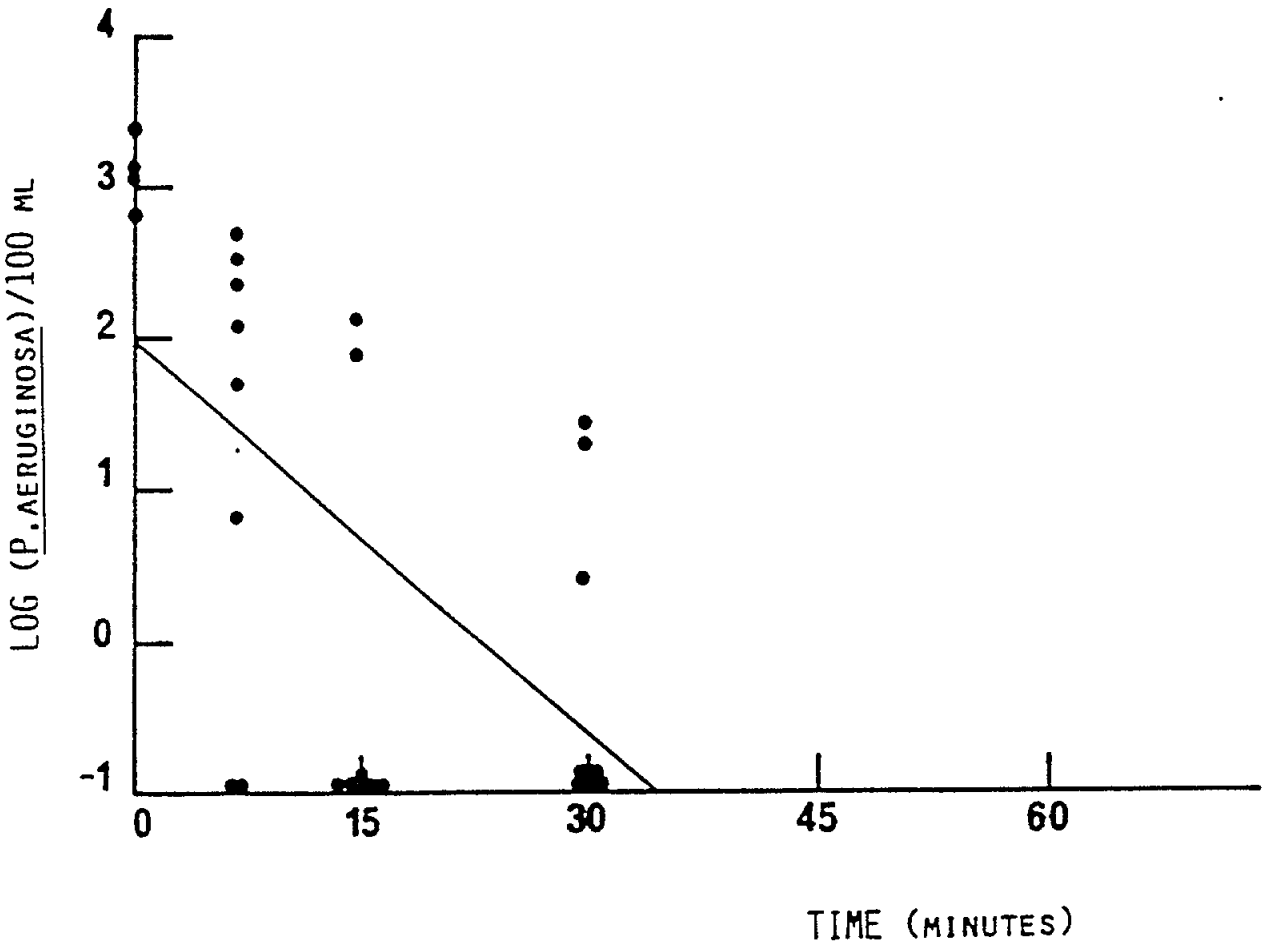


Figure 9

Disappearance kinetic of *P. aeruginosa* in the evolution zone of the Fuengirola submarine outfall

$y = 2.0135 - 0.0888\ x$; $r = 0.5675$; $p<0.001$

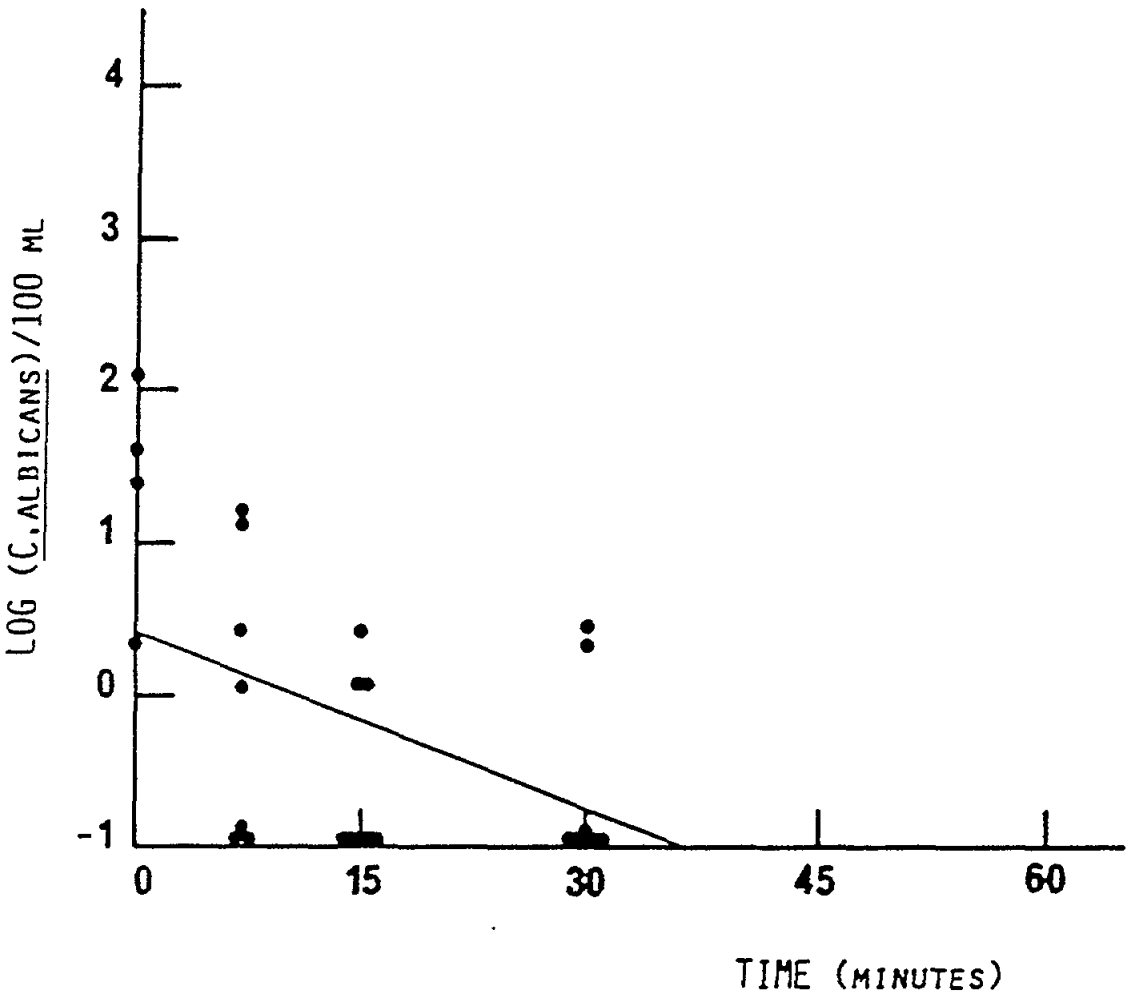


Figure 10

Disappearance kinetic of *C. albicans* in the evolution zone of the Fuengirola submarine outfall

$y = 0.4855 - 0.0440\ x$; $r = 0.5331$; $p<0.005$

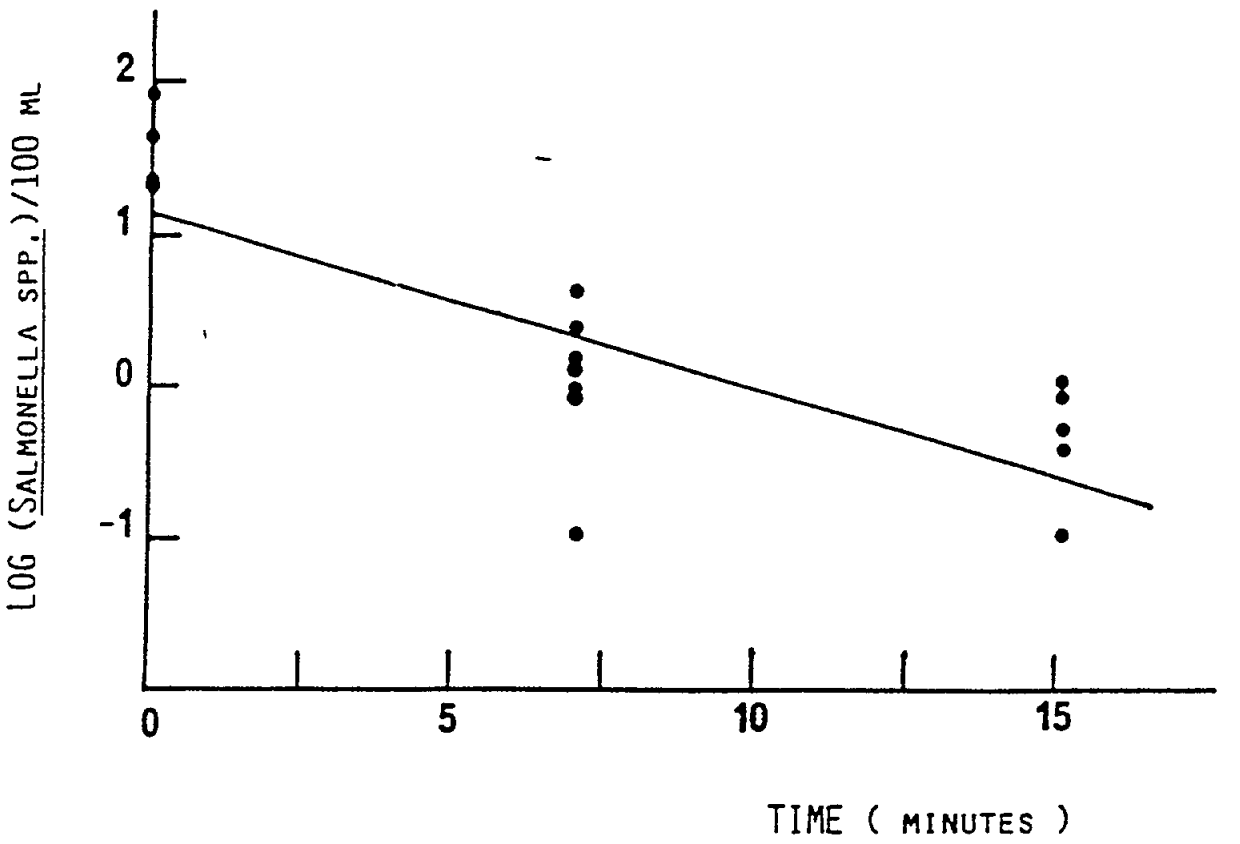


Figure 11

Disappearance kinetic of *Salmonella spp.* in the evolution zone of Fuengirola submarine outfall

$y = 1{,}1735 - 0.1212\,x$; $r = 0.7719$; $p<0.001$

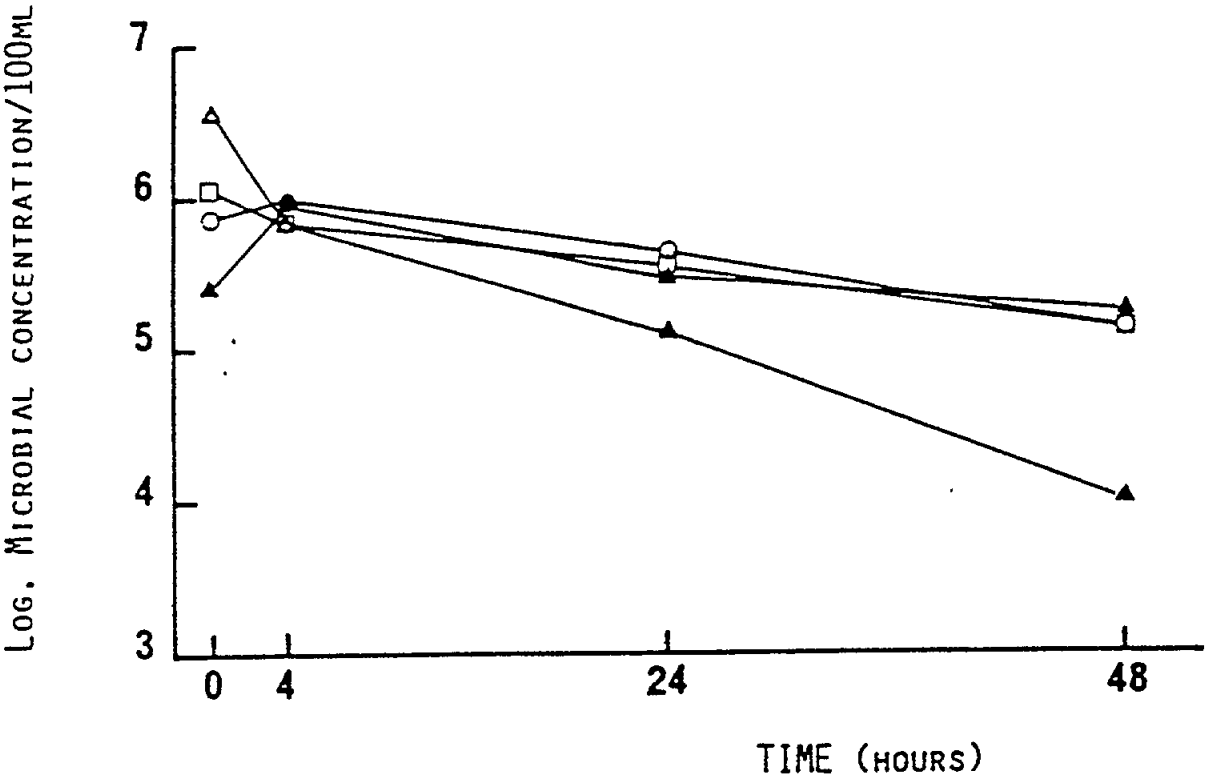


Figure 12

Partial experiments of survival of *E. coli* in diffusion chambers
Recounts on TSA

△——△	*E. coli*	19933	Experiment no 1
▲——▲	*E. coli*	19933	Experiment no 2
□——□	*E. coli*	21	Experiment no 3
○——○	*E. coli*	2	Experiment no 4

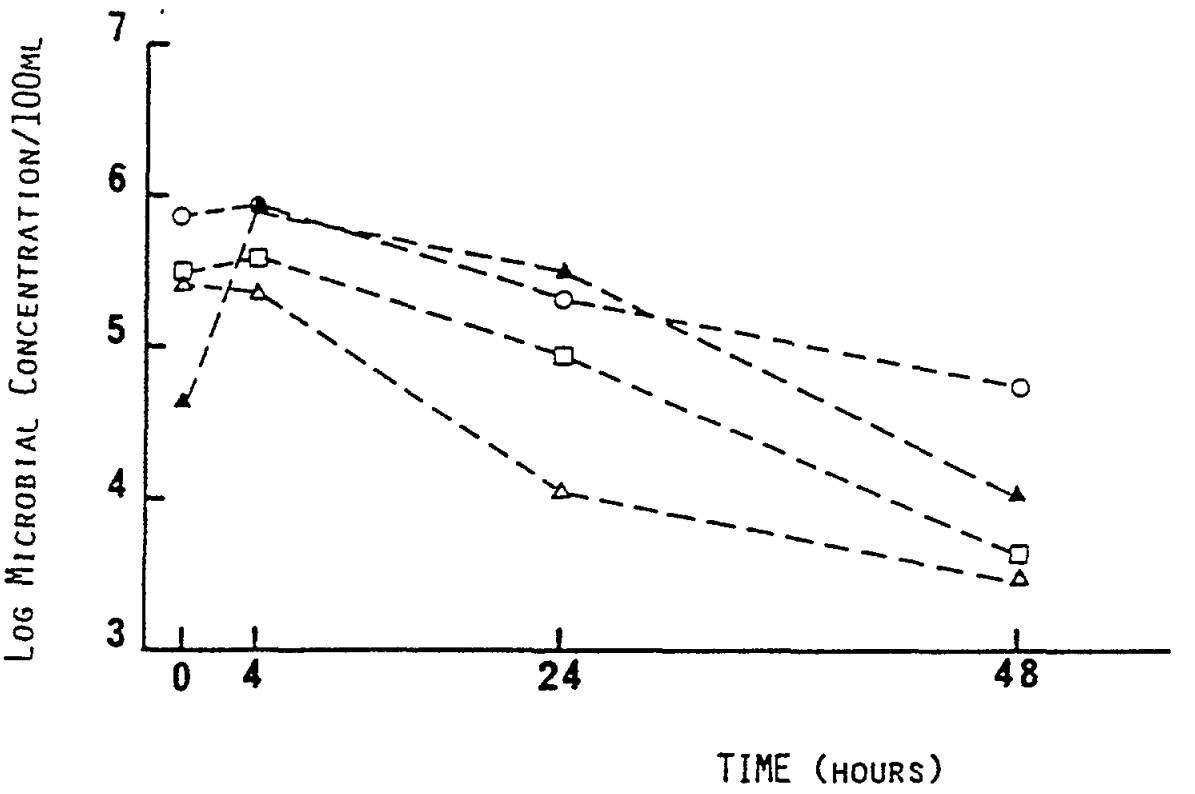


Figure 13

Partial experiments of survival of *E. coli* in diffusion chambers
Recounts on TSA

△------△	*E. coli*	19933	Experiment no 1
▲------▲	*E. coli*	19933	Experiment no 2
□------□	*E. coli*	21	Experiment no 2
○------○	*E. coli*	2	Experiment no 1

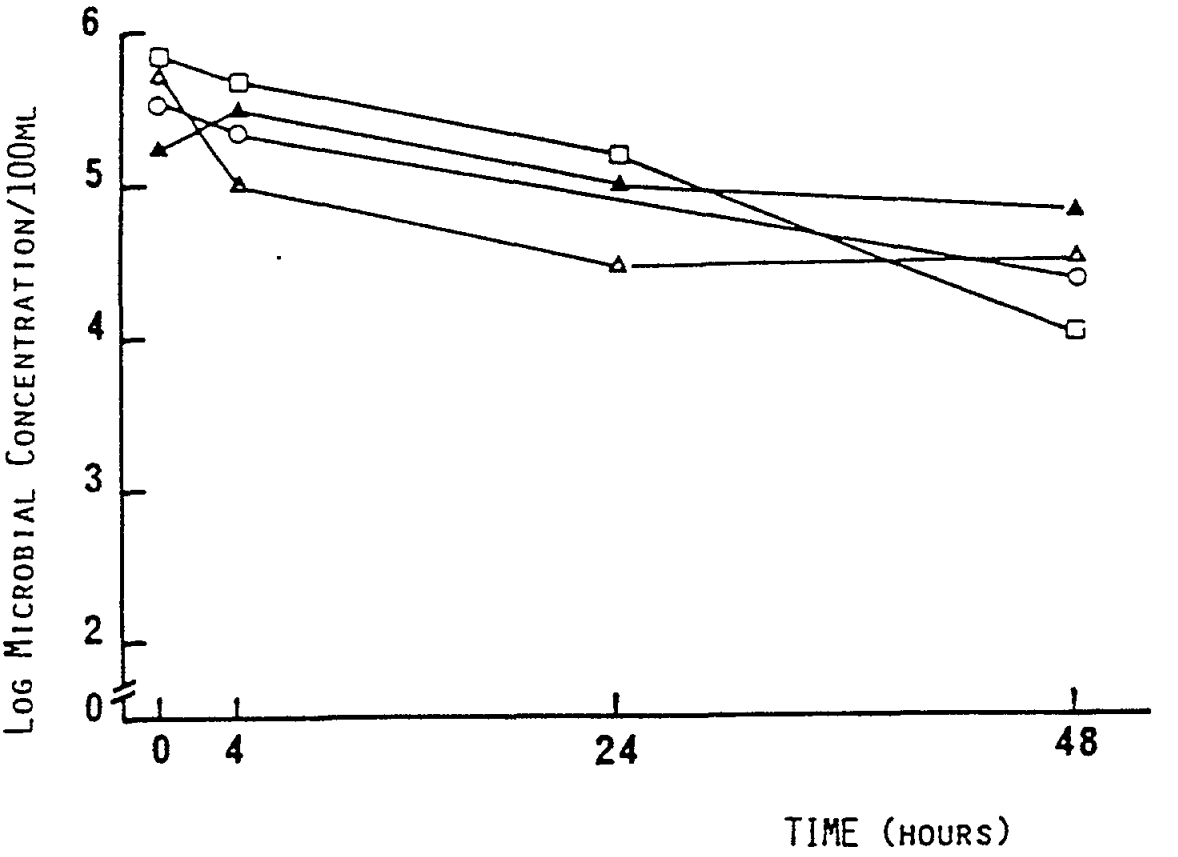


Figure 14

Partial experiments of survival of Faecal Streptococci
in diffusion chambers. Recounts on TSA

△——△	*S. faecalis*	19933	Experiment no 1
▲——▲	*S. faecalis*	19933	Experiment no 2
□——□	*S. faecalis*	21	Experiment no 2
○——○	*S . faecium*	2	Experiment no 1

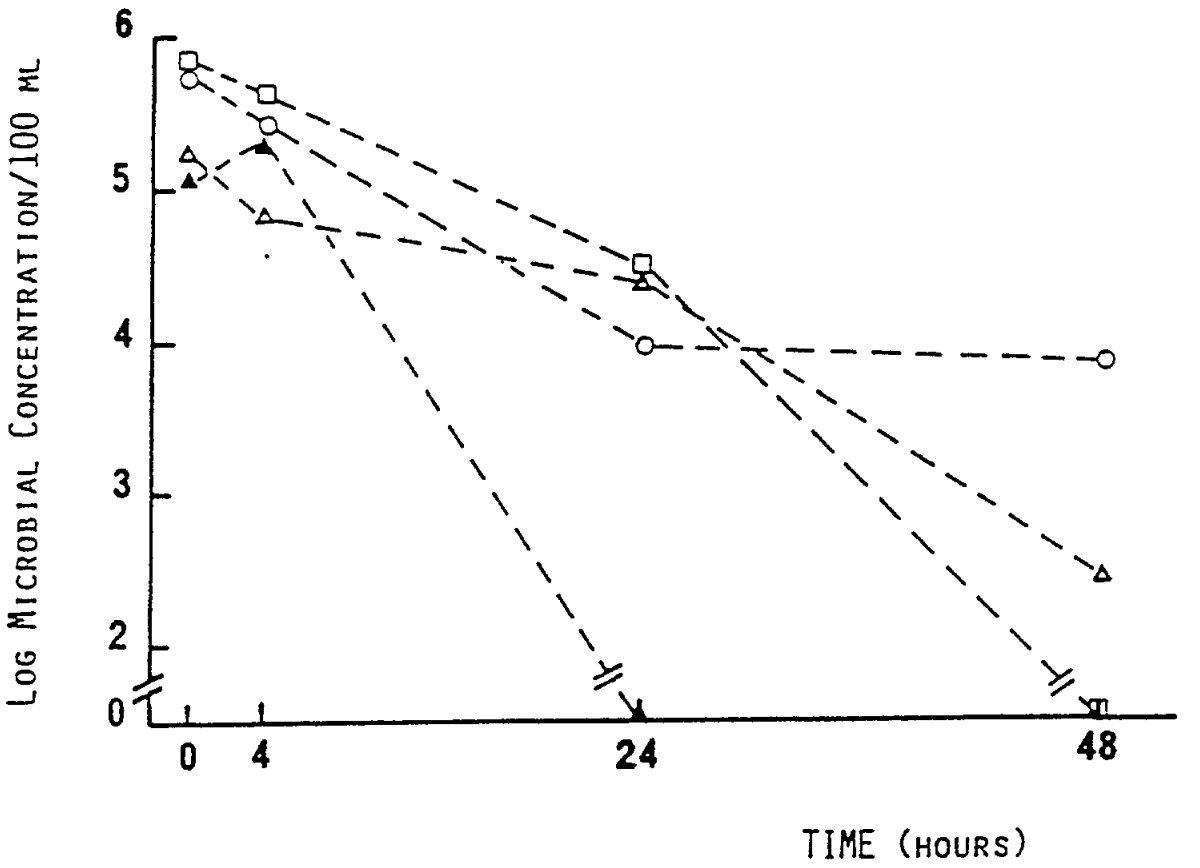


Figure 15

Partial experiments of survival of Faecal Streptococci in diffusion chambers. Recounts on selective medium

△------△	*S. faecalis*	19933	Experiment no 1
▲------▲	*S. faecalis*	19933	Experiment no 2
□------□	*S. faecalis*	21	Experiment no 2
○------○	*S. faecium*	2	Experiment no 1

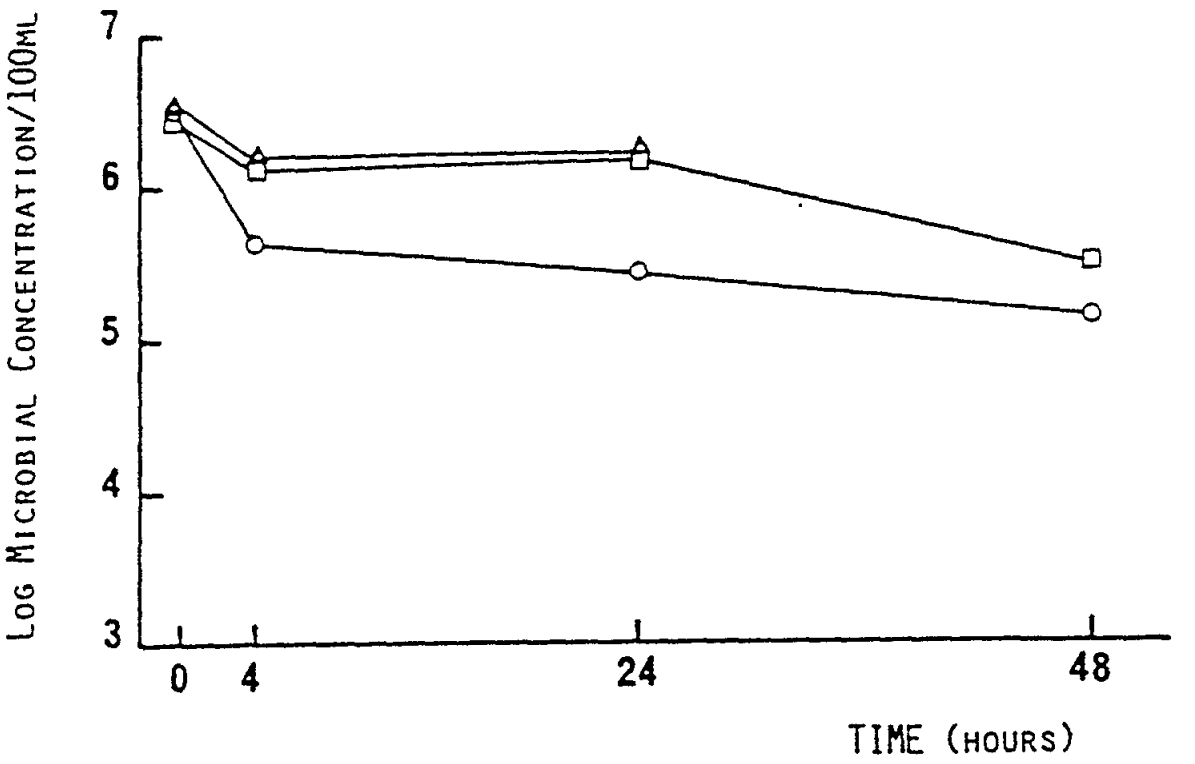


Figure 16

Partial experiments of survival of *P. aeruginosa* in diffusion chambers. Recounts on TSA

△——△	*P. aeruginosa*	110	Experiment no 1
□——□	*P. aeruginosa*	110	Experiment no 2
○——○	*P. aeruginosa*	J-75	Experiment no 1

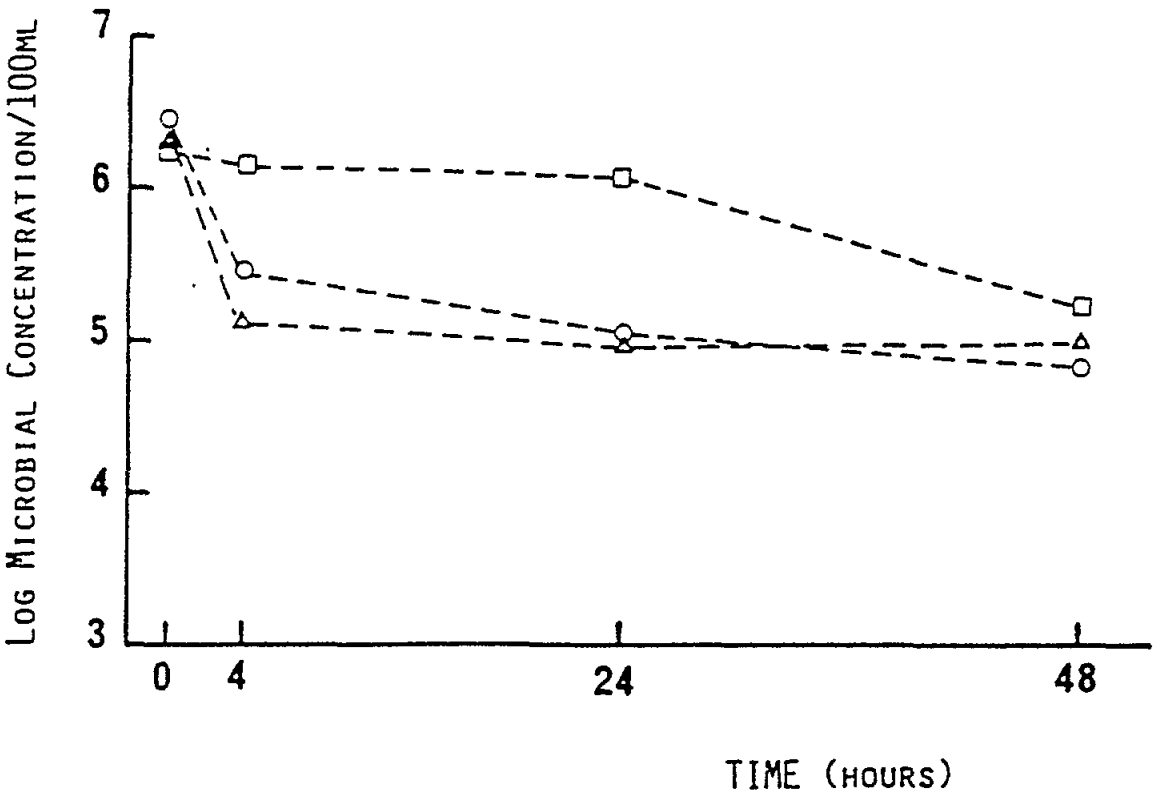


Figure 17

Partial experiments of survival of *P. aeruginosa* in diffusion chambers. Recounts on selective medium

△-----△ *P. aeruginosa*	110	Experiment no 1
□-----□ *P. aeruginosa*	110	Experiment no 2
○-----○ *P. aeruginosa*	J-75	Experiment no 1

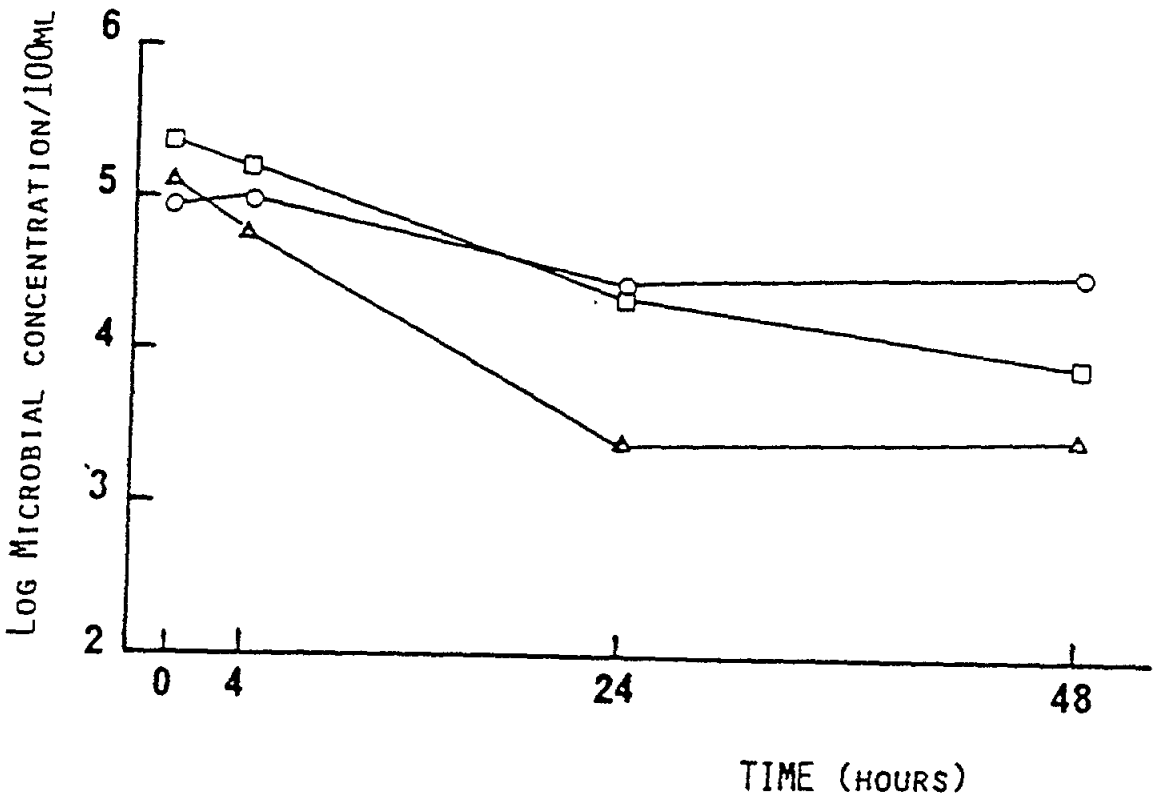


Figure 18

Partial experiments of survival of *C. albicans* in diffusion chambers. Recounts on TSA

△——△ *C. albicans*	1394	Experiment no 1
□——□ *C. albicans*	1394	Experiment no 2
○——○ *C. albicans*	1	Experiment no 2

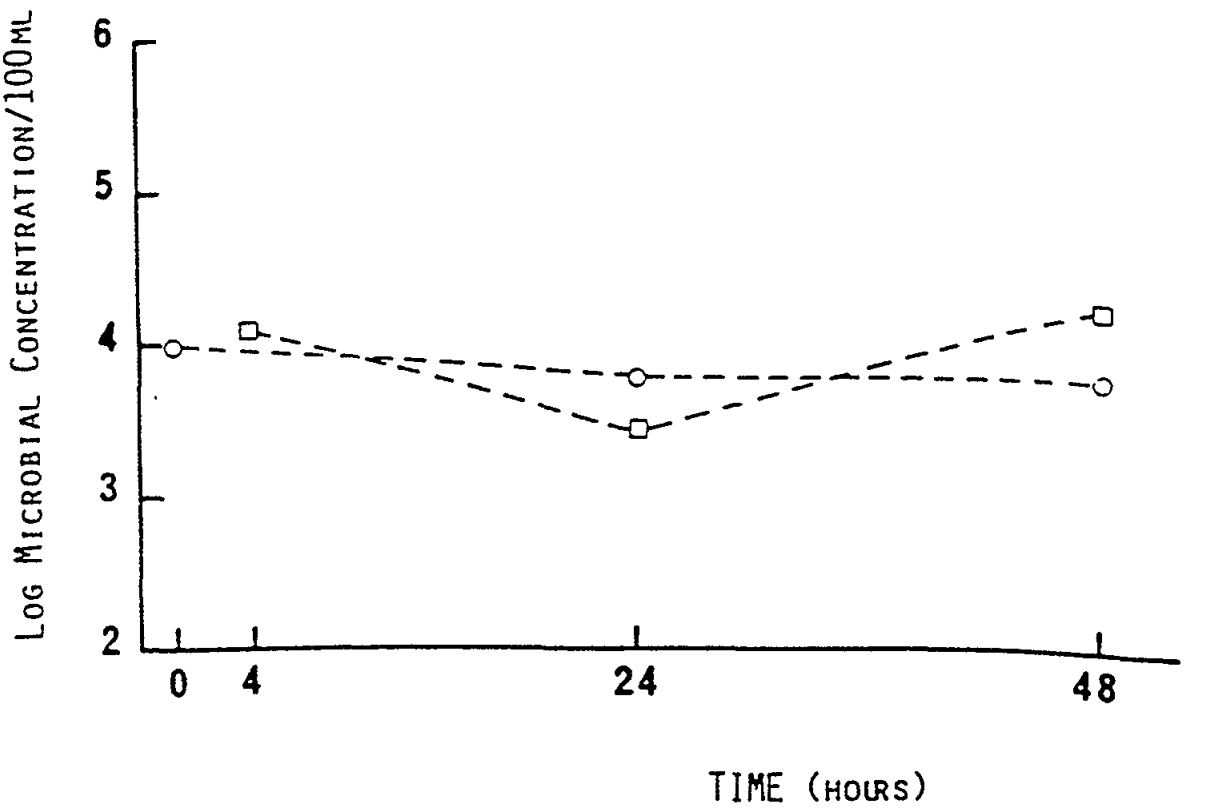


Figure 19

Partial experiments of survival of *C. albicans* in diffusion chambers. Recounts of selective medium

□-----□	*C. albicans*	1394	Experiment no 2
○-----○	*C. albicans*	1	Experiment no 2

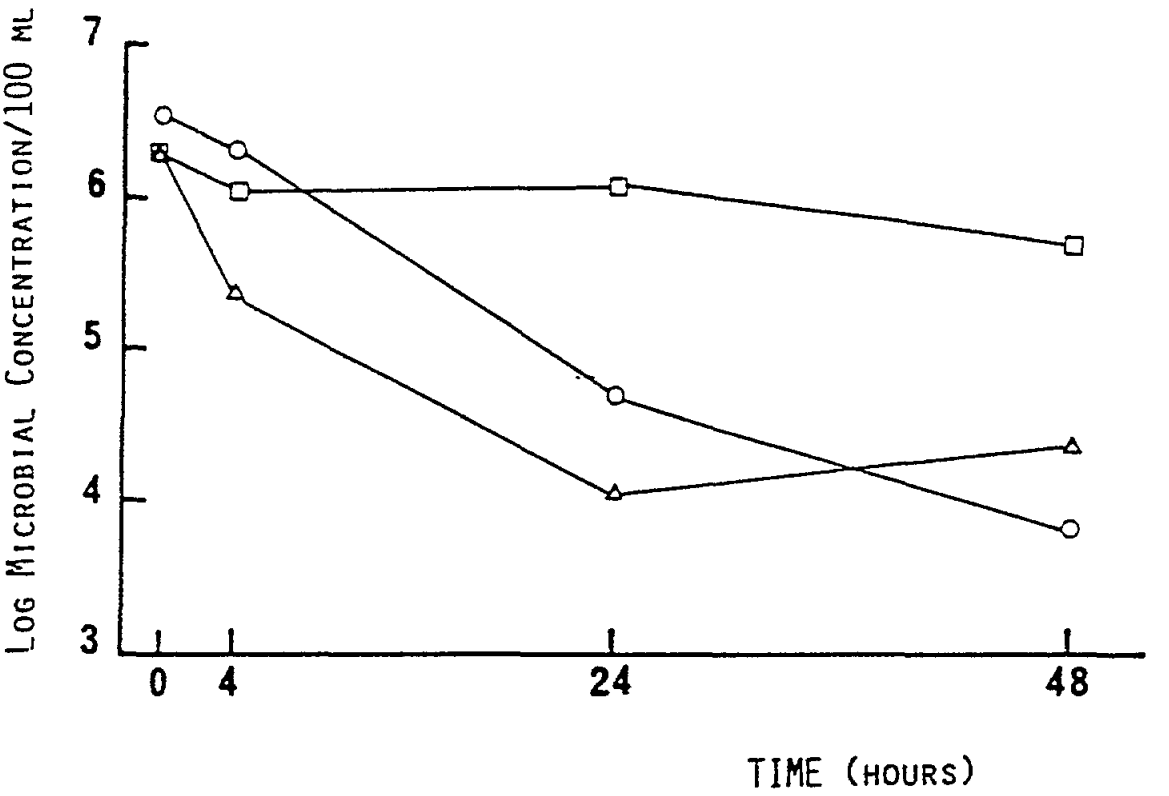


Figure 20

Partial experiments of survival of *S. aureus* in diffusion chambers. Recounts on TSA

△——△	*S. aureus*	29213	Experiment no 2
□——□	*S. aureus*	29213	Experiment no 1
○——○	*S. aureus*	15	Experiment no 2

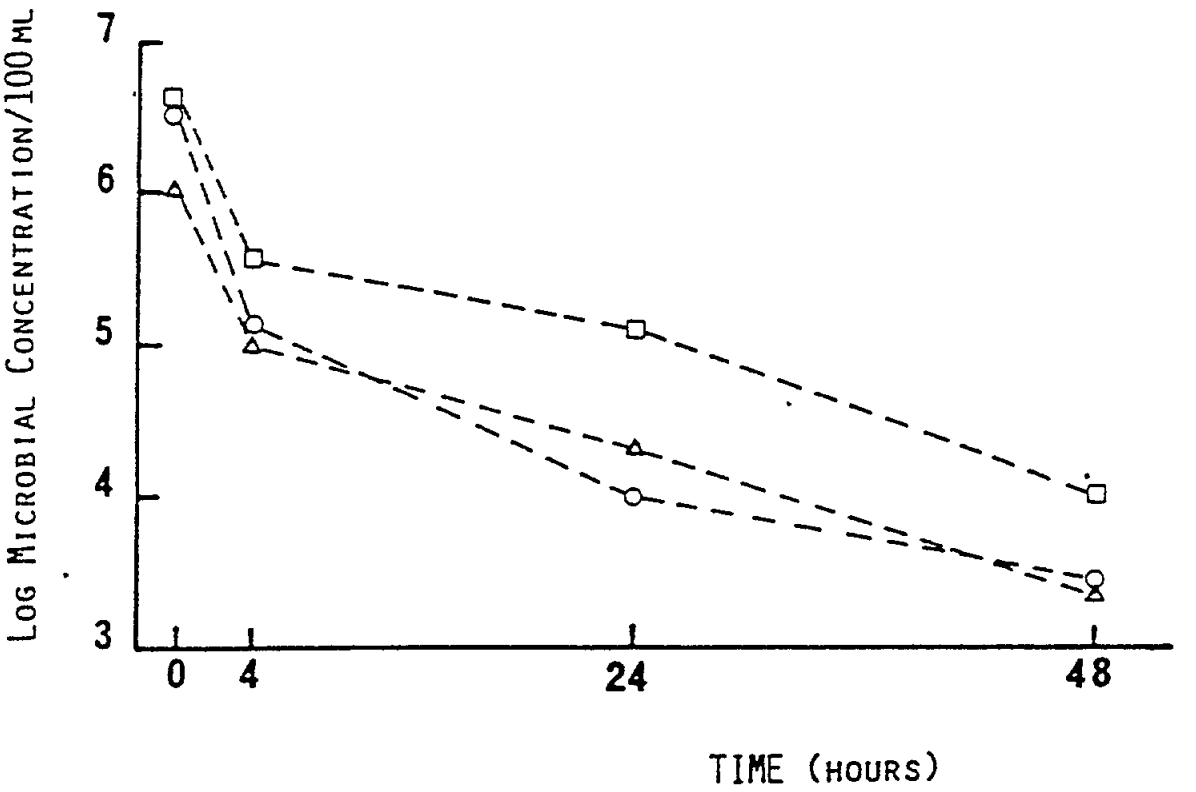


Figure 21

Partial experiments of survival of *S. aureus* in diffusion chambers. Recounts on selective medium

△------△	*S. aureus*	29213	Experiment no 2
□------□	*S. aureus*	29213	Experiment no 1
○------○	*S. aureus*	15	Experiment no 2

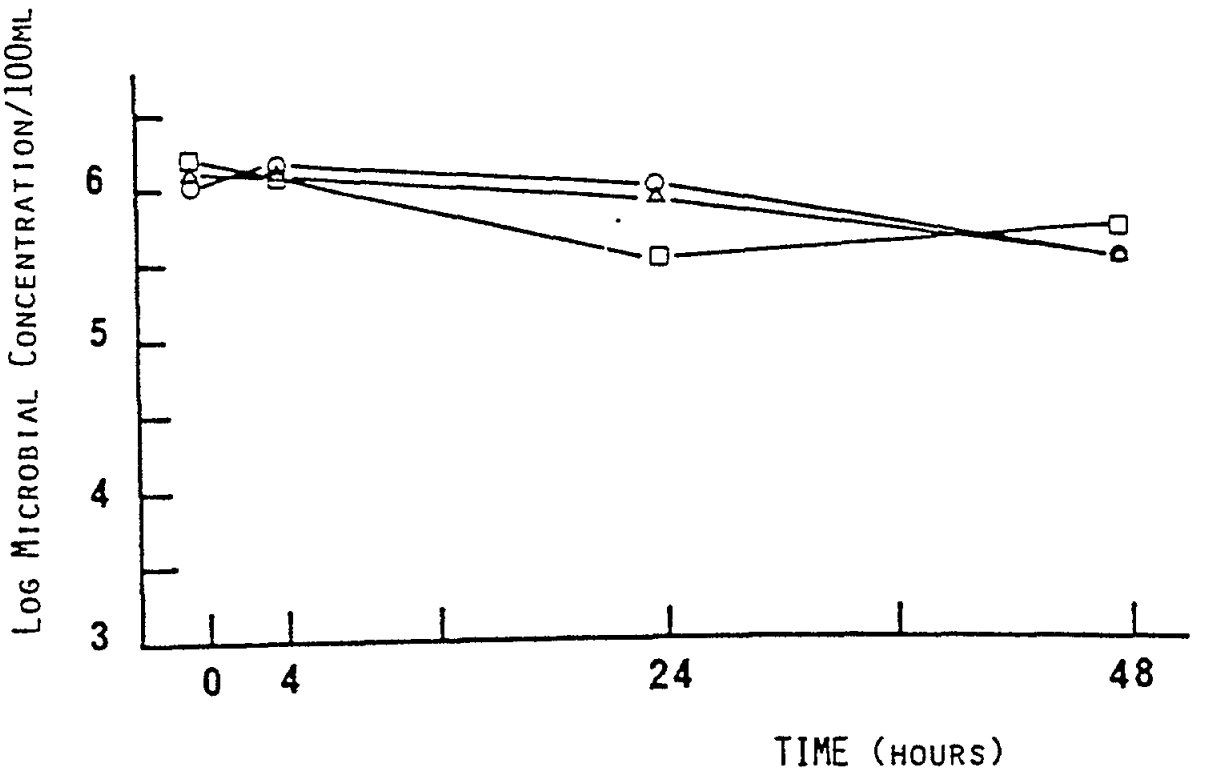


Figure 22

Partial experiments of survival of *Salmonella spp*
in diffusion chambers. Recounts on TSA

△——△ *Salmonella spp*	554	Experiment no 1
□——□ *Salmonella spp*	554	Experiment no 2
○——○ *Salmonella spp*	41-F	Experiment no 1

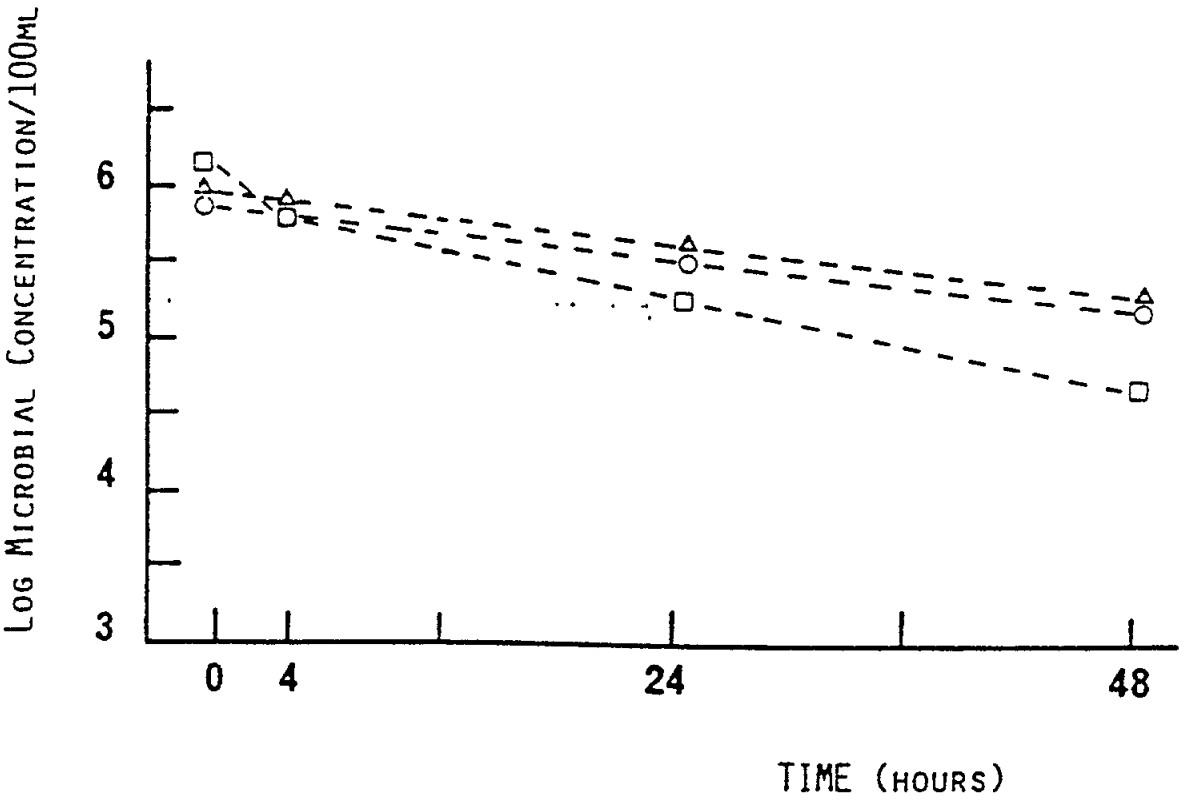


Figure 23

Partial experiments of survival of *Salmonella spp*
in diffusion chambers. Recounts on selective medium

△------△ *Salmonella spp*	554	Experiment no 1
□------□ *Salmonella spp*	554	Experiment no 2
○------○ *Salmonella spp*	41-F	Experiment no 1

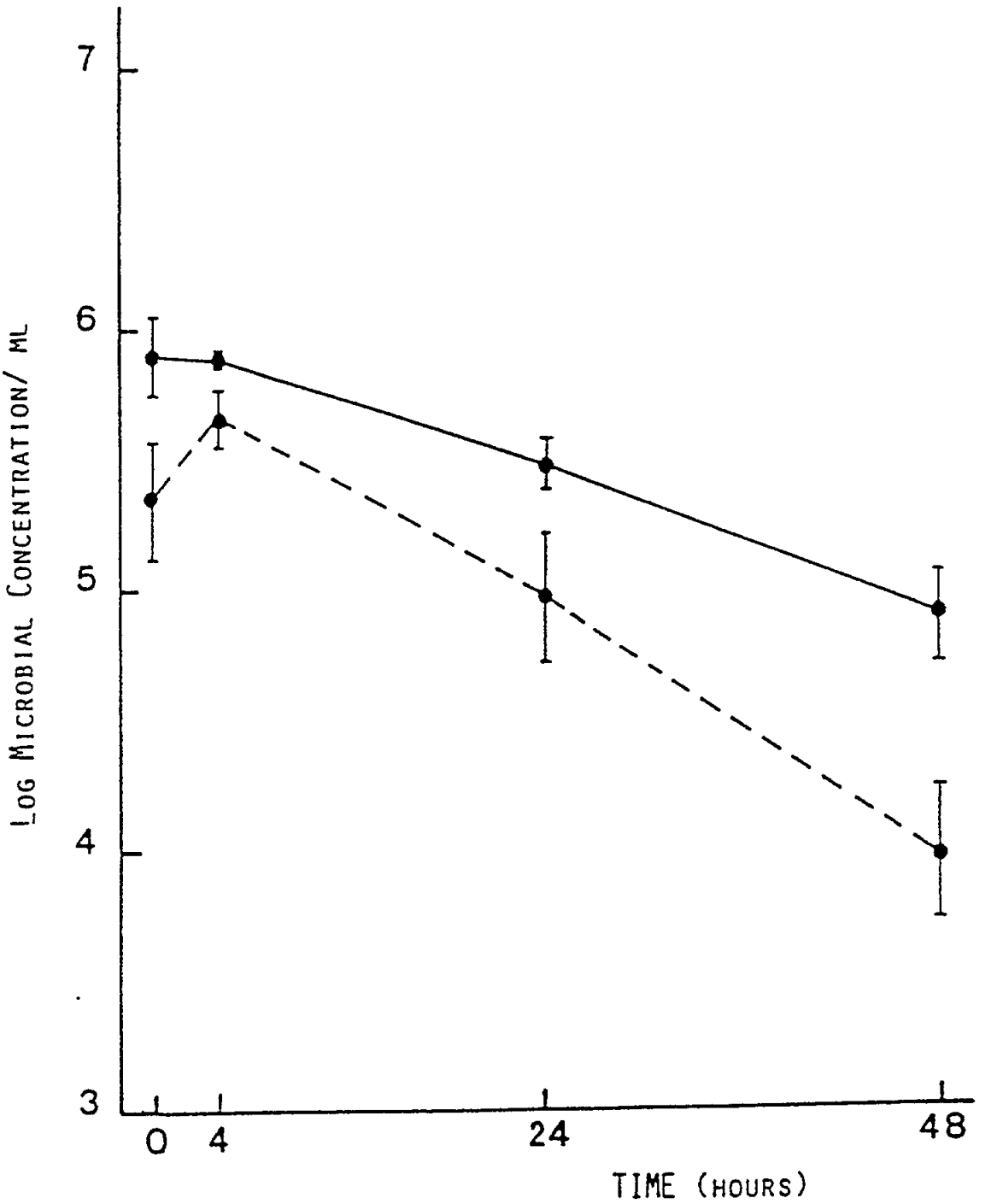


Figure 24

Global survival experiment of *E. coli* in diffusion chambers

●——● Recounts on TSA
●——● Recounts on selective medium

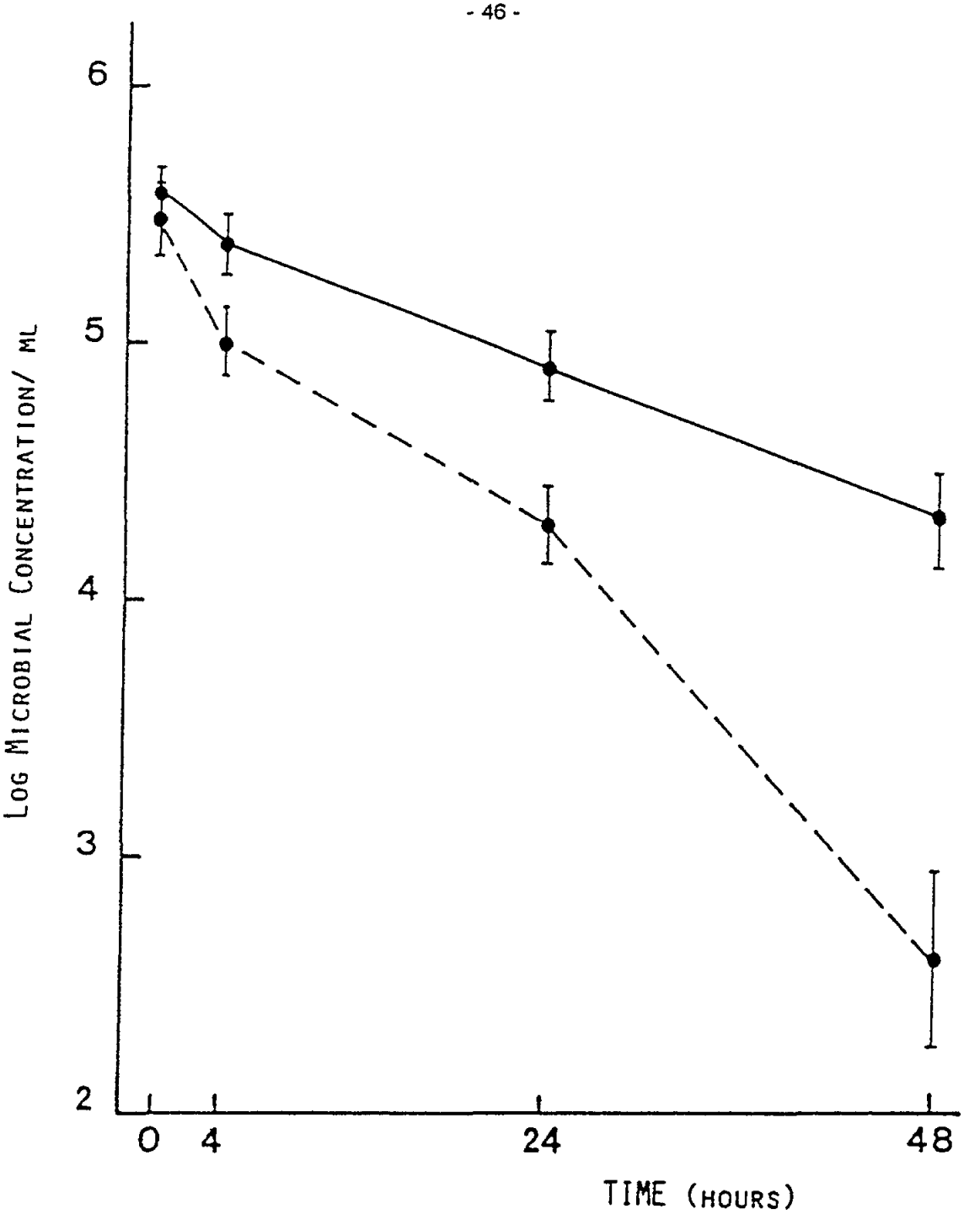


Figure 25

Global survival experiment of Faecal Streptococci in diffusion chambers

●——● Recounts on TSA
●- - -● Recounts on selective medium

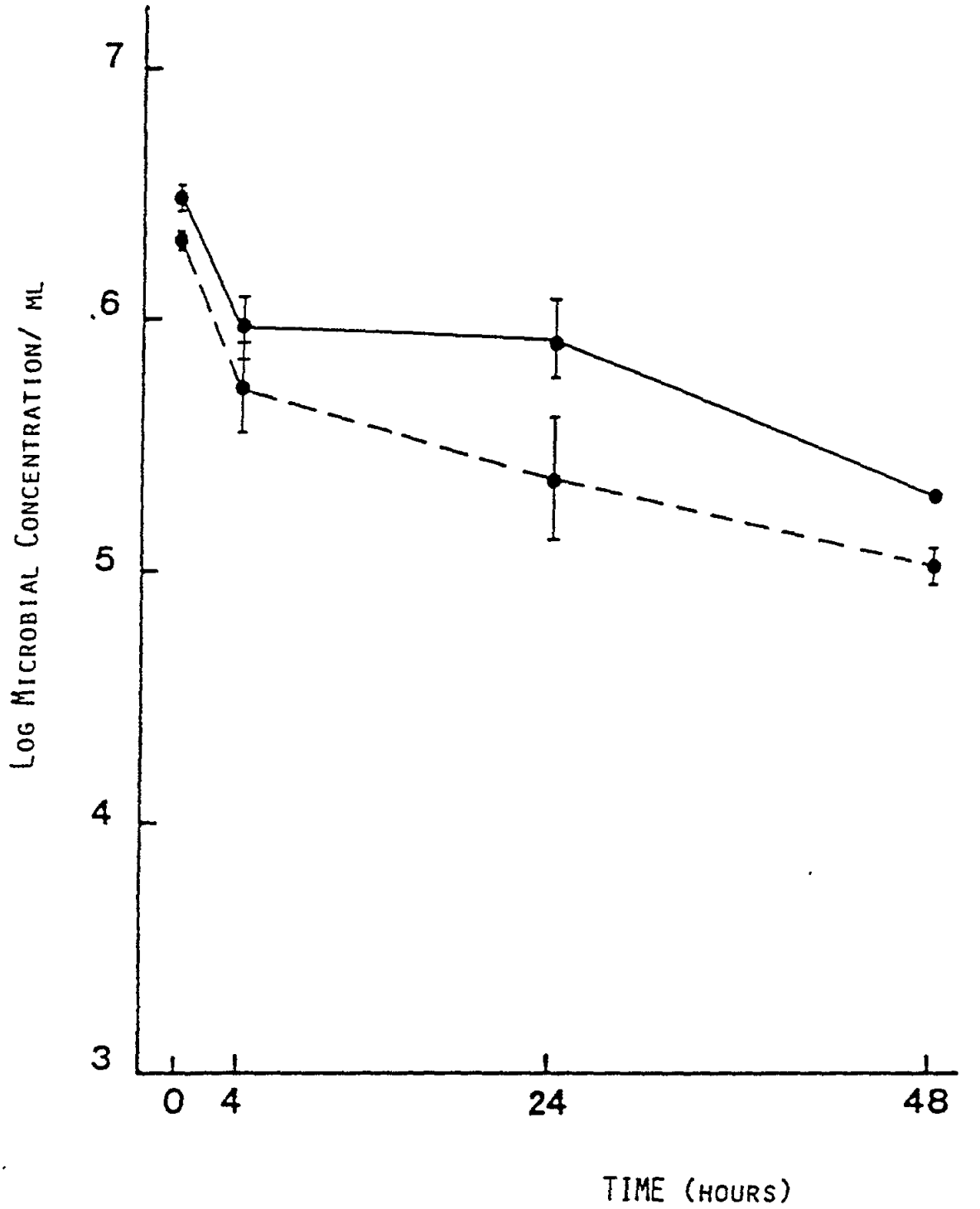


Figure 26

Global survival experiment of *P. aeruginosa* in diffusion chambers

●——● Recounts on TSA
●- - -● Recounts on selective medium

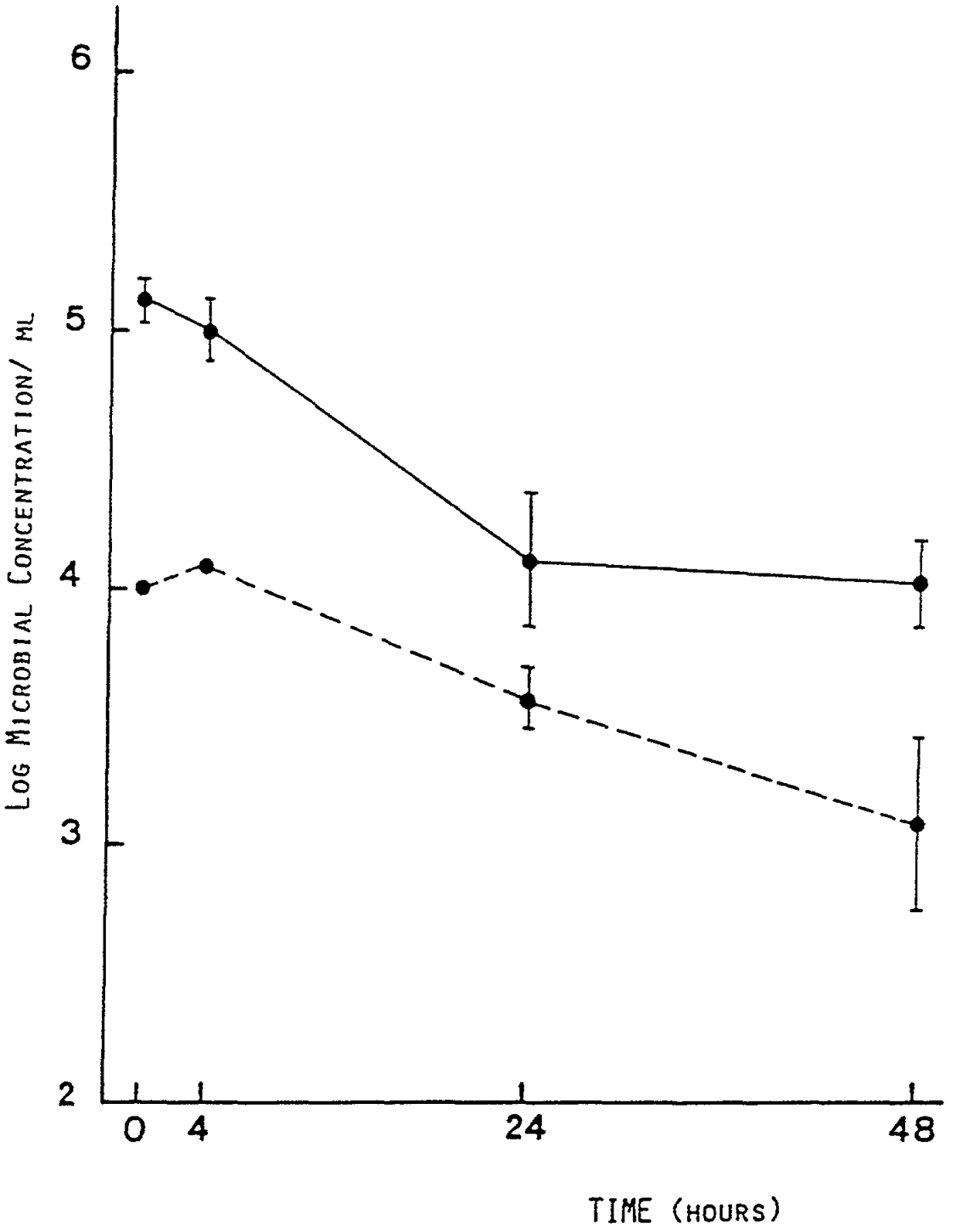


Figure 27

Global survival experiment of *C. albicans* in diffusion chambers

●——● Recounts on TSA
●——● *Recounts on selective medium*

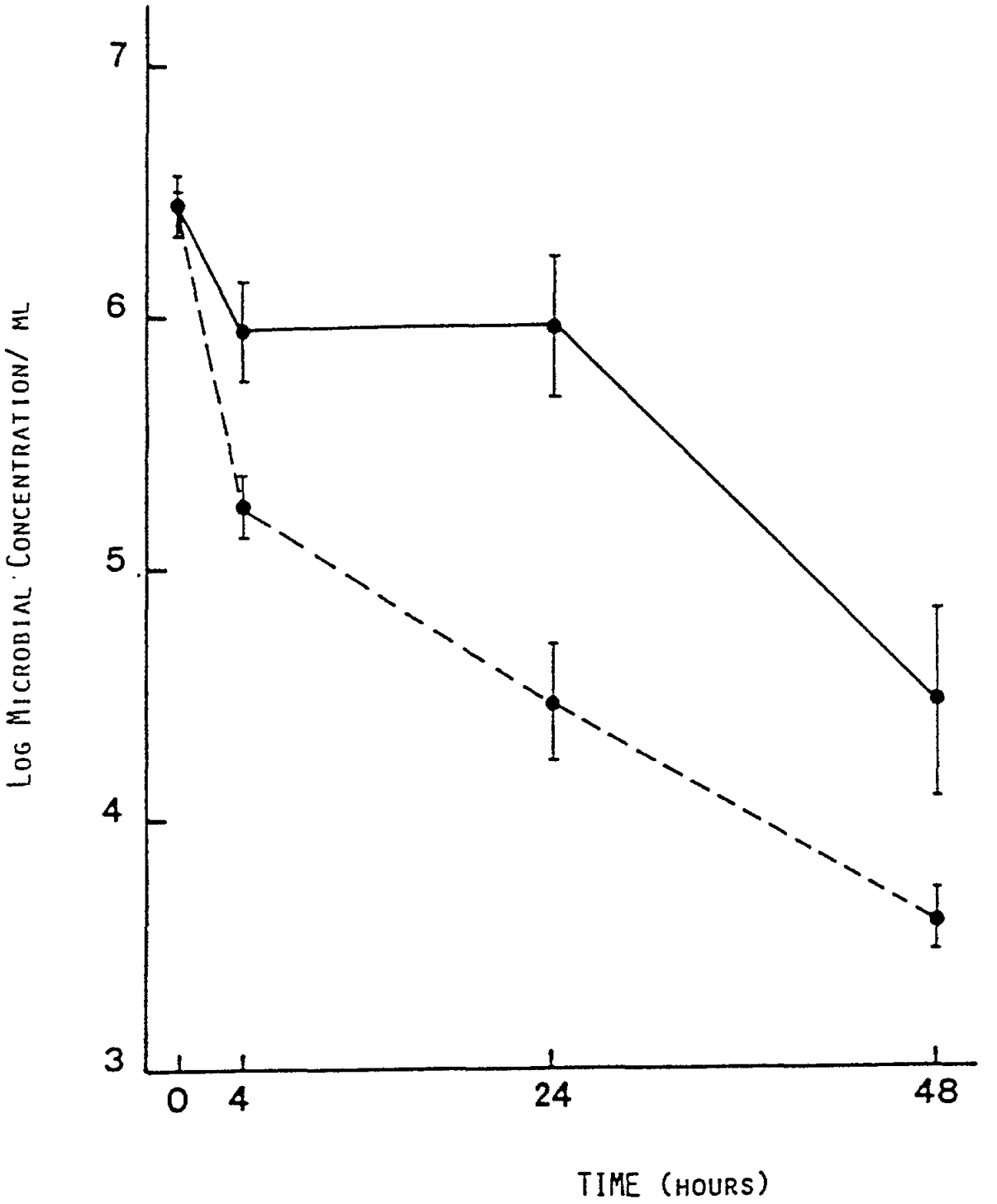


Figure 28

Global survival experiment of *S. aureus* in diffusion chambers

●——● Recounts on TSA
●- - -● Recounts on selective medium

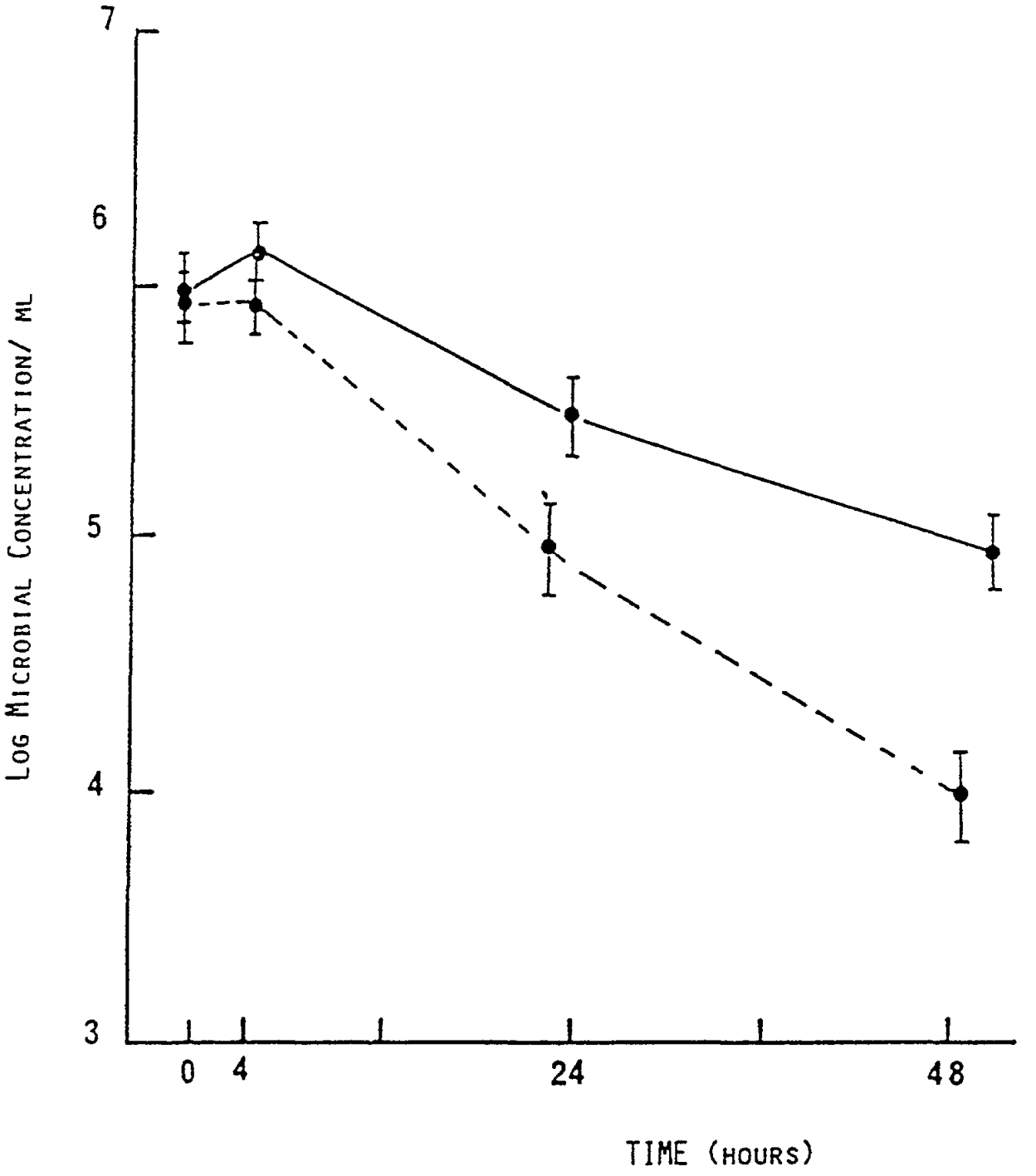
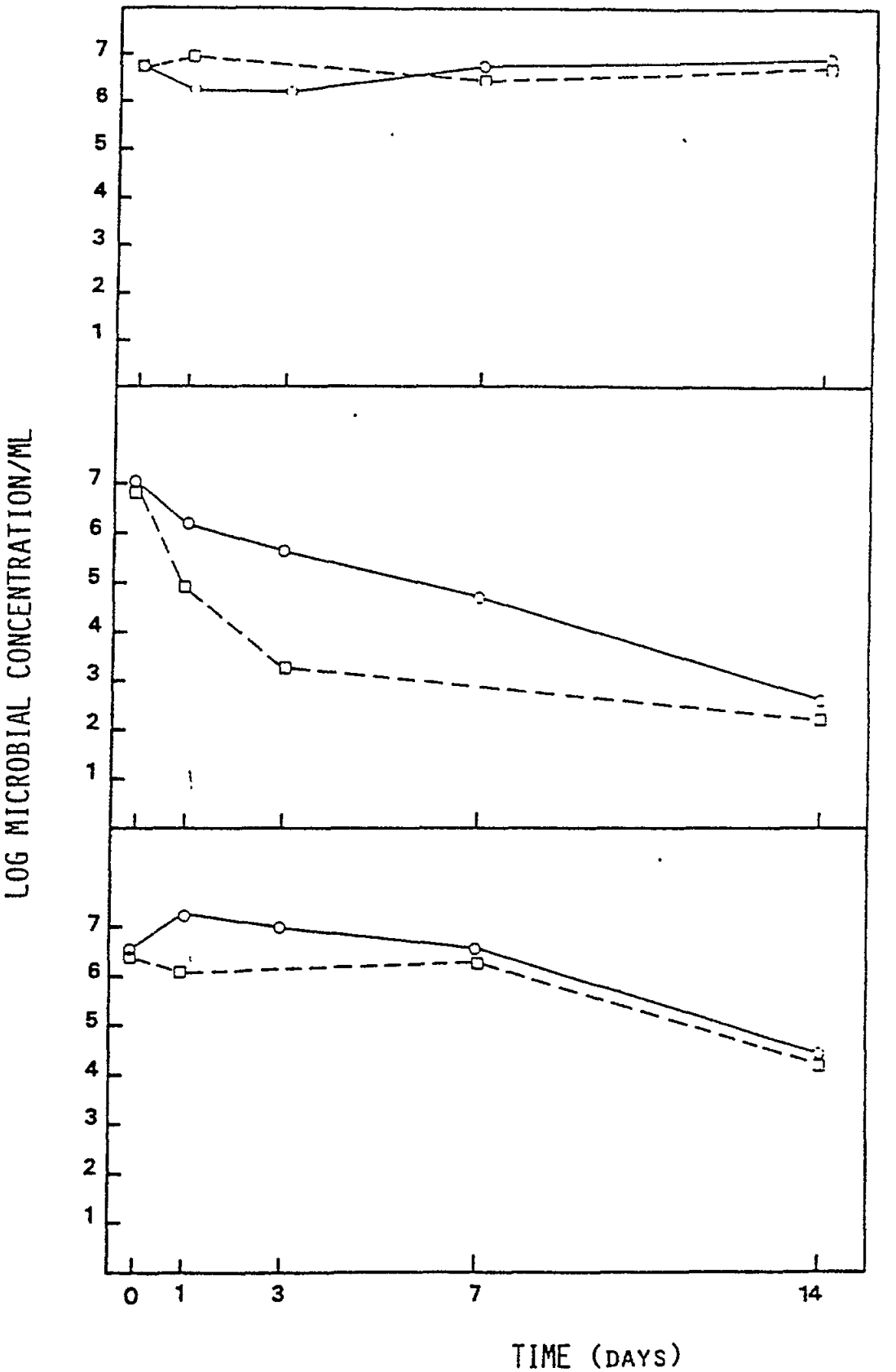


Figure 29

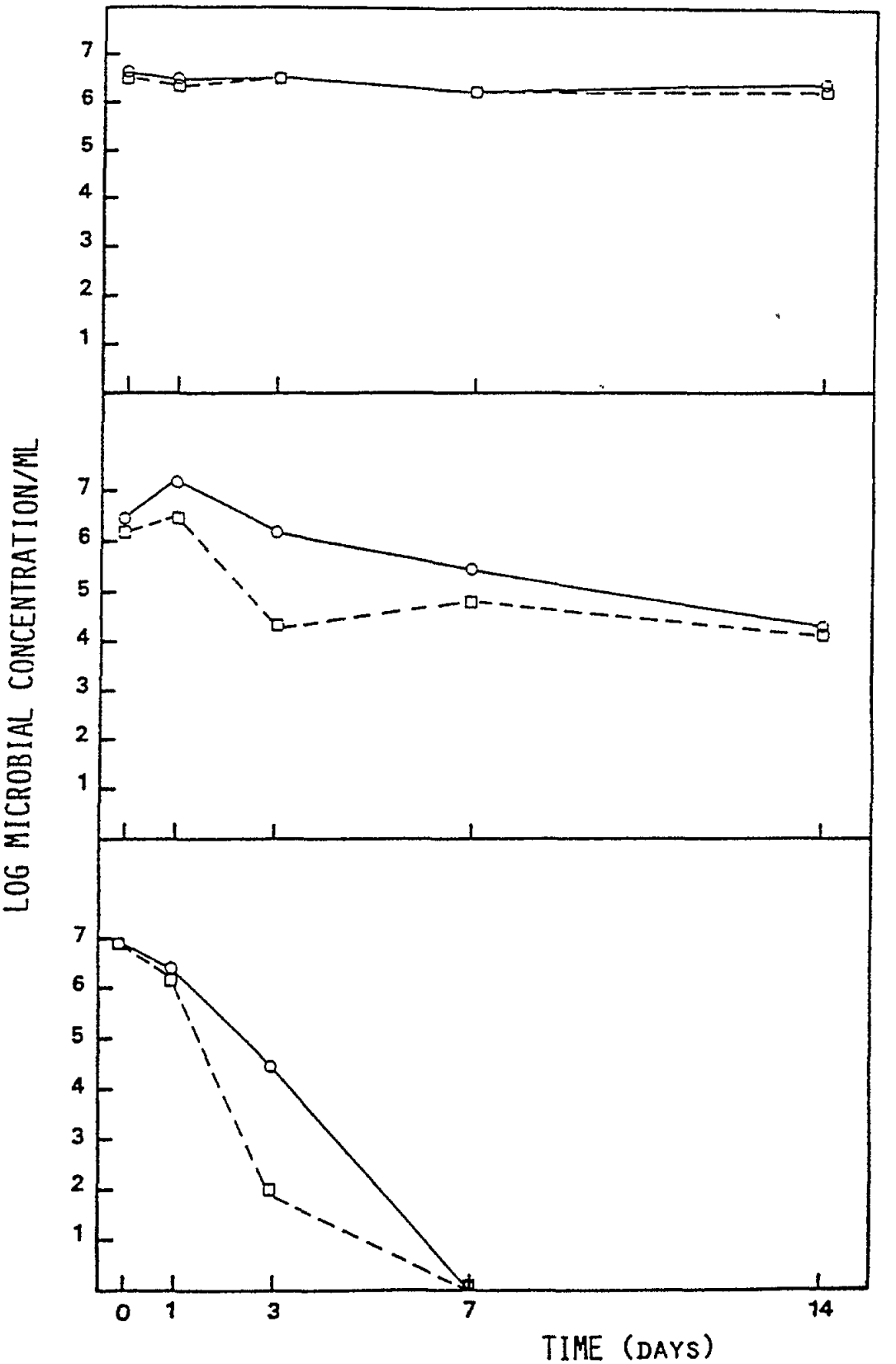
Global survival experiment of *Salmonella spp* in diffusion chambers

●——● Recounts on TSA
●- - -● Recounts on selective medium



Figures 30-32: Influence of (A) saline solution, (B) untreated seawater, (C) filtered seawater on the survival of *E. coli*

○——○ Recounts on double layer
□-----□ Recounts on selective medium



Figures 33-35: Influence of (A) artificial seawater, (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *E. coli*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium

Table 8

Percentage of injured and survival cells from each studied medium *E. coli*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	76.00	-	61.70	24.00	46.00	32.60	189.80	165.00
USW	94.00	99.65	-	52.90	16.20	5.75	0.30	0.003
FSW	92.00	-	43.00	55.40	406.00	152.00	79.40	0.75
ASW	12.10	0.00	0.00	23.68	60.95	82.85	36.42	45.23
ASWS	4.20	98.57	66.60	37.50	100.00	40.00	6.86	0.51
ASWL	30.50	99.75	-	-	29.50	3.40	0.00	0.00

S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater
ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days

The results of these experiments show that this supplement supposes an important decrease in the microbial population (99.49%) (Figure 34) in comparison with the results obtained from artificial seawater for the same microorganism.

Visible light (Figure 35) produced a very important decrease in the concentration of *E. coli*. This was the only factor which produced the total inactivativity of the population (day 7 of the experiment).

The results obtained in the study on survival in the different test media of the *S. faecalis* are given in Figures 36-41.

The reference solution (0.85% saline solution) produced a notable descent in the microbial concentration, reaching more than one order of magnitude (7.11% of surviving cells), as the proportion of damaged cells at the end of 14 days was very high (97.19%) (Figure 36). The testing medium which had the least noxious effect on this microorganism was artificial seawater which produced 44.5% of deaths after the time studied (Figure 39). The untreated seawater produced, as with *E. coli*, a high proportion of dead cells (Figure 37). The factors which principally affected the inactivation of this microorganism were the supplement of sewage and visible light (Figure 39 and 41). Visible light had a rapid effect, producing a 90% decrease of the population in the first 4.8 h.

In Figures 42-47 the survival curves obtained from the experimental study of the pathogen *P. aeruginosa* are represented. The most negative testing medium which acts on the survival of this microorganism was natural, crude seawater (0.0003% of survival cells) at the end of 14 days.

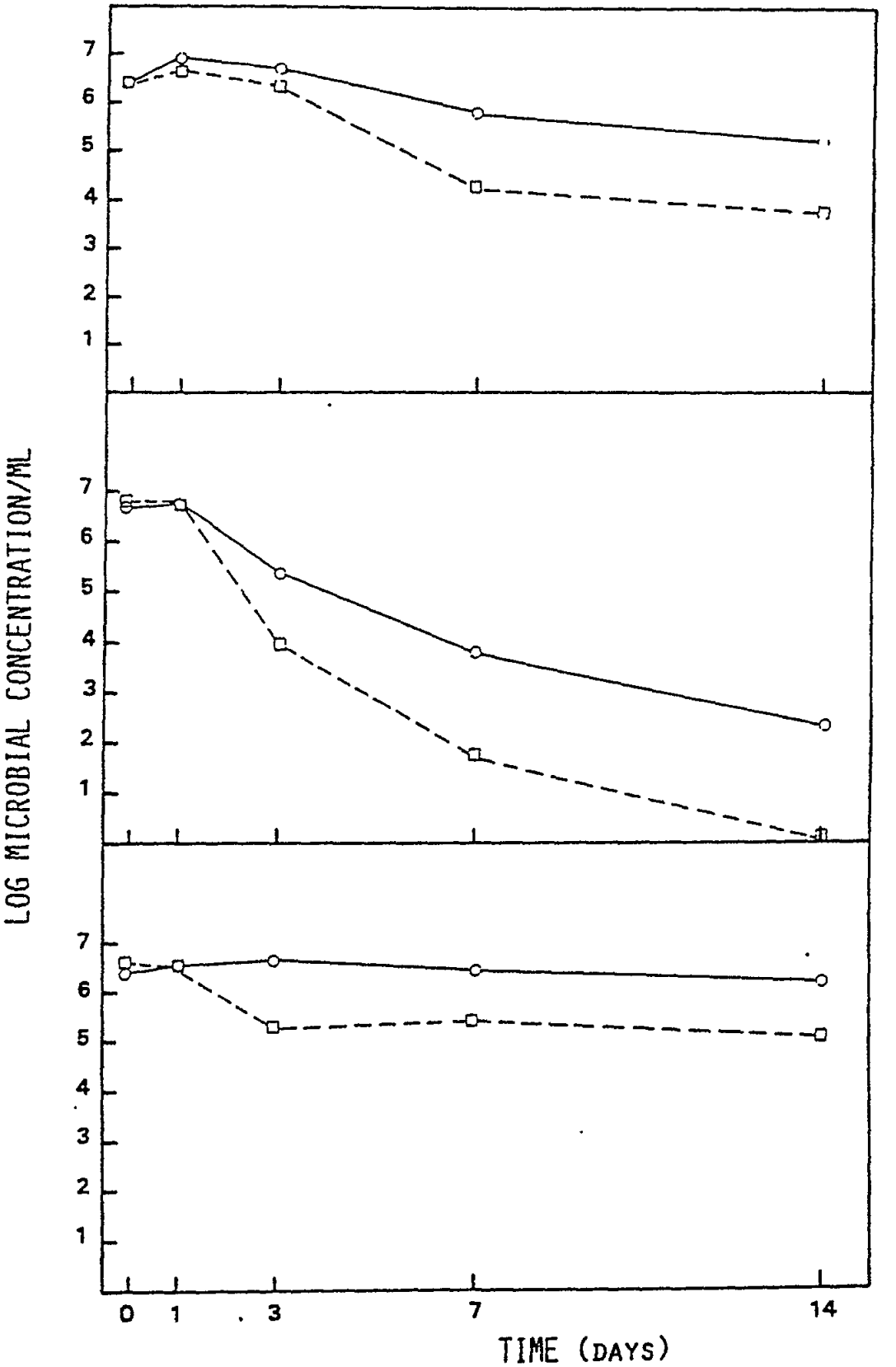
Visible light and toxic factors carried in sewage, also produce an important diminishing of the concentration which is far superior to that observed in the reference solution (saline solution) (Figures 42, 43, 46 and 47). It is important to emphasize the evaluated percentages of damaged or stressed cells observed in testing media.

In Figures 48-53 the different inactivating processes obtained by the pathogenic yeast *C. albicans* in the various suspensions tested are presented. This microorganism presented a high proportion of death in the reference solution (67.5%) (Figure 48), while in filtered seawater (Figure 50) no decrease was observed in the initial concentration during the time period studied. The conditions which produced the most accentuated number of deaths in this pathogen were crude seawater (0.28% of cells survived after 14 days and, principally, artificial seawater subjected to visible light which succeeded in inactivating almost the total of the initial inoculum (Figures 49 and 53).

The inactivation curves of *S. aureus*, taking into consideration the different factors studied, are shown in Figures 54-59. This pathogen showed a total inactivation in three of the media used (crude seawater, artificial seawater supplemented with sewage, and visible light). Figures 55, 58 and 59, respectively, reducing its initial concentration by more than 90% when tested in filtered seawater (Figures 56). No significant decreases of the microorganism were observed in saline solution at 0.85% (Figure 54), nor in artificial seawater (Figure 57).

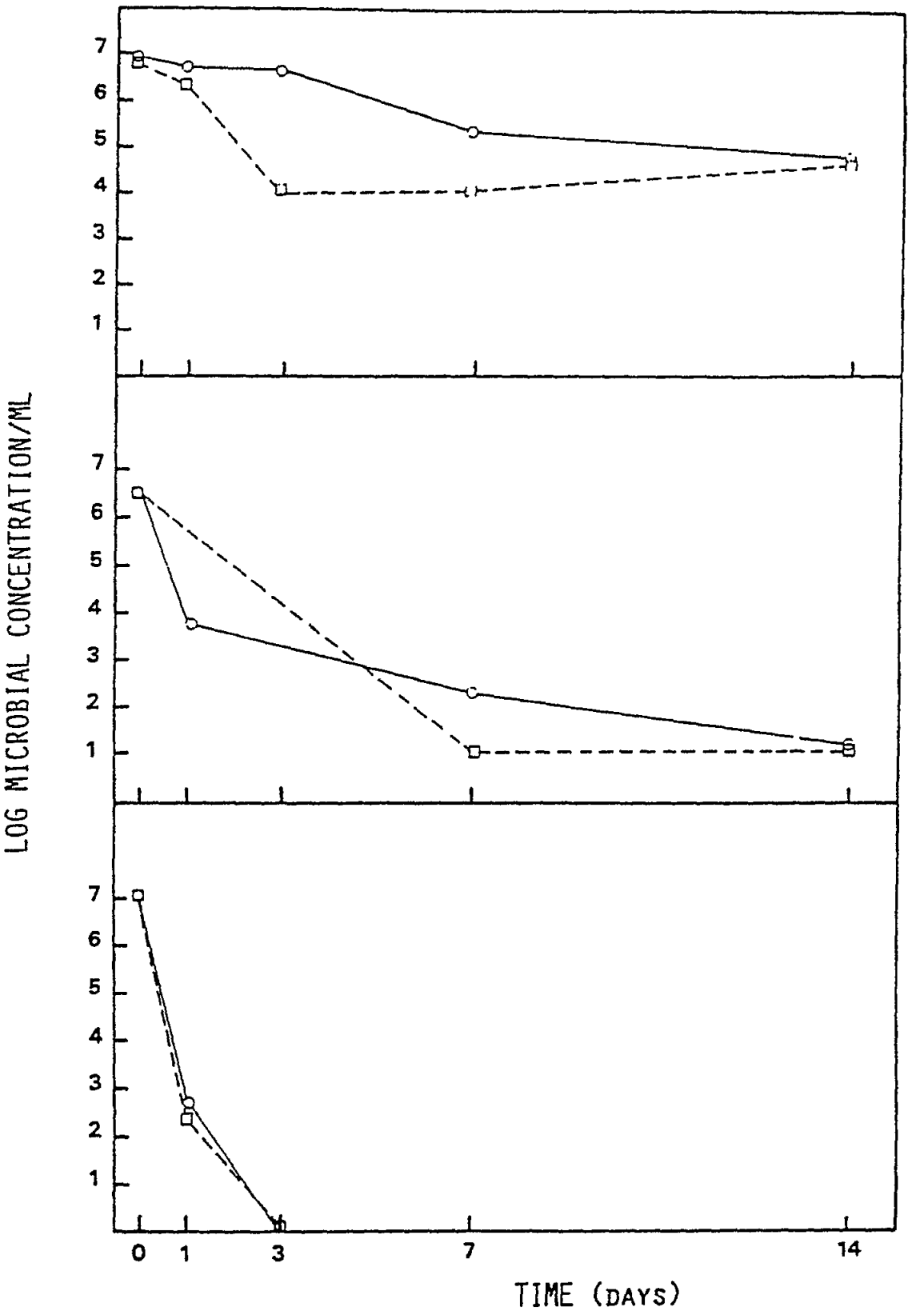
The results obtained by the *Salmonella* species: *S. thompson* and *S. paratyphi* C are presented in figures 60-71. Both species showed a similar behaviour against the sea factors, undergoing a greater inactivation when the suspension was made in untreated seawater or when they were subjected to visible light (Figures 61, 65, 67 and 71).

It is important to emphasize the slow inactivation of *S. thompson* when the artificial seawater was supplemented with sewage (with a survival percentage of 65.71%), factor which considerably influenced the survival of *S. paratyphi* C after 14 days of the test period (survival percentage of 5.43%) (Figures 64 and 70).



Figures 36-38: Influence of (A) saline solution, (B) untreated seawater, (C) filtered seawater on the survival of *S. faecalis*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium



Figures 39-41: Influence of (A) artificial seawater, (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *S. faecalis*

○——○ Recounts on double layer
□-----□ Recounts on selective medium

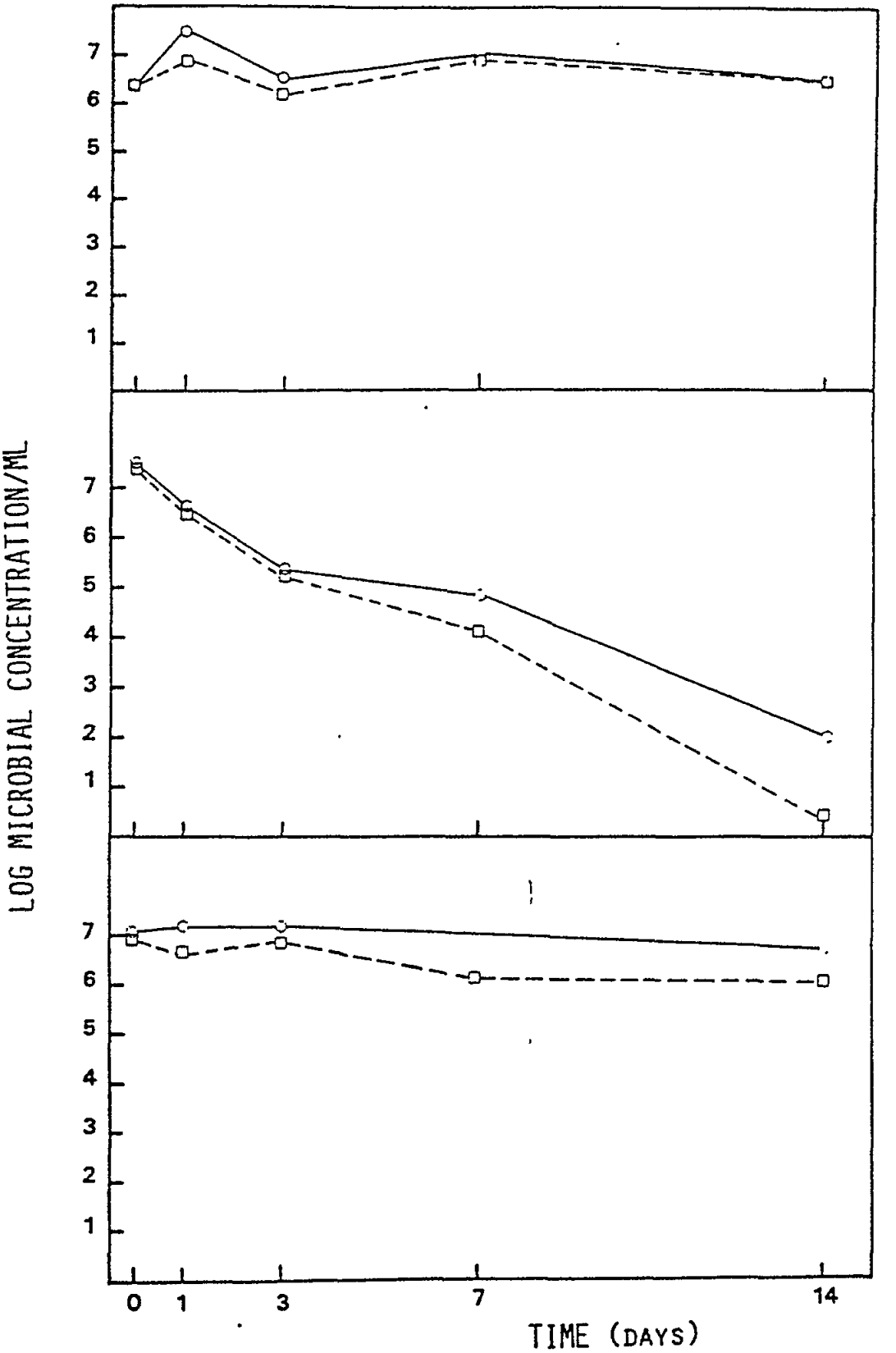
Table 9

Percentage of injured and survival cells from each studied medium for *S. faecalis*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	35.30	62.06	97.47	97.19	277.00	196.00	24.00	7.11
USW	0.00	99.66	98.95	100.00	112.00	51.92	0.10	0.005
FSW	22.40	96.28	93.19	93.35	163.00	185.00	121.00	65.50
ASW	13.60	4.50	95.11	38.12	103.30	54.54	7.27	1.32
ASWS	-	-	5.00	28.10	0.0014	-	0.0048	0.0008
ASWL	50.00	-	-	-	0.00	0.00	0.00	0.00

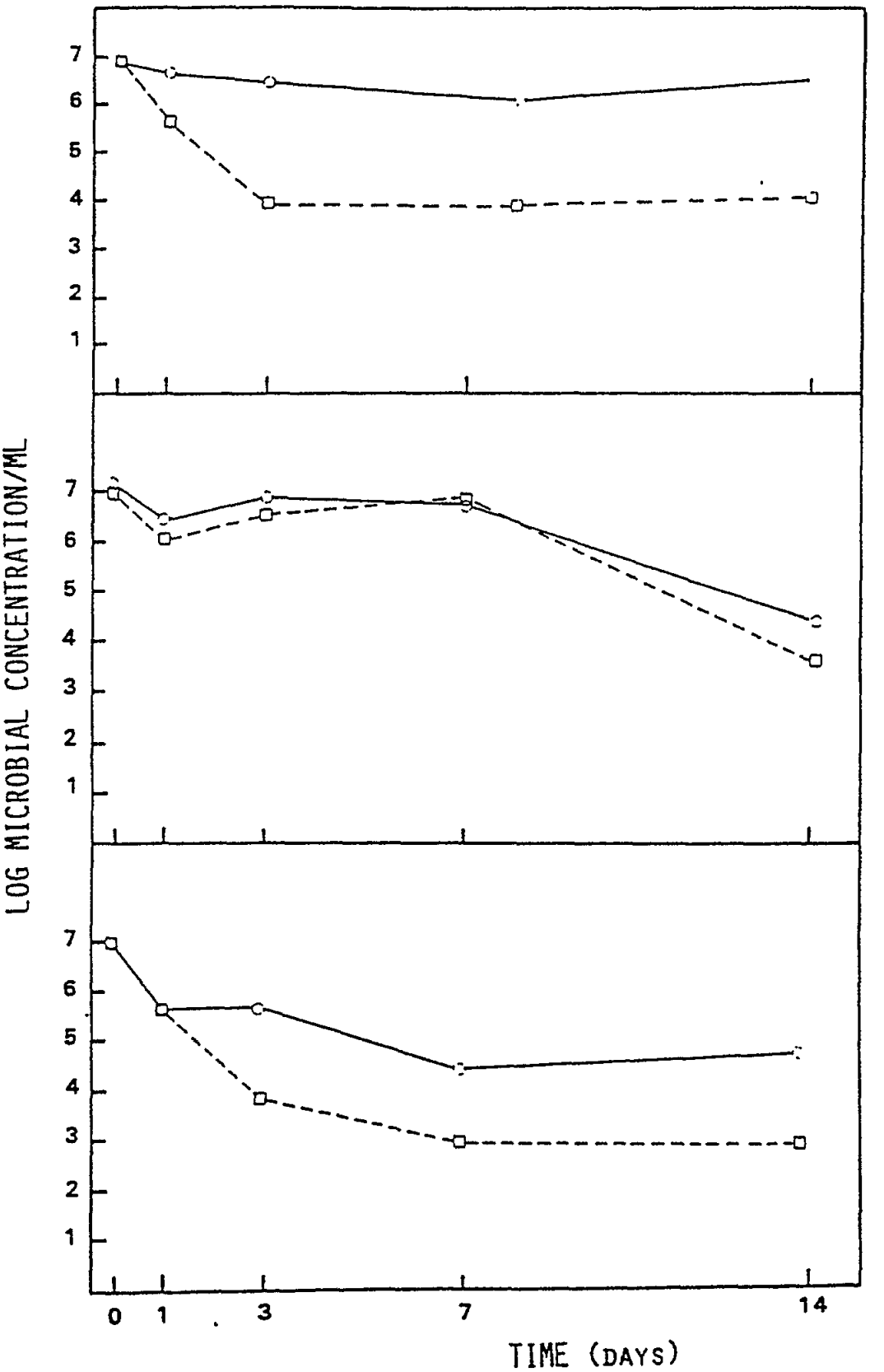
S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater
ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days



Figures 42-44: Influence of (A) saline solution, (B) untreated seawater and (C) filtered seawater on the survival of *P. aeruginosa*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium



Figures 45-47: Influence of (A) artifical seawater (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *P. aeruginosa*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium

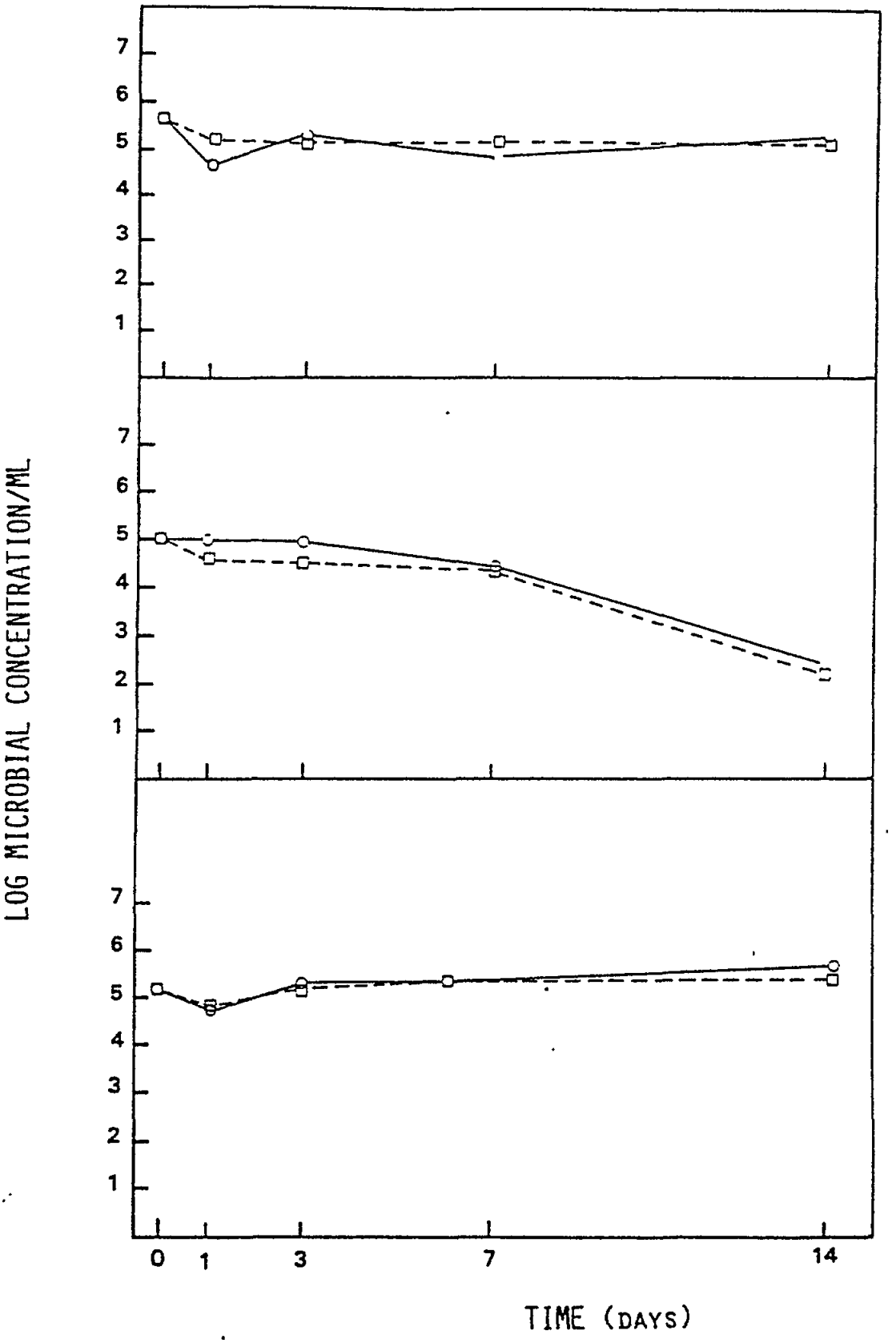
Table 10

Percentage of injured and survival cells from each studied medium for *P. aeruginosa*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	71.10	49.00	26.10	0.00	179.00	110.00	434.00	83.70
USW	12.30	76.24	83.66	100.00	21.40	0.76	0.26	0.0003
FSW	71.18	59.90	-	80.07	119.40	120.10	-	41.79
ASW	88.57	99.52	99.28	99.61	57.60	25.60	15.30	42.80
ASWS	59.00	39.74	0.00	84.84	18.10	41.64	37.84	0.14
ASWL	0.00	99.08	96.85	98.66	5.60	5.60	0.26	0.46

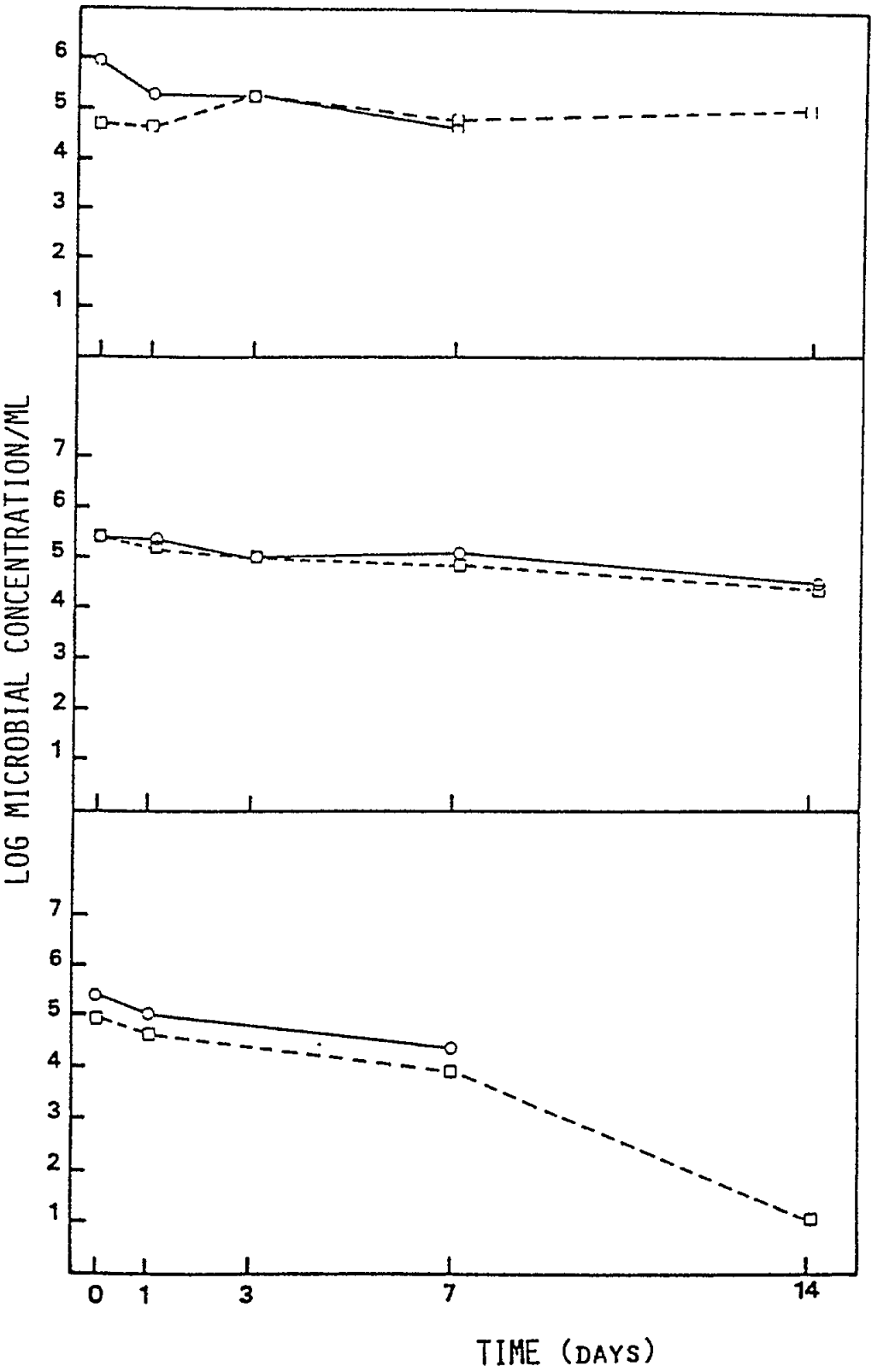
S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater
ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days



Figures 48-50: Influence of (A) saline solution, (B) untreated seawater and (C) filtered seawater on the survival of *C. albicans*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium



Figures 51-53: Influence of (A) artifical seawater (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *C. albicans*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium

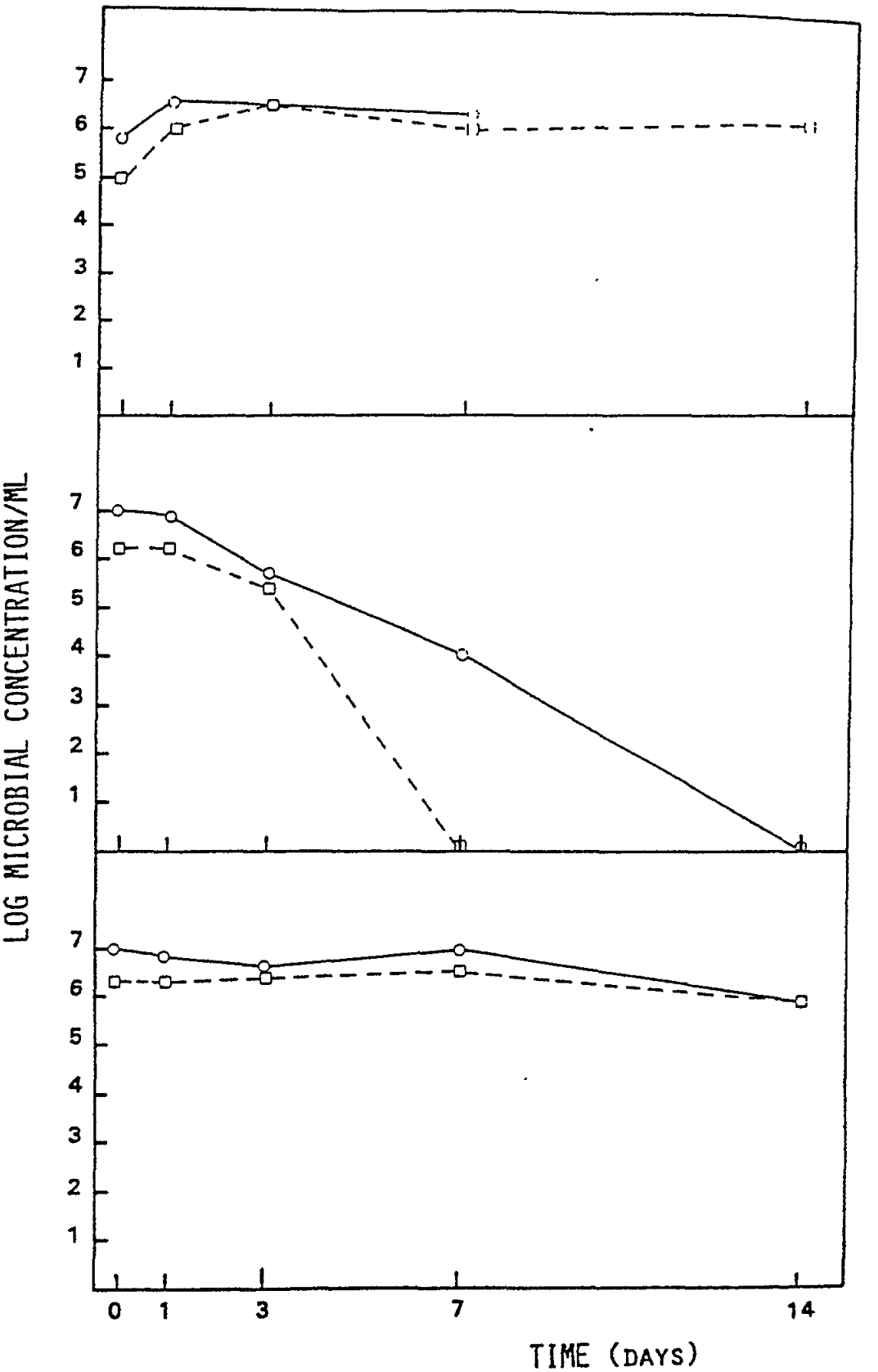
Table 11

Percentage of injured and survival cells from each studied medium for *C. albicans*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	0.00	26.47	0.00	11.76	40.00	51.00	36.25	42.50
USW	54.00	60.50	19.86	32.14	100.00	78.50	28.70	0.28
FSW	0.00	15.20	0.00	38.70	17.25	54.25	55.00	108.50
ASW	80.95	0.00	10.33	-	26.25	17.25	5.92	13.50
ASWS	15.62	10.63	62.30	30.83	96.00	58.75	63.	18.45
ASWL	54.54	60.00	59.23	-	3.97	0.36	1.00	0.0003

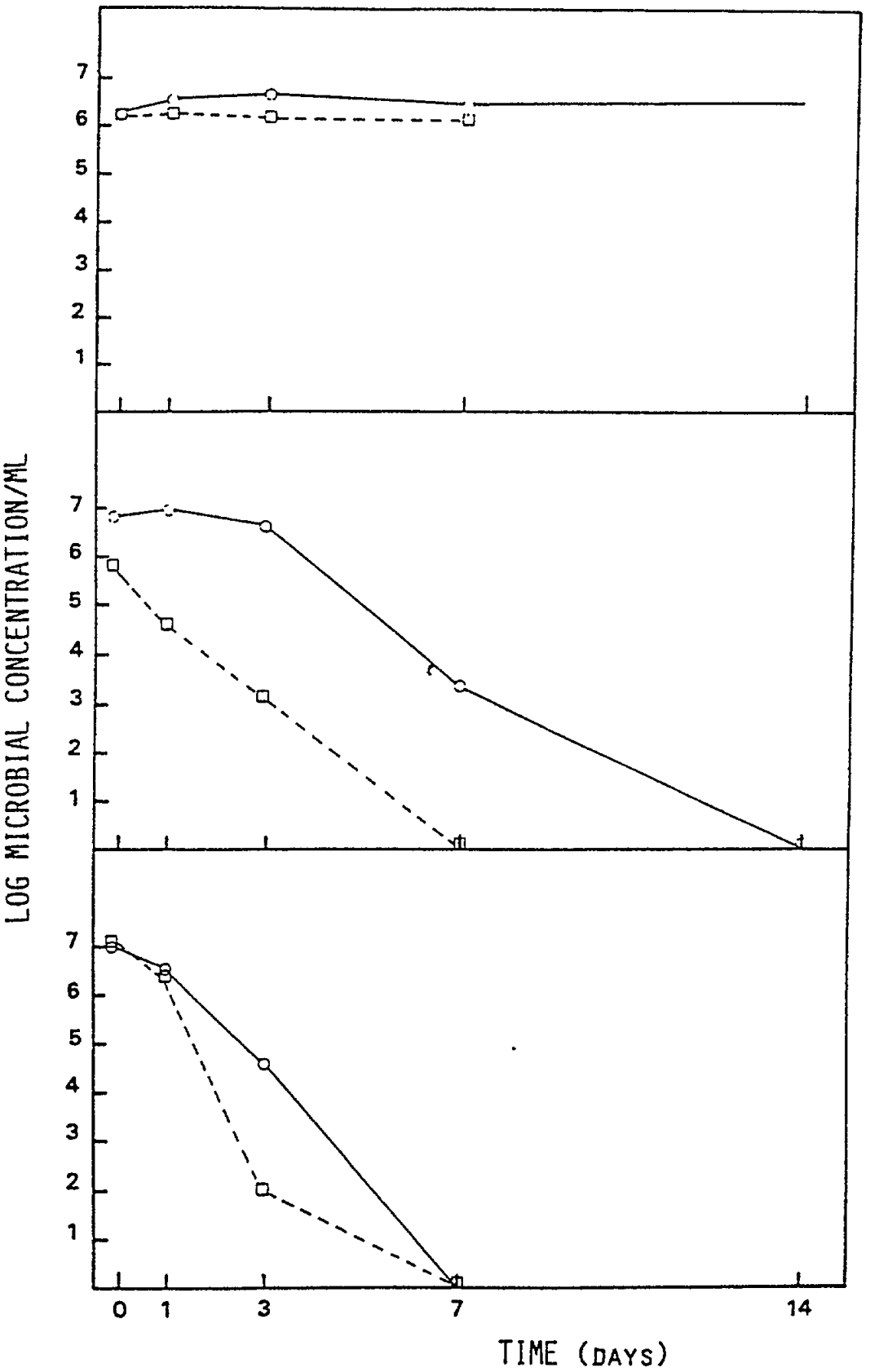
S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater
ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days



Figures 54-56: Influence of (A) saline solution, (B) untreated seawater and (C) filtered seawater on the survival of *S. aureus*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium



Figures 57-59: Influence of (A) artifical seawater (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *S. aureus*

○———○ Recounts on double layer
□-----□ Recounts on selective medium

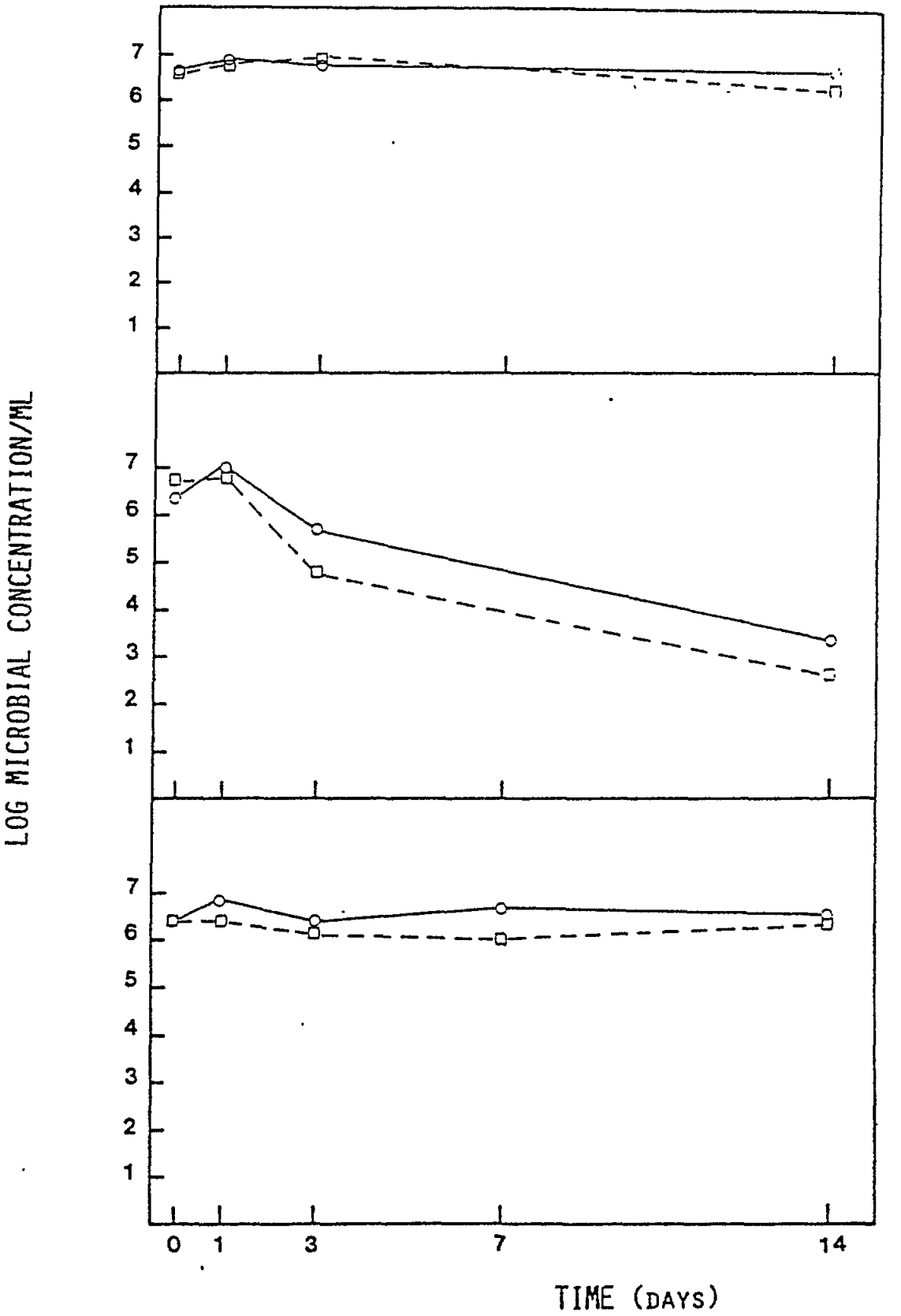
Table 12

Percentage of injured and survival cells from each studied medium for *S. aureus*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	74.46	100.00	43.86	-	195.80	170.80	88.30	102.50
USW	76.33	44.62	100.00	-	75.50	5.15	0.08	0.00
FSW	60.66	-	70.90	2.7	59.40	41.58	108.91	9.10
ASW	48.70	67.14	60.60	-	205.00	280.00	105.00	230.00
ASWS	99.39	99.57	100.00	-	144.00	86.22	0.03	0.00
ASWL	30.37	99.75	-	-	29.41	0.33	0.00	0.00

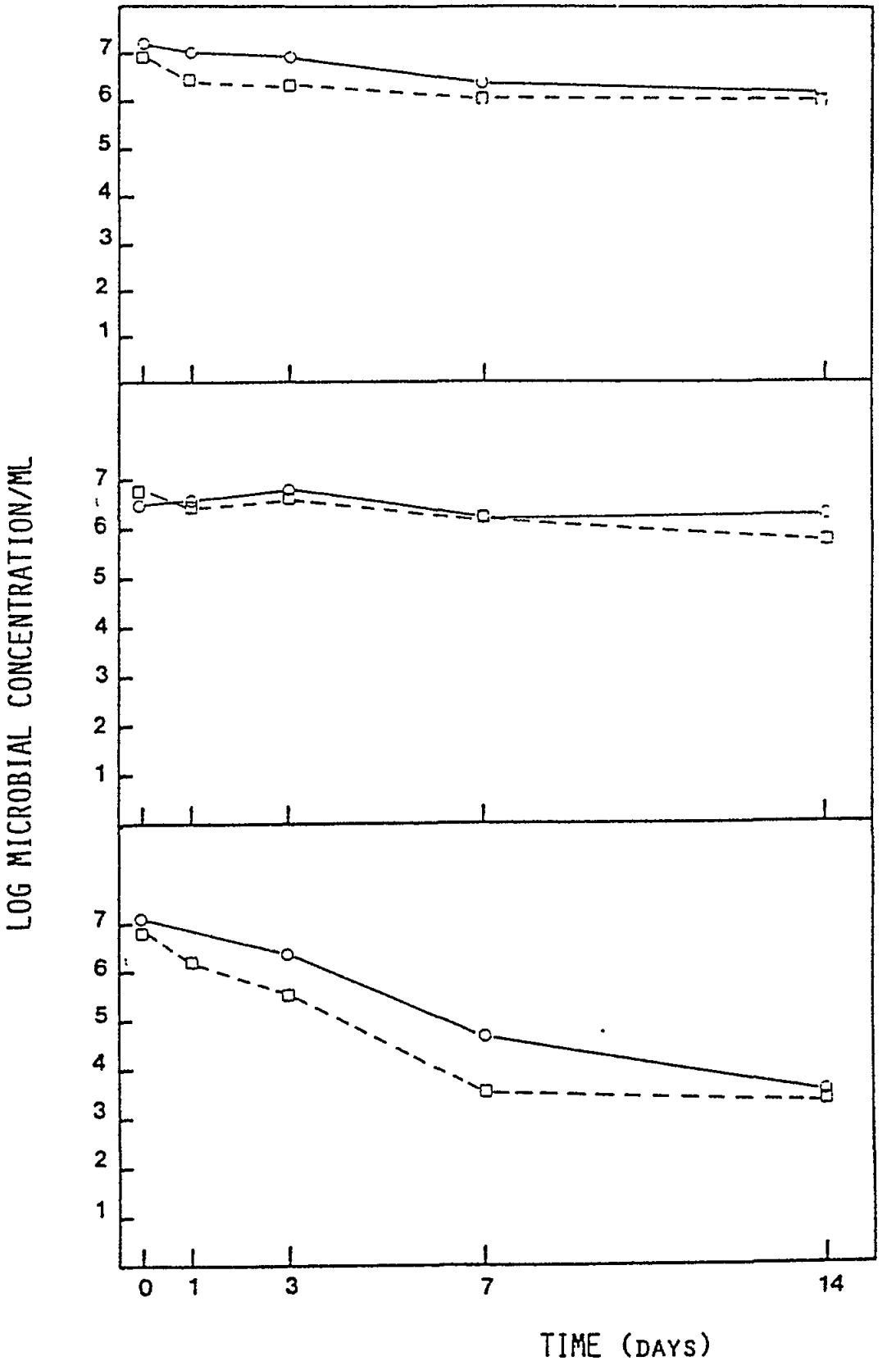
S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater
ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days



Figures 60-62: Influence of (A) saline solution, (B) untreated seawater and (C) filtered seawater on the survival of *Salmonella thompson*

○——○ Recounts on double layer
□------□ Recounts on selective medium



Figures 63-65: Influence of (A) artifical seawater (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *Salmonella thompson*

○——○ Recounts on double layer
□- - - -□ Recounts on selective medium

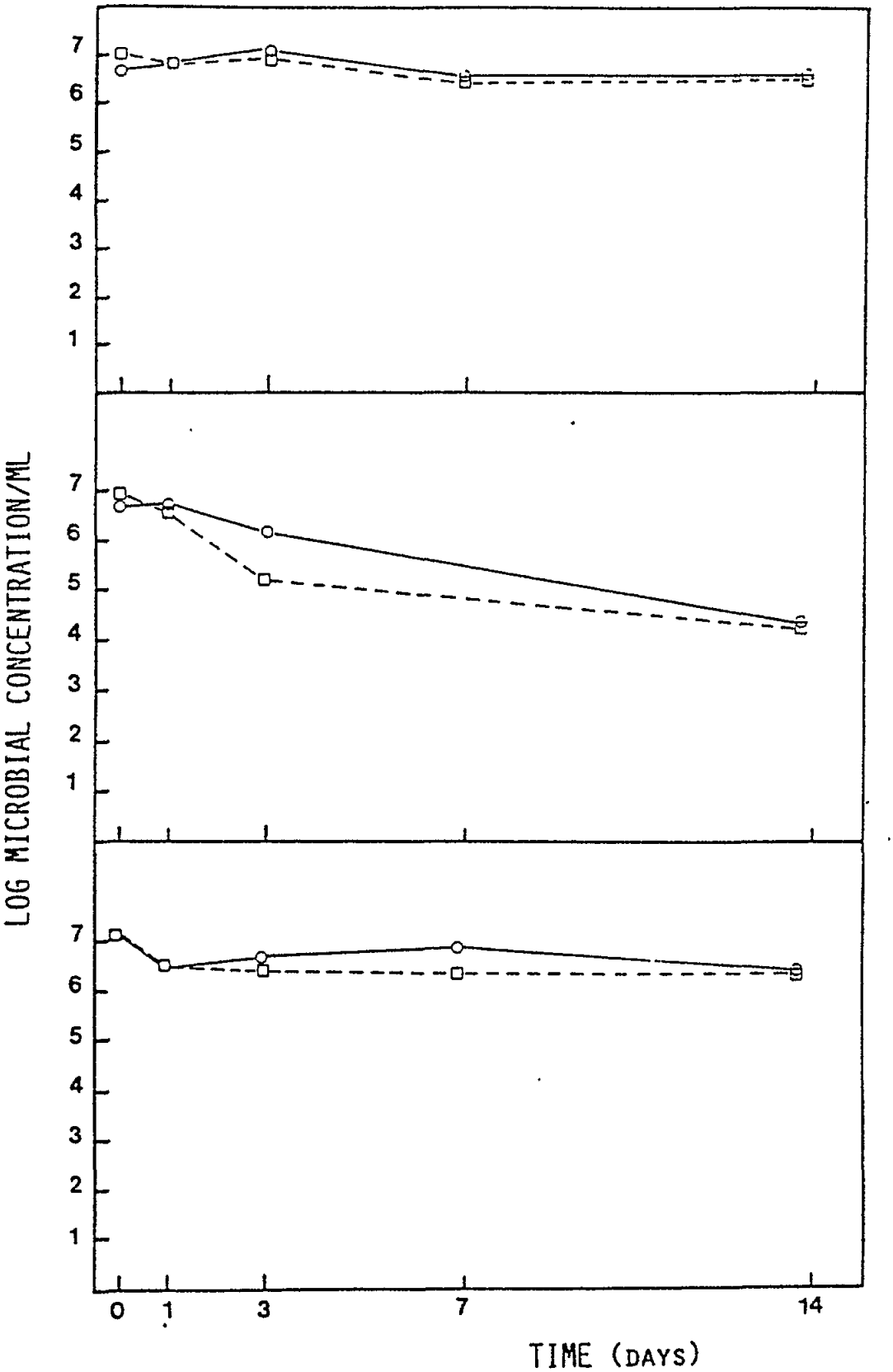
Table 13

Percentage of injured and survival cells from each studied medium for *S. thompson*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	13.10	0.00	-	51.46	161.00	170.00	-	91.11
USW	30.27	89.05	-	81.39	40.30	21.66	-	0.08
FSW	65.00	45.02	80.70	34.65	273.00	77.00	181.00	117.00
ASW	75.19	71.72	53.06	38.88	63.52	51.17	14.41	10.50
ASWS	28.31	32.41	0.00	70.00	110.00	188.00	40.57	65.71
ASWL	0.00	87.41	92.87	26.66	9.48	21.92	0.37	0.02

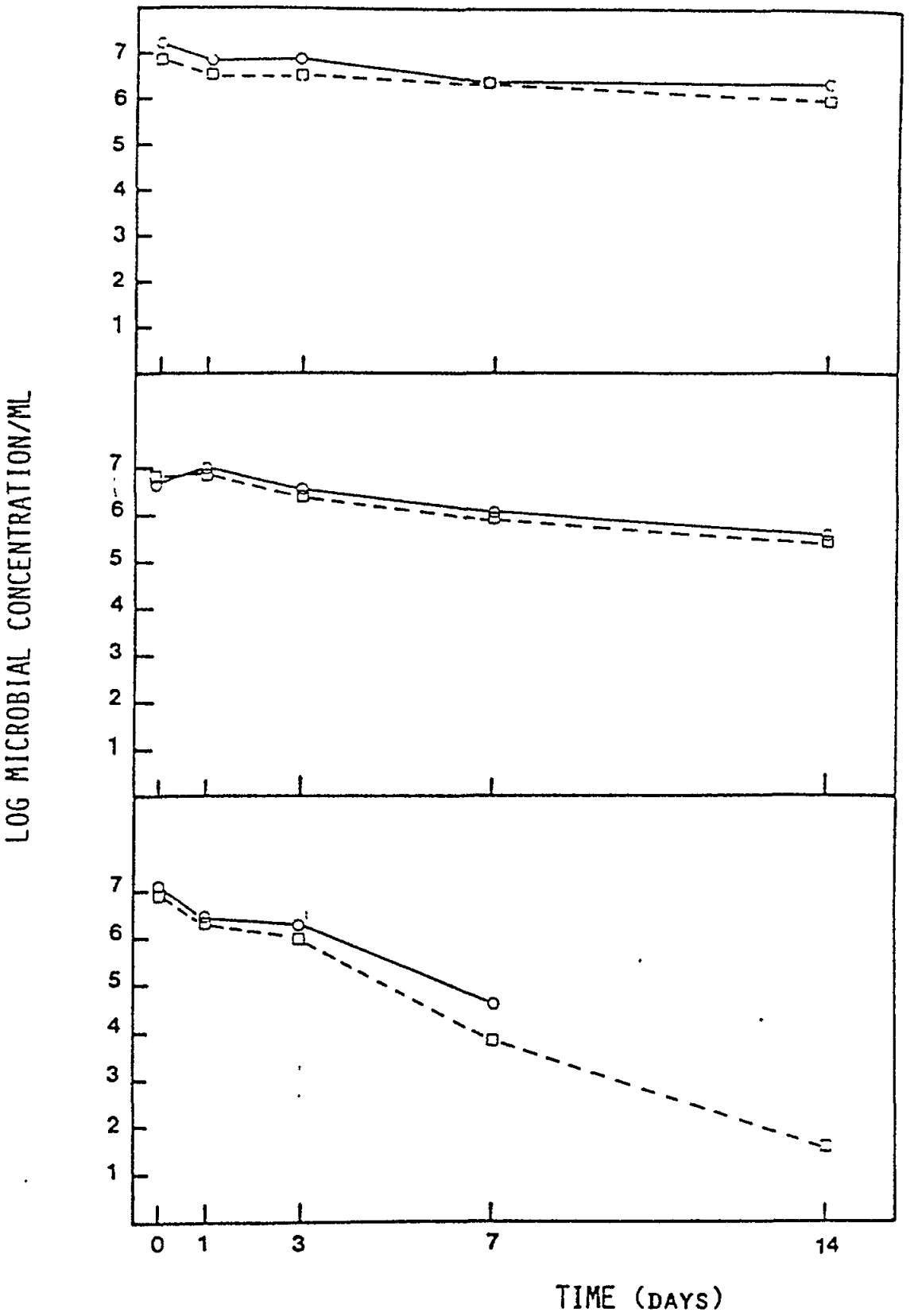
S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater
ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days



Figures 66-68: Influence of (A) saline solution, (B) untreated seawater and (C) filtered seawater on the survival of *Salmonella paratyphi*

○———○ Recounts on double layer
□-----□ Recounts on selective medium



Figures 69-71: Influence of (A) artifical seawater (B) artificial seawater plus sewage and (C) artificial seawater plus light on the survival of *Salmonella paratyphi*

○——○ Recounts on double layer
□-----□ Recounts on selective medium

Table 14

Percentage of injured and survival cells from each studied medium for *S. paratyphi*

	Percentage of injured cells				Percentage of injured cells			
	Sampling time[d]				Sampling time[d]			
	1	3	7	14	1	3	7	14
S.S.	0.00	19.50	16.00	20.56	113.00	197.00	42.85	74.57
USW	44.65	90.36	-	23.92	140.00	31.53	-	0.39
FSW	0.00	60.90	69.53	22.70	19.55	38.50	48.15	15.33
ASW	56.62	58.10	0.00	44.78	43.52	56.62	16.52	15.23
ASWS	14.28	37.77	32.06	34.04	186.00	75.00	24.16	5.43
ASWL	25.00	40.85	97.61	-	20.58	14.92	0.31	0.0003

S.S. : Saline solution
USW : Untreated seawater
FSW : Filtered seawater

ASW : Artificial seawater
ASWS: Artificial seawater + sewage
ASWL: Artificial seawater + Light

[d] expressed in days

4. DISCUSSION

The levels of microorganisms tested decrease in both the surface effluent and the dispersion plume when sewage is discharged into the sea. This inactivation or disappearance of microorganisms in the aquatic environment may be due to the combined effects of two mechanisms. The first, which involves dilution and sedimentation, is physical, it diminishes the concentration of microorganisms although they remain still viable. The other is a biological process of inactivation or microbial death.

Sewage discharged at a certain depth in the sea begins to move upwards because of its lower density and higher temperature. Its slope is fundamentally dependent on the speed at which the effluent is diffused by the underwater outlet, which produces a process of initial dilution.

The experimental results obtained from initial dilution using the technique of Borrego *et al*, (1983a) are superior to those theoretically estimated using the Cederwall's formula (1963). Other processes, apart from physical dispersion, act on the column of sewage as it ascends. These processes include precipitation, inactivation and sub-superficial dispersion which effectively decrease the microorganism concentrations, thereby increasing the initial dilution.

Microorganisms in sewage are generally adsorbed by colloidal particles of suspended organic materials, allowing their sedimentation (Jones, 1971; Dutka & Kwan, 1980) and their accumulation onto the sediments (Pellet *et al*, 1983).

In the summer period, thermal stratifications of the water mass may be produced which can slow up the ascent of the sewage, causing a phenomenon of subsurface dispersion (Sterregard, 1975), which diminishes the microbial concentrations with the self-purifying power of seawater, improving the condition of the seawater affected by these outlets at the season of greatest littoral recreative use.

Of all the microorganisms studied, the pathogenic yeast *C. albicans* and the *E. coli* parasitic viruses show the least degree of initial dilution due to their biological characteristics which confer them on a greater resistance to the self-purifying phenomena of seawater. The high dilution experienced by the total coliforms is probably due to its different faecal origin and to the different survival rates of the various species which belong to this group (Geldreich, 1975).

Despite the high decrease experienced by the indicator and pathogenic microorganisms on their way from the outlet to the zone where surface mixing takes place, the concentrations detected in this upwelling are still important. Therefore, it is of fundamental importance to establish and explain the dispersion mechanisms of these pollutants in the zone of marine evolution.

The T90 parameter has been used to find out the dispersion of polluting microorganisms in the sea. The values obtained in the present study differ from those quoted in specialized literature (Gunnerson, 1974), but agree with those determined by Mujeriego *et al*, (1980), Rodriguez *et al*, (1982) and Borrego *et al*, (1983a), with values varying between 4-92 minutes.

A possible explanation of the great variety of T90 values may be its subjection to the fluctuations of a series of factors such as the sewage load, the characteristics of the outlet studied, the geographical zone where it is found, the physical-chemical changes of seawater and environmental fluctuations (currents, winds, rains, temperature variations, degrees of sunlight, etc) (Borrego, 1982).

The resulting T90 values of the indicator microorganisms indicate a lower kinetic inactivation for coliphages (T90=180 min), showing faecal coliforms, faecal streptococci and clostridia sulphite-reducers kinetics to be very similar and the highest, while the total coliforms

exhibit an intermediate type of behaviour somewhere between the two groups of microorganisms mentioned.

Although there are differences, there are not significant, and it is therefore difficult, to determine a different survival rate for each microorganism in the sea. In the low times obtained with T90, for example, the process of physical dilution is the most important, comparing to other inactivating factors which might show more differences between the survival of the microorganisms (Carlucci and Pramer, 1959; Jones, 1971; McCoy, 1971; Castellvi *et al*, 1977).

The technique used in this work present several problems to the study of the "in situ" inactivation of pathogenic microorganisms: a) the concentrations of these microorganisms, detected in the sea, are generally inferior to those of the indicators, and, therefore, using the same drift times does not simulate the dispersion and survival processes of the pathogenic microorganisms adequately; b) if the values of a concentration of 0/100 ml are included in the mathematical calculations of the T90, fluctuations and over-valued T90 results are obtained. To avoid this problem the subsequent 0 values of concentrations, from the first obtained, are missing from the calculations.

A similar behaviour between *P. aeruginosa, Salmonella* spp and faecal bacterial indicators may be deduced from the values obtained for the former. On the other hand, the T90 values observed for *C. albicans* and *S. aureus* are superior to those of the indicators, thereby supposing that those pathogens can create a potential risk for public health in waters which found within the correct limits of the indicator microorganisms.

One may conclude that the technique of drifting by semi-submerged floats as proposed by Pearson (1956) is not the most adequate in the study of the inactivation of pathogenic microorganisms, as their rapid disappearance by physical dilution makes tracking impossible.

As previously discussed, the dilution process which the sewage undergoes makes accurate study of the survival of these microorganisms in the sea impossible. A series of experiments using diffusion chambers were used to solve this problem. This technique allows the study "in situ" of microbial populations, apart from confirming the microorganisms within a physical space which allows the dialysis of both substances and water through the membrane pores. Possible cryptic growth due to necrophagy is minimized in this way (McFeters, 1981).

The survival rates at the 48 h of study indicate the similar behaviour between faecal streptococci, *P. aeruginosa* and *Salmonella* species studied. *S. aureus*, contrary to the observations of the T90 experiments, was the microorganism which showed the lowest survival percentage. This phenomenon may be due to a greater sensibility to the inactivating processes of the sea which is not manifested in the short period of time used in the T90 technique. *E. coli* and *C. albicans* were shown to be the microorganisms with the greatest survival percentage at the end of the study period.

The numerical differences found between the survival percentages of the various microorganisms are incremented if the reduction times of 90% of the population are analyzed. These results graphically obtained from the kinetics of inactivation (Table 6) show the following of survival, analyzed on the control recovery medium (TSA).

E. coli > *Salmonella* spp > *P. aeruginosa* > faecal streptococci > *S. aureus* > *C. albicans.*

E. coli showed a greater survival rate than the enterococci, contrary to the findings of the majority of authors (McFeters *et al*, 1974; Vasconcelos & Swartz, 1976), but agreed with those of Dutka & Kwan (1983).

The species of *Salmonella* studied, as well as *P. aeruginosa,* possess an elevated resistance (Vasconcelos & Swartz, 1976; Lessard *et al*, 1983) superior to that shown by enterococci and similar to that of *E. coli.*

C. albicans showed the highest rate of inactivation of all the microorganisms tested on this medium but, on the contrary, showed the least limit in the tests on the selective sample medium (Table 6). This possible contradiction may be explained by this microorganism's greater sensitivity to experimental manipulation prior to its introduction into the diffusion chambers. In this way, the percentage of damaged cells in the initial moment (0 h) is superior to 95%. The rapid death of these damaged cells, which is not due to any negative effect of the sea, causes a decrease in the number of surviving cells.

A gradual process of cellular damage was observed in all microorganisms studied. It increased according to the time they were exposed to the sea. This metabolic damage was very important in the case of the enterococci and *S aureus,* which were severely affected by the self-purifying factors of the sea.

E. coli, C. albicans and *Salmonella* species showed an important recuperation of damaged cells with respect to the initial percentage, underlining the importance of experimental manipulation on the metabolic conditions of the microorganisms (Anderson *et al*, 1983).

Although the number of strains of each microorganism used in this study does not allow generalization, there were no significant differences observed between the behaviours of various strains of the different microorganisms. The only existing differences occured between the different experiments, which may suggest environmental fluctuations which are difficult to control. In any case it is interesting to investigate this aspect more slowly in an attempt to determine the possible existence of a direct relationship between the survival and origin of the strains (laboratory, natural medium, clinical sample, etc.).

The purifying role of the micro-predators, as is that of the protozoa in the process of microbial disappearance in the sea has been suggested by several authors. Effective predation depends upon a determined prey/predator ratio (McCambridge & McMeekin, 1980), although the diffusion chambers permit microorganisms with an elevated proportion surface/volume an "in situ" exposition to the marine environment, the concentration of microorganisms may be so high as to show prey/predator proportions which are improbable under natural conditions. This overrates the purifying power of the predators (Anderson *et al*, 1983). To avoid this, when studying inactivation in diffusion chambers, the suspension water which fills the chambers was filtered. However, it would be important to carry out more particular studies of this problem with mixed microbial populations of by adding untreated sewage or sea water.

Deductions may be made about the dispersion of pollutant bacteria and their global inactivation process from experiments carried out "in situ" dispersion and diffusion chambers experiences, respectively.

The effect of the different purifying factors of the sea may not be deduced directly, so, in a third part of this work, survival experiments were carried out on different microorganisms in the laboratory, under diverse conditions, to evaluate the individual effects of purifying factors.

On general terms, a more prolonged survival of the microorganisms was found on laboratory experiments than in those carried out "in situ". This is basically due to environmental factors, principally physical, which diminish the concentrations in the sea while having no inactivating effects (McFeters & Stuart, 1972; Pichot & Barbette, 1978; Dutka & Kwan, 1980).

No decrease in the initial concentration of microorganisms superior to one order of magnitude during experimental time, was found in the testing solution (saline solution 0.85%) (Figures 30, 36, 42, 48, 54, 60 and 66), with the exception of the *S. faecalis* which reached

a death percentage of over 93%. In almost all the microorganisms an initial phase of recuperation was observed. This may be due to a favourable division of the reverse substances accumulated during the incubation of the microorganisms in the inoculum medium. The lack of nutrients does not seem to be a fundamental factor affecting the inactivation process of the microorganisms. These results are opposed to those of Savage & Hares (1971) for coliforms and to those of Sinclair & Alexander (1984) for *S. faecalis* and *S. aureus* but are very similar to those observed by Fujioka *et al*, (1981) for coliforms and streptococci and those of de Vicente (1986) for *P. aeruginosa.*

The study of microbial survival in untreated seawater (Figures 31, 37, 43, 49, 55, 61 and 67) shows the global effect of all components of this medium on the allochtonous microorganisms contained in the sewage discharged. The negative effect on the survival of the microorganisms survival is very important and of great magnitude for all the microorganisms studied, resulting in the survival of less than 1% of the cells at the end of the study period, although *S. aureus*, in this medium, exhibited a total extinction of its population. The cellular damage exerted by this medium was also very high, showing a stressed effect in the cells of *S. faecalis, P. aeruginosa* and *S. aureus*.

The negative effect of seawater is due to diverse physical factors, both biological and chemical such as toxic substances, antibiotics, bacteriophages, predators, competence phenomena, etc. (Carlucci & Pramer, 1960a,b,c,; Mitchell, 1968; Geldreich, 1979).

The following experiments were designed to determine the role and rate of importance of each factor in the sea's self-purifying process on the survival of allochtonous microorganisms in this environment.

The effect of salinity in the sea was studied in suspensions of artificial seawater (Figures 33, 39, 45, 51, 57, 63 and 69). All the microorganisms tested suffered a decrease in their concentration with respect to the saline testing solution, except *S. aureus* which showed a higher level of survival in saline concentrations close to that of seawater (3.5%). The population decrease was not larger than one order of magnitude except that obtained for *S. faecalis,* showing a survival percentage of only 1.32%, data very inferior to that observed for other microorganisms, but near to that presented by this microorganism in a saline solution of 0.85%. Although salinity produces a decrease in population in microorganisms, it is not the principal factor responsible for the strong inactivating power of natural seawater. However, Carlucci & Pramer (1960a), Jones & Cobet (1975), Walker & Guarraia (1975), obtained greater inactivation with this factor.

The filtration of seawater eliminated the majority of biological agents and produced a differentiated effect on the survival of the microorganisms studied. *S. faecalis, C. albicans* and *S. thompson,* showed a lower inactivation than that obtained in the physiological saline solution (Figures 38, 50 and 62), while the other microorganisms studied underwent population decreases of greater magnitude than in the testing solution (Figures 32, 44, 56 and 68). The negative effect of filtered seawater is determined by filterable components such as toxic elements, antibiotics and heavy metals (Jones, 1971; Jones & Cobet, 1975), chemical pollutants (Verstrate & Voets, 1976; Castellvi *et al*, 1977); besides the osmotic effect due to its saline concentration, previously noted. These toxic factors principally affect *E. coli* and *S. aureus*, as their survival in this test solution after the study period (0.75% and 9.1%, respectively) was not only less than that observed in the saline solution but also less than that observed in natural seawater. Salinity therefore seems the factor responsible for the existing decrease in the populations of these two microorganisms when tested in filtered seawater.

The longer survival of *S. faecalis, C. albicans* and *S. thompson*, and also the increase of the initial population of *C. albicans* and *S. thompson* can be determined by the presence in the water of nutrients which do not exist in the physiological saline solution or in artificial seawater. This nutrients diminishes the inactivating effect, producing bacterial growth (Savage & Hanes, 1971; Gerba & McLeod, 1976; Borrego *et al*, 1983b; Lessard & Sieburth, 1983, Sinclair & Alexander, 1984).

The filterable factors of seawater are not those principally responsible for the major inactivation of the microorganism populations in the sea, either. It may therefore be assumed that the principal, but not the unique, inactivating effect is produced by biotic unfilterable factors in seawater, a deduction which coincides with that of other authors (Gauthier, 1980, McCambridge & McKeekin, 1981; Borrego *et al*, 1983b; Mallory *et al*, 1983; de Vicente, 1986).

These biotic factors are mainly constituted by predators amongst which the role of the protozoa stands out (Mitchell, 1971; Gauthier, 1980; Anderson *et al*, 1983; McCambridge & McKeekin, 1980, 1981; Mallory *et al*, 1983), while the named microdepredators (*Bdellovibrio bacteriovirus*, lytical bacteria and bacteriophages) are of minor importance in the purifying process (Carlucci & Pramer, 1960c; Aubert & Aubert, 1966; Jones, 1971, Gauthier, 1980; Geldreich, 1980). Another implied inactivating factor is the competition due to autochtonous microflora (Verstreate & Voets, 1976). The autochtonous microorganisms are adapted to the oligotrophic media (Sinclair & Alexander, 1984), thus they competing with the allochtonous bacteria for the nutrients.

The differences observed between the survival of the different microorganisms studied could be conditioned by selective depredation (Curds, 1977) or by adaptation to media of minimal recourses.

To adequately simulate the medium in which enteric microorganisms are found, 1% sewage was added to artificial seawater; a similar proportion to that obtained from the surface effluent of an underwater outlet after initial dilution. The effect of this addition was variable, according to the microorganisms studied. A strong negative effect was observed in the indicator microorganisms *E. coli* and *S. faecalis* (Figures 34, 40) and the pathogenic microorganisms *P. aeruginosa, S. aureus* and *S. paratyphi* (Figures 46, 58 and 70). The principal causes of this inactivation are the toxicity of sewage and the addition of competitive flora (Sinclair & Alexander, 1984). *C. albicans* and *S. thompson* (Figures 52 and 70) suffered a decrease in respect to that observed in the saline solution but inferior to that obtained in artificial seawater. The addition of organic material may favour microbial growth, as observed for these microorganisms in filtered seawater, although the increase of toxic substances and the existence of competitive flora produces a greater inactivation than that observed in this last medium.

Visible light has a strong inactivating effect on the microbial populations tested in artificial seawater. In Figures 35, 41, 47, 53, 59, 65 and 71 more than a 90% diminishing of the population was observed. This was especially important in the inactivation exerted on *E. coli, S. faecali* and *S. aureus,* whose populations were extinguished in the time studied. These results coincide with the strong effect of visible light observed on coliforms and faecal streptococci by Evison & Tosti (1980); Grigsby & Calkins (1980), Fujioka & Narikawa (1982) and Fattal *et al* (1983); on *P. aeruginosa* by Dutka (1984) and de Vicente, (1986), and on *Salmonella spp* by McCambridge & MaMeekin (1981).

Light action is produced by photooxidation of vital mechanisms of the cell, as for example the respiratory chain or the catalase system (Kapucinski & Mitchell, 1981; Dutka, 1984); other factors such as salinity may also have a synergic (Fujioka and Narikawa, 1982) or depredative effect (Mc Cambridge & McMeekin, 1981). The inactivating effect of light is proportional to the intensity of and time exposed to radiation (Gameson & Gould, 1975), its effect being determined by the transparency and concentration of organic matter dissolved in water (Fujioka *et al*, 1981).

The self-purifying factors of the sea have two different effects on the microorganisms: one is the inactivating or a damaging process which produces a physiological change (Bissonnette *et al*, 1975; Hoadley, 1971; Rhodes *et al*, 1983). The damaging process fluctuates, depending on the time of exposition. Three types of different dynamics may be determined from the results obtained: a) the percentage of damaged or stressed cells was not very high, remaining almost constant during the total experiment time, (Figures 72a), b) a proportional increase of damaged cells with respect to the time tested (Figure 72b), c) in a primary period of time, the proportion of cells damaged was increased without diminishing the

rate of the surviving cells. Later, the death of a large proportion of damaged cells, caused a decrease in the number of surviving cells, thereby reducing the index of damaged cells (Figure 72c).

The pathogenic microorganisms showed similar or superior resistances to those of the indicator microorganisms in several of the experiments. It would be easy to deduce a potential risk in waters which fulfill the sanitary rules based on the analysis of indicator microorganisms, while considering a series of considerations. Firstly, the diffusion chambers and laboratory experiments do not contemplate self-purifying factors of large importance such as the process of dilution and sedimentation. A pathogenic microorganism in the sea may survive for several days, but its potential danger is determined by its concentration in the water or by its minimum infective dose.

The concentration of pathogens in sewage are far inferior to those in the indicator microorganisms and to those used in laboratory and diffusion chamber experiments, this leading to their rapid disappearance in the sea. *S. aureus* is the only pathogen in the "in situ" experiments which remains in the sea for longer than 60 min, mainly due to being discharged in concentrations similar to those of the indicator microorganisms.

The exponential decrease mathematical model used in this study a first phase where there is a high population decrease produced in zones near to the effluent, includes caused principally by the physical processes of dilution and sedimentation. Later, the kinetic inactivation is established and in this phase the biological type factors are the most important in the self-purification process. In this way, those microorganisms with low concentrations in waste effluent will be the first affected by physical phenomena, obtaining minimum T90 values as the phase of exponential decrease is not stabilized.

In zones where the dilution process is very reduced, such as direct discharges into the beaches or zones with geographical characteristics which impede dilution and favour water recirculation phenomena, the greater resistance capacity of some pathogenic microorganisms may have an additional sanitary-epidemiological interest. A deeper localized study of this type of phenomena may be carried out.

The continuous sedimentation and precipitation processes of the discharged microorganisms produce an accumulation of pathogens both into marine sediment and benthonic organisms that filter the water. These last organisms, can be ingested by man and provoke food-toxic infections. The survival of pathogens in shellfish, therefore, can be of the great importance.

It is necessary to determine the minimum infective dose of each pathogenic microorganism to evaluate its danger through contact with water and through ingestion of contaminated sea products. It is also necessary to determine the infective capacity presented by damaged cells in the marine environment which are not included in conventional analysis, as they use selective culture media.

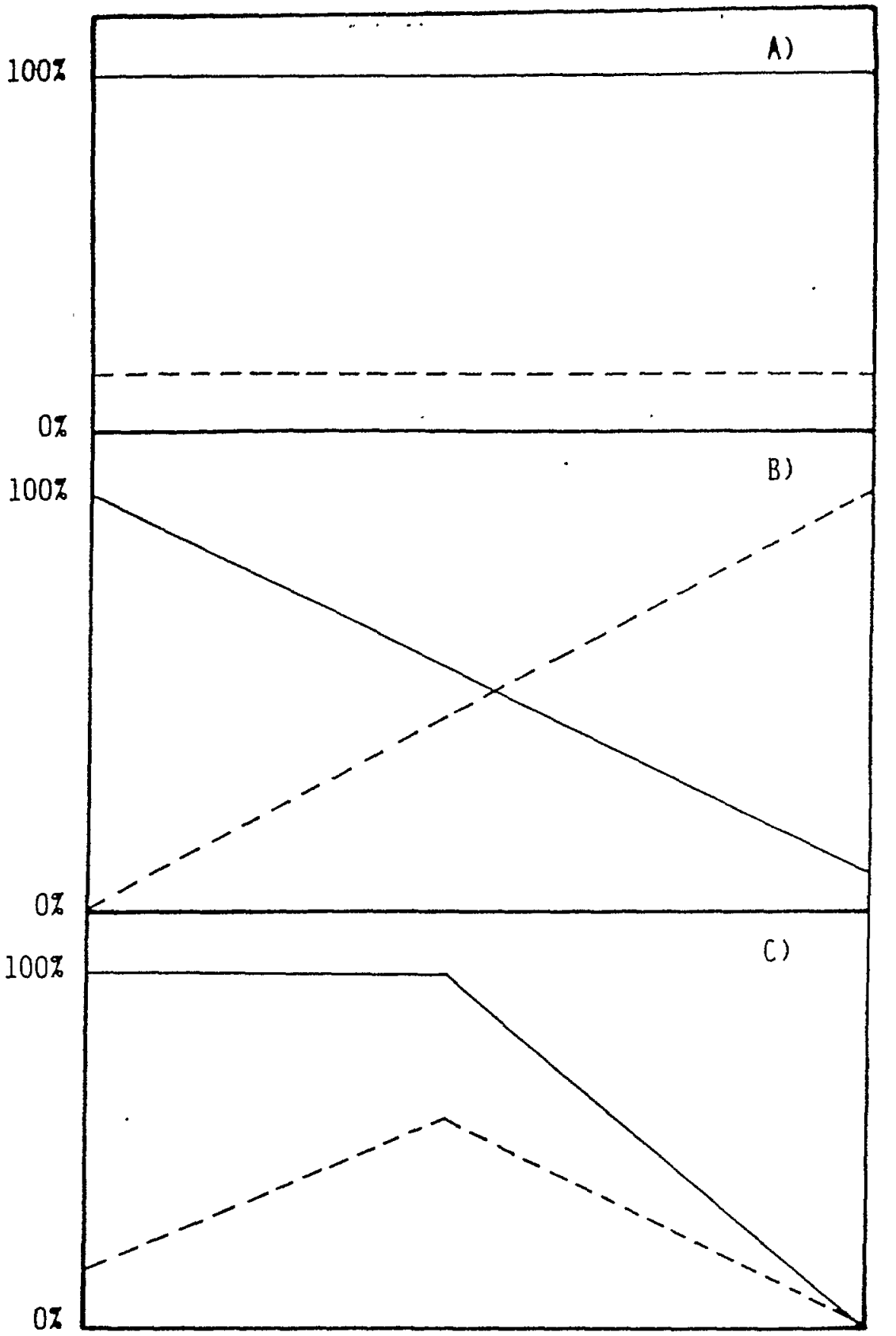


Figure 72 a,b,c

General behaviour of the injury processes produced by the different environmental factors

——— Percentage of survival cells
——— Percentage of injured cells

5. REFERENCES

ADAMS, M.H., 1959. *BACTERIOPHAGES*. Interscience Publishers. New York.

ANDERSON, I.C.; M.W. RHODES & H.I. KATOR, 1983. Seasonal variation in survival of *Escherichia coli* exposed in situ membrane diffusion chambers containing filtered and nonfiltered estuarine water. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **45**: 1877-1883.

A.P.H.A., A.W.W.A. & W.P.C.F., 1985. *STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER*, 16th Ed. American Public Health Association. Washington, D.C.

AUBERT, M & J. AUBERT, 1969. *OCEANOGRAPHIE MEDICALE*. Gauthier Villars. Paris.

BISSONNETTE, G.K.; J.J. JEZESKI; G.A. McFETERS & D.G. STUART, 1976. Influence of environmental stress on enumeration of indicator bacteria from natural waters. *APPLIED MICROBIOLOGY*, **29**: 186-194.

BONDE, G.J., 1977. Bacterial indicators of water pollution. In: *ADVANCES IN AQUATIC MICROBIOLOGY*, Vol. **1**. M. Droop and H.W. Jannasch (ed.). Academic Press, Inc. Londres. pp. 273-364.

BORREGO, J.J., 1982. *Estudio de los bacteriofagos de Escherichia coli en el agua de mar. Su relacion con la polución de dicho medio*. Doctoral Thesis, University of Malaga.

BORREGO, J.J.; F. ARRABAL; A. de VICENTE; L.F. GOMEZ & P. ROMERO, 1983a. Study of microbial inactivation in the marine environment. *JOURNAL WATER POLLUTION CONTROL FEDERATION*, **55**: 297-302.

BORREGO, J.J.; F.ARRABAL; A. de VICENTE; L.F. GOMEZ & P. ROMERO, 1983b. Decline of *Escherichia coli* cells in different water types. *RAPPORT COMITE INTERNATIONALE MER MEDITERRANEE*, **28**: 47-48.

BUCK, J.D. & P.M. BUBUCIS, 1978. Membrane filter procedure for enumeration of *Candida albicans* in natural waters, *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **35**: 237-242.

CABELLI, V.J., 1977. Indicators of recreational water quality. In: A.W. Hoadley & B.J. Dutka (ed.). *BACTERIAL INDICATORS/HEALTH HAZARDS ASSOCIATED WITH WATER.* American Society for Testing and Materials. Philadelphia. pp. 222-238.

CABELLI, V.J., 1978. New standards for enteric bacteria. In: R. Mitchell (ed.) *WATER POLLUTION MICROBIOLOGY*, Vol. 2. J. Wiley & Sons. New York. pp. 233-271.

CABELLI, V.J.; F.T. BREZENSKI; A.P. DUFOUR & M.A. LEVIN, 1974. *Microbiological methods for monitoring marine environment*. U.S.E.P.A. pp. 360-384.

CABELLI, V.L.; A.P. DUFOUR; M.A. LEVIN; L.J. McCABE & P.W. HABERMANN, 1976. Relationship of microbial indicators to health effects at marine bathing beaches. *ANNUAL MEETING OF AMERICAN PUBLIC HEALTH ASSOCIATION*. Chicago.

CARLUCCI, A.F. & D. PRAMER, 1959. Factors affecting the survival of bacteria in sea water. *APPLIED MICROBIOLOGY*, **7**: 388-392.

CARLUCCI, A.F. & D. PRAMER, 1960a. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. II: Salinity, pH and nutrients. *APPLIED MICROBIOLOGY*, **8**: 247-251.

CARLUCCI, A.F. & D. PRAMER, 1960b. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. III: Antibiotics. *APPLIED MICROBIOLOGY*, **8**: 251-254.

CARLUCCI, A.F. & D. PRAMER, 1960c. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. IV: Bacteriophages. *APPLIED MICROBIOLOGY*, **8**: 254-256.

CASTELLVI, J.; H. FERNANDEZ; J.M. PANADES; J. VALERO & A. OJEDA, 1977. Autodepuración de las aguas residuales en el litoral de Barcelona. *INFORMES TECNICOS DEL INSTITUTO DE INVESTIGAGIONES PESQUERAS*, No. 46.

CEDERWALL, K., 1963. The initial mixing on jet disposal into a recipient. *DIVISION HYDROLOGICAL, INSTITUTE TECHNOLOGY CHALMERS*, *14-15.*

CURDS, C.R., 1977. Microbial interactions involving protozoa. In: F.A. SKINNER & J.W. Shewan (ed.). *AQUATIC MICROBIOLOGY*. Society for Applied Bacteriology. Academic Press. London. pp. 69-105.

DIVE, D., 1973. Nutrition holozoique de *Colpidium campylum*, phenomènes de selection et d'antagonisme avec les bactéries. *WATER RESEARCH*, **7**: 695-706.

DUTKA. B.J., 1973. Coliforms are an inadequate index of water quality. *JOURNAL ENVIRONMENTAL HEALTH*, **36**: 39-46.

DUTKA, B.J., 1984. Sensitivity of *Legionella pneumophila* to sunlight in fresh and marine waters. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **48**: 970-974.

DUTKA, B.J. & K.K. KWAN, 1980. Bacterial die-off and stream transport studies. *WATER RESEARCH*. **14**. 909-915.

DUTKA, B.J. & K.KWAN, 1983. Environmental studies of enteric bacteria longevity in membrane filter chambers. *RESEARCH AND TECHNOLOGY JOURNAL, AWWA*. pp 380-382.

EDEL, W. & W.H. KAMPELMACHER, 1973. Comparative studies on isolation methods of sublethally injured salmonellae in nine european laboratories. *BULLETIN OF THE WORLD HEALTH ORGANIZATION*, **48**: 167-174.

EDWARDS, P.R. & W.H. EWING, 1972. *IDENTIFICATION OF ENTEROBACTERIACEAE*. 3rd ed. Minneapolis. Burgess Publ. Co.

EVISON, L.M., & E. TOSTI, 1980. An appraisal of bacterial indicators of pollution in sea water. *PROGRESS IN WATER TECHNOLOGY*, **12**: 591-599.

FATTAL, B.; R.J. VASL; E. KATZENELSON & H.I. SHUVAL, 1983. Survival of bacterial indicators organisms and enteric viruses in the mediterranean coastal waters off Tel-Aviv. *WATER RESEARCH*, **17**: 397-402.

FLIERMANS, C.B. & R.W. GORDEN, 1977. Modification of membrane diffusion chambers for deep-water studies. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **33**: 207-210.

FLORIDO, J.A.,; 1985. *Estudio comparativo de distintos medios de cultivo para cuantificación de Staphylococcus sp. a partir de aguas naturales*. Memoria de Licenciatura. Universidad de Málaga.

FLORIDO, J.A.; J.J. BORREGO & P. ROMERO, 1984. Nuevo medio para aislamiento de *Staphylococcus* sp. a partir de aguas. *IV Reunión Cientifica del Grupo de Alimentos de la S.E.M.*, **1**: 57.

FUJIOKA, R.S.; H.H. HASHIMOTO; E.B. SIWAK & R.H.F. YOUNG, 1981. Effect of sunlight on survival of indicator bacteria in sea water. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **41**: 690-696.

FUJIOKA, R.S. & O.T. NARIKAWA, 1982. Effect of sunlight on enumeration of indicator bacteria under field conditions. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **44**: 395-401.

GAMESON, A.L.H. & D.J. GOULD, 1975. Effects of solar radiation on the mortality of some terrestrial bacteria in sea water. In: A.L.H. GAMESON (ed.). *DISCHARGE OF SEWAGE FROM SEA OUTFALLS*. Pergamon Press. Londres. pp. 209-219.

GAUTHIER, M.J. 1980. Polución en el medio marino. In: J.M. Péres (ed.). *LA POLUCION DE LAS AGUAS MARINAS*. Omega S.A. Barcelona. pp. 127-141.

GELDREICH, E.E., 1975. *HANDBOOK FOR EVALUATING WATER BACTERIOLOGICAL LABORATORIES*. 2nd Ed. U.S. Environmental Protection Agency. Washington.

GELDREICH, E.E., 1978. Bacterial populations and indicators concepts in faces, sewage, stormwater and solid wastes. In: G. BERG (ed.) *INDICATORS OF VIRUSES IN WATER AND FOOD*. Ann Harbor Science. Michigan. pp. 51-97.

GELDREICH, E.E., 1979. Microbiology of Water. *JOURNAL WATER POLLUTION CONTROL FEDERATION*, **51**: 1721-1743.

GELDREICH, E.E., 1980. Microbiology of water. *JOURNAL WATER POLLUTION CONTROL FEDERATION*, **52**: 1775-1800.

GERBA; C.P. & J.S. McLEOD, 1976. Effect of sediments on the survival of *Escherichia coli* in marine waters. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **32**: 114-120.

GRIGSBY, P. & J. CALKINS, 1980. The inactivation of a natural population of coliform bacteria by sunlight. *PHOTOCHEMISTRY AND PHOTOBIOLOGY*, **31**: 291-294.

GUNNERSON, C.G., 1975. Discharge of sewage from sea outfalls. In: A.H.L. Gameson (ed.). *DISCHARGE OF SEWAGE FROM SEA OUTFALLS*. Pergamon Press. London. pp. 415-427.

HARMON, S.M. & D.A. KAUTTER, 1978. Media for confirming *Clostridium perfringens* from food and faeces. *JOURNAL OF APPLIED BACTERIOLOGY*, **41**: 626-630.

HOADLEY, A.W., 1981. Effect of injury on the recovery of bacteria on membrane filters. In: B.J. Dutka (ed.) *MEMBRANE FILTRATION: APPLICATIONS, TECHNIQUES AND PROBLEMS*. Marcel Dekker, Inc. New York. pp. 413-450.

JONES, G.E., 1971. The fate of freshwater bacteria in the sea. *DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY*, **12**: 141-151.

JONES, G.E. & A.B. COBET, 1975. Heavy metal ions as the principal bacterial agent in Caribbean sea water. In: A.L.H. Gameson (ed.). *DISCHARGES OF SEWAGE FROM SEA OUTFALLS*. Pergamon Press. Londres. pp. 199-208.

JONES, K.L. & M.E. RHODES-ROBERTS, 1981. The survival of marine bacteria under starvation conditions. *JOURNAL APPLIED BACTERIOLOGY*, **50**: 247-258.

KAPUSCINSKI, R.B. & R. MITCHELL, 1981. Solar radiation induces sublethal injury in *Escherichia coli* in sea water. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **41**: 670-674.

KOTT, Y., 1966. Estimation of low numbers of *Escherichia coli* bacteriophages by use of the most probable number method. *APPLIED MICROBIOLOGY*, **14**: 141-144.

LE MINOR, L., 1984. *Salmonella*. In: N.R. Krieg & J.G. Holt (ed.) *BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY.* Vol. 1. . Baltimore: Williams & Wilkins.

LESSARD, E.J. & J.M. SIEBURTH, 1983. Survival of natural sewage populations of enteric bacteria in diffusion and bath chambers in the marine environment. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **45**: 950-959.

LIN. S., 1977. Evaluation of methods for detecting coliforms and faecal streptococci in chlorinated secondary sewage effluents. In: Bordner, Frith & Winter (ed.) *PROCEDURE SYMPOSIUM ON THE RECOVERY OF INDICATOR ORGANISMS EMPLOYING MEMBRANE FILTER.* U.S. Environ. Prot. Agency. Cincinnati. pp. 113-129.

MALLORY, L.M.; C.S. YUK; L.N. LIANG & M. ALEXANDER, 1983. Alternative prey: A mechanism for elimination of bacterial species by protozoa. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **46**: 1073-1079.

McCAMBRIDGE, J. & T.A. McMEEKIN, 1980. Relative effects of bacterial an predators on survival of *Escherichia coli* in estuarine water samples. *APPLIED ENVIRONMENTAL MICROBIOLOGY*, **40**: 907-911.

McCAMBRIDGE, J. & T.A. McMEEKIN, 1981. Effect of solar radiation and predacious microorganisms on survival of faecal and other bacteria *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **41**: 1083-1087.

McCOY, J.H. 1971. Sewage pollution of natural waters. In: G. Sykes & F.A. Skinner (ed.). *MICROBIAL ASPECTS OF POLLUTION*. Academic Press, Inc. London, pp. 33-50.

McFETERS, G.A. & D.G. STUART, 1972. Survival of coliform bacteria in natural waters: Field and laboratory studies with membrane filter chambers. *APPLIED MICROBIOLOGY*, **24**: 805-811.

McFETERS, G.A.; G.K. BISSONNETTE; J.J. JEZESKI; C.A. THANSON, 1974. Comparative survival of indicator bacteria and enteric pathogens in well water. *APPLIED MICROBIOLOGY*, **27**: 823-829.

McFETERS, G.A. & D.G. STUART, 1981. In situ studies using membrane filter chambers. In: B.J. Dutka (ed.). *MEMBRANE FILTRATION; APPLICATIONS, TECHNIQUES AND PROBLEMS*. Marcel Dekker, Inc. New York. pp. 481-512.

MITCHELL, R., 1968. Factors affecting de decline of non-marine microorganisms in sea water. *WATER RESEARCH*, **2**: 535-543.

MITCHELL, R., 1971. Destruction of bacteria and viruses in sea water. *JOURNAL OF SANITARY ENGINEERING*, **97**: 425-432.

MITCHELL, R & C. CHAMBERLIN, 1975. Factors influencing the survival of enteric microorganisms in the sea: An overview. In: A.L.H. Gameson (ed.) *DISCHARGE OF SEWAGE FROM SEA OUTFALLS*. Pergamon Press. London pp. 237-251.

MOORE, B., 1971. The health hazards of pollution. In: G. Sykes & F.A. Skinner (ed.) *MICROBIAL ASPECTS OF POLLUTION*, Academic Press, Inc. London. pp. 11-32.

MORIÑIGO, M.A.; J.J. BORREGO; P. ROMERO, 1984. Método de detección y recuento de *Salmonella*. *IV REUNION CIENTIFICA DEL GRUPO DE ALIMENTOS DE LA S.E.M.*, **1**: 55.

MUJERIEGO, R.; J.M. BRAVO, F. CANOVAS; A. de VICENTE; M. PIÑAS; S. GRANE; A. HERNANDEZ & M.T. FELIU, 1980. *Calidad de las aguas costeras y vertido de aguas residuales en el mar: Sus aspectos sobre la salud pública*. Subdirección General de Sanidad Ambiental. Ministerio de Sanidad y Sehuridad Social. Madrid.

OLIVIERI, V.P., 1982. Bacterial indicators of pollution. In: W.O. Pipes (ed.) *BACTERIAL INDICATORS OF POLLUTION*. CRC Press, Inc, Boca Raton. pp. 21-41.

PAOLETTI, A., 1970. Facteurs biologiques d'autodépuration des eaux de mer. Points clairs et points obscurs d'une question discutée. *REVUE INTERNATIONALE OCEANOGRAPHIE MEDICALE*, **32**: 19-33.

PEARSON, A.E., 1956. An investigation on the efficacy of submarine outfall disposal of sewage and sludge. *CALIFORNIA STATE WATER POLLUTION CONTROL BOARD*, **14**: 31-43.

PELLET, S.; D.V. BIGLEY & D.J. GRIMES, 1983. Distribution of *Pseudomonas aeruginosa* in a riverain ecosystem. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **45**: 328-332.

PICHOT, G. & J. BARBETTE, 1978. Estimation des taux moyens de disparition (or dispersion) des bactéries fécales dans les eaux côtières belges de la Mer du Nord. *REVUE INTERNATIONALE OCEANOGRAPHIE MEDICALE*, **51-52**: 115-126.

PIPES, W.O., 1982a. Introduction. In: W.O. Pipes (ed.) *BACTERIAL INDICATORS OF POLLUTION.* CRC Press, Inc. Boca Raton. pp. 1-19.

PIPES, W.O., 1982b. Indicators and water quality. In: W.O. Pipes (ed.) *BACTERIAL INDICATORS OF POLLUTION*. CRC Press, Inc. Boca Raton. pp. 83-95.

PRESSWOOD, W.A. & D.K. STRONG, 1978. Modification of m-FC medium by eliminating rosolic acid. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **36**: 90-94.

RHODES, H.W.; I.C. ANDERSON & H.I. KATOR, 1983. In situ development of sublethal stress in *Escherichia coli*, effects on enumeration. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **45**: 1870-1876.

RODRIGUEZ, J.; J.M. BRAVO; V. RODRIGUEZ & A. CUEVAS, 1982. Influence of a submarine outfall on the surrounding pelagic and coastal system. *VI JOURNEES ETUDES POLLUTIONS CANNES*. pp. 775-777.

SAVAGE, H.P. & N.B. HANES, 1971. Toxicity of seawater to coliform bacteria. *JOURNAL WATER POLLUTION CONTROL FEDERATION*, **43**: 854-861.

SHUVAL, H.I., 1975. The case for microbial standards for bathing beaches. In: A.L.H. Gameson (ed.) *DISCHARGE OF SEWAGE FROM SEA OUTFALLS*. Pergamon Press. Londres. pp. 95-102.

SIEBURTH, J.M., 1979. *SEA MICROBES*. Oxford University Press. New York..

SINCLAIR, J.L. & M. ALEXANDER, 1984. Role of resistance to starvation in bacterial survival in sewage and lake water. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **48**: 410-415.

STARR, M.P. & R.S. SEIDLER, 1971. The bdellovibrios. *ANNUAL REVIEW MICROBIOLOGY*, **25**: 649-678.

STERREGAARD, B., 1975. The revelance of initial dispersion. In: A.L.H. Gameson (ed.) *DISCHARGE OF SEWAGE FROM SEA OUTFALLS*. Pergamon Press. London. pp. 285-295.

SUESS, M.J., 1977. Bacterial water quality and standards: The role of the World Health Organization. In: A.M. Hoadley & B.J. Dutka (ed.) *BACTERIAL INDICATORS/HEALTH HAZARDS ASSOCIATED WITH WATER*. American Society for Testing and Materials. Philadelphia. pp. 182-195.

VASCONCELOS, G.I. & R.G. SWARTZ, 1976. Survival of bacteria in sea water using a diffusion chamber apparatus in situ. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **31**: 913-920.

VASSILIADIS, P.; J. PATERAKI; N. PAPAICONOMOU; J. PAPADAKIS & D. TRICHOPOULOS, 1976. Nouveau procédé d'enrichissement de *Salmonella*. *ANNALES DE MICROBIOLOGIE (INSTITUT PASTEUR)*, **127B**: 195-200.

VERSTRAETE, W. & J.P. VOETS, 1976. Comparative study of *Escherichia coli* survival in two aquatic ecosystems. *WATER RESEARCH*, **10**: 129-136.

de VICENTE, A., 1986. *Estudio de Pseudomonas aeruginosa en aguas naturales contaminadas*. Doctoral Thesis, University of Malaga.

de VICENTE; A.; J.J. BORREGO & P. ROMERO, 1983. Estudio comparativo de diferentes medios de cultivo para el recuento de *Pseudomonas aeruginosa* de aguas naturales. *IX CONGRESO NACIONAL DE MICROBIOLOGIA. Valladolid*. pp. 929-930.

de VICENTE, A.; J.J. BORREGO; F. ARRABAL & P. ROMERO, 1986. Comparative study of selective media for enumeration of *Pseudomonas aeruginosa* from water by membrane filtration. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, **51**: 832-840.

WALKER, J.D. & L.J. GUARRIA, 1975. Other factors determining life expectancy of microorganisms in the marine environment. In: A.L.H. Gameson (ed.) *DISCHARGE OF SEWAGE FROM SEA OUTFALLS.* Pergamon Press. Londres. pp. 221-226.

WORLD HEALTH ORGANIZATION, 1977. *Health criteria and epidemiological studies related to coastal water pollution*. Report of a Group of Experts jointly convened by W.H.O. and U.N.E.P. W.H.O. Copenhagen.

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