ANTIFUNGAL DEFENCE MECHANISMS OF THE SEA URCHIN *ECHINUS ESCULENTUS*. L.

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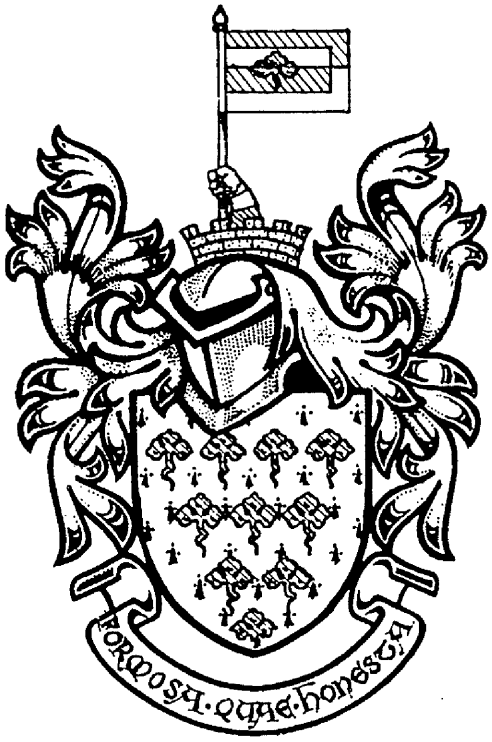
FORMOSA · QVAE · HONESTA

"Microbiology, like all the sciences, is founded upon the twin pillars of craft techniques and philosophical speculation. Without the empirical observations of the first, the subject would be but a mass of meaningless verbiage, and without the organising hypotheses of the second, would be but a collection of descriptions and recipes."

(Collard, 1976)

ACKNOWLEDGEMENTS

Foremost, I wish to thank Professor A.C. Wardlaw for his supervision, invaluable advice and encouragement during the research and preparation of this thesis.

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S U M M A R Y

This thesis describes an investigation of the antifungal defence mechanism of the common sea urchin, *Echinus esculentus*. Eleven species of marine yeasts, representing the four genera *Candida*, *Debaryomyces*, *Metschnikowia* and *Rhodotorula* were tested as target organisms.

The methods of study included : measurement of the antifungal activity of *E. esculentus* coelomic fluid (CF) *in vitro*, clearance of marine yeasts injected into the coelomic cavity, pathogenicity of marine yeasts, qualitative responses of the animals and quantitative changes in the coelomocyte profile.

Before commencing these studies it was necessary to establish maintenance facilities for the sea urchins in the laboratory in Glasgow. Three recirculating artificial-seawater aquaria (RASWA) of around 76 l working capacity were constructed. They had a system of seawater circulation, biological, chemical and physical filtration and were kept at 10°C under artificial, time-controlled illumination. A programme for the regular monitoring of water quality was developed and animals were kept in a healthy condition for up to 12 months. During this period the bactericidal activity of their CF was readily demonstrable.

All the eleven strains of marine yeasts were susceptible to antifungal activity in the CF. The activity was measured by the significantly lower viable count of yeasts that had been exposed for 24-48h to CF at 10°C as compared with similar exposure of the yeasts to various control fluids. A variety of control fluids was examined, notably MBASW (1% marine broth in artificial-seawater) and CFSN (coelomic fluid supernate). In supporting the growth of the marine yeasts, CFSN was markedly superior to MBASW and

was therefore used in most experiments.

As an additional control on the antimicrobial activity of individual samples of CF, the antifungal tests were all done with a mixed inoculum of approximately equal numbers of colony-forming units of the yeast and a previously investigated CF-sensitive marine Pseudomonad designated strain 111 (*Ps. 111*). This strain was much more sensitive to the killing activity of CF than were any of the yeasts.

Antifungal and antibacterial activities of individual CF's were variable but were not correlated with each other, suggesting that different mechanisms were involved in the killing of fungi and bacteria. However, both activities were temperature-dependent, with the same optimum in the range 5-10°C.

The requirement for coelomocytes in the antifungal and antibacterial activities of the CF was shown by the observations that removal of these cells by centrifugation, yielded a supernate (CFSN) which acted as a good growth medium for bacteria and yeasts at 10°C.

However, when coelomocytes were disrupted by ultrasonic treatment, the lysate lost all of its antifungal activity while retaining bactericidal activity. Indeed, the lysate was a better growth medium for the fungi than the CFSN.

Antifungal activity of the napthaquinone pigment echinochrome-A (Ech-A), extracted from the red spherule cells (RSC's), was absent at the normal physiological concentrations (3-60μg ml^{-1}) found in the CF. Conversely, these concentrations of Ech-A have been shown to be bactericidal. However, Ech-A of about four times this concentration did exert some

antifungal activity.

E. esculentus coelomocytes were separated into three bands on a step-wise, discontinuous density gradient of "Ficoll^R" and "Isopaque^R". At the top of the gradient the most prominent band was composed of the phagocytic leucocytes (PL's), the second band below the middle of the gradient was composed of a mixture of vibratile cells (VC's) and colourless spherule cells (CSC's) and the third band formed at the interface of the "Ficoll^R" "Isopaque^R" "cushion" consisted of a narrow band of the RSC's. Only the PL fraction exerted pronounced antifungal activity, whilst the main locus of bactericidal activity was concentrated in the RSC function.

Having established fungicidal activity of *E. esculentus* CF *in vitro*, the clearance of injected yeasts from the coelomic cavity of the intact animal was studied. High doses of (10^9) *Ps.111* were rapidly cleared from the coelom of *E. esculentus* within 24h, indicating a parallel in the bactericidal activity *in vitro* and *in vivo*. This bacterium was included in all clearance experiments as a "sentinel" control. The yeast strains *Rhodotorula rubra* (NCYC 63) and *Metschnikowia zobelli* (NCYC 783) were selected on the basis of their distinctive colony-characteristics and their growth at 10°C in MBASW and CFSN control fluids. Large doses of the marine yeasts (10^7) were cleared in two stages : a primary rapid clearance of about 95% of the inoculum, within hours after injection and a secondary stage in which the residual yeasts persisted within the coelom over a period of several weeks or until death of the animals. The secondary stage was invariably accompanied by heavy background bacterial contamination of the CF.

Different doses of yeast and bacteria were injected into the coelom of *E. esculentus* in an attempt to quantitate clearance. All dose sizes of *Ps. 111* (range 6 x 10^4 - 2 x 10^{10}) were completely cleared within a week. In contrast, only relatively low doses of marine yeasts (10^4 - 2 x 10^6) were completely cleared from the coelomic cavity. Higher doses of yeasts (10^7 - 10^9) were incompletely cleared and accompanied by a higher incidence of mortality and bacterial contamination of the CF. The marine yeasts did not show net multiplication, but they may have acted by depressing the host defence mechanisms, thereby allowing secondary bacterial infection to supervene and kill the animals.

Several qualitative changes in the animals were observed during the course of experimental infection with yeasts and bacteria, particularly the formation of red/black gelatinous lesions on the exterior test surface of *E. esculentus*. Observation by light microscopy revealed a meshwork of RSC's, PL's, CSC's and VC's in order of decreasing abundance and were infected with algae, bacteria and organisms of the experimental infection.

Quantitative analyses of the coelomocyte-profile of healthy animals indicated a major proportion of the CF to be composed of PL's (66%), followed by combined VC's and CSC's (23%) and RSC's (11%). The relative percentages remained fairly constant with evidence of only minor changes (±1-2%). However, there was a decline in the differential coelomocyte counts, particularly the fraction containing CSC's and VC's, (40%). There was also a 17% decline in the PL's and 10% drop in the RSC's all from infected animal CF. Moreover, the total coelomocyte count was also observed to decline by about 40% in the CF from "sick" animals. This decline in total and differential coelomocyte count may be attributed to

lysis, clotting inaccessible to routine sampling methods or coelomocyte migration to the test or infected areas.

This investigation therefore demonstrates antifungal defence mechanisms in the sea urchin *E. esculentus* illustrated by fungicidal activity of the CF *in vitro* and clearance of infected yeasts from the coelomic cavity of the whole animal. The study confirms conclusions made by previous workers that the main locus of bactericidal activity resides in the whole or lysed RSC's. However, the mechanism of antifungal activity, requiring cellular integrity, appears to be concentrated in the phagocytic leucocytes of the coelomic fluid.

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ABBREVIATIONS

a.c.	almost confluent (equivalent to an SI of ≥200)
c.	confluent (equivalent to an SI of ≥200)
ASW	artificial seawater
ATCC	American Type Culture Collection, Rockville, Maryland, U.S.A.
BGG	bovine gamma globulin
CBS	Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands.
CF	coelomic fluid
CFL	coelomic fluid lysate
CFSN	coelomic fluid supernate
cfu	colony forming units
CSC	colourless spherule cells
Ech-A	echinochrome-A
EGTA	ethylene glycol bis-(β-aminoethyl ether)N,N,N',N'-tetra acetic acid
HA	haemagglutinin
HL	haemolysins
IFO	Institute for Fermentation, Osaka, Japan.
MA	marine agar
MB	marine broth
MBASW	1% marine broth in artificial seawater
NCMB	National Collection of Marine Bacteria, Aberdeen. U.K.
NCYC	National Collection of Yeast Cultures
NSW	natural seawater
OD	optical density
OU	opacity units

PL	phagocytic leucocytes
Ps.111	*Pseudomonas* strain number 111
pwc	partial water change
RASWA	recirculating artificial-seawater-aquaria
RSC	red spherule cells
SCUBA	self-contained underwater breathing apparatus
SEM	scanning electron microscopy
SG	specific gravity
SI	survival index (%); SI = 100. Ct/Co
TAME	2mM p-tosyl-L-arginine methyl ester
VC	vibratile cells
w/v	weight for volume
w/w	weight for weight
YMA	yeast marine agar

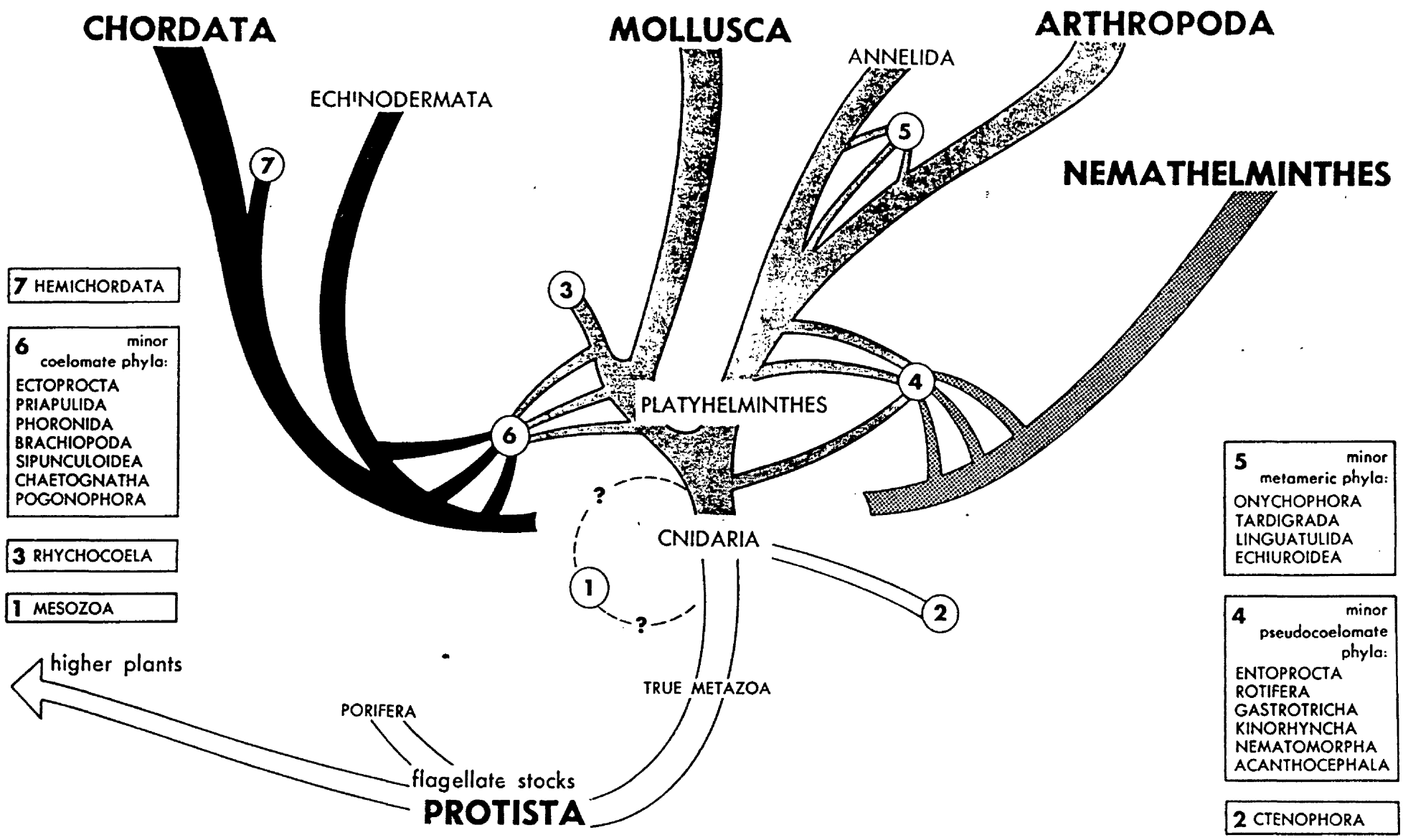
I N T R O D U C T I O N

1. GENERAL INTRODUCTION

As experimental animals, sea urchins have been little studied by microbiologists but, as this thesis illustrates, they do not deserve such neglect. The particular aspect of prime interest is their position on the phylogenetic tree just below the level of the chordates (Figure 1). In the words of Burnet, (1974) the Nobel laureate in physiology and medicine: "Since Darwin, much of the 'fun' of biological research has been to interpret what interests one in evolutionary terms". Such a position near the chordates suggests that sea urchins may possess, in primordial or vestigial form, some of the antimicrobial defence mechanisms which reach great elaboration in the higher animals. Indeed, in this context, it is of great interest to note the discovery of a complement C3ib receptor on sea urchin phagocytes (Bertheussen and Seljelid, 1982) and the report of similarities in amino acid composition between starfish haemagglutinin and vertebrate immunoglobulin (Carton, 1974). These aspects are discussed more fully below.

Written accounts of sea urchins go back to 380 B.C. when Aristotle wrote about sea urchin mouth parts in his "Historia Animalium": "I seem to see the lantern itself, like a street-lamp, in the shape of an inverted cone, with its panes of horn set in a frame all round". The mouth apparatus of the sea urchin was thus named "Laterna Aristotelis" by Klein in his "Naturalis Dispositio Echinodermatum" in 1734 (cited by Peck, 1965). "Echinoderma" is a Greek word and means "prickle-skinned", and the name "Echinodermata" applied to the phylum, means "sea urchin skins" and was invented in 1734 by Klein to denote only the empty shells or tests of sea urchins (cited by Bather, 1929). According to Nichols (1969), the first book about echinoderms was probably that written by Linck in 1733, "De

FIGURE 1. Summary scheme of how the main invertebrate phyla may be related.
(From Russell-Hunter, 1969)

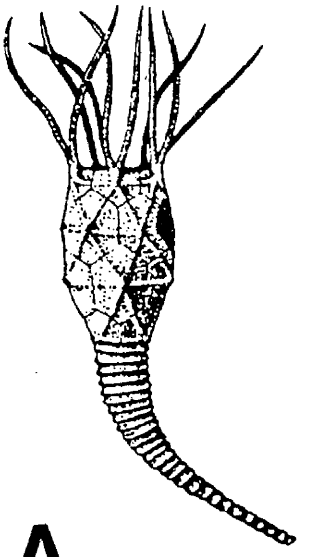

CHORDATA
MOLLUSCA
ARTHROPODA
ANNELIDA
ECHINODERMATA
NEMATHELMINTHES
7
5
3
4
6
PLATYHELMINTHES
?
CNIDARIA
1
2
?
higher plants
TRUE METAZOA
PORIFERA
flagellate stocks
PROTISTA
7 HEMICHORDATA
6 minor coelomate phyla:
ECTOPROCTA
PRIAPULIDA
PHORONIDA
BRACHIOPODA
SIPUNCULOIDEA
CHAETOGNATHA
POGONOPHORA
3 RHYCHOCOELA
1 MESOZOA
5 minor metameric phyla:
ONYCHOPHORA
TARDIGRADA
LINGUATULIDA
ECHIUROIDEA
4 minor pseudocoelomate phyla:
ENTOPROCTA
ROTIFERA
GASTROTRICHA
KINORHYNCHA
NEMATOMORPHA
ACANTHOCEPHALA
2 CTENOPHORA

Stellis Marinis" ("The Sea Stars"). On a different time scale from their literary antiquity, sea urchins are also of great geological antiquity. Echinoderms, have representatives from as early as the Cambrian epoch approximately 500 million years ago (Figure 2). This long persistence implies great survival value, including effective defence mechanisms against the numerous types of microorganisms which inhabit the sea. This conclusion is strengthened by the longevity of modern sea urchins. *Echinus esculentus* Linnaeus (1758; cited by Bell, 1892) for example, has a lifespan of up to 10 years (Boolootian, 1966).

The other major component of this thesis comprises the marine yeasts, the organisms used by Metschnikoff in his classic demonstration of phagocytosis (by another invertebrate *Daphnia*) (Figure 3). Yeasts, like bacteria, are widely distributed in the world's oceans, estuaries and sediments, and sea urchins must therefore be exposed to them. This thesis focuses on the potential pathogenicity of marine yeasts for *E. esculentus* and the effectiveness of antifungal defence mechanisms in the sea urchin. The wider context of this study is the much more general question of whether sea urchins do indeed resemble vertebrates in their antimicrobial defence capabilities, or whether during the long span of evolutionary separation they have developed different, but perhaps equally effective, immunity mechanisms.

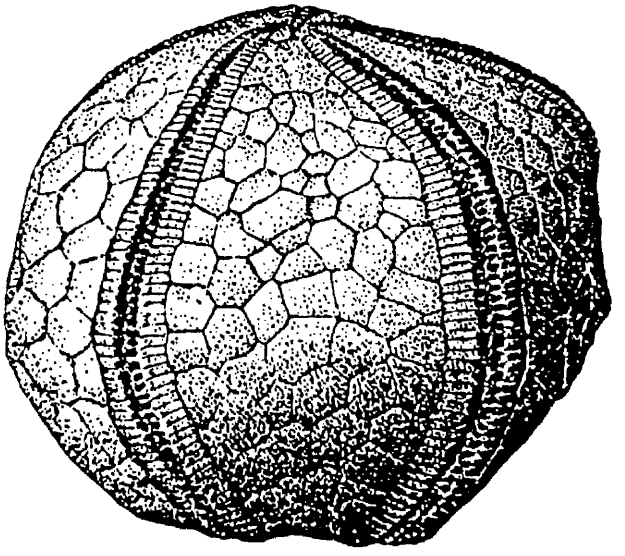
To introduce the experimental studies the following pages present reviews on marine yeasts, on the sea urchin *E. esculentus* and microbial pathogenicity and sea urchin defences.

FIGURE 2. Examples of Cambrian (A) and Ordovician (B) echinoderms, approximately 500 and 400 million years old respectively.
(By courtesy of the British Museum (Natural History), Ball, 1975)



A

Macrocystella mariae Callaway. Artificial cast (×1.) Tremadoc Series; Sheinton, Salop. RANGE: Tremadoc Series.



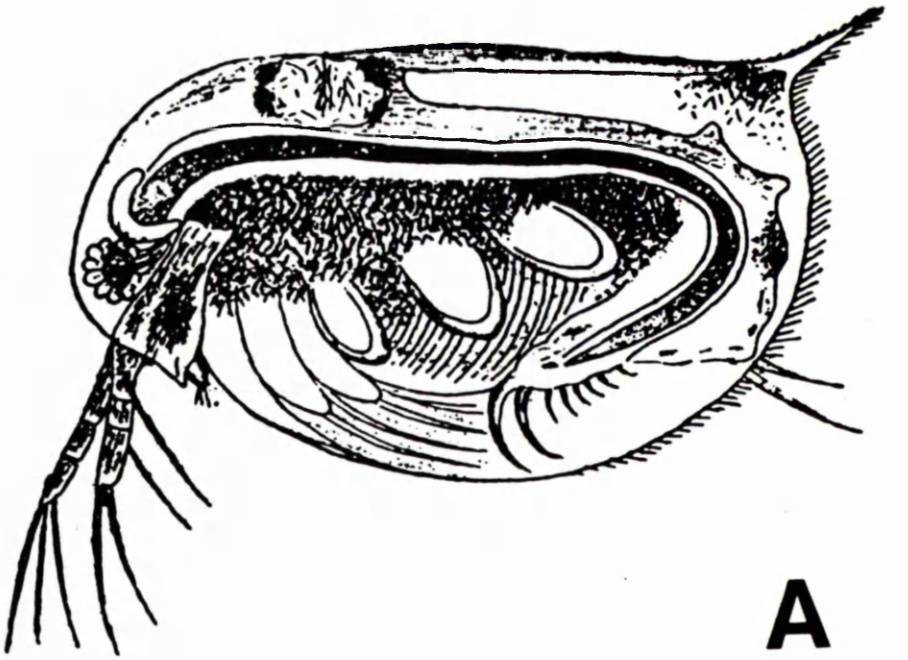
B

Aulechinus grayae Bather & Spencer. (× 1¼.). Ashgill Series; Thraive Glen, Girvan, Ayrshire. RANGE: Ashgill Series.

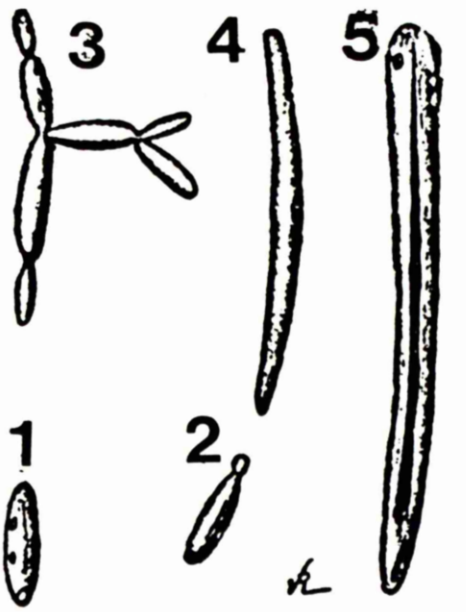
FIGURE 3. Metschnikoff's classic illustrations (1893) of *Daphnia* infested by *Monosporae* (A) and *Monospora* in various stages (B)

1. Young conidium
2,3. Budding conidia
4. Elongated conidium
5. Spore

(From Metschnikoff, 1893)



A



B

2. MARINE YEASTS

With the invention of the microscope by Antonie van Leeuwenhoek in the seventeenth century the systematic study of fungi began, and the man who deserves the honour of being called the founder of the science of mycology is Pier' Antonio Micheli, the Italian botanist, who in 1729 published "Nova plantarum genera", in which his researches on fungi were included (Alexopoulos, 1962).

A vast literature exists on various aspects of the yeasts, therefore only selected topics are included in the discussion below.

2.1. Yeasts Within the Fungi

The fungi constitute a large number of eucaryotic organisms and are heterotrophic organisms which depend on previously elaborated organic materials on which they live as saprophytes or parasites (Rheinheimer, 1974; Evans and Gentles, 1985). In water, saprophytic forms are found as well as parasites which attack a great variety of aquatic plants and animals (Rheinheimer, 1974). They show considerable diversity in size and morphology, but no matter how large or small a fungal mass may be, it is never vascularized and is composed of elements of the same basic form. These cells consist of a firm, mainly polysaccharide wall with an inner cell membrane surrounding cytoplasm which encloses the nuclei (usually two or more), food reserves (fat, oil or glycogen) and vacuoles filled with sap. Such cell-units may exist separately as yeasts, but are more often joined together to form filaments (hyphae) as in moulds; and in certain instances the hyphae may become closely associated to form a pseudo-tissue, such as toadstools and mushrooms.

The mycological classification of fungi is based primarily on the method of sexual reproduction. However, one group is an artificial one and contains all the fungi for which no method of sexual reproduction has been discovered, the Fungi Imperfecti (Evans and Gentles, 1985). An outline of the classification of the fungi and the major characteristics of each group are presented in Table 1. Among the Ascomycetes, Basidiomycetes and Fungi Imperfecti (Deuteromycetes), the characteristic vegetative structure is the coenocytic mycelium. Nonetheless, there are a few groups in these classes that have largely lost the mycelial habit of growth and have become unicellular. Such organisms are known collectively as the yeasts (Stanier *et al.*, 1979).

Yeasts are predominately unicellular and uninucleate with round, oval or elongate cells. A few species reproduce by fission but most yeasts propagate by a process of budding, with a daughter cell or blastospore arising on the surface of the parent (Figure 4A). Usually the daughter cells separate from the parent but, under certain conditions, they may elongate and remain attached to form a chain of cells or pseudomycelium (Figure 4B). True mycelium may also be formed by a number of yeast species.

Therefore, the group of microorganisms known as the yeasts are by traditional agreement limited to the fungi in which the unicellular form is predominant and reproduction is by budding or fission (Kreger-van Rij, 1969; Sieburth, 1979 and Alexopoulos, 1962). This group does not constitute a taxonomic unity, although it comprises subdivisions of narrowly related species. The diversity of the yeasts is illustrated by the fact that 39 genera and 350 species are recognized (Kreger-van Rij, 1969). The features by which the species are distinguished are of the smaller part morphological and of the greater part physiological.

TABLE 1. Outline classification of the fungi and major characteristics of the groups (after Ainsworth and Sussman, 1973)

Main Grouping	Subdivisions	Characteristics
Lower Fungi (Phycomycetes)	Mastigomycotina	Mycelium, if formed, coenocytic Asexual spores formed in sporangium. Spores and/or gametes motile. Sexual spores varied in form.
	Zygomycotina	Mycelium, coenocytic. Asexual spores in sporangia. Spores non-motile. Sexual reproduction by gametangial copulation. Sexual spores, zygospores.
Higher fungi	Ascomycotina (Ascomycetes)	Mycelium septate or single cells (yeasts). Asexual spores (condia) borne exogenously. Sexual spores (ascospores) borne within sac or ascus; usually eight in number. Asci may be borne singly or in groups within a fruiting body (ascocarp).
	Basidiomycotina (Basidiomycetes)	Mycelium septate or single cells (yeasts). Asexual cells if formed, exogenous. Sexual spores (basidiospores) borne exogenously on a basidium - often within macroscopic fruiting body (basidiocarp).
	Deuteromycotina (Fungi Imperfecti)	Mycelium septate* or single cells (yeasts). Asexual spores borne exogenously, sometimes within pycnidium. Sexual reproduction not known.

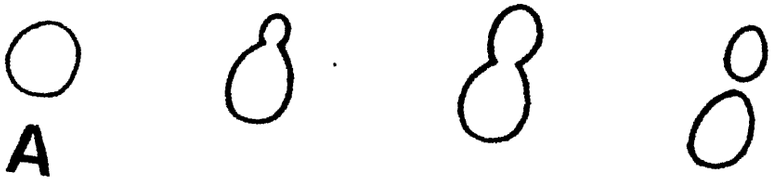
* Coenocytic mycelial fungi without a sexual phase and classified in Lower fungi.

FIGURE 4. Diagrammatic representation of vegetative forms of the yeasts.

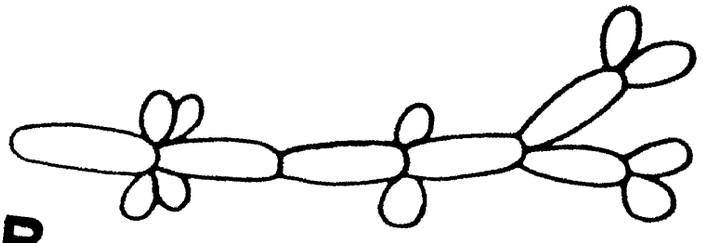
A. Yeast cells showing stages in budding,

B. Yeast pseudomycelium (pseudohypha).

(Reproduced with the kind permission of Dr. G.V. Evans and Dr. J.C. Gentles, 1985, Churchill Livingstone, Edinburgh).



A



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Therefore, even though the yeasts are considered higher fungi, they have been avoided by the mould specialists (Kohlmeyer and Kohlmeyer, 1971) because they cannot be characterized by morphology alone, requiring biochemical tests, similar to those used for bacteria. A standard set of tests and substrates are used to test for fermentation and assimilation, characteristics that are used for generic and for species differentiation (van der Walt, 1970).

The basis for the taxonomy and the systematics of the yeasts have been discussed by Kreger-van Rij (1969, 1984). The ultimate source book on the taxonomy of the 39 genera of the yeasts is the text edited by Lodder (1970). The three volumes edited by Rose and Harrison (1969, 1970, 1971) contain discussions on yeast growth, cytology, life cycles and genetics.

The germ tube test is the main criterion used to screen for the presence of *Candida albicans*. If the test is positive, the identification of *C. albicans* may be made and further testing is not required. A schema for the practical laboratory identification of yeasts is presented in Figure 5 (Koneman and Roberts, 1983). This assumes a germ-tube negative result.

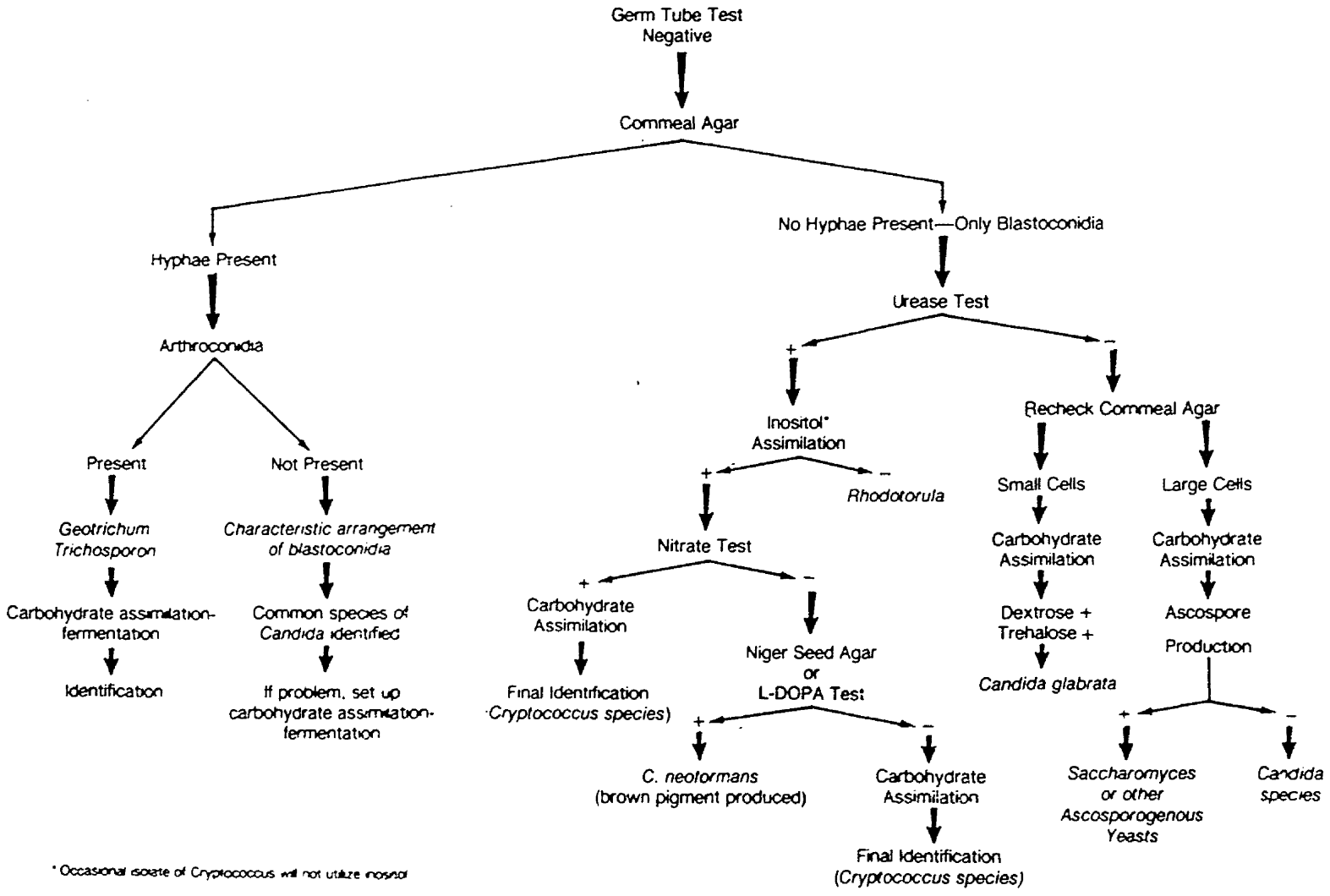
2.2. Yeasts in the Marine Environment

The major emphasis of the literature on marine yeasts is largely of an ecological nature. Evidence has accumulated that marine yeasts frequently occur as constituents of marine microbial populations (van Uden and Fell, 1963), although knowledge of the aquatic yeasts is much more limited than that of the terrestrial yeasts (Phaff *et al.*, 1966).

FIGURE 5. Schema of practical laboratory identification of yeasts.

(Reproduced with kind permission of the Williams and Wilkins Co., Baltimore. (Koneman and Roberts, 1983)).

Yeast Identification Schema



* Occasional isolate of Cryptococcus will not utilize inositol

The earliest observations on marine yeasts were made by Bernard Fischer during his pioneering work on bacteria in the 1880's. Interest in this aspect of marine ecology has resulted from chance observations of yeasts reported at various times since the beginning of marine microbiology. The history of these findings was recorded by Zobell (1946), Kriss (1959) and Johnson and Sparrow (1961).

The definition of "marine yeasts", as suggested by van Uden and Fell (1963), included all yeasts and yeast-like organisms that are capable of building up self-perpetuating populations in marine environments. This definition did not exclude yeasts that are also capable of occupying non-marine ecological niches or even preferentially do so. Morris (1975) suggested a modification of Kochs' postulates, namely, a yeast can be regarded as a marine yeast when it can be isolated in pure culture from a marine environment and then can be shown to be capable of reproducing in such an environment. The fact that tests have not indicated any major metabolic or physiological differences in yeasts isolated from terrestrial and marine environments has raised questions as to what constitutes a marine yeast (van Uden and Fell, 1963). However, Bhat *et al.* (1955) studied a collection of marine yeasts for their ability to utilize various carbon and nitrogen compounds as sole sources of these elements. The results indicated that the assimilatory powers and utilization of such compounds by marine yeasts were poor as compared to terrestrial yeasts. However, they also reported that as a group, marine yeasts exhibit a perceptibly higher tolerance to common salt and phosphate than their terrestrial-counterparts as did Ross and Morris (1962). Investigations into salt-tolerance of marine yeasts in relation to the regulation of potassium and sodium ratio and osmotic potential (Norkrans, 1966, 1968; Norkrans and Kylin, 1969) and morphological alterations of *C. albicans* in

the presence of seawater (Safer and Ghannoum, 1983) indicated that salinity factors do not appear to be sufficiently deleterious or restrictive to prevent the continued existence of yeasts in the marine environment (Roth *et al.*, 1962).

For a bacterium to be considered as a "true" marine form demands that in primary culture it shows demonstrable optimal growth in media prepared with seawater and conversely growth fails in fresh water. This criterion has been successfully employed in delineating most marine bacteria. However, in the experience of van Uden and Fell (1963) no yeast satisfied the same criterion, growing well in either medium. Therefore, with this definition, the yeasts encountered by the latter authors in their studies were suggested as representing terrestrial species of transitory status in the marine situation *or,* adaptive forms which survived in both environments. The genus *Metschnikowia* appears to be the only well adapted group to live in the sea (Fell and van Uden, 1963) and could be regarded with some certainty as marine yeasts in the strictest sense (Rheinheimer, 1974). Ferguson Wood (1965) summarized early work and he too concluded that the authors could not designate a true marine yeast flora and were therefore, transients in the marine environment. Kriss *et al.* (1967) listed 91 species isolated from the marine environment belonging to the genera *Candida, Cryptococcus, Saccharomyces, Torulopsis,* and *Trichosporon* and concluded they were salt-tolerant forms of terrestrial origin.

2.3. Role of Yeasts in the Marine Environment

Exclusive of the parasitic varieties, the importance of yeasts in modifying the marine environment is strictly secondary to that of the ubiquitous, more versatile marine bacteria (Zobell, 1946). However, the

yeasts and yeast-like fungi participate in a range of ecologically significant processes in the sea, especially in estuarine and near-shore localities (Meyers and Ahearn, 1974). Among such activities are, decomposition of plant substrates (van Uden and Castelo-Branco, 1963; Seshadri and Sieburth, 1971), nutrient recycling phenomena (Meyers and Nicholson, 1970, Meyers *et al.*, 1970), biodegradation of oil (Ahearn *et al.*, 1971; Ahearn, 1973; Fedorak *et al.*, 1984) and recalcitrant compounds, e.g. breakdown of cellulose by *Trichosporon* spp. (Dennis, 1972) and parasitism of marine animals (van Uden and Castelo-Branco, 1961), (discussed more fully later).

2.4. Distribution and Abundance

Yeast populations decrease in numbers with distance from land (van Uden and Fell, 1968) falling off more rapidly than bacteria (Hoppe, 1972a and b). Yeasts, however, are still the dominant fungi in the open ocean (Fell, 1968; Bahnweg and Sparrow, 1971). The filamentous fungi are mainly confined to nearshore waters, due to the distribution of substrates for example, the remnants of land plants and terrestrial "run-off" (van Uden and Fell, 1963). Terrestrial "run-off" is a major factor that influences the character of the yeast population e.g. some isolates from estuarine waters, *Candida guilliermondii*, *C. krusei* and *T. cutaneum* are often associated with terrestrial substrates such as cultivated soils. These findings suggested that certain yeasts which are normally associated with terrestrial sources are probably pollution organisms when occurring in marine locales (van Uden and Fell, 1963). This is discussed more fully later in section 2.4.1.4.

The yeasts in the inshore waters belong to a variety of species in all three divisions of the higher fungi; the Ascomycetes, the Basidiomycetes and the Deuteromycetes (Fell, 1976). Population densities are contingent upon the availability of organic substrates for growth, since as distance from land decreases, the load of organic debris is less. The variety of yeast species decreases, and the Ascomycetes become more rare, except for the ubiquitous species *Debaryomyces hansenii* (Fell, 1976; Phaff *et al.*, 1966). In inshore waters, a majority of the population of yeasts (a 1:100 ratio of yeasts to bacteria, Hoppe 1972a) is normally dominated by species of *Candida*, *Rhodotorula* and *Debaryomyces* (van Uden and Fell, 1968; Hoppe, 1972b).

2.4.1. Oceans, estuaries and sediments

2.4.1.1. *Ascomycetous yeasts*

The genera with marine representatives fall into two of the four families of *Endomycetales*, the *Spermophthoraceae* and *Saccharomycetaceae*.

The species in the family *Spermophthoraceae* are distinguished by needle- or spindle-like ascospores, which are often larger than the vegetative yeast cells from which the ascus is formed. The marine isolates belong to the genus *Metschnikowia* and have budding vegetative cells, rudimentary pseudomycelium and elongated asci with characteristic needle-like spores. The checkered taxonomy of the five recognized species is discussed fully by Miller and van Uden (1970). The type species *M. bicuspidata*, with its asci and needle-like spores pointed at both ends, was observed in the body cavity of the common freshwater crustacean *Daphnia magna* by Metschnikoff in 1884. The first *Metschnikowia* yeasts obtained in

pure culture and the first marine species, described as *M. zobelli* and *M. krissii* were obtained off Southern California by van Uden and Castelo-Branco (1961). *M. krissii* was only isolated from seawater, and even here it occurred in numbers ranging between 10-570 l^{-1}. The population estimates of *M. zobelli* were 20-580 l^{-1} in seawater, 25-5730 ml^{-1} of fish contents and 520-39200 g^{-1} of the giant kelp *Macrocystis pyrifera* (van Uden and Castelo-Branco, 1963). A number of haploid and heterothallic isolates of marine variety of Metschnikoff's microorganism, *M. bicuspudata* var. animalis, were obtained near the South Shetland Islands in Antarctica by Fell and Hunter (1968).

Most of the ascogenous yeasts are in the family *Saccharomycetaceae*, which reproduce vegetatively mainly by the budding of single cells, although fission occurs in two genera. The ascospores occur in a variety of shapes other than needle-shaped and are the main distinguishing feature of the genera. Species of *Debaryomyces* which have round ascospores in an oval ascus, are quite common in some environments. *Debaryomyces globosus* was isolated by Kriss (1963) in the Pacific Ocean, Greenland and Norwegian Seas, and *D. hansenii* was reported as common in Biscayne Bay (Roth *et al.*, 1962) off the coast of Bombay (Bhat and Kachwalla, 1954) and in the waters around Helgoland (Meyers *et al.*, 1967a), being a ubiquitous species (Fell, 1976). Species of *Pichia* have vegetative cells of various shapes, reproduce by multiple budding, and form spherical or hat-shaped spores. The genus *Saccharomyces* which is vigorously fermentative has a few representatives in the sea. Other genera recovered from the sea include *Hansenula*, *Hanseniaspora*, *Endomycopsis* and *Kluyveromyces*.

2.4.1.2. *Basidiomycetous yeasts*

The marine forms of the Basidiomycetous yeasts are the perfect stages of *Rhodotorula* and *Candida;* they are placed in *Rhodosporidium* and *Leucosporidium,* respectively (Sieburth, 1979).

The genus *Rhodosporidium* formed by Banno (1967), now contains eight species of which seven were isolated from the sea, including the perfect form of the red-pigmented *R. glutinis* which he named *Rhodosporidium toruloides.* Thirty seven strains of *R. glutinis* were isolated by Newell and Fell (1970) from the Antarctic and Carribean waters, with large numbers of thick-walled spores. This led to the discovery of the sexual phases similar to *R. toruloides* but different enough to create the species *Rhodosporidium sphaerocarpum* (Sieburth, 1979).

Studies on the mating systems of the genus have clarified the taxonomic status of the species and have strengthened the evidence of their relationship to the Basidiomycetes by the finding of both tetrapolar and multipolar allelic bipolar systems in several species (Fell *et al.*, 1973).

The study of the metabolic attributes of marine and terrestrial counterparts of the genus *Rhodotorula* (Ahearn *et al.*, 1962) failed to disclose any major differences between the yeasts from the two environments. Moreover, the physiological characteristics of the marine isolates were so similar to those of the established taxa that the creation of a species was not justified (Sieburth, 1979).

The genus *Leucosporidium* was created by Fell *et al.* (1969) to include the *Candida*-like heterobasidiomycetous yeasts. The essential difference between *Rhodotorula* and *Candida* is that the former are coloured orange to red by carotenoid pigments, whereas the latter are non-pigmented, being

white to cream. Pigmentation is also the distinguishing feature between *Rhodosporidium* and *Leucosporidium*. The life cycles of species in the two genera are essentially identical. The species of *Leucosporidium* have a distinct budding yeast phase and produce pseudomycelium. Some species are heterothallic and homothallic, having both a mating phase and a self-sporulating phase. The *L. scottii* and *L. antarcticum*, which have both phases, are differentiated by their ability to intermate and by their different physiology. The four strictly self-sporulating species are differentiated on the basis of fermentation and assimilation of carbon compounds. The names of several species of the genus, *L. scottii*, *L. nivalis*, *L. frigidum*, *L. antarcticum* and *L. gelidum* indicated they were isolated from cold Antarctic waters (Fell *et al.*, 1969; Sieburth, 1979).

2.4.1.3. *Deuteromycetous yeasts*

The Deuteromycetous yeasts also occur in the sea and are placed either in the family *Sporobolomycetaceae*, with characteristic ballistospores, *or* in the family *Cryptococcaceae*, which lack ballistospores (Sieburth, 1979).

The *Cryptococcaceae* include cream, yellow orange and red yeasts which bud, with some forming pseudomycelium (by budding), and some form true mycelium (by fission) or arthrospores (by hyphal fragmentation). The major yeast population of many nearshore materials is often the cryptococcoid yeasts. The genera in the *Cryptococcaceae* (discussed in Lodder, 1970) are distinguished mainly by morphological, rather than physiological differences.

The genus *Torulopsis* is differentiated from *Candida* only by the poor development, or lack, of pseudomycelium. The genus *Cryptococcus*, not only lacks pseudomycelium, but is also non-fermentative and assimilates inositol.

The "pink" yeasts are immediately recognized as *Rhodotorula* by their visible carotenoid pigments and lack of fermentation. Species of *Trichosporon* are distinguished by the formation of true mycelium and arthrospores. Two genera of unusual yeasts which reproduce by conidia, were isolated by Fell (1966) from the Indian Ocean (*Sterigmatomyces*) and Fell and Statzell (1971) from the open waters of Antarctica (*Sympodiomyces*) (Sieburth, 1979).

Many surveys on the distribution of yeasts in the oceans, seas, estuaries and rivers have been done worldwide, some of which are presented in Table 2.

2.4.1.4. *Pollution*

Many of the recent reports of yeasts isolated from seawater were associated with their presence in polluted areas. For example, Ahearn (1973) observed waters, heavily polluted with domestic wastes, often contained large populations of species of *Candida*, *Rhodotorula* and *Trichosporon*. Hagler and Mendonça-Hagler (1981) and Hagler *et al.* (1981) have presented several reports on their studies of yeasts isolated from the polluted marine and estuarine areas of Rio de Janeiro, Brazil. *Candida krusei* and phenotypically similar yeasts as a group were prevalent in polluted estuarine water, but rare in unpolluted seawater. The prevalent genera in the polluted estuary were *Candida*, *Debaryomyces*, *Hansieniaspora*, *Rhodotorula*, *Torulopsis*, and *Trichosporon* and strains of the oppurtunistic pathogens, *C. albicans*, *C. guilliermondii*, *C. krusei* and *C. tropicalis* were frequently found in water with coliform counts above 1000 per 100ml (Hagler and Mendonça-Hagler, 1981). Studies on the sodium chloride tolerances of these yeasts isolated from polluted seawater were done and most of the 121 yeasts isolated, representing 51 species of yeasts, had sodium chloride

TABLE 2. Origin of yeasts from various marine locations.

Yeast species	Places of isolation	Reference(s)
Cryptococcus, Debaryomyces, Rhodotorula, Saccharomyces, Torulopsis, Trichosporon	Indian Coast	Bhat and Kachwalla (1954)
Candida marina, Torulopsis torresii, T. maris	Torres Strait	van Uden and Zobell (1962)
Candida, Debaryomyces, Rhodotorula, Torulopsis	Estuaries, River Tagus and Sado, Portugal	Taysi and van Uden (1964)
C. diddensii, D. hansenii, R. glutinis, R. rubra	The Black Sea	Meyers *et al.* (1967b)
C. atmospherica, C. polymorpha, R. crocea, R. glutinis, R. rubra, Sporobolomyces hispanicus, Sp. adorus	The Indian Ocean	Fell (1967)
C. diddensii, D. hansenii R. rubra	The North Sea	Meyers *et al.* (1967a)
Candida, Cryptococcus, Rhodosporidium, Sterigmatomyces	The Antarctic	Fell (1968)
Thirty three species from the three classes Ascomycetes, Basidiomycetes and Deuteromycetes	South Indian, South Pacific and Antarctic Oceans	Fell (1968, 1974, 1976)

TABLE 2. (continued).

Yeast species	Place of isolation	Reference(s)
Candida, Cryptococcus, Debaryomyces hansenii, Rhodotorula	South Florida	Ahearn *et al.* (1968)
R. glutinis	St. Lawrence River	Simard and Blackwood (1971)
Candida, Cryptococcus, Debaryomyces, Rhodotorula	The Pacific Ocean	Goto *et al.* (1972, 1974a) Yamasato *et al.* (1974)
Candida, Cryptococcus, Hansenula, Rhodotorula	Suwanee River Florida Estuary	Lazarus and Koburger (1974)
Candida spp., *C. informo-miniatus, D. hansenii, R. marina, R. rubra, Torulopsis* spp.	Aburatsubo Inlet, Japan	Goto *et al.* (1974b)
Candida, Cryptococcus, Debaryomyces, Rhodotorula, Saccharomyces, Torulopsis, Trichosporon	Abaco Island, The Bahamas	Volz *et al.* (1974)
Candida, Cryptococcus, Debaryomyces, Rhodotorula, Saccharomyces	The Atlantic and Mediterranean	Briscou (1976)
Sporobolomyces	Lorraine, France	Hinzelin and Lectard (1976)

tolerances of 6-12% and none below 4%. Inhibition of growth below the tolerance limit should be an important selective factor in marine conditions since yeasts prevalent in seawater had sodium chloride tolerances of 9% or more and increased lag phases were observed with increasing sodium chloride concentration.

A survey of yeast populations at various points along the St. Lawrence River indicated that certain yeast blooms occur after bacteria have degraded the easily metabolizable constituents of the raw sewage and that their numbers could be used as an indicator of pollution. Over 50% of all isolates belonged to *Candida, Cryptococcus, Pullularia* (Black Yeasts) *Rhodotorula spp.* and *Torulopsis* (Simard, 1971; Simard and Blackwood, 1971). Several yeasts from polluted and non-polluted beaches of São Paulo, Brazil, were isolated by de Paula *et al.* (1983); five hundred species were distributed in nine genera *Candida, Cryptococcus, Debaryomyces, Hansenula, Pichia, Rhodotorula, Sporobolomyces, Torulopsis,* and *Trichosporon.* The results pointed to *Candida* as a probable pollution indicator for coastal seawater. Although *C. albicans* occurs in high densities in human excrement (Ahearn *et al.*, 1966; Cohen *et al.*, 1969), it is not commonly found in natural waters or treated sewage effluents. Hence, its isolation from water has been associated with recreational bathing or recent contamination with raw sewage (Ahearn *et al.*, 1971; Cook, 1970). Several studies have also demonstrated that pure cultures of *C. albicans* survive and maintain their pathogenicity for prolonged periods in both fresh and seawater (Fell and Meyer, 1967; Buck *et al.*, 1977). Other human-associated yeasts *C. parapsilosis, C. tropicalis* and *T. glabrata* were frequently isolated from quahogs, oysters and mussels collected from four estuarine areas along the northern shore of Long Island Sound. Generally, these yeasts in the animals sampled reflected the overall pollution-status of the estuary. These studies represent a clear demonstration of potentially pathogenic yeasts in a valuable marine resource.

2.4.2. Association with marine plants and animals

2.4.2.1. *Marine plants*

There are a few accounts of the association of yeasts with marine plants.

In the review by Morris (1968) only sixteen species representing seven genera, could be recognized as being definitely isolated from marine plants. These included *Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Metschnikowia zobelli* and *Torulopsis famata*. A high incidence of *Cryptococcus* spp. may be related to the fact that most samples of seaweeds had been taken from littoral waters which may have been contaminated by yeasts from terrestrial "run-off".

It is probable that healthy marine plants do not serve as reservoirs for yeasts and that the latter are incidental contaminants from the plants' environment (Fell and Roth, 1961; Capriotti, 1962; Roth *et al.*, 1962; Siepmann and Höhnk, 1962). This view is supported by the fact that van Uden and Castelo-Branco (1961, 1963) showed that strains of *M. zobelli* were present on the surface of decomposing Giant kelp (*Macrocystis pyrifera*) in numbers of about 530, 200 g^{-1} from regions where this yeast is common in seawater. *C. pulcherrima* was also isolated from one sample (800 g^{-1}). Seawater normally contains tens or hundreds of yeasts per litre. However, in regions of grass or algal beds there are five to six thousand yeasts per litre. Thus, the organic constituents present in algae and seaweeds become accessible to yeasts only after death and dissolution of the plants. The complex polysaccharides are probably reduced to simpler molecules by bacteria and actinomycetes before serving as energy sources for most yeasts (van Uden and Fell, 1963; Morris, 1955a and b). Most heaps of decomposing kelp are sooner or later washed back into the sea, thereby releasing their

yeast populations into the seawater. Thus, decomposing kelp may constitute a reservoir for *M. zobelli* and possibly other marine-occurring yeasts (e.g. *C. pulcherrima*) (van Uden and Castelo-Branco, 1963). Seshadri and Sieburth (1971) gave a detailed account on the cultural estimation of yeasts on different types of seaweeds located at Rhode Island, U.S.A. They found maximal numbers on Rhodophytes and Chlorophytes, while lower numbers were found on Pharophytes and this was attributed to the release of inhibitory phenolic materials. Only colourless yeasts of the genus *Candida* were observed, except for brief occurrences of the pink yeast *Rhodotorula* in the late Spring. Yeast populations were detected on decomposing seaweeds by Suehiro (1962) and Suehiro and Tomiyasu (1962, 1964) on Japanese beaches and Patel (1979) from the Gopnath coastal area, India. The dominant yeast genera isolated were composed of *Candida*, *Rhodotorula* and *Torulopsis*. Gunkel *et al.* (1983) reported increased yeast populations associated with the degradation in *Desmarestia viridus*, (a marine brown alga), in seawater enclosures. The yeast populations increased from less than 10 to approximately 10^6 ml^{-1}, within three weeks, in most cases. The increase in yeast populations was accompanied by a decline in bacterial populations and pH. The yeasts were found to be exclusively *Rhodotorula* spp.

Of all the potential habitats and sources of yeasts in coastal waters, seaweeds are the most obvious due to their rich content and variety of carbohydrates (Percival and McDowell, 1967). Suehiro and Tomiyasu (1964) undertook *in vitro* studies on the availability of the organic matter of the phytoplankton and seaweeds as nutrients for yeasts occurring in marine environments. They concluded that yeasts may develop in the sea in close association with living algae, using excreted organic compounds as nutrients, and yeasts may also develop on decaying plankton and seaweeds.

The former was confirmed by reference to seaweeds of India by Patel (1975) and the latter by Patel (1979). Thus, the reports of Suehiro (1962), Suehiro and Tomiyasu (1962), van Uden and Castelo-Branco (1961) and Patel (1975, 1979) indicate that yeasts are the important component of the complex ecology of marine environments and are engaged in the conversion of algal material into their own biomass.

2.4.2.2. *Marine mammals and birds*

By comparison with the number of reports on yeasts from seawater, much less attention has been paid to their distribution in either animals from within the marine environment or those normally associated with it such as marine birds. Furthermore, most studies relating to yeasts from such animals are qualitative, rather than of a quantitative nature (Morris, 1968).

Gulls and terns, which are carnivorous birds, appear to maintain an intestinal flora of a relatively high population density of yeasts. *Torulopsis glabrata* was isolated up to 344,000 g^{-1} intestinal contents of 6 out of 14 gulls studied (van Uden and Castelo-Branco, 1963). *T. glabrata* is an obligatory saprophyte of the intestinal tract of man (Artagaveytia-Allende and Garcia-Zorron, 1957; van Uden, 1958; Mackenzie, 1961) and only rarely has it been isolated from sources other than warm-blooded animals. Phaff *et al.* (1952) obtained it from the surface of shrimps from the Gulf of Mexico and Bhat *et al.* (1955) isolated *T. glabrata* from the Indian Ocean. Possibly in both of these cases the sample had been polluted by gulls or other warm-blooded hosts of *T. glabrata*. This led to the suggestion that there may be a role of marine birds in the dispersal and replenishment of marine-occurring yeasts in the sea (Taysi and van Uden, 1964). Kawakita and van Uden (1965) isolated 62 strains of yeasts from

four species of gulls and three species of terns. The yeasts belonged to 16 species including *Candida*, *Saccharomyces* and *Torulopsis*. The average numbers of these yeasts were between 50-2000,000 colony-forming units per gram of intestinal contents.

Despite the frequency of colonization of marine plants by yeasts, not all animals feeding on this vegetation carry yeasts in their intestines. In contrast, no yeasts were found in the intestinal samples from cormorants and Californian sea lions. These two species apparently do not constitute reservoirs of marine-occurring or other yeasts (van Uden and Castelo-Branco, 1963). They are both fish eaters and there is evidence that a high intake of protein e.g. fish and insects may render them unsuitable as hosts for intestinal yeasts (van Uden 1960; van Uden and Carmo-Sousa, 1962).

2.4.2.3. *Marine fish*

There are few reports in the literature on the yeast flora of marine fish and these tend to deal mainly with the gut flora (Fell and Roth, 1961; Roth *et al.*, 1962; Capriotti, 1962; Siepmann and Höhnk, 1962; van Uden and Castelo-Branco, 1963; Fell, 1967), and are summarized in Table 3. Since yeasts appear to be relatively common in the sea, they are undoubtedly ingested during the normal feeding activities of the fish (van Uden and Fell, 1963). However, Ross (1963) found that the gills and slime layers of fish could harbour a yeast flora as great as that found in the faeces. The gut contents of fish caught off the coast of California, were found to contain *M. zobelli* of between 25-5,730 ml^{-1} gut contents (van Uden and Castelo-Branco, 1961, 1963), counts much higher than those found in the surrounding water. The authors suggested that the fish species examined may constitute a reservoir of *M. zobelli* and consequently serve as a

TABLE 3. Yeasts isolated from marine fish.

Location of isolation	Fish body region	Main genera and species isolated	Reference(s)
Biscayne Bay, Florida	Gut	*Candida guilliermondii* *C. parapsilosis*, *Rhodotorula mucilaginosa*, *Rhodotorula* spp., *Trichosporon cutaneum*, *Debaryomyces kloeckeri*, *D. minuta*, *Hanseniaspora valbyensis*, *H. anomala*	Roth *et al.* (1962)
Atlantic Ocean Biscayne Bay, Florida	Gut	*Candida* spp.	Capriotti (1962)
North Atlantic	Gut and skin	Mainly *D. hansenii*, *T. cutaneum*	Siepmann and Höhnk (1962)
Pacific Ocean off La Jolla, California	Gut	*Metschnikowia zobelli*	van Uden and Castelo-Branco (1963)
Bimini, The Bahamas	Gut	*R. minuta*, *C. parapsilosis*, *R. glutinis*, *C. tropicalis*, *R. pilimanae*, *Torulopsis* spp., *T. cutaneum*	Roth *et al.* (1962); Fell and van Uden (1963)

TABLE 3. (continued).

Location of isolation	Fish body region	Main genera and species isolated	Reference(s)
Tyrrhenian Sea, The Mediterranean	Gut	*D. hansenii*, *C. natalensis*, *P. ohmeri*, *C. parapsilosis*, *Pichia guilliermondii*, *S. cerevisiae*, *C. mesenterica*	Picci and Verona (1964)
The Clyde Estuary and North Sea	Mouth, gills, skin and gut	*D.hansenii*, *Torulopsis inconspicua*, *C. parapsilosis*, *Candida* spp., *Cryptococcuss* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Torulopsis* spp., *Trichosporon* spp., *Rhodotorula infirmio-miniata*, *T. pullulans*	Ross and Morris (1965); Bruce and Morris (1973)
Atlantic Ocean off Iceland	Mouth, gills, skin and gut	*D. hansenii*, *C. parapsilosis*, *R. glutinis*, *Torulopsis* spp., *Pichia* spp., *Cryptococcus* spp.	Ross and Morris (1965)

vector of the dispersal of this yeast in the sea. Other fish species harbour relatively low numbers of yeast not exceeding 380 ml^{-1} gut contents. These yeast species, encountered in fish gut contents, were low in number and both qualitative and quantitative studies suggested that this yeast flora reflects only recent feeding habitats of the fish (van Uden and Kolipenski, 1962; Fell and van Uden, 1963; Roth *et al.*, 1962). So apparently, *M. zobelli* differs from other marine-occurring yeast in its capacity to establish higher population densities in fish guts, which may be an expression of its truly marine or aquatic nature (van Uden and Castelo-Branco, 1961).

However, it appears that the number of yeasts in marine animals are small compared with those of the bacteria (Morris, 1968) for example, early reports, Snow and Beard (1939) reported that only 1% of their total microbial isolates from the North Pacific salmon were yeasts. However, these workers used media more suitable for the isolation of bacteria and therefore, yeasts may not have grown. Ross (1963) and Ross and Morris (1965), with improved isolation procedures, obtained yeasts from 66-100% of the fish examined.

2.4.2.4. *Marine invertebrates*

Yeasts obtained from the surface-washings of crustaceans contained those species of yeasts found in adjacent water and sediments (Roth *et al.*, 1962). A number of tube-inhabiting amphipods e.g. *Podocerous brasiliensis* yielded pure cultures of *Rhodotorula rubra*. An identical strain was isolated from the pink shrimp, *Panaeus duorarum* (Roth *et al.*, 1962). Phaff *et al.* (1952) also reported *Rhodotorula* spp. and *Trichoderma* spp. from the shrimp *P. setiferus* and *Metschnikowia kamienskii* from the brine shrimp, *Artemia salina* (Spencer *et al.*, 1964). Koburger *et al.* (1975)

recovered yeasts and yeast-like fungi from both fresh and stored samples of rock shrimp, *Sicyonia brevirostris* from the East Coast of Central Florida. The species and genera found were the same as those reported by Phaff *et al.* (1952) as being present on the Gulf shrimp (*Panaeus*) and the genus *Rhodotorula* were the only yeasts surviving the period of iced storage. Kobayashi *et al.* (1953) found the most common yeasts from the liver of the long-necked clams to be *Candida* spp. and *Rhodotorula*. Of the samples of the invertebrates including members of *Porifera*, *Polychaeta*, *Cnidaria* and *Echinodermata* all were found to harbour yeasts, possibly because of chance-contamination from adjacent environments (Roth *et al.*, 1962). In those cases where the incidence of yeasts are absent or scanty, it is not known whether this is due to physiology of the animals itself, or due to inhibitory factors produced by other microbial flora normally associated with that animal (Morris, 1968). For example, van Uden and Castelo-Branco (1963) failed to isolate yeasts from the guts of the sea urchins, *Strongylocentrotus franciscanus* and *S. purpuratus* collected from Point Loma, San Diego, California. It was suggested by Johnston and Mortimer (1958) that yeasts entering the guts of sea urchins were digested by enzymes similar to those found in snails (van Uden and Castelo-Branco, 1963).

2.5. Pathogenicity of Yeasts

Records on the pathogenicity of the yeasts towards marine animals are sparse. As a result, the review below summarizes reports of the yeasts, with marine-occurring counterparts in terrestrial and freshwater environments as well as the marine niche.

2.5.1. Terrestrial

The commonest terrestrial yeasts which have marine counterparts are the asporogenous genera *Candida*, *Cryptococcus*, *Rhodotorula* and *Torulopsis*, (Rippon, 1974). These yeasts occur as contaminants of bacterial cultures, growing on foods, as airborne organisms, or as transient flora of the human skin. Of these *C. albicans* is the most important as a human commensal and pathogen. Their pathogenicity has been reported in several aspects of human medicine. However, there has been no attempt to summarize such a mass of information.

System ic mycoses are basically pulmonary diseases in that the site of infection is almost invariably the lungs, usually with grave consequences. All of the vital organs may be affected and lesions extensive, cutaneous and sub-cutaneous forms of these diseases do occur as a result of dissemination or as a consequence of direct inoculation following injury. For example, the species responsible for Candidiasis include *C. albicans*, *C. guilliermondii*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* (Chandler *et al.*, 1980).

Rhodotorula spp. namely *R. rubra*, have on rare occasions been isolated from human patients in the terminal stages of debilitating diseases. Of these yeasts *R. rubra* is most frequently involved, and its presence has been documented in several fatal infections of the lung, kidney and central nervous system (Rippon, 1974).

Species of the genus *Cryptococcus*, such as *C. albidus*, have been implicated in human disease (Rippon, 1974) and members of the genus *Trichoderma* which are primarily soil organisms, *T. beiglii* (*T. cutaneum*) is the well known agent of "white piedra" (Speller, 1980) and has been

implicated as the cause of a brain abscess in cancer victims (Chandler *et al.*, 1980).

2.5.2. Freshwater

Fungal diseases of freshwater aquatic animals were reviewed by Alderman (1982), and of marine hosts by Johnson and Sparrow (1961) and Alderman (1976). However, there are very few reports on the pathogenicity of the yeasts towards the aquatic animals, especially of marine origin.

One of the earliest observations was by Metschnikoff (1884 and 1893) who established the genus *Monospora* (later renamed *Monsporella*) to designate a parasitic fungus which he discovered in a freshwater crustacean, *Daphnia magna*. This monocellular fungus, of which he only described one species, *M. bicuspidata* lived in the body cavity of its host, where it multiplied actively by budding in a yeast-like manner. When the body cavity of the host was entirely invaded by parasites, they increased in size, became elongated, and formed club - or sausage - shaped asci, in each of which developed a single, needle-like spore pointed at both ends (Figure 3). When the parasitized host died, it was observed to be filled with ripe spores, and healthy *Daphnia* which fed on the detritus of their dead and diseased fellows, became infected by ingesting the asci. The latter, when they entered the hosts' alimentary canal, set free the needle-shaped spores, which penetrated the gut wall and invaded the body cavity. The spores then germinated laterally, thus initiating the new infection. The genus *Monospora* or *Monosporella* had hitherto described a, the species of *M. bicuspidata* Metschnikoff and b, a yeast-like fungi found by Bütschli (1876) in the coelom of the free-living nematode, *Tylenchus pellicidus*. The authors description and figures were insufficient, however, for

determining more than the genus to which the parasite belonged. Keilin (1920) later found a new species of *Monosporella*, called *M. unicuspidata* living in dipterous larvae, (*Dasyhelea obscura*). Kamienskii (1899) pointed out that *Monospora* was a later homonym of a genus of algae and he therefore changed *Monospora* to *Metschnikowia*. The generic name *Metschnikowia kamienskii* has been challenged a number of times by various authors, but a careful survey of the pertinent literature by van Uden (1962) showed that the name *Metschnikowia* was available when Kamienskii introduced it in 1889 and it has priority over the frequently used name *Monosporella keilin* (Lodder and Kreger-van Rij, 1952; Genkel, 1913).

Metschnikowia zobelli and *M. krissii* were observed by van Uden and Castelo-Branco (1961) as club-shaped asci containing a single needle-shaped ascospore, and were capable of parasitizing *Daphnia magna* under experimental conditions. The asci and cells of both yeasts resembled Metschnikoff's drawings of *M. bicuspidata*. As cultures of the latter do not exist and its physiological properties unknown, the possible identity of *M. zobelli* and *M. krissii* with *M. bicuspidata* cannot be verified and is therefore considered "nomen dubium".

Two other genera of yeast, *Candida* and *Cryptococcus*, were reported as being isolated from other non-marine aquatic animals. In 1975, Hatai and Egusa reported a disease, gastro-tympanites of the salmonid, *Onchorynchus rhodurus* in Japan. The characteristic symptom of the disease, was a distended stomach containing up to tens of millions of yeasts per millilitre. These were identified as *Candida sake* (Lodder, 1970). Also Pierotti (1971) reported a particular mycosis of the green tench *Tinca tinca*, and a microscopic examination identified the filaments surrounding the eyes of the fish as almost certainly a *Cryptococcus* species.

2.5.3. Marine

Whereas a wide variety of yeast species can act as pathogens of terrestrial and freshwater animals, in the marine environment it seems that only members of the genus *Metschnikowia* have this capability. In 1964, Spencer *et al.* described a yeast *M. kamienskii* associated with the brine shrimp *Artemia salina* collected from the saline Lake Saskatchewan, Canada. In addition, a strain originally isolated by Wickerham (1951) from sporocysts of the trematode *Diplostomum flexicalidium*, parasitizing the lymnaeid snail, found in Lake Douglas, Michegan. The strains were identical, containing the characteristic single needle-shaped spores, pointed at both ends.

Another species of *Metschnikowia* appeared to infect species of marine copepod, and to play a significant role in the control of their populations. Seki and Fulton (1969) described an infestation of *Calanus plumchrus*, a predominate species in the Straits of Georgia, observed in February and March of 1968. The yeasts were found to parasitize the marine copepods both under natural and experimental conditions. Yeasts identified as *Metschnikowia* spp. were isolated and their physiology studied. Vegetative cells and asci with their single spores were found both as epibionts on the surface and endobionts between the bodywall and the wall of the gastrointestinal tract.

Fize *et al.* (1970) described a similar infection of the calanoid copepod, *Eurytemora velox* with *M. kamienskii* in the brackish waters of the Golfe du Lion, of the Mediterranean Sea, during April 1969. The yeast cells filled the host's bodies, killed them, and decimated the population, thereby influencing the food chain. There appears to have been no similar observations in the oceanic copepods. Nevertheless, in this

context, Seki and Fulton (1969) suggested that the empty copepod exoskeletons in the deep aphotic zone (Wheeler, 1967) reflected kills by *Metschnikowia*. However, normal moulting and efficient stripping of flesh from dead and dying copepods by carniverous macrophagous ciliates could also account for this effect. Subsequently, Seki and Hardon (1970) in studies in Fatty Basin, British Columbia, observed that *M. krissii* could become a destructive fungal parasite of the crab, *Cancer productus*.

It is of interest to note here that the terrestrial species of *Metschnikowia* are usually isolated from fruits and flower nectar, probably carried by bees (Phaff *et al.*, 1966).

There appears to be no information however, regarding the pathogenicity of marine yeasts towards the echinoderms.

3. THE SEA URCHIN *ECHINUS ESCULENTUS*

3.1. General Characteristics

The echinoderms occupy a phylogenetically strategic position in the Animal Kingdom, near the chordates (Russell-Hunter, 1969; Nichols, 1971). The Echinodermata are a major component of fossil associations. The majority have a strong calcaereous skeleton (test) composed of regular (in the primitive types) or irregularly arranged plates, so they preserve well as fossils (Prokop, 1981).

Echinoderm fossils occur only in marine deposits, and they are a very old group whose beginnings possibly date back to the Proterozoic era. Sea urchins appeared not to play a very important role in the Palaezoic Seas. Their period of abundance began in the Mesozoic and Cainozoic eras, and they are still a flourishing group today (Prokop, 1981).

The present day Echinodermata is represented by about 6,000 species of worldwide distribution (Russell-Hunter, 1968), divided into five classes within the subphyla;

a. Crinozoa

Contains only one living class, the Crinoidea comprising the sea-lilies and the feather-stars. This is the most primitive class, its adult members being mostly sessile on the sea bed.

b. Asterozoa

Composed of Asteroidea, the starfish (or sea-stars), and the Ophiuroidea, the brittle-stars and basket-stars. These two classes are sometimes combined as the class Stelleroidea.

c. Echinozoa

Includes the Echinoidea, the sea-urchins, sand dollars and heart-urchins, and the Holothuroidea, the sea-cucumbers (Nichols, 1969). The diagram in Figure 6 illustrates the interrelationships of the genera in the families Echinidea of which there are fourteen species, Strongylocentrotidea and Echinometridea (Clark, 1925).

3.2. Basic Anatomy

The sea urchin *Echinus esculentus* (Plate 1) belongs to the class Echinoidea in the subphylum Echinozoa of the phylum Echinodermata. It is a regular urchin, having pentameric symmetry. The transverse section through the body of *E. esculentus* shows (Figure 7) that it has a rigid, perforated test. Through the holes protrude the tube feet with a variety of suggested functions: locomotory, tactile or chemosensory, feeding, burrow-building or respiratory, the latter probably being the original function (Nichols, 1969). The calcaereous test is covered in spines which are used

FIGURE 6. Diagram to illustrate the interrelationships of the genera in the families Echinidae, Strongylocentrotidae and Echinometridae.

(From Clark, 1925)

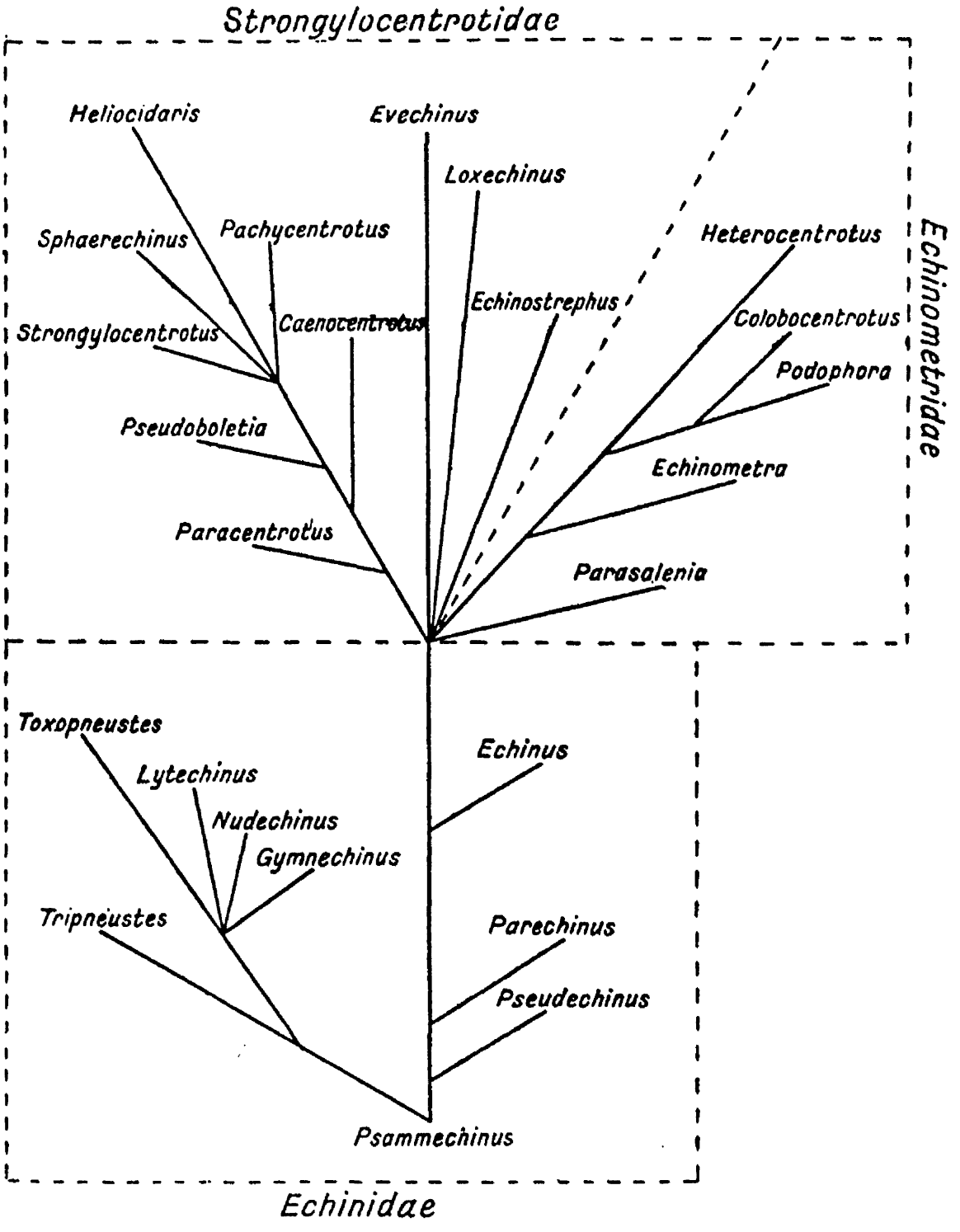

Strongylocentrotidae
Heliocidaris
Evechinus
Loxechinus
Sphaerechinus
Pachycentrotus
Heterocentrotus
Echinostrephus
Caenocentrotus
Strongylocentrotus
Colobocentrotus
Podophora
Pseudoboletia
Echinometra
Paracentrotus
Parasalenia
Echinometridae
Toxopneustes
Echinus
Lytechinus
Nudechinus
Gymnechinus
Tripneustes
Parechinus
Pseudechinus
Psammechinus
Echinidae

PLATE 1. Healthy specimens of *Echinus esculentus* maintained in recirculating artificial-seawater aquaria. Note the bright pink test colouration, regular spine arrangement and extended tube feet.

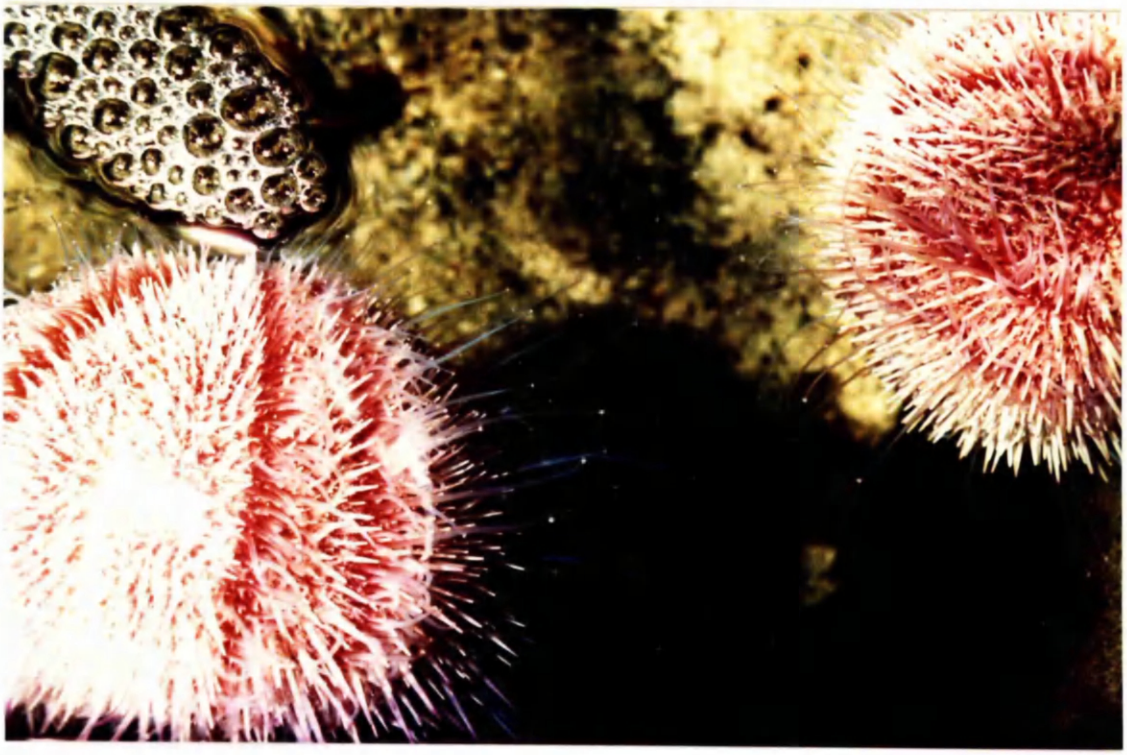
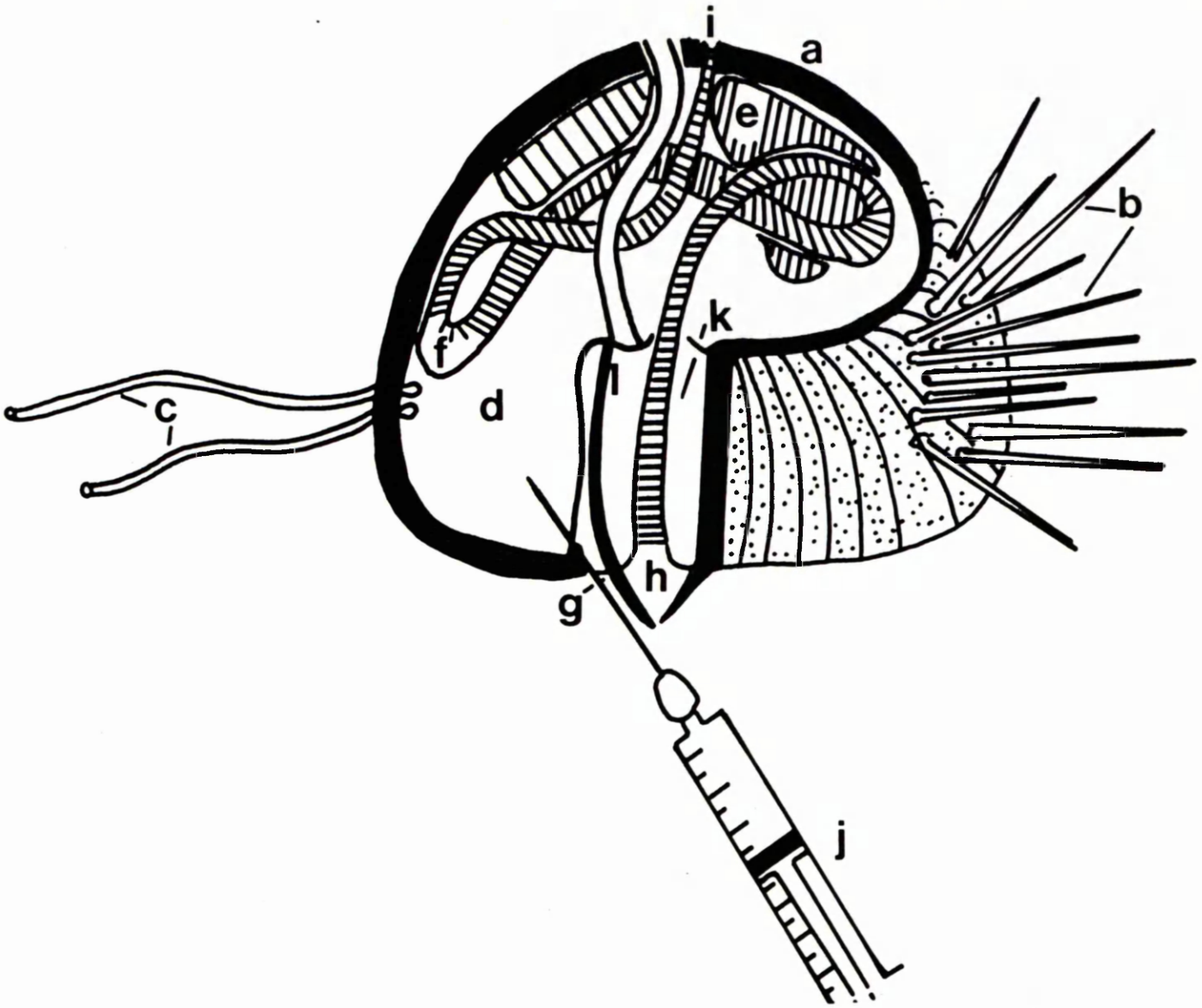


FIGURE 7. Cross-sectional diagram of *Echinus esculentus* showing point of insertion of needle and syringe, for extraction of coelomic fluid or injection of microbial-inocula.

(Adapted from Nichols, 1969)

a. Calcareous test
b. Spines
c. Tube feet
d. Coelomic cavity
e. Gonads
f. Intestine
g. Peristomial membrane
h. Mouth
i. Anus
j. Syringe
k. Lantern
l. Tooth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in locomotion. To protect themselves from parasitic invasion and particle settlement, the pincer-like organs, the pedicellariae, are situated amongst the spines. The mouth pieces, Aristotle's Lantern consist of a complex masticatory apparatus consisting of forty skeletal pieces intricately bound with muscles and connective tissue. It carries five strong teeth which are used to rasp encrusting organisms, such as algae from the seabed. The oesophagus leads from the mouth to the intestine which runs one and a half times around the inside of the test before ascending to the anus (Nichols, 1969).

Coelomic fluid fills the body cavity of the sea urchin and is more extensive in the echinoids than other classes of echinoderms (Nichols, 1969). *E. esculentus* are osmoconformers in that their body fluids osmotically match the surrounding seawater (Binyon, 1972). The coelomic fluid contains a number of cell types which will be discussed later (Section 4.3).

3.3. Ecology

Echinus esculentus, the common or edible sea urchin, is a major component of the fauna of the kelp forest in the shallow, sub-littoral waters around the shores of British Isles (Rodhouse and Tyler, 1978). Its range extends from Northern Norway to Portugal and westwards towards the Greenland Coast, but it does not appear to reach the American continent nor does it occur south of Portugal or in the Mediterranean. It may therefore, be regarded as an Atlantic-European species (Reid, 1935).

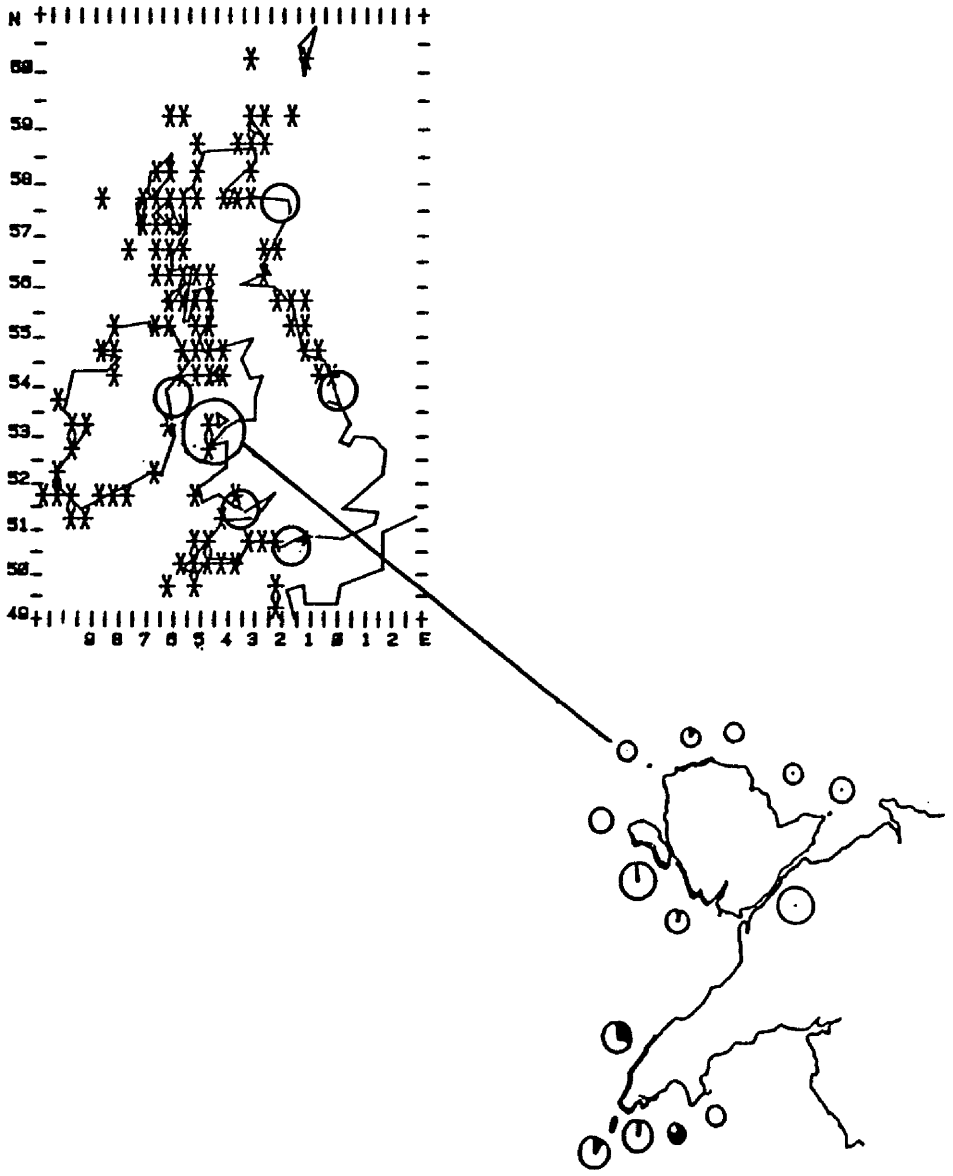
Several studies have been done in recent years on the distribution of *E. esculentus* around the British Isles (Nichols, 1978, 1979; Rodhouse and Tyler, 1978; Cranmer, 1985; Nichols *et al.*, 1985).

During the four year period, 1977-1980 a nationwide diving survey of the populations of *Echinus* in the seas of the British Isles was conducted by the Marine Conservation Society (Earll, personal communication, 1985) as shown in Figure 8. *E. esculentus* was most abundant in the shallow Scottish waters, an observation confirmed by Cranmer (1985). The importance of *E. esculentus* in determining the lower limit of the kelp forest was demonstrated by Jones and Kain (1967). Earlier accounts of the distribution of this species are those of Elmhurst (1922), Moore (1935a and b) and Forster (1959). The latter author observed on a 4 mile long, 1/2 mile wide strip off the coast of Plymouth, there was a population of nearly 1.4 million *E. esculentus* giving an average density therefore, of one animal per 4.7 square metres.

Growth of the populations of sea urchins depends on a complex of factors, including seawater quality, temperature and food availability. Seawater temperatures in the English Channel are generally higher than those in the Firth of Clyde, which might favour the Plymouth populations. On the other hand, food availability may favour certain Cumbrae populations. For example, the Keppel Pier area on Cumbrae Island, Firth of Clyde, may be nutritionally enriched, since returning fishing boats deposit scraps, deck washings etc. into the sea (Nichols *et al.*, 1985) and Service (1982) reported the pier piles to be encrusted with barnacles and byrozoans grazed by *E. esculentus*.

E. esculentus are collected fairly extensively in several coastal areas of the country, such as the extreme South-West; the curio trade offers the tests for sale as ornaments. This is not threatening to the survival of the species, but is said to be reducing the number of large urchins in certain popular diving areas (Nichols, 1978).

FIGURE 8. The results of the 1977-1980 Nationwide Diving Survey of *E. esculentus* by The Marine Conservation Society, (4 Gloucester Road, Ross-on-Wye, Herefordshire, HR9 5BU). The presence of *E. esculentus* represented by X.



Anglesey is one area of the country where urchins have been studied in detail. It is quite clear that they are far from common. The empty circles show areas with no urchins.

Several species of sea urchins are a culinary delicacy, their roe (the gonads) being eaten in many parts of the world. For example, *Strongylocentrotus franciscanus* and *S. purpuratus* are consumed in California and *Paracentrotus lividus* in France (Southward and Southward, 1975; Nichols, 1979). The Japanese are the largest producers and consumers of sea urchin roe, over 20,000 tons are harvested annually (Kato, 1972). *E. esculentus* are not exploited for this to any extent in Britain, possibly possibly because it reaches its prime condition in winter when collecting by divers is more difficult (Nichols, 1979).

Of the large invertebrates *E. esculentus* is the most conspicuous species known to graze on benthic macrophytes in British coastal areas. It also browses on encrusting zoobenthos (Rodhouse and Tyler, 1978). These sea urchins were observed to feed readily on the algal film on the glass of an aquarium tank, browsing steadily on one spot until after a few hours a small, clear area was observed. The same species was also observed to "scrub" clean by grazing the filamentous algal growth encrusted by diatoms on the surface of rocks on the sea bed (Forster, 1959). Sea urchins are omnivorous opportunists and eat a wide variety of foods (Kinne, 1977). The food preferences of *E. esculentus* are summarized in Table 4 as reported by earlier and more recent workers.

3.4. Laboratory Maintenance of Adult Sea Urchins

There is an extensive literature on various aspects of the development of sea urchins, from the fertilized egg to the adult form (for reviews see Hinegardener, 1969; De Angelis, 1976; Kinne, 1977; Leahy *et al.*, 1978; Hinegardener and Tuzzi, 1981). However, almost without exception, such studies on sea urchins until recently were made with natural seawater at

TABLE 4. Food of the regular sea urchin *Echinus esculentus* in the field (Extended from Lawrence, 1975).

Food (Analysis of gut contents or direct obvervation)	References
Mainly worms; also echinoderms, crustaceans hydroids, macroalgae; bottom material.	Peterson, in Eichlbaum (1909)*
Shell (probably of Balanus); algae. Stomach contents in balls.	Roaf (1910)*
Only animal food; Balanus, Pomatoceros, gastropods, hydroids.	Blegvad (1914)*
Membranipora; Polyzoa; *Laminaria*, particularly if encrusted with *Membranipora*; balanids; young primarily on foods such as *Membranipora*.	Elmhirst (1922)*
Gut filled with mixture of sand, small stones, and detritus, with numerous fragmented animal remains (tubiculous worms, Polyzoa, hydroids and barnacles).	Hunt (1925)*
Balanus and algae in shallow water; drift algae in deep water.	Moore (1934*,1935a and b)
Mainly carniverous	Mortensen (1943)
Attached algae	Forster (1959)
Primarily algae, with very little animal material	Jorde & Klavestad (1963)*
Laminaria, *Saccorhiza*	Kain and Jones (1966); Jones and Kain (1967); Kain (1971)
Laminaria	Larsson (1968)*
L. saccharina encrusted with *Membranipora membranacea*.	Bonsdorff & Vahl (1982)

* cited by Lawrence (1975).

marine stations. For example, as early as 1909, Shearer *et al.* described the rearing of hybrids of *Echinus* from the fertilized egg to the mature animal (1cm in diameter) (cited by Fuchs, 1914). However, this work was done with flowing natural seawater as the medium. More recently, Leahy *et al.* (1978) gave a detailed account of the large-scale laboratory maintenance of several thousand gravid sea urchins (*Strongylocentrotus purpuratus*). The system contained fifteen plexiglass tanks (163 x 102 cm, depth 19 cm) each containing 200 gravid-adult sea urchins in natural running seawater, cooled to 11°C piped from the local harbour and exhausted back into the harbour. However, if an interruption to the normal system occurred it automatically switched to a mode of recirculation. On a smaller scale, Hinegardener and Tuzzi (1981) described in detail the laboratory culture of *Lytechinus pictus* from the larvae to sexually mature adults (1cm in diameter). The larvae were cultured in various sized polystyrene dishes (300 - 8,000 ml capacity) allowing at least 1 millilitre of seawater per larva and transferred to the larger vessels during maturation, and mature animals (10-15) kept in 80 l aquaria. Filtered natural-seawater was used throughout.

Hinegardener (1969) was one of the first to compare an artificial salt mixture "Instant Ocean^{R}" with natural seawater for raising five species of sea urchins (*Arbacia punctulata, Lytechinus pictus, L. variagatus, S. purpuratus* and *Echinometra mathaei*) from the fertilized egg to the reproductive adult (approximately 6 cm in diameter). Growth of the sea urchins was found to be consistently better in natural seawater than in the artificial seawater.

There are surprisingly few articles (Table 5) dealing with the long-term maintenance of adult sea urchins in artificial, as distinct from natural seawater. Indeed, the earliest report appears to be Fridberger *et*

TABLE 5. Long-term maintenance of adult sea urchins in natural and artifical-seawater aquaria.

Type of seawater (and supplier if relevant) & type of aquaria	Species of sea urchin kept	Duration of maintenance	Comments and reference
Natural seawater and "Instant Ocean^{R}" (Aquarium Systems Inc., 1450E, 289 Street, Wickliffe, Ohio) stirred in petri dishes.	*Arbacia punctulata, Lytechinus pictus, L. variagatus, Strongylocentrotus purpuratus, Echinometra mathaei.*	At least complete life cycle, 6 months	Natural seawater compared with artificial seawater, latter found to be less satisfactory, Hinegardener (1969)
Natural seawater, running and recirculating seawater aquaria.	*S. purpuratus*	Over two years	Large-scale up to 3,000 gravid sea urchins kept, Leahy *et al.* (1978)
Artificial Seawater "hv-meeresalz^{R}" (Wiegandt, GmbH & Co., KG, 4150 Krefeld 1 Germany). Recirculating seawater aquaria.	*Psammechinus miliaris, Echinus esculentus, S. droebachiensis, Paracentrotus lividus, A. lixula.*	Five years experience in maintaining these sea urchins	Fridberger *et al.* (1979)
Natural filtered sea-water. Recirculating-seawater aquaria.	*L.pictus*	From larvae to sexually mature adults.	Hinegardener & Tuzzi (1981)

al. (1979) from Uppsala, Sweden. These authors reported five years experience in carrying and raising several species of sea urchin, including *E. esculentus* under strict artificial conditions.

The noteworthy features of the aquaria and maintenance procedures of Fridberger *et al.* (1979) were : transport of *E. esculentus* in natural seawater from the coast to the aquaria within 8 hours; aquaria of capacity 75 or 150 l; aquarium water made from the artificial sea-salts "hv-meeresalz^{R}" reconstituted in distilled water to a salinity range of 2.7 - 3.5%; and the pH adjusted to 8.2 with Na_2CO_3 or HCl, and allowed to equilibriate at 16°C. The tanks were illuminated from 10-40cm above the water surface with two different light sources, one "gro lux^{R}" fluorescent tube and one 20-40W white tube. The light period was 14/24 hours. The tanks were left like this for four to six months before introducing either newly metamorphozed animals or adults. The authors reported that the presence of brown algae, for example, reduced the time for establishing the "proper microflora and microfauna" by 2-3 months. The water in the tanks was recirculated by "Eheim^{R}" power filter pumps with pumping capacities of 100l h^{-1} in the 75l tanks and 250l h^{-1} in the 150l tanks. Water changes of a one third volume were done every two weeks and the filters were changed when the pH dropped below 7.4. The first months the animals fed on red and green algae growing on the walls and bottom, as a result of intense illumination. Thereafter, sea urchins were fed with *Laminaria*, *Fucus* and commercially available boiled, deep-frozen spinach, but there was no indication which food was preferable. Other noteable omissions from the described procedures were: whether there was a biological filter bed; if so, its nature and method for maturation; routine methods of water quality monitoring and management. Moreover, the authors did not report the size of adult sea urchins kept, and the seawater volume allowance per animal.

4. MICROBIAL PATHOGENICITY AND SEA URCHIN DEFENCES

4.1. Sea Urchin Disease

In contrast to other sea urchins (4.1.1.), *E. esculentus* does not seem to be subject to mass mortalities. There are however, reports of two types of disease in this species, one which is noticeable by a blackening of the aboral surface. This occurred with low frequency in certain populations around the British Isles (Earll, 1985, pers. comm.). The other type, causing the "bald sea urchin" disease, in which the animals lose their spines from a large area of the test, has been recorded around Millport (Earll, 1985, pers. comm.) and the N.E. Atlantic, Brittany, France (Maes and Jangoux, 1984). This disease was communicable (Maes and Jangoux, 1984) in that necrotic tissues could act as sources of infection. Similar symptoms were recorded for several other species of sea urchin, notably *E. acutus*, *Paracentrotus lividus*, *Psammechinus miliaris*, *Sphaerechinus granularis*, *S. droebachiensis*, *S. franciscanus* and *S. purpuratus* (Table 6). The disease was not specific and it was easy to infect echinoids of several other species e.g. *Arbacia lixula* and *Cidaris cidaris* (Maes and Jangoux, 1984). Disease transmission was accomplished by scarification, or merely by placing unaffected echinoids in the vicinity of diseased individuals. The pathogenic agent was not an algal agent, as suggested by Mortensen and Rosevinge (1934); but histological and electron microscopic investigations revealed numerous bacteria in the necrotic tissues, but no protozoa or fungi. The authors therefore suggested a bacterial agent for this "bald sea urchin" disease. Service (1982) also recorded a high incidence of *E. esculentus* disease in Loch Riddon in 1981 and Loch Sunnart in 1982 which did not appear to be attributable to pollution.

4.1.1. Mass mortalities

The mass mortalities of different sea urchins in several geographical areas are presented in Table 6. Mass mortalities, attributed to disease, have occurred in *Strongylocentrotus* species and in phylogenetically/ecologically similar echinoid species in other areas, but none on the scale observed in Nova Scotia. Mass mortalities of the green sea urchin, *S. droebachiensis* were reported by Miller and Colodey (1983) and the causative agent was suggested by the authors to be a microorganism rather than a physical or chemical factor. A labrynthomyxa-like protozoan, also reported by Li *et al.* (1980) isolated from diseased oysters was abundant in gonadal and digestive tract tissue of sick sea urchins, but rarely found in healthy individuals. This was considered to be perhaps a result of secondary rather than a primary infection (Li *et al.*, 1980; Miller and Colodey, 1983). Later Jones and Schiebling (1985) suggested the involvement of *Paramoeba* sp. as the causative agent of sea urchin mortality in Nova Scotia. The mass mortalities occurred at a seasonal maximum and cessation at winter temperatures. Disease symptoms in the mass mortality episodes of the red sea urchin *S. franciscanus*, California (Johnson, 1971; Pearse *et al.*, 1977) were found to be similar to those of *S. droebachiensis* found in Nova Scotia. The sea urchins released their hold of the rock substratum, spines dropped and were absent from areas of the test (Miller and Colodey, 1983). The disease was also found to be species-specific affecting no other echinoids in the area (Pearse *et al.*, 1977; Schiebling and Stephenson, 1984). The pathogen has promise for use as a tool for biological control because it is virulent, apparently species-specific, can be maintained and transferred in the laboratory and is waterborne. The most immediate effect expected from complete or near sea urchin mortality is the colonization by macroalgae, especially kelp and a substantial increase in nearshore primary productivity (Breen and Mann, 1976; Chapman,

TABLE 6. Records of sea urchin disease and mass mortility.

Geographical area	Species of sea urchin involved	References
North West Atlantic, Nova Scotia	*Strongylocentrotus droebachiensis.*	Hooper, 1980; Li & Cornick , 1982; Miller & Colodey, 1983; Moore & Miller, 1983; Schiebling, 1984; Schiebling & Stephenson, 1984; Jones *et al.*, 1985; Miller, 1985.
North East Pacific, California	*S. franciscanus, S. purpuratus.*	Johnson, 1971; North *et al.*, 1970-71; Pearse *et al.*, 1977; Pearse & Hines 1979; Tegner & Dayton, 1981.
Puerto Rico & The Caribbean	*Lytechinus variegatus, Tripneustes ventriosus, Diadema antillarum.*	Glynn, 1968; Bak *et al.*, 1984; Lessios *et al.*, 1984; Murillo & Cortés, 1984; Hughes *et al.* 1985; Liddell & Ohlhorst, 1986.
Western Mediterranean (Alicante, Spain, French coast, Southern Italy, Sicily, Rijeka, Yugoslavia); North East Atlantic (Brittany, France) and North Sea (Normandy, France).	*Psammechinus lividus, P. miliaris, Echinus esculentus, Sphaerechinus granularis, Arbacia lixula*	Boudouresque *et al.,1981;* Höbaus *et al.*, 1981; Maes and Jangoux, 1984; Fenaux 1984 (cited by Maes and Jangoux 1984).
North Sea, Norway	*Echinocardium cordatum, S. droebachiensis, E. acutus.*	Mortenson & Rosevinge, 1934; Kravig, 1975; Hagen, 1983.

1981; Moore and Miller, 1983).

Localized mass mortalities of *S. droebachiensis* occurred off Newfoundland (Hooper, 1980) and *S. franciscanus* off California (Johnson, 1971; Pearse *et al.*, 1977). The symptoms of the two outbreaks were found to be similar and Johnson (1971) suspected a fungal pathogen.

A marked decline in *Paracentrotus lividus* off the Mediterranean Coast of France was also attributed to disease (Boudouresque *et al.*, 1981). The pathogenic basis of these mortalities was not identified.

Dungan *et al.* (1982) observed mass mortalities of sea stars, *Heliaster kubinji*, in California and hypothesized that unusually high sea temperatures rendered them susceptible to infection by an unidentified pathogen. Localized deaths of *Lytechinus variagatus* have been attributed to periods of low salinity (Goodbody, 1961; Lawrence, 1975) and tidal exposure (Glynn, 1968).

4.1.2. Sea urchin control

Sea urchin mortality due to disease is well documented, particularly in Nova Scotia (Table 6). The possibility of using the causative agent as a tool for biological control of population explosions of these animals in overgrazing kelp beds was suggested by Miller *et al.* (1971). Kelp beds represent some of the most productive biocommunities in the world (Miller *et al.*, 1971; Mann, 1972a and b). They function as habitat, shelter, and nursery for some coastal fish, a large number of invertebrates and a rich variety of associated algae, in addition to being a considerable biosource in their own right (Breen and Mann, 1976; Breen, 1980; Duggins, 1981). Large areas suitable for kelp development are however, occupied by grazing sea urchins which prevent kelp establishment (Lawrence, 1975; Duggins, 1980). This phenomenon has been described as being in the "Isoyake" (barren of fleshy seaweeds) condition, named thus because this has been

studied in Japan since the turn of the century (Yendo, 1903). This condition has been observed in California (North *et al.*, 1970-1971), Japan (Noro *et al.*, 1983), Norway (Hagen, 1983) and predominately at the Atlantic Coast of Canada (Mann and Breen, 1972; Arnold, 1976; Breen and Mann, 1976; Lang and Mann, 1976). The latter region used to support some of the most productive kelp beds in the world (Michanek, 1975).

The most common explanation of sea urchin population explosions and subsequent overgrazing of kelp beds is decreased predation of the sea urchins (Mann, 1977). One suggestion is that this may be due to excessive removal of sea urchin predators such as sea otters, lobsters, wolfish, flounders and halibut (Pringle *et al.*, 1980; Wharton and Mann, 1981).

Artificial methods of controlling sea urchins have included the employment of SCUBA divers to destroy sea urchins with hammers or to spread quicklime, over aggregations of sea urchins (North 1974). Because these methods are expensive and must often be repeated to produce lasting effects, they are therefore only practical on a small scale (Miller, 1985).

4.2. Experimental Infections of Sea Urchins and Microbial Clearance

Clearance of injected bacteria from echinoderm coelomic fluid (CF) has been reported (Unkles, 1976; Wardlaw and Unkles, 1978; Kaneshiro and Karp, 1980; Bertheussen, 1981a; Service, 1982; Yui and Bayne, 1983). Also the clearance of other foreign particles have been reported as follows : bacteriophage T4 (Coffaro, 1978); red blood cells, latex beads and yeast cells (Bertheussen, 1981a); xenogenic cells, carborundum (Reinisch and Bang, 1971) and bovine and serum albumin (Hilgard and Phillips, 1968).

Wardlaw and Unkles (1978) found that *E. esculentus* cleared injected doses of 10^7 bacterial cells (*Pseudomonas* strain number 111, *Ps.111*) from the coelomic cavity, within 24h. However detailed kinetics were not presented. Service (1982) stated that an initial inoculum of 10^9 *Ps. 111* was reduced to ten or fewer organisms per millilitre within 48h. Yui and Bayne (1983) noted that *S. purpuratus* efficiently cleared three species of bacteria (2 Gram-negative and 1 Gram-positive). Initial viable counts of 10^6-10^7 bacteria per millilitre of CF were reduced by 90-99% in 3-6 hours after injection and were not detected 4-8 days after injection. This clearance was similar to the clearance in mammals (and other invertebrates) rapid and exponential, with 90-99.9% reduction in circulating-bacteria (Rogers, 1960). Similarly, four mollusc species (Bayne and Kime, 1970; Pauley *et al.*, 1971; Bayne, 1973; van der Knaap *et al.*, 1981) and a crustacean (Smith and Ratcliffe, 1980) cleared 90-99% of injected bacteria (10^6 - 10^9 bacteria per animal) in 2-3 hours after injection. Gram-negative and Gram-positive bacteria may be recognized by different coelomocyte subpopulations in the marine annelid, *Arenicola marina* (Fitzgerald and Ratcliffe, 1982) and Johnson (1969b) noted more active phagocytosis of Gram-positive than Gram-negative bacteria in *Strongylocentrotus* spp.

Clotting, due to aggregation of phagocytes, no doubt contributes to some of the observed decline in numbers of cells and bacteria (Johnson, 1969a). Cellular clots were often observed in CF samples which were taken after injection of bacteria (Bertheussen, 1981a). Bertheussen (1981a) demonstrated 70% clearance of a dose of 2 x 10^8 baker's yeast, *Saccharomyces cerevisiae* from the coelomic cavity of *S. droebachiensis* within 2h after injection. Complete clearance was observed within 24h, and the main mechanism of this clearance was shown by scanning electron microscopy (SEM) to be phagocytosis.

However, clearance of marine yeasts from the coelomic cavity of sea urchins has not been reported.

4.3. Antimicrobial Activity of Coelomic Fluid

4.3.1. The coelomic fluid

Although many studies of echinoderm biology have been concerned with understanding the structure and function of the coelomic systems, some aspects are still poorly understood. In particular, there is confusion about the structure and function of the cellular components of the CF (for reviews see Kindred, 1921, 1924; Boolootian and Giese, 1958, 1959; Boolootian, 1962; Hetzel, 1963; Burton, 1966; Endean, 1966; Smith, 1981).

The coelom of the echinoids is filled with a watery, cell-rich fluid, the coelomic fluid (CF), the ionic composition of which resembles that of seawater (Binyon, 1972). It has, however, a higher concentration of potassium ions, undissolved lipid, protein (Holland *et al.*, 1967; Giga and Ikai, 1985), non-protein nitrogen and reducing sugars (Endean, 1966).

There is lack of agreement on the terminology of the types of coelomocytes recorded by different workers (Table 7), but part of this may be due to there being several developmental stages of particular cell types. A consistent feature is the basic division into phagocytes, spherule or morula cells and vibratile cells. This was confirmed in a review by Smith (1981), with the inclusion of progenitor cells as stem cells from which other coelomocytes are derived. The coelomocytes of the echinoderms carry out a number of important physiological functions, such as host defence, clotting, transport, storage, synthesis and excretion (Endean, 1966). However, the precise role of each cell type in these

TABLE 7. Summary of cell-types and their synonyms in various echinoids.

References	Species of echinoid	Phagocytes	Spherule cells		Vibratile cells
			Colourless	Coloured	
Kindred (1924)	*Arbacia* spp.	Leucocytes	Spherule cells	Spherule cells	Vibratile cells
Bookhout & Greenburg (1940)	*Mellita quinquiperforatus*	Lobed/Granular leucocytes	Colourless spherule cells	Brown & red spherule cells	
Liebman (1950)	*Arbacia punctulata*	Amoeboid/ Petaloid phagocytes. Fibroblasts	Colourless trephocytes	Nephrocytes	
Boolootian & Giese (1958)	Various species	Bladder/ Filiform/ Fusiform amoebocytes	Colourless amoebocytes	Eleocytes. Hyaline haemocytes	Vibratile cells
Burton (1966)	*Diadema antillarum* and *Psammechinus miliaris*	Phagocytes	White morula cells	Red morula cells	Vibratile cells
Endean (1966)	Various species	Amoebocytes	Spherule cells	Eleocytes	

TABLE 7. (continued).

References	Species of echinoid	Phagocytes	Spherule cells		Vibratile cells
			Colourless	Coloured	
Johnson (1969a)	*S. purpuratus*	Phagocytic leucocytes	Colourless spherule cells	Red spherule cells	Vibratile cells
Chien *et al.* (1970)	*S. franciscanus*	Phagocytic leucocytes	Colourless spherule cells	Red spherule cells	Vibratile cells
Stang-Voss (1971)	*P. miliaris*	Amoeboid phagocytes	Colourless spherule amoebocytes	Eleocytes	
Vethamany & Fung (1972)	*S. droebachiensis*	Phagocytic leucocytes, Lymphocytes, Granulocytes	Colourless spherule cells	Red spherule cells	Vibratile cells
Bachmann & Goldschmidt (1978)	*Sphaerechinus granularis*	Phagocytes, Leucocyte-type cells	Empty morula cells	Full morula cells	Fibrocytes
Bertheussen & Seljelid (1978)	*S. droebachiensis*	Phagocytes	White morula cells	Red spherule cells	Vibratile cells
Messer & Wardlaw (1979)	*Echinus esculentus*	Phagocytic leucocytes	Colourless spherule cells	Red cells	Vibratile cells

processes has not been clearly defined (Smith, 1981). Table 8 shows the characteristics and suggested functions of each cell type.

In regard to bacterial contamination of the sea stars, *Asterias*, was found to be sterile, provided the animals were healthy. Bacteria were found only in animals undergoing autotomy, which had been traumatized dermally, or collected from stagnant water, or if signs of muscular weakness and sluggish movement were present (Bang and Lemma, 1962). The CF from *E. esculentus* in the majority of cases (75%) was also found to be sterile (Unkles, 1977) and rarely had more than 40 bacteria ml^{-1} CF.

4.3.2. Clotting

Clotting of echinoderm coelomic fluid occurs rapidly in response to injury, trauma, or contact with foreign materials (Donellon, 1938; Boolootian and Giese, 1959; Bang and Lemma, 1962; Abraham, 1964; Endean, 1966; Bang, 1970; Bertheussen, 1981a).

The calcium chelator ethylene glycol bis-(β-Amino ethyl ether) N,N,N',N' - tetra acetic acid (EGTA) was an effective anticoagulant for *E. esculentus* CF (Messer and Wardlaw, 1979) indicating that clotting is a calcium-mediated process. The main coelomocyte types involved in the clotting process are the phagocytic amoebocytes or leucocytes (Donellon, 1938; Johnson, 1969a), but some workers (Vethamany and Fung, 1972; Bertheussen and Seljelid, 1978) stated that the vibratile cells also participate, and are often seen in association with coelomocyte clots (Johnson, 1969a) and appear to release acid mucopolysaccharide substances in freshly drawn coelomic fluid (Johnson, 1969a; Chien *et al.*, 1970; Bertheussen and Seljelid, 1978).

TABLE 8. Summary of main features of echinoderm coelomocytes (adapted from Smith, 1981).

Coelomocyte type	Size, shape and special features	Function	Key references
Phagocytic Leucocytes	Large, approx. 20-30 µm diameter. Variable shape, either petaloid, filopodial or transitional.	Phagocytosis	Kindred (1921); Bookhout & Greenburg (1940); Hilgard *et al.* (1967); Hilgard & Phillips (1968); Johnson (1969c); Bertheussen (1981a and b).
		Cell clumping/ encapsulation	Bang & Lemma (1962); Johnson (1969c); Reinisch & Bang (1971).
		Clotting/ coagulation	Kindred (1921); Donnellon (1938); Bookhout & Greenburg (1940); Boolootian & Giese (1959); Vethamany & Fung (1972).
		Graft rejection	Hildemann & Dix (1972); Coffaro & Hinegardener (1977); Bertheussen (1979).
		Wound repair	Heatfield & Travis (1975a)
		Transport	Kindred (1921).

TABLE 8. (continued).

Coelomocyte Type	Size, shape and special features	Function	Key References
Vibratile Cells	Small, approx 7 μm diameter. Usually spherical	Clotting Immobilization of invading microbes, probably by the release of acid mucopolysaccharides.	Johnson (1969 a, b and c); Chein *et al.* (1970); Vethamany & Fung (1972); Bertheussen & Seljelid (1978)
Colourless and Red Spherule Cells	Approx. 10-15 μm diameter. Usually round. Napthaquinone pigments (= echinochrome) present in the red spherule cells, absent in the colourless spherule cells.	Cell clumping, encapsulation and wound repair. Probably acts in bactericidal capacity.	Ghiradella (1965); Johnson (1969c); Reinisch & Bang (1971); Heatfield & Travis (1975b).
		Graft Rejection	Coffaro & Hinegardener (1977);
		Bactericidal activity	Vevers (1963); Johnson & Chapman (1970); Wardlaw & Unkles (1978); Messer & Wardlaw (1979); Service, (1982); Service & Wardlaw (1984).

TABLE 8. (continued).

Coelomocyte type	Size, shape and special features	Function	Key references
		Algistat	Fitzgerald & Skoog (1984); Vevers (1966).
		Synthesis-pigment. Chiefly red spherule cells.	MacMunn (1883); Millott (1950, 1956 & 1957); Jacobson & Millott (1953).
		Storage/transport. Probably transport of elaborated materials and not oxygen.	MacMunn (1883); Liebman (1950); Boolootian & Lasker (1964).
		Collagen. Chiefly colourless cells.	Endean (1966)
Progenitor Cells	Small, approx 7 μm diameter.	Stem cells	Hetzel (1963).

4.3.3. Antibacterial factors

In addition to cellular defence such as phagocytosis and cell-clumping, many invertebrates are capable of protecting themselves against microbial invasion by the production of antibacterial factors, or humoral components, such as agglutinins, bacteriolysins and bacteriocidins, all of which may be found free in the body fluids (Sindermann, 1970). Unkles (1976), Wardlaw and Unkles (1978), Service (1982) and Service and Wardlaw (1985), found strong bactericidal activity in the CF of *E. esculentus*. This was not due to phagocytosis or cell clumping since the effect was still obtained in cell-free extracts after ultrasonic-disruption of the cells. Subsequent fractionation of *E. esculentus* coelomic fluid on density gradients, revealed that the main cells responsible for bactericidal activity were the red spherule cells (Messer and Wardlaw, 1979). Subsequently, the extract, the red napthaquinone pigment, echinochrome-A was found to be highly bactericidal especially towards the marine *Pseudomonas* strain number 111 (Service and Wardlaw, 1984), (see later, Section 4.4).

Further evidence for the participation of the red spherule cells in bactericidal activity are the observations of Johnson (1969a) who reported that in *S. strongylocentrotus* and *S. purpuratus*, the spherule cells *in vitro* were strongly attracted to some strains of bacteria. In conjunction with the phagocytic leucocytes the bacteria were effectively phagocytozed and killed. Höbaus (1979) reported that wounded body walls of *P. lividus*, injected with foreign organisms, resulted in an increase of phagocytes and red spherule cells which were observed to increase within and below the wound region, suggesting these cell types were mainly involved in the defence mechanisms of these sea urchins. Red spherule cells were found in the internal organs and regenerating spine tips of *S.*

franciscanus, infected with diatoms and other microorganisms (Johnson and Chapman, 1970). Similar observations were made with *S. purpuratus* (Heatfield and Travis, 1975a and b).

4.3.4. Phagocytosis

So far, no observations have been reported on the uptake of bacteria or yeasts by the phagocytic cells of *E. esculentus*. However, Bertheussen (1981a) observed and quantitated by SEM the uptake of *Escherichia coli*, *Vibrio anguillarum*, *Saccharomyces cerevisiae* (baker's yeast) and other injected particles (sheep red blood cells and latex) *in vitro* and *in vivo* by phagocytes of *S. droebachiensis*. The question of promotion of phagocytosis by opsonins has been considered by Bertheussen and colleagues who showed that human complement C_3ib cooperated with sea urchin phagocytes (Kaplan and Bertheussen, 1977; Bertheussen and Seljelid, 1982) which is a key factor in the human bodys' resistance against microbial invasion. The recognition of mouse complement by echinoid phagocytes may reflect a preservation of essential structures of complement during phylogenesis (Kaplan and Bertheussen, 1977; Bertheussen, 1981a). Bertheussen (1983) also described complement-like activity in echinoid (*S. droebachiensis*) coelomic cell-free fluid. It was found to be heat-labile, inhibited by Ca^{2+} concentration below 10mM and low pH. Human-complement inhibitors also inhibited the lytic and opsonic activities in the coelomic fluid.

Valuable information of basic animal cellular defence against diseases may be obtained by investigating echinoid phagocytes *in vitro* (Bertheussen, 1981a). Studies by Bertheussen (1981a and b) showed that echinoid coelomic phagocytes recognized foreign matter in a manner strongly resembling mouse peritoneal macrophages.

Injection of bacteria into the coelom of *Arbacia punctulata*, produced gross structural changes in the animals' axial organ observed by Millott (1966) who suggested that the organ may clear material ingested by the phagocytes of the CF. Similarly, Bachmann *et al.* (1978 and 1980) detected intense phagocytic activity in the axial complex of *Sphaerechinus granularis*.

4.3.5. Immune reactions

Because of the ancestral relationship of the echinoderms to the vertebrates, several studies have been directed towards the search in this phylum for precursors of vertebrate immunocompetent cells and molecules (Smith, 1981) none of which however, relate to *E. esculentus*. Coffaro and Hinegardener (1977) found an accelerated second set rejection of allografts in the sea urchin *Lytechinus pictus*, indicating that the coelomocytes can not only distinguish between "self" and "non-self", but are also capable of a specific, adaptive response. This was similar to that reaction observed in the Holothurian, *Cucumaria tricolor* (Hildemann and Dix, 1972) and were probably directly responsible for the destruction of foreign tissues.

The exact mechanism(s) by which graft rejection is achieved in echinoderms is (are) still largely unknown. However, Bertheussen (1979) concluded that the cytotoxicity of the echinoid phagocytes to allogenic and xenogenic mixtures *in vitro* indicates that phagocytic coelomocytes are recognition and effector cells in transplant reactions. The participation of other cell types in graft rejection however, cannot be ruled out, especially since Coffaro and Hinegardener (1977) detected the accumulation of massive numbers of red spherule cells at the site of both first and second set allografts in *L. pictus* which may possibly have been behaving as general disinfecting agents.

The adult seastar *Asterias* was taken as a model for the study of phagocytosis and inflammation by Bang (1982). These animals were shown to recognize foreign grafts, but the role of the amoebocytes was not clear. An inflammatory response to bacterial suspensions and sea urchin amoebocytes, injected into the coelom, was also observed.

4.3.6. Haemagglutinins and haemolysins

The CF of some echinoids possess powerful haemagglutinins (HA) and haemolysins (HL) against mammalian red blood cells (Ryoyoma, 1973, 1974a and b; Giga *et al.*, 1985). Parker (1975) showed that the CF of *E. esculentus* possessed HA for three types of mammalian erythrocytes and the HA was found to be localized in the colourless spherule cells (Messer and Wardlaw, 1979). Tripp (1966) reported that the protein HA of the oyster *Crassostrea virginica* acted as an opsonin, enhancing adhesion and phagocytosis of erythrocytes by the phagocytic cells and McKay *et al.*, 1969 demonstrated a similar activity with HA of the crayfish. It is possible, therefore, that the HA of *E. esculentus* may function as an opsonin also, enhancing phagocytosis by the phagocytic leucocytes. Conversely, Fuke and Sugai (1972) found that HA in ascidians did not enhance or activate phagocytosis.

Attempts have been made to determine if the evolution of the vertebrate immune system can be traced through to the echinoderms. The HA of the asteroid, *Asterias rubens*, has an amino-acid sequence which shows some correlation with the heavy chains of vertebrate immunoglobulins (Carton, 1974). It has been suggested, therefore, the HA is a possible candidate as an evolutionary precursor of the vertebrate immunoglobulins (Carton, 1974; Burnet, 1974). Ryoyoma (1974a and b) found that calcium

ions were essential for the development of high haemagglutinating potency in the coelomic fluid of three species of sea urchin, *Anthocidaris crassipina*, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*.

4.4. Echinochrome A and Other Napthaquinones

Echinoderms are often highly pigmented. The pigments may be present in the epidermal layers of the body wall or deposited in a more permanent form in the exoskeleton as calcium salts of the polyhyroxynapthaquinones (Binyon, 1972; Millott, 1957). It was shown that the pigments do not occur in the free state in *Arbacia* eggs but are conjugated with a protein of high molecular weight (Thomson, 1957).

The calcareous test and the red spherule cells of the CF of *E. esculentus* and *Paracentrotus lividus* (and other species) contain the napthaquinone pigment, echinochrome-A (Figure 9) and was first discovered by Macmunn in 1883 (Goodwin and Srisukh, 1950; Thomson, 1957). The napthaquinone pigments present in the spines were named spinochromes (Goodwin and Srisukh, 1950; Thomson, 1957; Nishibori, 1959, 1961) and much work on the spinochromes of Japanese sea urchins has been done by Kuroda and Okajima (1950, 1951, 1954, 1955, 1960, 1962). (Figure 9). The distribution of spinochrome pigments in the spines and test of over thirty species of echinoids was surveyed by Anderson *et al.* (1969). Echinoids are virtually unique in the animal kingdom in possessing substances of this type (Vevers, 1966).

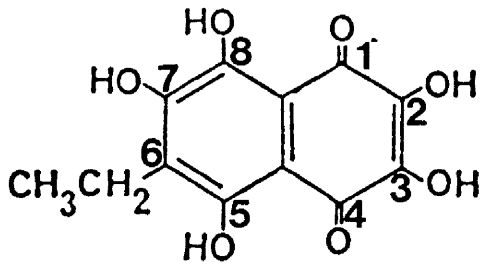
4.4.1. Distribution and uses

Napthaquinones have been reported in areas of medicine in their use as antibiotics and specifically, inhibiting the growth of *Staphylococcus aureus* by Fairbank, 1860; Benj, 1961 and Hoffmann-Ostenhof, 1947 (all cited

FIGURE 9. The six common spinochrome pigments, Echinochrome-A (Ech-A) (1) and spinochromes A to E (2-6).

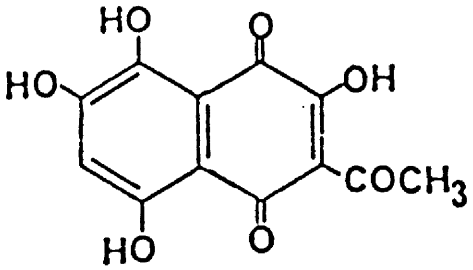
(Reproduced with the kind permission of Dr. H.A. Anderson, Dr. J.W. Mathieson and Prof. R.H. Thomson, 1969).

1



(EchA)

2



(A)

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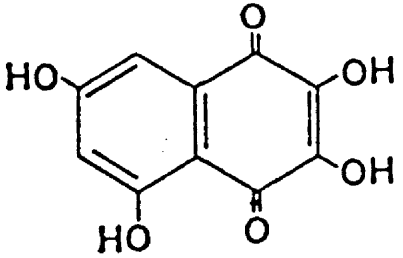

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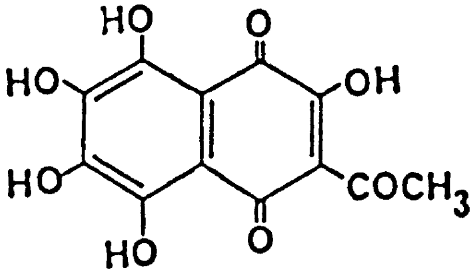

(C)

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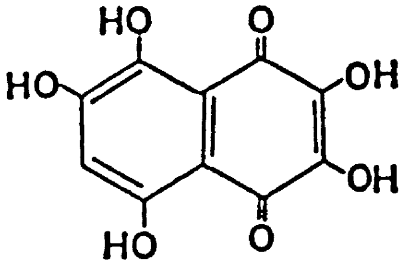
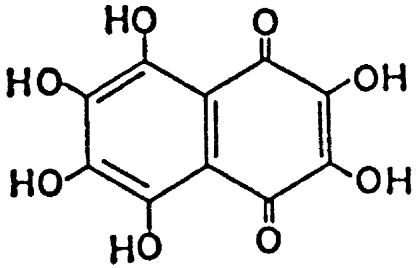


(D)

6



(E)

by Thomson, 1957). They have also been used in the treatment of rheumatism, whooping cough, leprosy, ringworm, dyspepsia, piles, diarrhoea, skin disease, jaundice and used as an emetic and a potent fungicide (Thomson, 1957; Gershon *et al.*, 1972). A few synthetic napthaquinones are of practical value e.g. in vitamin K therapy and as a plant fungicide (Thomson, 1957).

Apart from the occurrence of napthaquinones in echinoids few references record the presence of these substances in other animals apart from the Pacific sea otter, *Enhydra lutris*, the bones of which are coloured purple by the calcium salt of polyhdroxynapthaquinone, similar to that isolated by Goodwin and Srisukh (1950) from *E. esculentus* and *Paracentrotus lividus*. The colouration of the bones may be due to the fact that the animals feed largely on *S. droebachiensis* (Mertens, 1935, Fox, 1953). The pigments have also been isolated from certain insects (Thomson, 1957). The napthaquinones have also been shown to occur in bacteria (Ciegler *et al.*, 1981; Gast *et al.*, 1985), cyanobacteria (Allen *et al.*, 1967) and actinomycetes (Eckardt, 1966).

The quinone pigments as a whole, occur amongst at least thirty families of higher plants, usually masked by other pigments present. These pigments especially *Rubiaceae*, have been used for dyeing textiles in ancient craft in many countries (Thomson, 1957). The napthaquinone pigment, henna (lawsone), dyes protein fibres an orange shade, and has been used for dyeing hair, skin and fingernails. It is of very ancient lineage going back to the Egyptian times and Egyptian mummies are usually a reddish colour owing to this custom. However, the quinones may also be present in the reduced form with little or no colour as a result (Thomson, 1957). Ech-A was suggested to be in a colourless reduced form *in vivo*, (Cannan, 1927).

4.4.2. Function

The most characteristic feature of the napthaquinones is their ease of reduction and reoxidation. This is virtually the only chemical reaction common to all the natural quinones. This in turn suggests that these substances may play a part in the oxidation-reduction processes of living matter (Thomson, 1957). For example, the diquinone, pheonicin, according to Friedham in 1933 (cited by Thomson, 1957) functions as a respiratory catalyst, and so may be the function of the quinones of the lower fungi. A quinone, pthiocol, in low concentrations has been found to accelerate the respiration of algae, conversely, a number of napthaquinones inhibit various respiratory substances (Ball *et al.*, 1947) and therefore, the function of the quinones may be considerably variable due to their structure and function.

Certain napthaquinones are known to behave as algistats, and have been used to control the growth of Cyanophyceae in freshwaters in North America (Fitzgerald and Skoog, 1954). The 2,3-dichloronapthaquinone was used in the form of a paste containing 50% active ingredient with wetting agent and 50% water. The chemical was applied to wide areas of the lake as a water spray by the means of mechanical pump. Vevers (1966) showed that pigments leached from the dried test of an unspecified echinoid placed on a culture plate of cyanobacteria inhibited their growth.

4.4.3. Napthaquinones in sea urchins

The echinoderms are able to synthesize the napthaquinones *de novo* (Fox and Hopkins, 1966). In some sea urchin species polyhydroxynapthaquinones are contained in the oocytes (Ball, 1934; Griffiths, 1965; Gibson and Burke, 1985) and embryos (Asashima, 1972; Ryeberg, 1979; Koltsova *et al.*, 1981). The latter author suggested a possible synthesis of echinochrome.

Hence, sea urchins have a need for these substances already in the early stages of development, and napthaquinones may take part in major physiological processes.

Several hypotheses have been made for the function of these substances but only a few of the suggested functions have been confirmed experimentally (Binyon, 1972). Echinochrome pigment after liberation from the protein-binary complex in *Arbacia* eggs (by treatment with dilute hydrochloric acid) was found to be a very powerful chemotactic principle and activated sperm at a dilution of one in 2.5×10^9 parts water by Kuhn and Wallenfels in 1940 (cited by Thomson, 1957). Echinochrome is a highly active inhibitor of fat peroxidation (Maximov *et al.*, 1977) and calcium stimulated O_2 consumption of *Arbacia punctulata* egg homogenates resulted from the oxidation of echinochrome-A (Perry and Epel, 1981).

Cannan (1927) questioned the respiratory function, although he stated that Ech-A could act as an "activator" for oxygen transport in the CF. Perry and Epel (1981) suggested that Ca^{2+}-mediated Ech-A oxidation may also function in an antibacterial role. The function of Ech-A in the adult sea urchin may also be related to H_2O_2 production, perhaps mediating the antibacterial activity of the substance (Johnson, 1969b and c; Johnson and Chapman, 1970; Service and Wardlaw, 1984). Pearse *et al.* (1977) recorded that several specimens of *S. franciscanus* had a middle "red- friable" layer present due to Ech-A and Coffaro and Hinegardener (1977) noted damaged tissues of *Lytechinus pictus*, during grafting experiments, became reddened by an influx of red spherule cells. It would therefore appear that one function of Ech-A is that of a general disinfectant, but little is known about the release of this substance from the red spherule cells, or its chemical mode of action.

OBJECT OF RESEARCH

Although there is now a useful body of information on the antibacterial properties of *Echinus esculentus* coelomic fluid and the ability of the animal to clear bacteria from the coelomic cavity, there is no information on the antifungal capabilities of these animals or the factors in the coelomic fluid which may contribute to antifungal defence.

The main objects of the present investigation were to determine :

a. whether coelomic fluid from *E. esculentus* was able to kill marine yeasts *in vitro*, in addition to its known lethal effect towards marine bacteria,

b. the response of the animal to infection, with marine yeasts, specifically :

i. clearance of injected marine yeasts from the coelom of the intact animal,

ii. whether marine yeasts are pathogenic towards *E. esculentus*, and

iii. to define cellular response to infection with marine yeasts, in terms of the total numbers and relative types of coelomocytes.

To facilitate the pursuit of these main objectives, it was first necessary to set up recirculating artificial-seawater aquaria (RASWA) so that experiments could be done in a laboratory which did not have access to running seawater.

MATERIALS AND METHODS

1. MICROBIOLOGICAL MATERIALS AND METHODS

1.1. Culture Media

1.1.1. Bacteriological culture media

Marine agar, Zobell agar 2216E (MA) and marine broth (MB) both from "Difco^{R}" Laboratories, Detroit, U.S.A., were used. The powdered media were dissolved in distilled water, according to the manufacturer's instructions and sterilized by autoclaving for 15 min at 103.4 kPa (121°C).

1.1.2. Mycological culture media

Marine agar and marine broth were enriched with glucose ("Formachem^{R}" Research International Ltd., 80, Kirk Street, Strathaven, Scotland, ML10 6BA) to a final concentration of 50mM, to encourage growth of the yeasts. The glucose was dissolved in 10ml of distilled water to allow sterile-filtration through a 0.45 µm disposable filter ("Swinnex^{R}", Millipore SA, Molsheim, France) and added to the medium after autoclaving. Allowance was made for the 10ml volume of sugar solution per litre when reconstituting the powdered media. The MA media was also enriched with chloramphenicol ("Sigma^{R}" Chemical Company, Fancy Road, Poole, Dorset), to inhibit bacterial growth arising from the coelomic fluid contaminants and to suppress the growth of *Pseudomonas* strain number 111 (*Ps.111*) in mixed inocula *in vitro* experiments. The antibiotic was dissolved as 0.5% (w/v) in 95% (v/v) ethanol, filter sterilized, then 10ml added to the autoclaved, cooled agar (980 ml) to give a final concentration of 50µg ml^{-1}.

1.2. Microbial Strains

1.2.1. Marine yeast strains

Eleven strains of marine yeast were obtained from the National Collection of Yeast Cultures (NCYC) (Agricultural and Food Research Council, The Food Research Institute, Colney Lane, Norwich NR4 7UA).

They were grown at approximately 22°C (room temperature) on YMA for 48 to 72h and maintained on YMA slopes at 4°C. Stocks of the marine yeast strains were kept as freeze-dried cultures. A list of the yeast strains and their place of origin are presented in Table 9, and the characteristics of each strain in Appendix 1A and B. A few strains were rejected from a longer list because they did not meet some of the required criteria (see later).

1.2.2. Bacterial control strain

The bacterial control strain *Pseudomonas* strain number 111, (*Ps.111*), was originally isolated from sand at Kames Bay, Millport, Isle of Cumbrae (Wardlaw and Unkles, 1978).

Ps. 111 was grown at room temperature (approx. 22°C) on MA for 24 to 48h and maintained on MA slopes at 4°C.

1.3. Assay of *in vitro* Antimicrobial Activity of *Echinus esculentus* Coelomic Fluid

1.3.1. Coelomic fluid

To obtain coelomic fluid (CF), a sea urchin was removed from the recirculating artificial-seawater aquaria (RASWA) and the water allowed to

TABLE 9. List of NCYC marine yeast strains and their source of isolation.

NCYC Strain no.	Genus and species	Isolation source and Reference
798	*Candida famata*	NCMB (*Torulopsis candida*, NCMB 42, from seawater) 1974. Ross & Morris (1965).
799	*Candida famata*	NCMB (*Torulopsis famata*, NCMB 308s, from seawater) 1974. Ross & Morris (1965).
145	*Candida guilliermondii*	(*Torula fermentati*). Harrison (1928).
787	*Candida haemulonii*	NCMB (*Torulopsis haemulonii*, 3025 from seawater, Torres Strait) 1974. van Uden and Zobell (1962).
784	*Candida marina*	NCMB (NCMB 1130, CBS 5235, ATC 22974, T, from seawater, Torres Strait) 1974. van Uden and Zobell (1962).
785	*Candida maris*	NCMB (*Torulopsis maris*, NCMB 1131, CBS 5151, ATCC 22997, T, from seawater, Torres Strait) 1974. van Uden and Zobell (1962).
786	*Candida torresii*	NCMB (*Torulopsis torresii*, NCMB 1132, CBS 5152, ATCC 22999, T, from seawater, Torres Strait) 1974. van Uden and Zobell (1962).

TABLE 9. (continued).

NCYC strain no.	Genera and species	Isolation source and Reference
792	*Debaryomyces hansenii*	NCMB *(Debaryomyces kloeckeri*, strain 43, from seawater) 1974. Ross and Morris (1965).
783	*Metschnikowia zobelli*	NCMB (NCMB 2892, from the Pacific Ocean) 1974. van Uden and Zobelli (1961).
63	*Rhodotorula rubra*	(NCTC 2622, CBS 316, IFO 0890 and IFO 0909.) Harrison, 1928.
797	*Rhodotorula rubra*	NCMB (450P, from seawater) 1974. Ross and Morris (1965).

drain away. The peristomial membrane was then cleaned by swabbing with 95% (v/v) ethanol and the surface dried with a cotton wool bud. The sea urchin was held with the oral surface downwards and the peristomial membrane punctured, at an angle of approximately 45° to the oral surface, with a 26 gauge 13mm length needle attached to a sterile pre-chilled 10ml syringe. Coelomic fluid was withdrawn slowly, so as not to disrupt the coelomocytes and was carefully dispensed immediately into test tubes held in an ice bath.

When large amounts (up to 100ml) of fluid were required for the preparation of stock coelomic fluid supernate (CFSN), for example, the peristomial membrane was washed first with sterile-seawater and then slit with a sterile scalpel. The sea urchin was inverted and the fluid allowed to drain, via a funnel, into a chilled vessel.

1.3.2. Control fluids

The fluid used as an innocuous control, in experiments where antifungal agents were tested, was 1% (w/v) MB in artificial seawater, (ASW) "Tropic Marin^{R}", designated MBASW. It consisted of 0.37g of MB powder dissolved in 1 l artificial seawater and was sterilized by autoclaving at 103.4 kPa pressure (121°C) for 15 minutes.

1.3.3. Artificial and natural seawaters

The artificial seawater (ASW) used throughout this work, except where otherwise stated, was prepared by dissolving approximately 30g of "Tropic Marin^{R}" (Dr. Biener Aquarientechnik, 6423 Wartenberg, Germany) in distilled water. Extra salt or extra distilled water was added so as to give a final specific gravity (SG) of 1.0240 at room temperature (approx. 22°C). The method for determining SG is described in section 2.1.3.2.

For a few experiments, ASW was also prepared from "Sea Salt^{R}" ZGV-110-T (Griffin and George Ltd., Gerrard Biological Centre, East Preston, West Sussex, BN16 1AS) and "Instant Ocean^{R}" (Aquarium Systems Inc., 33208 Lakeland Boulevard, East Lake, Ohio 44094) by dissolving respectively 36-38g l^{-1} (SG 1.022-1.025) and natural seawater (NSW) was obtained from the Clyde Estuary, Millport, Isle of Cumbrae, KA28 OEG. When required for use as sterile diluents, all of the above seawaters were sterilized by sterile filtration (0.45μm).

1.3.4. Coelomic fluid supernate

Coelomic fluid supernate (CFSN) consisted of pooled or individual fresh coelomic fluids from which the coelomocytes had been removed by centrifugation at 2000g for 10 min. The CFSN was then filter sterilized by passing through a 0.45μm pore size filter ("Millipore^{R}").

1.3.5. Coelomic fluid lysate

Coelomic fluid lysate (CFL) was prepared by disrupting the coelomocytes, on ice, in an MSE 100 Watt Ultrasonic Disintegrator. Sonication of coelomic fluid (10ml) was done at 0°C in three "bursts" at full power, each of two min duration and with 30 s intervals inbetween. To minimize contamination, the probe (end diameter 2mm) was dipped in boiling distilled water for 5 min and immersed in sterile distilled water which was sonicated for 30 s before each run. After sonication, the coelomic fluid lysate was inspected by eye for reduction in opacity and microscopically for the absence of intact cells. It was then centrifuged at 2000g for 10 min at 4°C, to remove cellular debris and the supernate sterile-filtered through a 0.45μm pore size "Millipore^{R}" filter into

sterile, capped, plastic universals held in an ice bath. The coelomic fluid sonicate was stored at -20°C.

1.3.6. Density gradient separation of coelomocytes

Coelomocytes were separated by density gradient centrifugation by a modified version of the method of Messer and Wardlaw (1979) which in turn was based on the Boyum (1968) procedure for separating leucocytes from human blood. Ethylene glycol bis-(β-Aminoethyl ether) N,N,N',N',-tetracetic acid (EGTA) ("Sigma^R" Chemical Company, Fancy Road, Poole, Dorset) was dissolved in 1.6% sodium chloride solution ("Formachem^R" (Research International) Ltd., 80, Kirk Street, Strathaven, Scotland, ML10 6BA) at a final concentration of 140mM. The EGTA does not dissolve in the 1.6% NaCl solution without prior adjustment to the pH of the solution. The pH of initially 2.50 was adjusted to the region of 7.40. This was accomplished by the dropwise addition of 1.0N NaOH whilst stirring continuously until the EGTA was completely in solution. This was sterile-filtered through a 0.45μm filter.

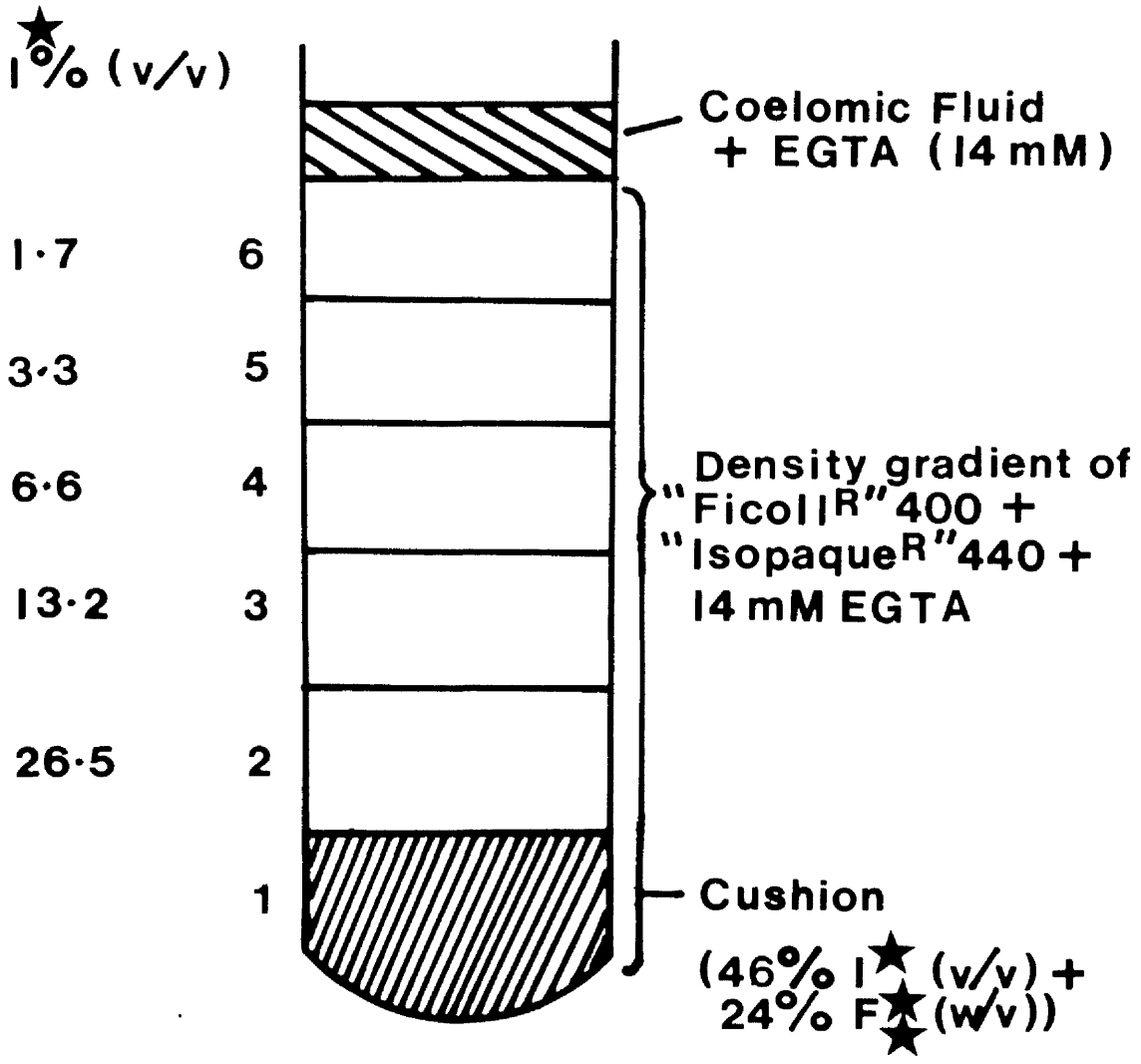
Density gradients were made by layering solutions of "Ficoll^R" 400 (Pharmacia Fine Chemicals AB, Laboratory Separation Division, Box 175, S-751 04 Uppsala 1, Sweden.) and "Isopaque^R" (Nyegaard and Co., Oslo, distributed by Vestric Ltd., Runcorn, Cheshire) as set out in Table 10 and Figure 10. "Ficoll^R" 400 is a synthetic polymer made by the copolymerization of sucrose and epichlorhydrin. A stock solution of 24% (w/v) "Ficoll^R" 400 was prepared by dissolving the high molecular weight (400,000) hydrophilic polymer of sucrose in ASW by gentle heating over a bunsen burner and stirring continuously until complete dissolution had occurred. From this 24% (w/v) stock solution of "Ficoll^R", an 8% (w/v) solution was made by diluting 10ml of 24% "Ficoll^R" to 30ml with ASW.

TABLE 10. Constituents of the "Ficoll^{R}"/"Isopaque^{R}" density gradient for the separation of *E. esculentus* coelomocytes.

Gradient layer	24% (w/v) "Ficoll^{R}" in ASW (ml)	8% (w/v) "Ficoll^{R}" in ASW (ml)	"Isopaque^{R}" (Undiluted) (ml)	140mM EGTA* in 1.6% NaCl (ml)	Total Volume (ml)
6	-	8.83	0.17	1.0	10
5	-	8.67	0.33	1.0	10
4	-	8.34	0.66	1.0	10
3	-	7.68	1.32	1.0	10
2	-	6.35	2.65	1.0	10
1 ("Cushion")	4.40	-	4.60	1.0	10

* pH adjusted to 7.4

FIGURE 10. Composition of the "Ficoll^{R}"/"Isopaque^{R}" density gradient for the separation of *E. esculentus* coelomocytes. See Table 10 for the composition of the "cushion" (3ml) and of each gradient layer numbered 2 to 6 (2ml of each). The "cushion" is shaded to emphasize its high content of "Ficoll^{R}" and "Isopaque^{R}". The density gradient was loaded with 2ml of CF and EGTA (1.8ml CF and 0.2ml 140mM EGTA).



I★ "Isopaque^R" 440

F★★ "Ficoll^R" 400 dissolved in ASW 14 mM EGTA mixture.

Gradient steps 1–5 consisted of 8% Ficoll in ASW/EGTA.

The other component of the density gradient was "Isopaque^R, an intravenous contrast medium, which was supplied in the form of 20ml sterile ampoules. "Isopaque^R" is a solution of balanced salts of tri-iodinated organic acid, metrizoic acid. The package slip stated that each 1.0ml of "Isopaque^R" contained sodium metrizoate, 660mg, calcium metrizoate, 35mg, magnesium metrizoate, 38mg, sodium calcium edetate, q.s., all dissolved in aqueous solution. "Isopaque^R" replaced the intravenous contrast media, "Triosil^R" used in the method of Messer and Wardlaw (1979). "Isopaque^R" was added to each gradient step as shown in Table 10.

All of the prepared solutions used to separate coelomocytes for Coulter counting were filtered through a 0.45μm pore size filter ("Millipore^R") to remove debris and thereby minimize the "background count" of the instrument. Also, where sterile conditions were needed, the filtration also ensured sterility. All solutions were pre-chilled at 4°C before use.

The five gradient steps were arranged above the 24% (w/v) "Ficoll^R" and "Isopaque^R "cushion". Each layer had a final concentration of 14mM EGTA in 1.6% NaCl as shown in Figure 10. The complete gradient was pre-chilled at 4°C before loading with a mixture of 1.8ml freshly drawn coelomic fluid (see 1.3.1) and 140mM EGTA, the (0.2ml) EGTA being present in the 2ml syringe when withdrawing fluid from the sea urchin. An air bubble was allowed to enter the syringe which was then inverted to allow thorough mixing of the anticoagulant, EGTA, with the coelomic fluid. The loaded gradient was centrifuged at 600g for 6 min at 4°C in a swing-out rotor. The bands were carefully removed from the gradient with a Pasteur pipette and transferred to capped, plastic, sterile universals held in an ice bath. The separated fractions were washed in sterile MBASW (to remove EGTA) and again centrifuged at 1200g for 15 mins at 4°C to pellet the

coelomocytes and the supernates decanted. The cells were then resuspended to the original volume of whole coelomic fluid (1.8ml) in sterile MBASW and kept on ice until required.

1.3.7. Echinochrome-A

Crystalline echinochrome-A (Ech-A), extracted from the red spherule cells of *E. esculentus* was obtained from Professor R.H. Thomson, Department of Chemistry, University of Aberdeen. This compound is only sparingly soluble in seawater, but dissolves in the presence of various mammalian proteins (Johnson, 1970). Bovine Gamma Globulin (BGG), (Koch Light Laboratories Ltd., Colnbrook Ltd., Colnbrook, England) was selected since this was used previously in the investigation of bactericidal activity of Ech-A (Service and Wardlaw, 1984) and gave consistent results. A standard solution of Ech-A was prepared by dissolving a known weight (typically 1mg) in few drops of 0.1N NaOH, mixing thoroughly and then diluted with 2mg ml^{-1} BGG dissolved in MBASW. The pH was adjusted to 8.0-8.3, although usually little adjustment was needed.

It appeared visually that the Ech-A did not completely dissolve, and some of it was retained in the filter. Therefore, a spectrophotometric scan, between the wavelengths 250 and 700nm, was done using a "Unicam^{R}" SPI 800 spectrophotometer, before and after the solution was passed through a 0.45μm filter ("Swinnex^{R}"), to remove debris and a 0.22μm filter to sterilize the solution. MBASW containing a 2mg ml^{-1} BGG was used as the blank solution to zero the instrument. Comparison of the spectrophotometric scans (Chart speed, $20scm^{-1}$; band width, 10nm; absorbance scale, 0.5; wavelength speed $5nms^{-1}$) before and after filtration allowed an estimation of the percentage of undissolved Ech-A lost in the

filter during sterilization, as shown in Figure 11 . The amount of Ech-A passed through the filter was estimated to be between 90 and 92% (8-10% retained).

1.3.8. The microbiocidal tests

Marine yeasts and *Ps.111* were grown on YMA and MA respectively at approx. 22°C for 48-72h and were harvested in sterile MBASW. To standardize the concentration of yeast and bacterial cells, each suspension was adjusted to OD = 1.0 with a "Unicam" SPI 800 spectrophotometer at a wavelength of 610nm, using MBASW as the blank. The OD = 1.0 suspension was then diluted in sterile MBASW by a factor determined in a preliminary test so as to give a suspension of approximately 5000 colony forming units (cfu) ml^{-1} as shown in Figures 12 and 13.

Since many of the tests involved a deliberately-mixed inoculum of yeasts and bacteria, the suspensions were prepared at double-strength so as to produce approximately 5000 cfu of each organism in a dual-inoculum test mixture as shown in Figure 14.

The prepared suspensions were dispensed as 0.2ml volumes into 12 x 100mm sterile, capped, glass test tubes. To each tube containing the microbial suspension, 1.8ml of freshly-drawn coelomic fluid or other test fluid or 1.8ml of MBASW (control fluid) was added and gently mixed. A zero-time sample was taken only from the control tubes and 0.1ml spread onto both YMA and MA to obtain the initial count of marine yeast and *Ps. 111*, respectively. Between 50 and 100 colonies at zero-time were considered satisfactory for counting. A zero-time sample was not taken from tubes containing whole or density gradient coelomic fluid so as not to disturb clotting and avoid possible disruption of the coelomocytes.

FIGURE 11. Estimation of dissolved Echinochrome-A. Spectrophotometric scan between 250 and 680nm wavelength before (A) and after (B) filter (0.45μm) sterilization of 250μg ml^{-1} echinochrome A (1:10 dilution in MBASW) in MBASW-BGG solution. Approximately 8-10% of the Ech-A (undissolved) Ech-A lost during filtration.

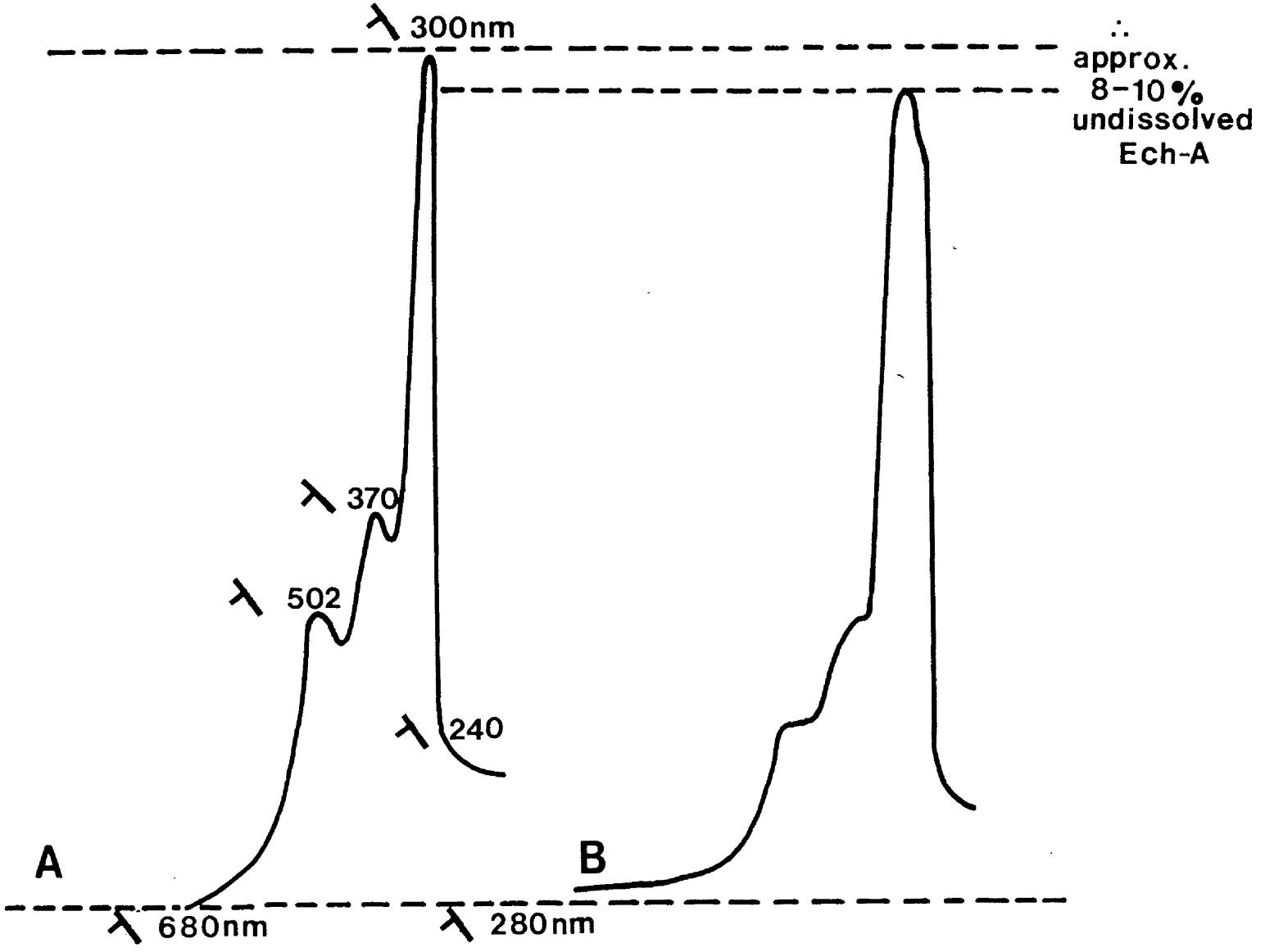

λ 300nm
∴
approx.
8–10%
undissolved
Ech-A
λ 370
λ 502
λ 240
A
B
λ 680nm
λ 280nm

FIGURE 12. Scheme of dilution of the marine yeast strains in MBASW diluent.

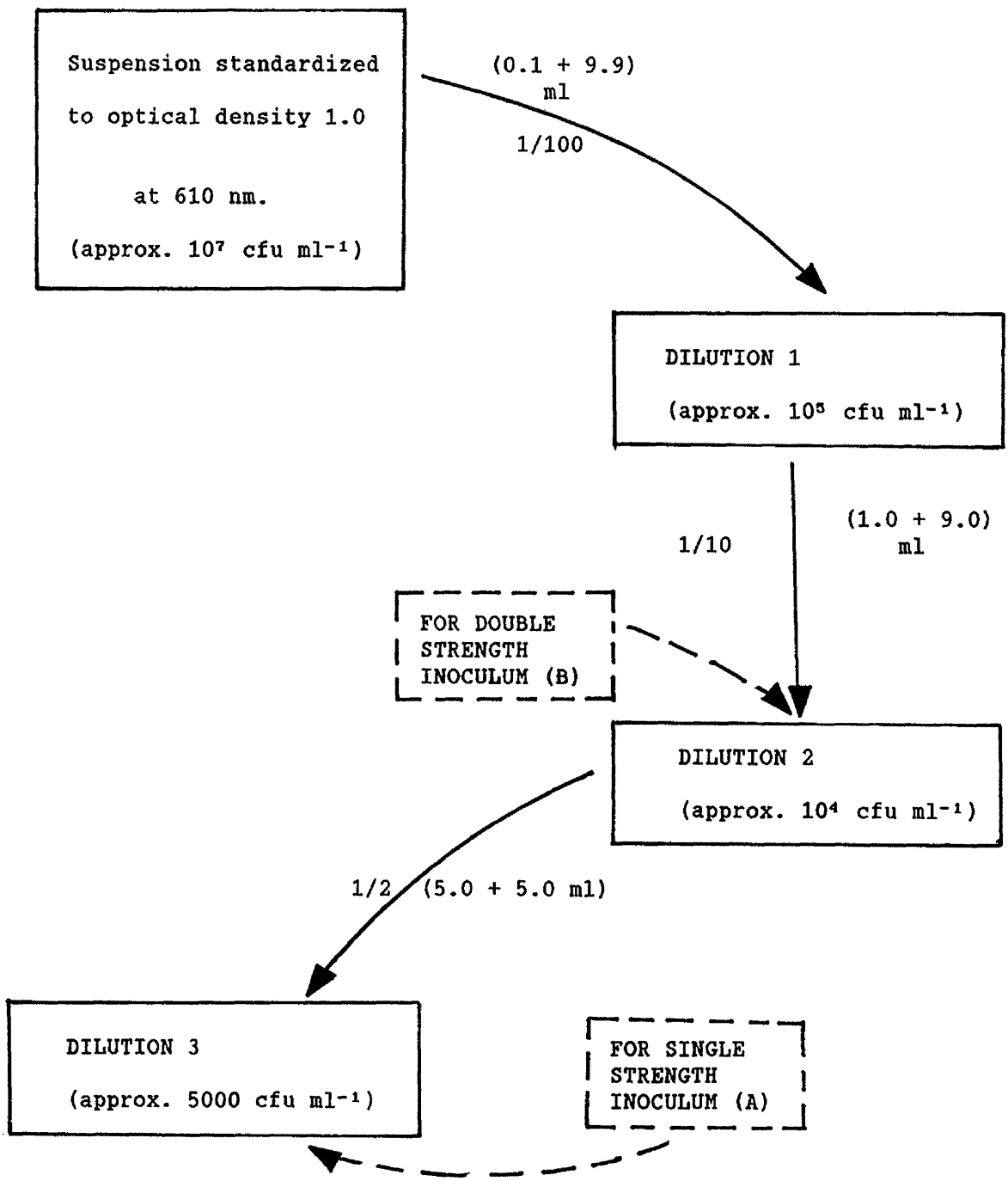

Suspension standardized
to optical density 1.0
at 610 nm.
(approx. 10^7 cfu ml^{-1})
(0.1 + 9.9)
ml
1/100
DILUTION 1
(approx. 10^5 cfu ml^{-1})
1/10
(1.0 + 9.0)
ml
FOR DOUBLE
STRENGTH
INOCULUM (B)
DILUTION 2
(approx. 10^4 cfu ml^{-1})
1/2 (5.0 + 5.0 ml)
DILUTION 3
(approx. 5000 cfu ml^{-1})
FOR SINGLE
STRENGTH
INOCULUM (A)

FIGURE 13. Scheme of dilution of the bacterial control strain *Pseudomonas* strain number *111* in MBASW diluent.

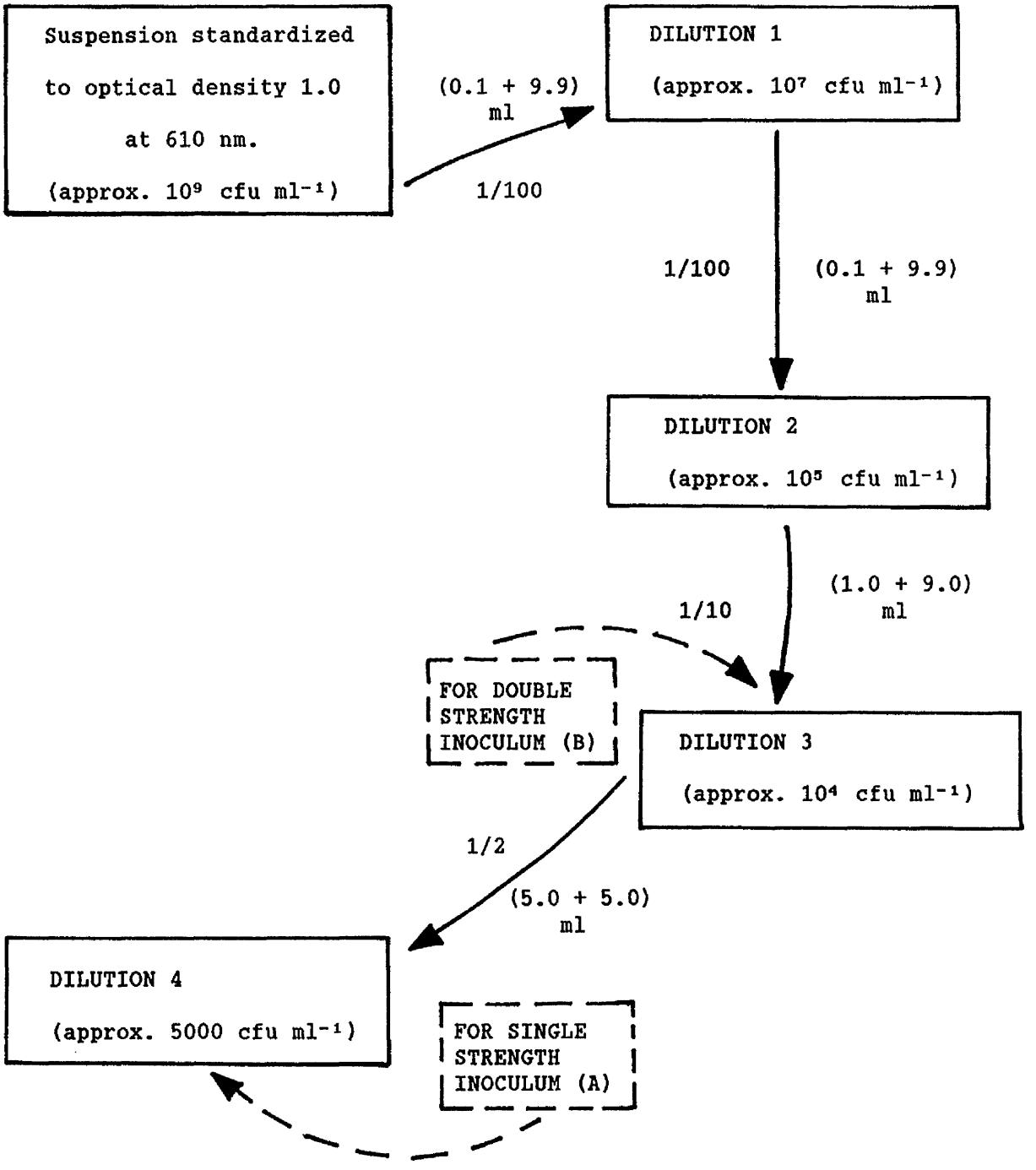
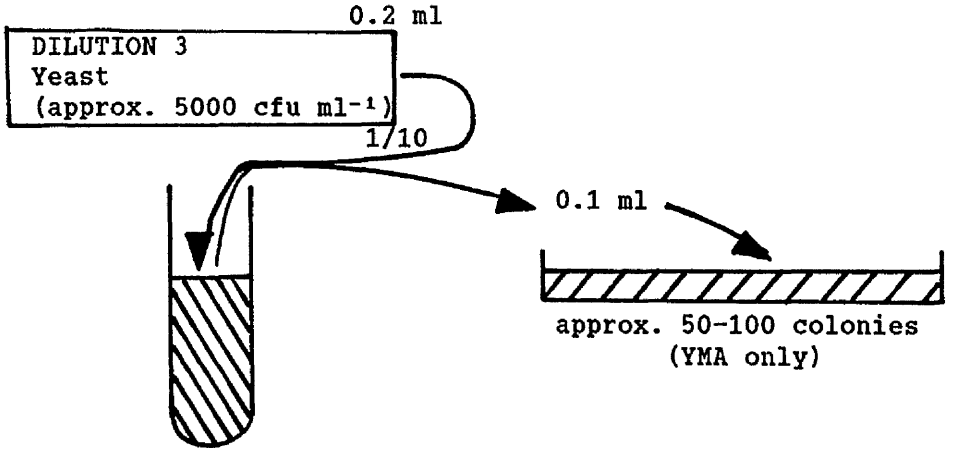

Suspension standardized
to optical density 1.0
at 610 nm.
(approx. 10^9 cfu ml^{-1})
(0.1 + 9.9) ml
1/100
DILUTION 1
(approx. 10^7 cfu ml^{-1})
1/100
(0.1 + 9.9) ml
DILUTION 2
(approx. 10^5 cfu ml^{-1})
(1.0 + 9.0) ml
1/10
FOR DOUBLE STRENGTH INOCULUM (B)
DILUTION 3
(approx. 10^4 cfu ml^{-1})
1/2
(5.0 + 5.0) ml
DILUTION 4
(approx. 5000 cfu ml^{-1})
FOR SINGLE STRENGTH INOCULUM (A)

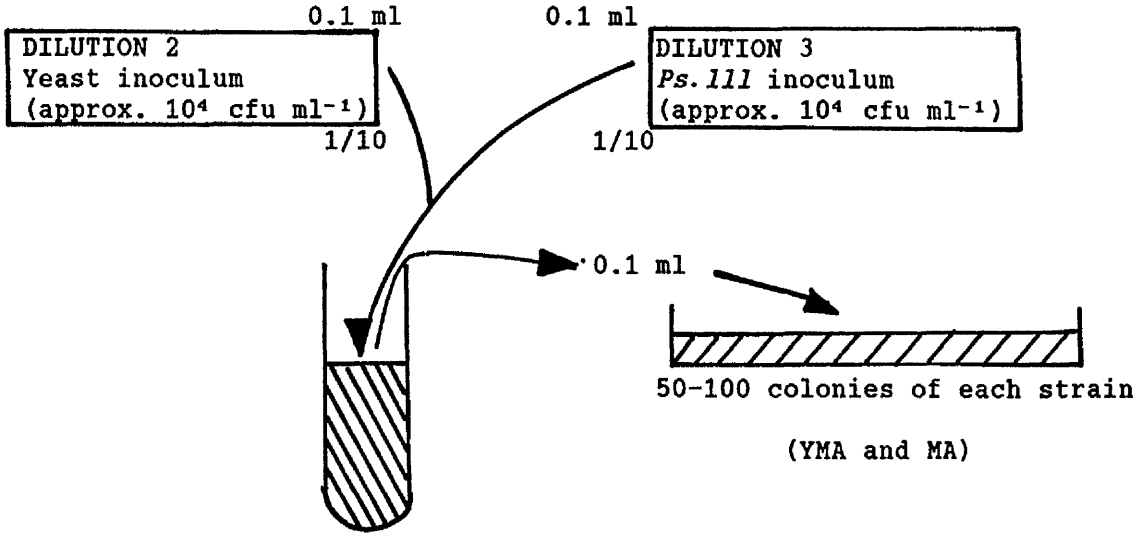
FIGURE 14. Single-strength inocula (A) or double-strength (B). They were inoculated into the test mixtures so that the final petri dish, shown opposite, grew between 50-100 colonies if there was neither growth nor killing.

(see Figures 12 and 13).

A. Single inoculum experiment



B. Mixed inoculum experiment



The tubes were incubated at 10°C in a rack held in the water of an aquarium tank maintained at this temperature. All tubes were sampled at some, or all of the times 24, 48,72 and 96h. Undiluted samples (0.1ml) and serial dilutions were plated out on both YMA and MA and incubated at room temperature (approx. 22°C) for 48 to 96h and the colonies counted.

1.3.9. Marine yeast and bacterial agglutination tests

The marine yeasts and *Ps.111* were harvested from YMA and MA slopes respectively, after incubation at approx. 22°C for 48 to 72h with sterile MBASW. To determine suitable cell concentrations for agglutination tests, 25µl volumes of serially diluted cell suspensions in ASW were pipetted into wells of a microtitre tray ("Sterilin^{R}" Ltd., Sterilin House, Clockhouse Lane, Felltham, Middlesex, TW14 8QS). A volume of 25µl of either CFL or control fluids MBASW or CFSN were added to each well and the contents thoroughly mixed. The concentration of cells which gave the "sharpest" end point (10^{7}) were used in further investigations.

The CFL was titrated against the yeast or bacterial suspensions of suitable density, by serial dilution with sterile ASW ("Tropic Marin^{R}"), and with MBASW and CFSN as control fluids.

The agglutination titres were read initially, after incubating the covered trays at room temperature (approx. 22°C) for 2h and in the second instance, after overnight refrigeration. The titration end-point was taken as the highest dilution of the test fluid which produced definite agglutination of the cells.

1.3.10. Growth characteristics of marine yeasts and *Ps.111*

For the determination of mean generation time (MGT), flasks containing 100ml YMB and MB were inoculated with 0.2ml mid-exponential phase broth cultures of marine yeasts and *Ps.111*, respectively. The flasks were incubated at 10°C in a water bath with rotating flask-clip base. Samples were taken from each broth culture (about 2.0ml) and the OD read at 610nm at convenient, pre-determined intervals along the growth curve.

1.4. Whole Animal Infection Studies

1.4.1. Background contamination of the coelomic fluid

The bacterial background contamination of each sea urchin CF was determined before experimental infection, by spreading a 0.1ml sample of undiluted CF on MA. The plates were incubated 48 to 72h at room temperature (approx. 22°C) and the colonies counted.

1.4.2. Standardization of microbial suspensions

Suspensions of marine yeasts and *Ps.111* were prepared in sterile MBASW diluent as described in section 1.3.8. Materials and Methods. The cell concentrations used in the virulence titration experiments are represented in Table 11. Control animals were injected with sterile, MBASW diluent (1.0ml) only and placed alongside microbial-injected animals in the "infection" tank or alone in a separate "storage" tank.

1.4.3. Heat-killed yeast suspensions

The marine yeast strains *Ps.111* were harvested in sterile MBASW from YMA slopes and standardized as previously described to 10^7 yeasts. A

TABLE 11. Doses of bacteria and yeasts used in virulence titrations.

Organism injected	Viable count of microbial cells at dose			
	High (H)	Medium (M)	Low (L)	Very low (VL)
Ps.111	$1 - 2 \times 10^{10}$	$5 \times 10^{7} - 10^{9}$	5×10^{5}	6×10^{4}
R. rubra (NCYC 63) and *M. zobelli* (NCYC 783)	$5 \times 10^{8} - 10^{9}$	$1 - 5 \times 10^{7}$	$4 \times 10^{5} - 2 \times 10^{6}$	10^{4}

maximum volume of 5.0ml of each cell suspension was heat treated by immersing the tubes in a water bath, equilibrated at several temperatures and for various time periods. The temperature and duration period were determined which would kill the microbial suspensions. This was done by taking periodic 0.1ml samples of the treated suspensions and spreading 0.1ml on YMA and incubating at approx. 22°C for 48-72h to test for viable microorganisms. From these preliminary investigations a temperature of 65°C and 30 min exposure period was found to be suitable for sterilization.

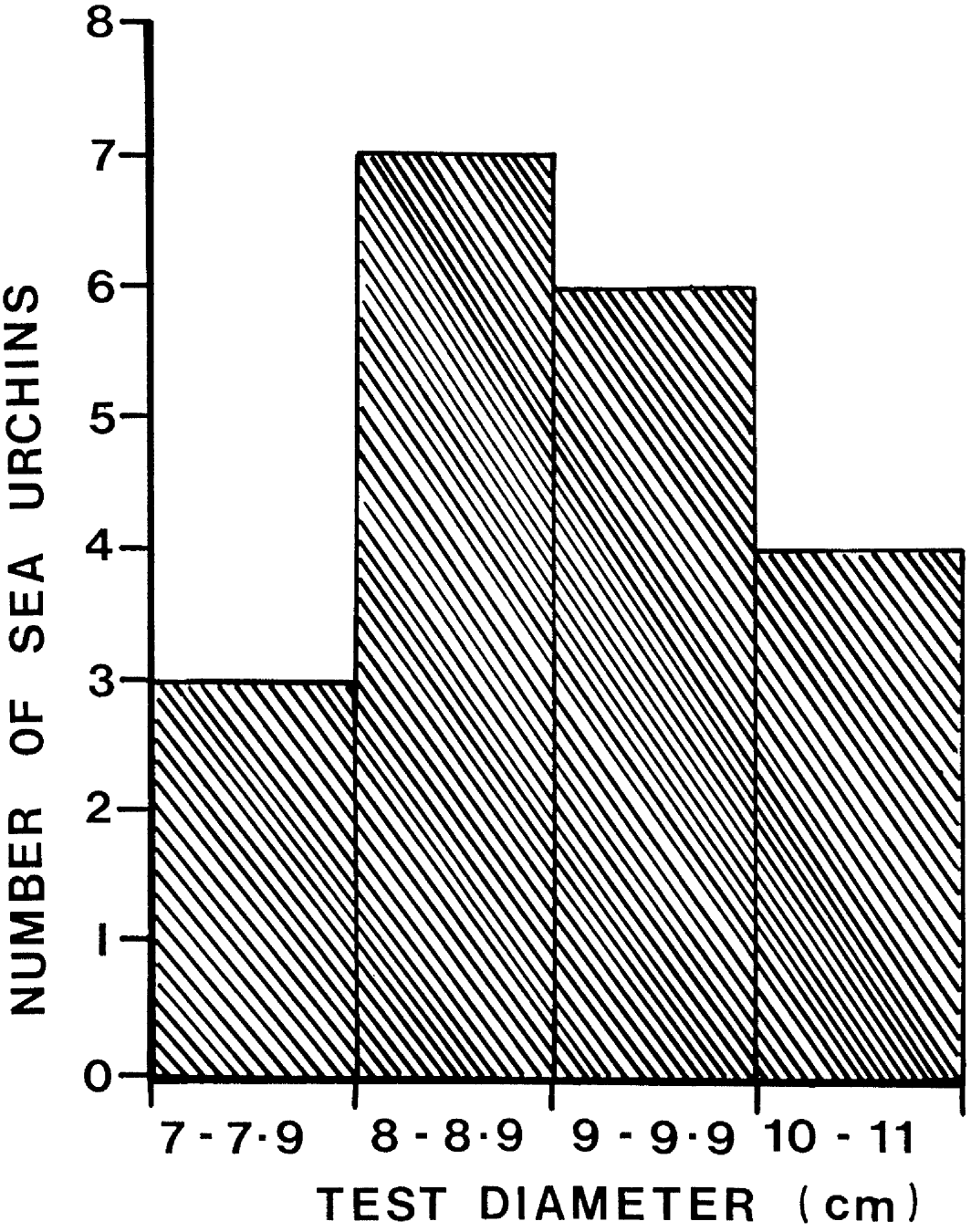
1.4.4. Diameter of *E. esculentus* selected for clearance experiments

Sea urchins of approximately 9cm in diameter (measured with Vernier calipers) were selected for whole animal experiments. This was borne in mind when collecting the specimens from Millport. Very large and very small (11 and 7cm) animals were rejected. A random selection of 20 specimens of *E. esculentus* from a typical delivery are presented as a histogram in Figure 15. The volume range of coelomic fluid found in these sized animals was 50 to 150 ml (average 100 ml).

1.4.5. The injection procedure

The animal to be injected was prepared by swabbing the peristomial membrane with 95% (v/v) ethanol and dried with a cotton wool bud. The standardized marine yeast suspension (live or heat-killed, Table 11) or sterile diluent (MBASW) was injected (1.0ml) into the coelomic cavity with a 25 gauge 15mm sterile needle and 1.0ml syringe assembly. The injected animals were placed in allocated positions in the "infection" tank, and control animals were placed both in the "infection" tank with the microbial-infected animals and in a "storage" tank with uninfected animals.

FIGURE 15. Histogram of the diameters (cm) of the widest part of the tests of twenty randomly-selected specimens of *E. esculentus* used in whole-animal infection experiments.


NUMBER OF SEA URCHINS
8
7
6
5
4
3
2
1
0
7 - 7·9
8 - 8·9
9 - 9·9
10 - 11
TEST DIAMETER (cm)

Typically 0.3ml samples of CF were taken from the experimentally-infected and control animals, via the peristomial membrane, at daily or weekly intervals. The 0.1ml volumes of undiluted or serially diluted fluid were spread onto both YMA (to recover injected yeasts) and MA (to detect background bacterial contamination or recovered injected bacteria). The CF from animals injected with heat-killed yeast suspensions was spread onto both agar types to ensure thorough heat treatment and to detect bacterial contamination.

The YMA and MA plates were incubated at room temperature for (approx. 22°C) 24 to 72h and the colonies counted.

1.4.6. External appearance of *E. esculentus* and treatment of dead animals

The external appearance of each specimen was noted before, during and after an experimental injection. Particular attention was paid to : attachment, tube feet display, test colouration, algal colonization, lesions, spine arrangement and denudation.

In some cases after a lethal dose of marine yeasts or bacteria, the animals were dissected. The coelomic fluid was drained from the coelom by slitting the peristomial membrane with a sterile scalpel and collecting the fluid in a sterile vessel held on ice. The calcareous test was cut in two beginning at the peristome and cutting with scissors towards the mouth. Selected organs, for example, the gonads, gut, test lining inner and outer lesion surfaces and internal peristomial membrane were examined for the presence of injected microorganisms and bacterial contamination. This was done by homogenizing, swabbing, or washing the organs in small volumes of sterile MBASW and spreading 0.1ml volumes of undiluted and serially diluted

samples on to both YMA and MA. All plates were incubated at approx. 22°C for 24 to 72h.

1.4.7. Enumeration of coelomocytes

1.4.7.1. *Calibration of Coulter counter*

The Coulter counter model "FN" (Coulter Counter Electronics Ltd., High Street South, Dunstable, Bedfordshire, England) was flushed through with ASW ("Tropic Marin^{R}") which was prefiltered through a 0.45µm pore size filter before use to remove debris and therefore minimize the background interference count.

Whole coelomic fluid containing 14mM EGTA anticoagulant (1.8ml CF and 0.2ml 140mM EGTA) was used to calibrate the Coulter counter. A 0.1ml volume of this was diluted in 19.9ml filtered "Tropic Marin^{R}". A volume of 20ml was routinely used in a plastic Coulter counter vessel which could be capped to prevent contamination of the sample with dust and debris. This contained the coelomocyte suspension to be counted.

To calibrate the instrument and to obtain the threshold setting value (T), the attenuation setting (B, 0.707) and aperture current (D, 8.0) (values recommended for counting mouse red blood cells), were kept constant whilst the threshold setting control was altered from 0 to 5 in increments of 2 and the Coulter count recorded in each case. This allowed selection of a threshold setting from the centre of the "threshold plateau" suitable for counting sea urchin coelomocytes.

1.4.7.2. *Background count*

To minimize background interference the system was routinely flushed through with filtered "Tropic Marin^{R}" several times before use. The background count was then recorded for the filtered ASW in triplicate, the average value of which was subtracted from the count obtained for the test sample.

1.4.7.3. *Total coelomocyte count*

Samples of CF were taken from specimens of *E. esculentus* maintained in recirculating artificial-seawater aquaria (RASWA). Whole CF samples or CF fractions separated by density gradient centrifugation (0.1ml volumes) were diluted 1:200 in filtered, chilled "Tropic Marin^{R}" and 20ml volumes counted in triplicate with the settings; attenuation (B: 0.707), aperture current (D: 8.0) and the threshold setting, T as determined above (1.4.7.1.). Since the volume occupying the U-tube of the Coulter counter was 0.5ml, the coelomocyte counts were corrected by a factor of 2 and the dilution factor to obtain the number of coelomocytes per millilitre.

The counts obtained in the above method were compared with those of the Neubauer counting chamber. The coelomocytes were diluted 1:10 in filtered "Tropic Marin^{R}".

The number of coelomocytes per 5, 16-small square blocks were counted, and the counts converted to coelomocytes per ml; if there are n cells per smallest square the number of cells per ml of suspension = $4n \times 10^{6}$.

2. SEA URCHINS: THEIR MAINTENANCE AND USE AS EXPERIMENTAL ANIMALS.

2.1. Recirculating Artificial-Seawater Aquaria

Recirculating artificial-seawater aquaria (RASWA) were established using the guidelines on general marine aquaria (Mills, 1985) and water quality management (King and Spotte, 1974; Committee on Marine Invertebrates, 1981).

Three RASWA were set up as described below:

2.1.1. Equipment

A list of the equipment and materials used, and the supplier of each item are listed in Table 12.

The aquarium tanks were inert, non-toxic (seawater is highly corrosive and can leach out toxins that might kill or debilitate the animals) containers ("Bellco^{R}") made from tough opaque, polyropylene of dimensions 47 x 47 x 47 cm and with a "working" capacity of approximately 76l. An aquarium with its fittings is shown in Figure 16. In each tank two plastic filter bases ("Interpet^{R}") were cut and fitted to the entire tank floor, and an airlift tube inserted. The filter-base was then covered with a depth of 25-50mm coral sand ("Underworld^{R}"). The design of the undergravel filter was such that the weight of the coral sand and water sealed the outer edge of the filter plate to the bottom of the aquarium, thus enabling only filtered water to enter the subgravel area via the slots in the filter plate (Figure 17). The plastic filter bases were ribbed for added strength to support the gravel and water. Sealing the edges of the filter plate accomplishes two functions : a) eliminates dead areas where

TABLE 12. Sources of equipment and supplies for recirculating artifical-seawater aquaria (RASWA).

Equipment/Materials	Description	Name & address of supplier or manufacturer
Aquarium Tank	Opaque polypropylene casing. Dimensions 47 x 47 x 47 cm	"Bellco^{R}, U.S.A. or, WCB Mailbox Ltd., Bayley Street, Stalybridge, Cheshire, SK15 1QQ
Plastic Filter Bases	Constant velocity sub-gravel no. 5, size 590 x 285 mm (2 needed per tank)	"Interpet^{R}", Dorking, Surrey
Coral Sand	1 1/2 mm grain size Depth 50 mm (2") (4.5K)	M & R (Dog-Fish) Shop, 466, Paisley Road West, Glasgow, G51
Power Filter Pumps	"Eheim^{R}" type 2011 51. Filter capacity: 80 - 150 l	M & R (Dog-Fish) Shop, 466, Paisley Road West, Glasgow, G51
Charcoal Filter Refills	"Eheim^{R}" Ehfikarbon 250110	M & R (Dog-Fish) Shop, 466, Paisley Road West, Glasgow, G51
Filtration Wool and Polymer Wool Sheets	"Interpet^{R}" re-useable polymer wool	M & R (Dog-Fish) Shop, 466, Paisley Road West, Glasgow, G51
Thermostatic Water Heater	"Atlantis^{R}" T250 "Sicce^{R}" 250 Watt, 220/240 V	Atlantis Customer Services Dept., Thomas's, Pelton Lane, Halifax
Artificial Seawater	"Tropic Marin^{R}" Artificial salts, 4Kgs per 120 l	Dr. Biener, Aquarientechnik, 6423 Wartenberg, Germany

TABLE 12. (continued).

Equipment/Materials	Description	Name & address of supplier or manufacturer
Hydrometer	"SeaTest^{R}"	Aquarium Systems, 8141 Tyler Boulevard, Mentor Ohio 44060, USA
Aquarium Maturation	"Sea Mature^{R}" inoculation concentrate	Waterlife Research Industries Ltd., Bath Road, Longford, Middlesex. UK
Seawater pH Buffer	"Sea Buff ^{AN}" pH buffer crystals and "Sea Buff" liquid	Waterlife Research Ltd., Heathrow, U.K.
Nitrite and pH Test Kits	"TetraTest^{R}" rapid indicator test solutions	Tetra Werke, Dr. rer. nat. Ulrich Baensch GmbH, D4520 Melle 1, West Germany
Aquarium Lids and Aquarium Dividers	0.5 cm thick perspex sheets	Glenaden Plastic Co. Ltd., 230 London Road, Glasgow G40 1PD

FIGURE 16. Diagram showing the equipment and the direction of water flow in a recirculating artificial-seawater aquarium (RASWA).

a. Artificial-seawater

b. Electric power filter pump

c. Transparent perspex lid

d. Water aeration spray

D. Direction of water flow

e. Plastic filter base

f. Coral sand layer

g. Polymer wool sheet

h. Thermostatic water heater

i. Light source (60 Watt)

k. Thermometer

l. Plastic airlift tube

D
D
D
D
D
D
a
b
c
d
e
f
g
h
i
k

FIGURE 17. Construction of biological filter bed at the base of RASWA.

a. Mounted air lift tube

b. Undergravel filter plate

c. Coral sand layer (25-50mm)

D. Direction of water flow

a
D
b
D
D
c

anaerobic bacteria can proliferate, and b) prevents gravel from working under the gravel plate and therefore impeding water flow. The calcium-rich coral sand layer, when mature, formed the biological filtration system and pH stabilizer on which the success of the aquarium was largely dependent.

Electric filter pumps ("Eheim^{R}") were fitted with coarse plastic mesh ("Eheim^{R}"), polymer wool("Interpet^{R}") and pre-washed charcoal filters ("Eheim^{R}") which were employed for the biological, chemical and mechanical filtration of the recirculating seawater. This power-filter was of the external variety with the motor and centrifugal pump mounted on the top. It delivers a water flow rate of 270 l h^{-1}. The filter medium was placed in the canister body according to the diagram shown in Figure 18. The filter removed suspended matter from the aquarium water as it passed through. One of the filter media used was polymer fibres, and to prevent clogging of the fibres a "prefilter" medium in the form of plastic filter mesh was placed ahead of the floss in the water flow as shown in Figure 18. Activated charcoal was used to remove dissolved wastes from the water by adsorption. This was contained in a nylon mesh bag to prevent it being drawn into the aquarium. The filter materials were renewed and replaced regularly about every 6 weeks to prevent redissolving of filtered debris back into the water. The polymer wool and charcoal materials were changed on alternate occasions since nitrifying bacteria also colonize them and therefore require time to recolonize new material.

The pump was positioned so that at all times the top of the filter was at least 15cm below the aquarium water level. The recirculated filtered seawater was sprayed onto the surface of the aquarium, maintaining maximum oxygenation, by surface agitation, and thereby aerobic conditions for the bacteria of the biological filter bed. The oxygenation saturation of

FIGURE 18. Packing plan of power filter-pump, incorporating biological, chemical and physical filtration material.

a. Pump head

b. Filter bowl

c. Pressure side hose connection

d. Upper lattice screen

D. Direction of water flow

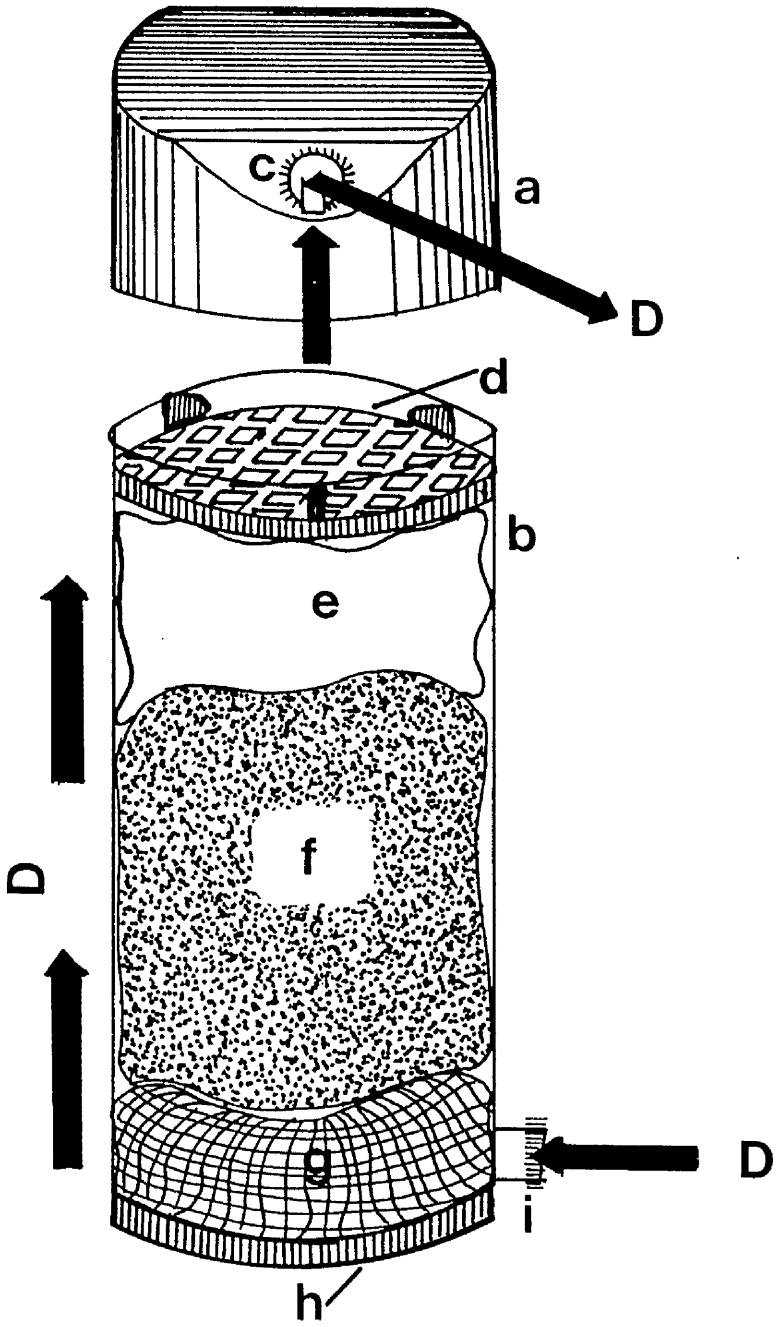
e. Polymer wool "Ehfisynth^{R}" removal of smaller particles of suspended material and bacterial colonization.

f. Activated charcoal contained in nylon mesh "Ehfikarbon^{R}" removal of coarse suspended material and adsorption of toxic materials and bacterial colonization

g. Coarse plastic mesh, "pre-filter" medium.

h. Lower lattice screen

i. Suction side hose connection


c
a
D
d
b
e
D
f
g
D
i
h

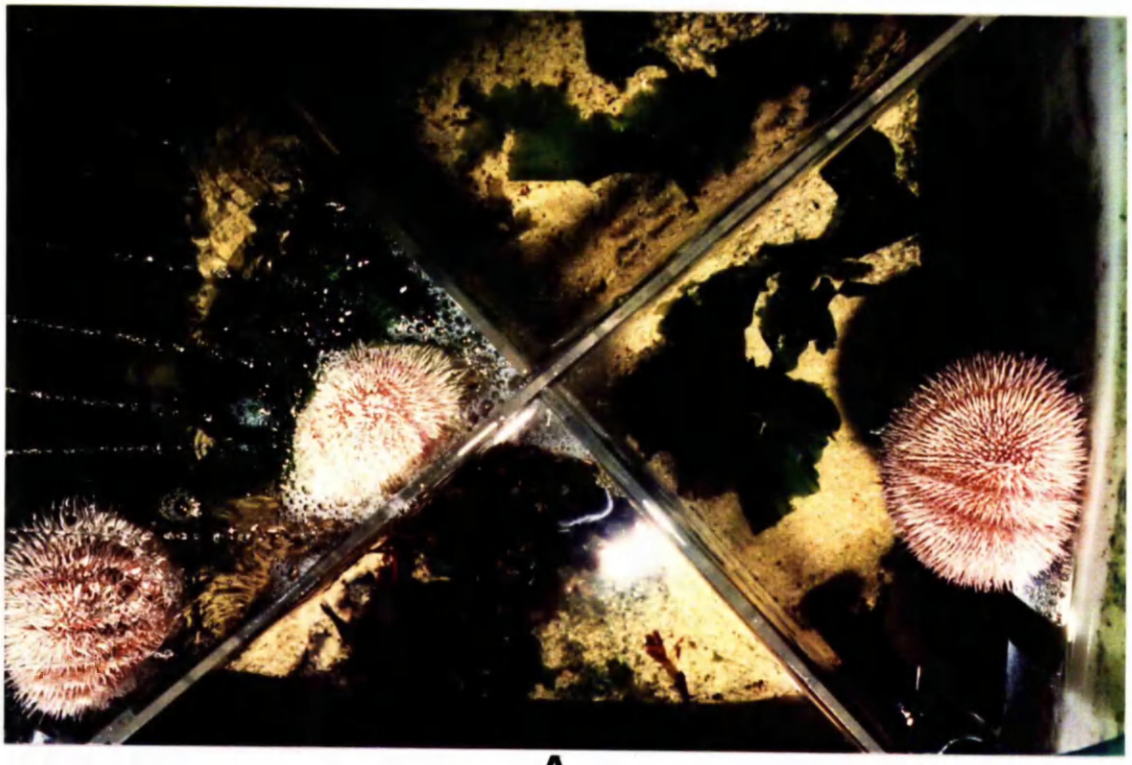
seawater with a salinity of between 28 to 35 ‰ is between 6 and 6.5ml^{-1} (Appendix 2). The aquarium water was maintained at a constant temperature 10 ± 2°C by a thermostatically controlled 150 Watt heater ("Sicce^{R}"), the aquaria themselves being located in a refrigerated room at 8°C (range 4 to 12°C).

The thermostatic heater was in the form of a submersible heating coil mounted in a tubular-glass container with a neon power indicator. Heaters selected were constructed of salt water-tolerant materials such as plastic, since rubber would perish rapidly and metal was avoided due to corrosion and possible contamination of the water. An allowance of 2 Watts of heat per litre was made (150W "Sicce^{R}"), (Mills, 1985). The heater units were fixed vertically to the tank wall by the means of plastic suction caps positioned on the tube at two places recommended by the manufacturers. The heater was submersed to the recommended level and mounted clear of the coral sand layer to prevent local boiling action which would crack the thermostatic heater casing.

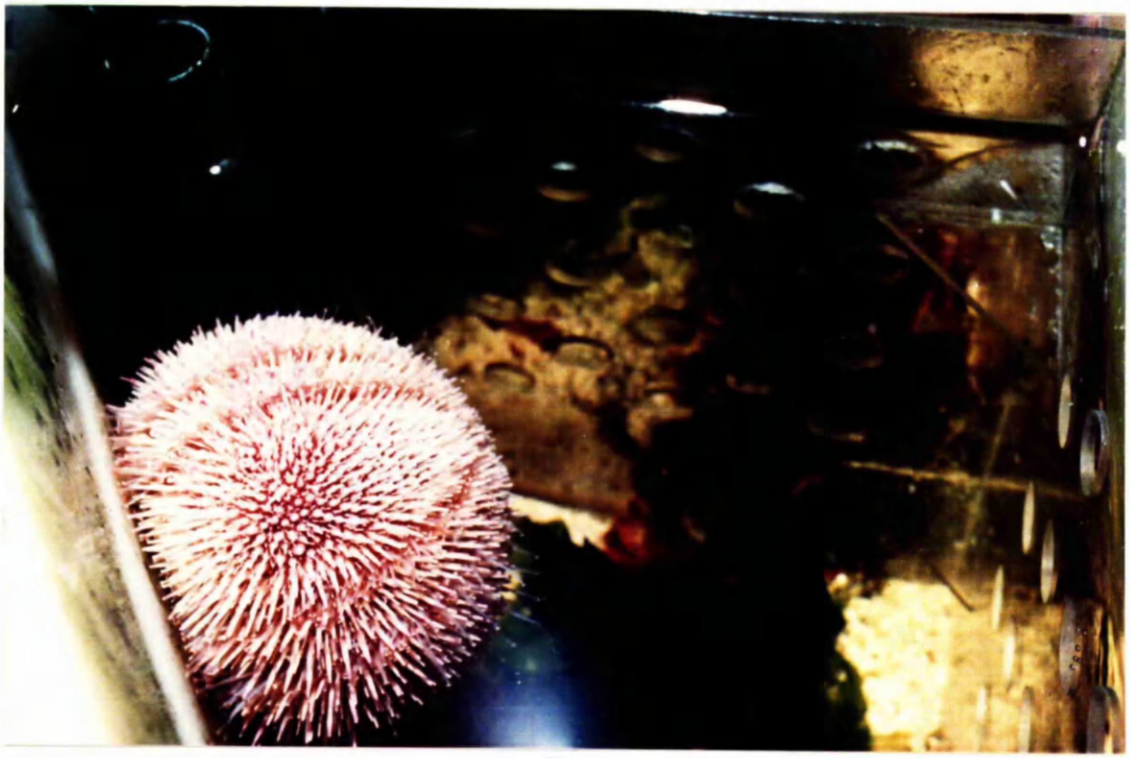
Each aquarium was illuminated with a 60 Watt tungsten lamp on an electrical time switch set to provide 14h of light per day between 1st April and September 30th and 12h of light daily during the rest of the year. The lamps were positioned at no less than 30cm from the water surface, to minimize heating effects. The aim of the lighting was to allow some growth of algae which was considered desirable for water purification and feeding for the *E. esculentus*.

In experiments where individual sea urchins had to be kept apart, the tanks were partitioned with 5mm thick perspex dividers in which 2.2cm holes were made (so as not to retard water circulation) as shown in Plates 2A and B. Each aquarium was fitted with a perspex lid to reduce evaporation.

PLATES 2A and B. Perforated perspex dividers installed in aquaria to separate individual, or pairs of *E. esculentus*.



A



B

2.1.2. Preparation of artificial seawater

Artificial-seawater of approximately the correct salinity (35%) was prepared by adding 0.5Kg of "Tropic Marin^{R}" to 15l of freshly distilled water, in a 25l plastic container. When first setting up the aquaria complete packs of "Tropic Marin^{R}" salts were dissolved. This ensured that all the necessary trace elements were present in the preparation. The salts were dissolved by thorough mixing, and the specific gravity measured with a "swing-needle" unit hydrometer "Sea Test^{R}" (accurate to $\pm$ 0.001 AF unira) as shown in Plate 3. The ASW was prepared at room temperature (approx. 22°C) to a specific gravity (SG) of between 1.0200 to 1.0240 (salinity 28 to 36‰). Figure 19 shows the relationship between the specific gravity, selected temperatures and salinity. The SG was increased by the addition of "Tropic Marin^{R}" or decreased by the addition of distilled water. To obtain accurate results, the hydrometer was filled to the line marked and care taken to prevent attachment of air bubbles to the plastic swing needle.

To fill an aquarium, the freshly-prepared artificial seawater was poured into the tank slowly so as not to disturb the coral sand layer. This was best achieved by pouring the water onto a saucer resting on the substrate, the overflow gently filling the tank without causing any disturbance. Approximately 76 l, made in 25 l batches as described, were added to the two 80 l tanks and 72 l to the 75 l tank. The exact composition of "Tropic Marin^{R}" was not given on request to the manufacturers, except to say the salt mixture contained materials of the highest purity with 70 trace elements present.

PLATE 3. "Sea Test[R]" swing-needle unit hydrometer for measurement of specific gravity of artificial-seawater solutions.

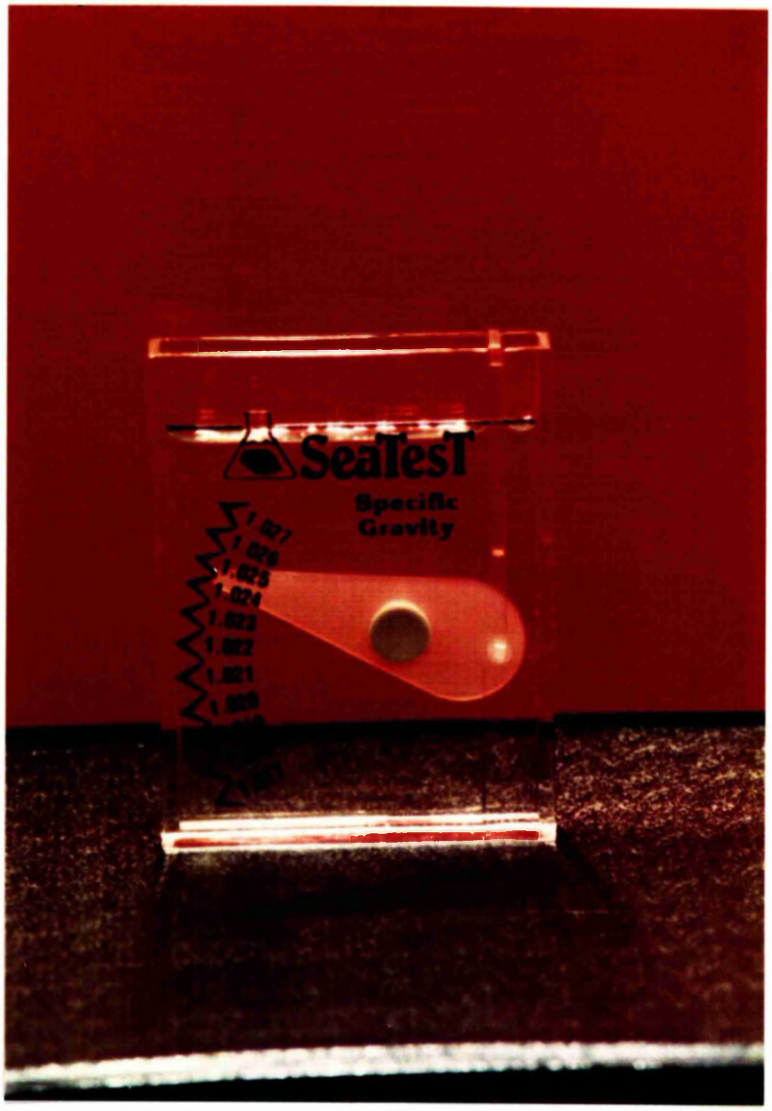
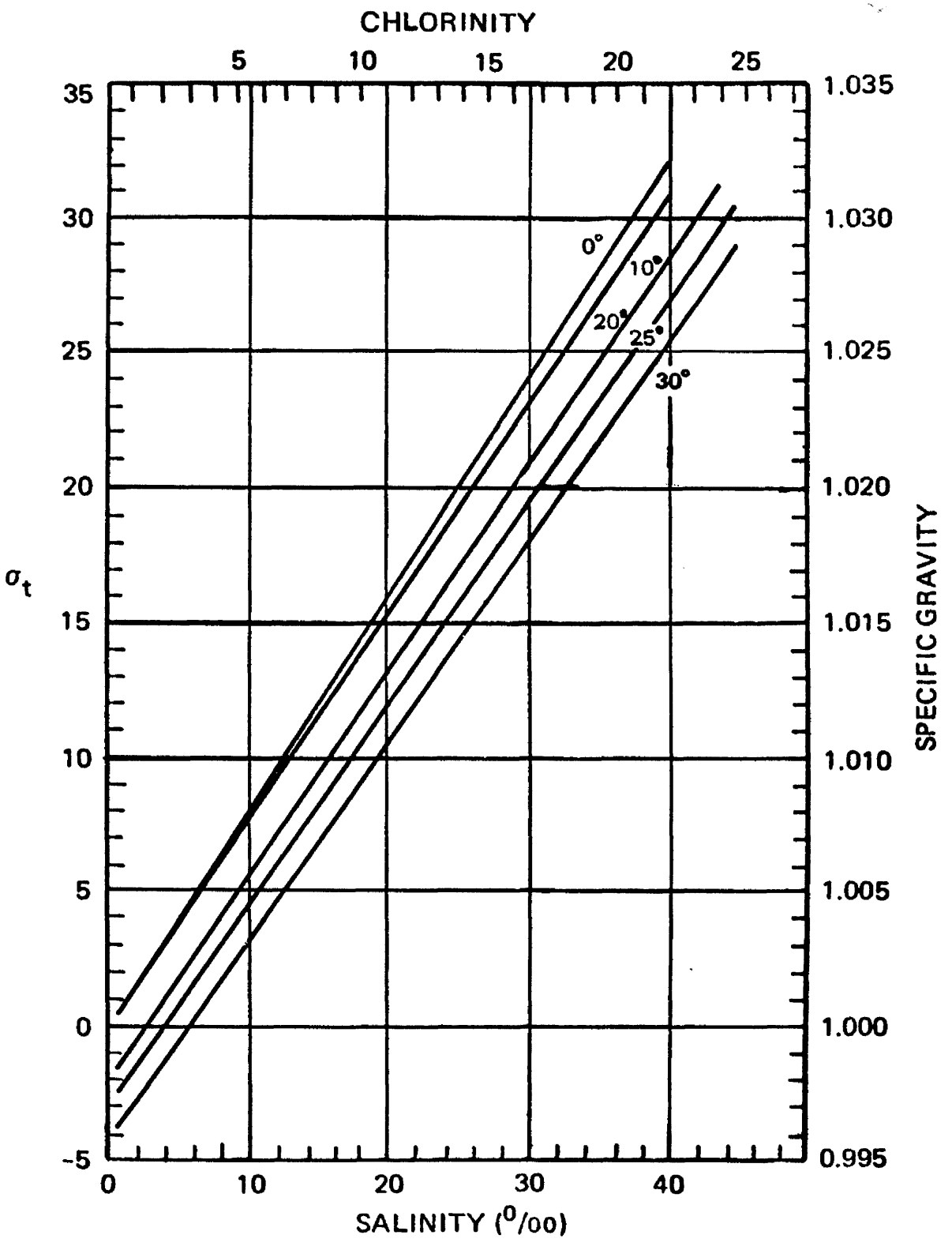

SeaTesT
Specific
Gravity

FIGURE 19. The relationship between the specific gravity salinity (‰) and chlorinity of artificial-seawater at selected temperatures.

(from Committee on Marine Invertebrates, 1981).


CHLORINITY
5
10
15
20
25
35
30
25
20
15
10
5
0
-5
σ_t
1.035
1.030
1.025
1.020
1.015
1.010
1.005
1.000
0.995
SPECIFIC GRAVITY
0°
10°
20°
25°
30°
0
10
20
30
40
SALINITY (°/oo)

2.1.3. Water quality management

2.1.3.1. *Temperature*

The temperature of the water was taken daily with an ordinary mercury thermometer and adjustments made, if required, to the thermostatic heater +/- control to maintain the temperature at 10 ± 2°C. The temperature varying a few degrees on either side of that pre-set on the thermostat did not harm the sea urchins since they have a reasonable tolerance to temperature change. They are able to tolerate moderate extremes as long as the upper or lower limits are arrived at gradually.

2.1.3.2. *Specific gravity*

The specific gravity (SG) of the aquarium water at 10°C was measured with a hydrometer to within ± 0.001, and maintained within the range 1.0200 and 1.0240 by the addition of freshly distilled water, to replace that lost by evaporation, or by the addition of "Tropic Marin^{R}" when the SG fell below 1.020 during a large partial water change, for example. An SG of 1.0200 to 1.0240 at 10°C corresponds to a salinity range of 26 to 32‰. Partial water changes of up to 25% water volume were made routinely at approximately monthly intervals, to replace depleted minerals and to dilute toxic materials which might have accumulated. Larger water changes of up to 80% of the total volume were made after sea urchins died or had spawned.

2.1.3.3. *pH*

The pH was measured at least weekly with a salt water pH test kit "Tetra Test^{R}" which contained a rapid colour indicator. The test vial was

filled until the bottom line of the meniscus was even with the 5ml line, 7 drops of indicator were added, gently shaken and the test compared with the colour card. This measured the pH of between 7.5 and 9.0 to 0.3pH units in the middle of the scale. The "Tetra Test^{R}" kit was limited to measurement by five indicator colours at pH's of 7.5, 8.0, 8.3, 8.6 and 9.0. The pH's between these values could not be measured accurately due to the limitations of the indicator kit.

Two pH buffer solutions "Sea Buff" and "Sea Buff^{AN}" were periodically added to each aquarium. "Sea Buff^{AN}" is a pH buffering compound used for replacing the anionic reserve materials which are continually lost from captive seawater and cause a falling pH. "Sea Buff" replaces cationic reserve elements lost from closed-circuit seawater. A quarter of a teaspoonful of "Sea Buff^{AN}" or 5 drops of "Sea Buff" per 45l seawater was added and the pH rechecked after 4h, additional "Sea Buff" was added if required. Both of these products were added to each aquaria, but not within the same 24h period.

2.1.3.4. *Nitrite (NO_2^-)*

The nitrite (NO_2^-) level of the water was also measured at least weekly, and more often if necessary, by the means of a NO_2^- test kit ("Tetra Test^{R}"). The desired level of NO_2^- (mg l^{-1}) is $\leq$0.33mg l^{-1} as found in natural seawater. The kit contained a colour indicator which allowed readings of between $\leq$0.1mg N l^{-1} and 1-10mg N l^{-1}. The test vial was filled to 5ml with water to be tested. Seven drops of reagent 1 were added, gently shaken and 10 s later seven drops of reagent 2 added and again shaken. After 2-5 min the colour indicator was compared with the

colour chart. Conversion of the scale readings from nitrite (N) to nitrite content (NO_2^-) in mg l^{-1} were made by multiplying the readings by 3.3. The "Tetra Test^R" kit produced four indicator colours in the presence of $\leq$0.33, 0.495, 1.65 and 3.3 - 33mg l^{-1} (NO_2^-). The indicator kit was not capable of reading levels between these values. The main causes of high nitrite levels ($\leq$0.495mg l^{-1}) may be attributed to the following: overcrowding; overfeeding; presence of putrefying dead animals; or the system has not matured, therefore no bacterial degradation activity. The problem may be reduced by : a partial water change; removal of animals; reduce/stop feeding until biological filtration system has matured; addition of "Sea Mature^R" providing nitrite does not exceed 0.495mg l^{-1}.

2.1.3.5. *Microbial viable counts*

Samples of the water from each aquarium were taken about every fortnight and the bacterial and yeast viable counts determined by spreading undiluted and serially diluted samples (0.1ml) on MA and YMA respectively, and incubating at room temperature (approx. 22°C) for 48 to 72h and the colonies counted.

Table 13 shows the schedule for biological, chemical and physical sampling of the aquaria, together with the desired ranges or limits.

TABLE 13. Schedule of times for routine maintenance sampling and desirable limits of water quality for each RASWA.

Measurement	Frequency of measurement	Desirable limits
Temperature °C	Daily and after partial water change, or the introduction of sea urchins into the aquaria.	10°C ± 2°C
Specific Gravity	Weekly and after partial water change.	1.0200 - 1.0240
pH	Weekly and more often on death of an animal or after partial water change.	8.0 - 8.3
Nitrite (NO_2^-)	As above	$\leq$0.33 - 0.495 mg l^{-1}
Microbial Viable Count (ml^{-1})	Fortnightly and after the initiation of infection experiments.	

2.1.4. Collection and maintenance of *E. esculentus*

2.1.4.1. *Collection*

Specimens of *E. esculentus* were obtained from the University Marine Biological Station, Millport, Isle of Cumbrae, KA28 OEG. The entire process of collection from the sea bed, transport to Glasgow and transfer to the aquaria was usually accomplished within two hours.

The specimens were carefully handpicked from the sea floor beside the Keppel Pier, Millport, by a SCUBA diver. They were collected in a bucket, transferred to a 50l carboy containing fresh seawater and given minimal exposure to the air during the transfer procedure. A maximum of ten animals per vessel were transported to prevent overcrowding and consequent spine damage and trauma. A sealed plastic bag containing a block of ice was floated on the water surface of each carboy and the drum lid firmly closed. This maintained the water temperature at, or below, 10°C during the Summer months.

The sea urchins were transported by road, and on arrival at the laboratory (The Alexander Stone Building, Bearsden, Glasgow) about 35 miles from Millport, they were removed from the side of the carrier vessel by very gently rocking from side to side to encourage tube feet retraction and therefore, minimize damage. During the Winter period, the Clyde Estuary water temperature falls to as low as 6°C. Since the aquaria were maintained at 10 $\pm$ 2°C the drums containing the sea urchins were allowed to equilibrate to just below 10°C by standing at room temperature for about an hour before transfer of the animals to the aquaria.

2.1.4.2. *Distribution in aquaria*

A maximum of 12 sea urchins were placed in each of the 76l or 72l working-capacity tanks at any one time. This allowed more than 6l of water per specimen. The number of live sea urchins per tank was recorded daily and dead animals were removed promptly to minimize damage to the biological filters through overloading with organic materials.

2.1.4.3. *Feeding*

Fresh seaweeds, such as *Ulva lactuca* and *Laminaria digitata*, were supplied with each delivery of sea urchins and they were fed *ad libitum*. The animals seemed mainly to use the weeds for the characteristic "covering reaction" displayed by sea urchins in the *marine milieu*, although there was evidence of the weeds being eaten also. This was noticed as ridges on the surface of the seaweeds caused by scraping by the teeth of the sea urchins when attempting to rasp encrusting byrozoans from the surface of the seaweeds. The animals also fed on the algal growth on the walls of the aquaria.

R E S U L T S

1. MAINTENANCE OF *ECHINUS ESCULENTUS* IN RECIRCULATING ARTIFICIAL-SEAWATER AQUARIA

1.1. Preliminary Observations

The finally adopted method for successful maintenance of *Echinus esculentus* in recirculating artificial-seawater aquaria (RASWA) was developed only after many months of trial and error. In early attempts to keep sea urchins in the laboratory, it was common for every animal in the group to die within 24h of placement in the tank (Figure 20B, days 1-10). However, this initial system differed from that described in Materials and Methods (2.1) in the following respects :

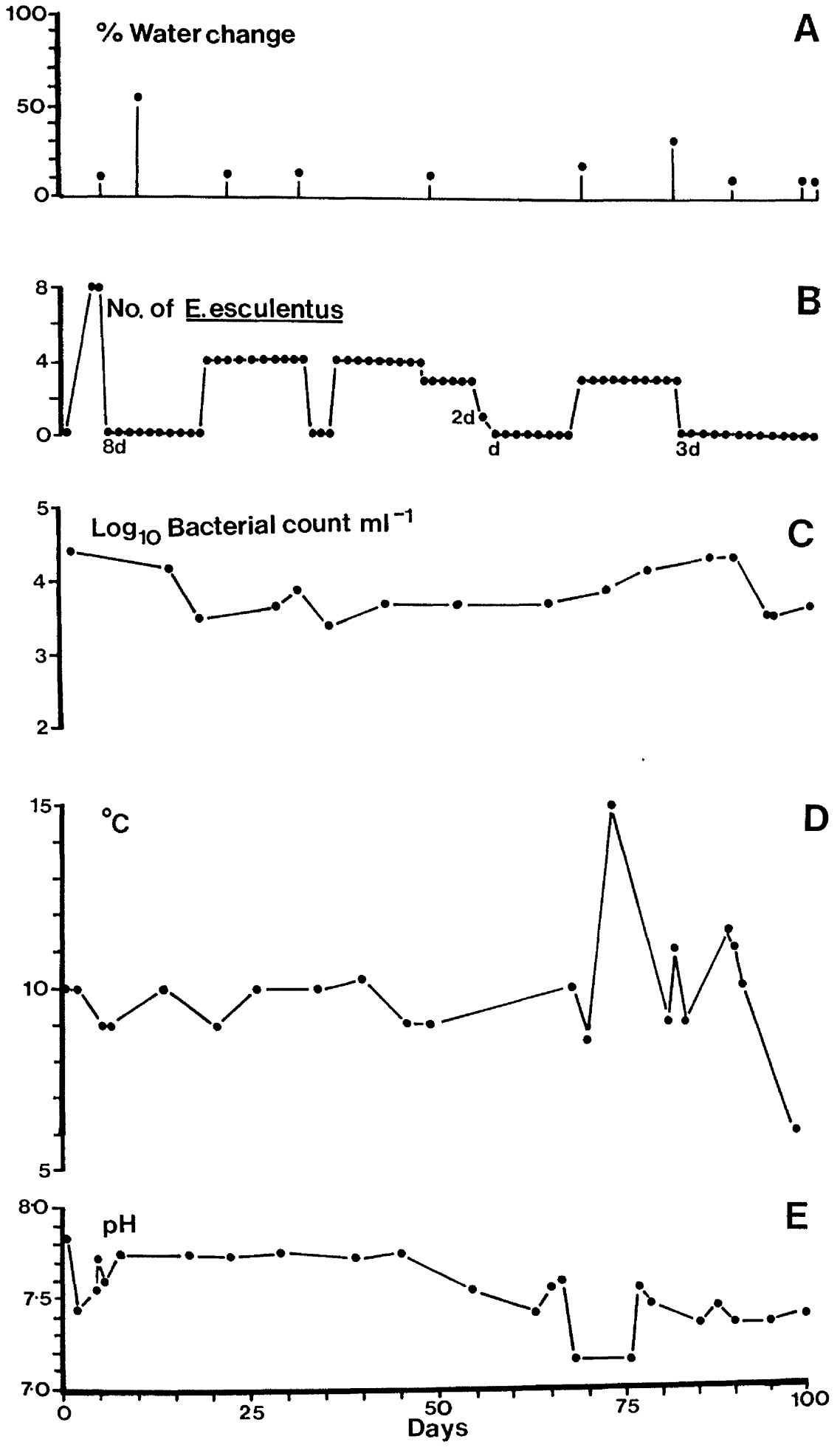
a. An external refrigeration system (Grant Instruments Ltd., Barrington, Cambridge) was employed to cool the recirculating-water before returning to the main body of water in the aquarium. The filtered water from the power-pump was piped through 2.3cm plastic tubing of approx. one and a half metres in length, which was in turn accommodated in the external refrigeration unit (Appendix 3). Adjusting the cooler thermostat between 0 and 5°C allowed maintenance of the recirculating aquarium water at 10 ± 2°C. However, because the whole apparatus was housed in the laboratory at room temperature (approx. 22°C) this sytem was unreliable because of occasional electrical failures and freezing of the water in the cooling system which was replaced by commercially available car antifreeze.

b. Use of "Sea Salt^{R}" (Griffin and George Ltd., Gerrard Biological Centre, Worthing Road, East Preston, West Sussex, BN16 1AS) instead of "Tropic Marin^{R}" used routinely in the final system.

FIGURE 20. Five routinely-measured water parameters, recorded over a 100-day period, in the preliminary aquarium system for the maintenance of *E. esculentus*.

This differed from the system eventually developed in a) having an external refrigeration system, b) using smooth quartz pebbles rather than coral sand, and c) not monitoring SG or nitrite (see Appendix 3).

(d = Death of an animal)


100
% Water change
A
50
0
8
No. of E. esculentus
B
4
0
8d
2d
d
3d
5
Log10 Bacterial count ml-1
C
4
3
2
15
°C
D
10
5
8·0
pH
E
7·5
7·0
0
25
50
75
100
Days

c. Artificial sea salts were reconstituted by weight (36-38g l^{-1}) or all the contents of a whole 50 l pack, by dissolving all of the salts to a final volume of 50 l (the latter method was preferable since this ensured all trace elements were present in the resulting seawater, if only half a bag was used there was a chance some of the trace elements would be left in the packet) in laboratory tap water instead of in distilled water, and the specific gravity of the prepared solution was not measured. However, the manufacturers estimated a final specific gravity (SG) of 1.022 - 1.025 at 15°C, with the use of the above specified proportions (w/v).

d. Smooth, quartz pebbles of approximately 10mm diameter were used to give an approx. 6cm depth over the plastic filter base in the tank. This gravel filter bed was not "matured" by the addition of a source of biological filter bacteria and nutrients required for their development. Instead, the tank was allowed to recirculate for several weeks to allow stabilization (temperature and complete solution of the salts) of the system before introducing the sea urchins.

In attempting to improve the equipment and procedures several changes were next made :

a. "Sea Salt^{R}" was reconstituted by weight with distilled water in place of laboratory tap water. The latter was thought to be contaminated.

b. Smaller, smooth quartz pebbles of approx. 8mm in diameter ("Dorset Pea") were used of approx. 6cm in depth.

This gave a system in which 4 animals (plus several transient specimens) were successfully kept for over 3 months (Figure 20, days 10-100).

Reference to available literature (King and Spotte, 1974; Mills, 1985) suggested an improved aquarium could be produced by installing a better biological filter system to enhance the biological filter activity and filtration of the polymer wool and charcoal filter in the power filter pump outside the aquarium. This and other improvements were done by introducing the equipment and procedures as described in the Materials and Methods (2.1), of which the key elements are :

a. Use of an approx. 6.5cm layer of coral sand spread over a polymer wool sheet-covered plastic filter base, in place of the previously used gravel.

b. "Tropic Marin^{R}" artificial sea salt replaced "Sea Salt^{R}", and reconstituting the former in distilled water to a SG of between 1.020 - 1.024 at 10°C.

c. Routine measurement or control of the following : numbers of *E. esculentus*, partial water changes, bacterial viable count (ml^{-1}), SG, temperature (°C), nitrite (mg l^{-1}) measured with NO_2^- "Tetra Test^{R}" indicator kit and pH (electrical WPA digital pH meter later replaced by the more convenient "Tetra Test^{R}" pH indicator kits).

d. Two pH buffer preparations "Sea Buff^{R}", were added to replenish the depleted alkali reserve when the pH fell below 8.0.

e. The entire aquaria systems were housed in a refrigerated room at approx. 8°C (range 4 to 12°C) and thermostatically controlled heaters were installed in each aquarium, allowing a stable water temperature of 10 ± 2°C.

When the electricity supply failed, as happened occasionally, the water temperature increased at a slower rate, reducing the possibility of heat stressing the animals present in the tank (until the electrical problem was rectified).

f. "Sea Mature^{R}", an inoculation-concentrate, was added to the water in the aquaria to initiate the development of the bacteria in the biological filtration bed at the base of the aquaria.

1.2. Maturation of the Biological Filter

To produce a mature biological filter in an aquarium tank required :

a. Coral sand

b. "Sea Mature^{R}" (see Materials and Methods, Table 13)

c. A source of organic nitrogenous material e.g. a sea urchin

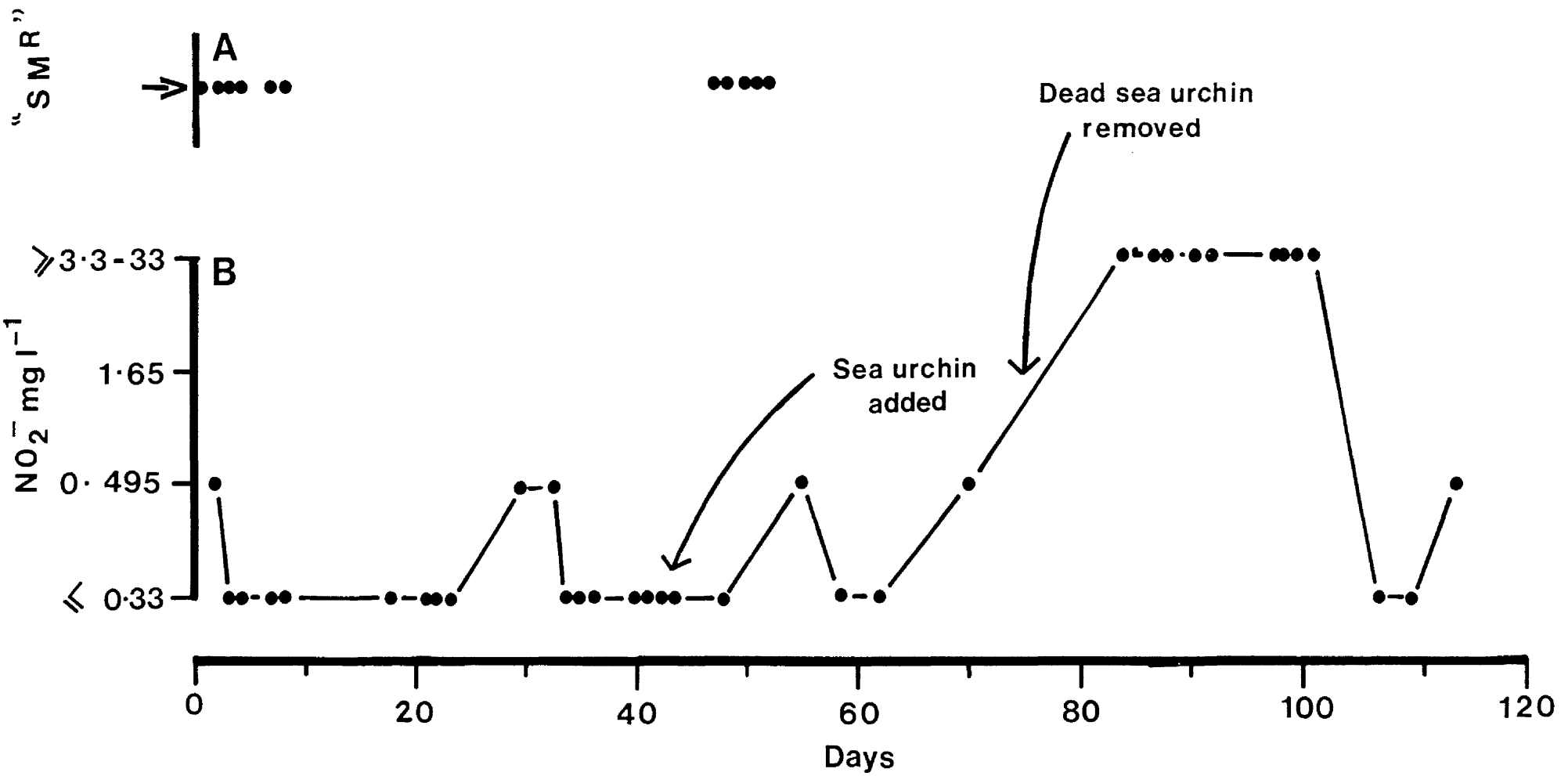
d. Maturation of "run-in" period

Figure 21 (B) shows the maturation of the biological filter by the ability of the tank to reduce nitrite rapidly. Thus, on day zero the "Sea Mature^{R}" ("SM^{R}") was added in the recommended amounts Figure 21 (A) and was added daily until the nitrite level of the water reached 0.495mg l^{-1} (as indicated by the "Tetra Test^{R}" NO_2^- colour indicator kit), as observed

FIGURE 21. Maturation of the RASWA biological filter bed over a 110-day period at 10 ± 2°C.

A. Record of additions of "Sea Mature^{R}" ("SM^{R}").

B. Recorded levels of nitrite (NO_2^-) and addition and removal of a sea urchin (organic source).


"SMR"
A
B
Dead sea urchin removed
Sea urchin added
≥3·3-33
1·65
0·495
≤0·33
NO_2^- mg l^{-1}
0
20
40
60
80
100
120
Days

about day 25. At about day 41 a further source of bacteria and nitrogenous organic material was added in the form of a healthy specimen of *E. esculentus* and further addition of "Sea Mature^{R}". The animal died at day 75 (Figure 21B) and the recorded nitrite level increased from the basal level of $\leq$ 0.33mg l^{-1} to 3.3 - 33mg l^{-1} NO_2^-. During the next 30 days the nitrite level returned to $\leq$0.33mg l^{-1} indicating activity of the biological filter bed. Typically an aquarium required about 15 weeks to reach a "mature" or "run-in" condition at 10 $\pm$ 2°C nitrite of $\leq$0.33mg l^{-1} and a pH of 8.3 (Appendix 4H) signifying "maturation" of the filter bed and suitability for the acceptance of fresh specimens of *E. esculentus* (Appendix 4). The maturation period of further biological filter beds was reduced by over two weeks by adding a small amount of "mature" filter bed to the new aquarium and/or maturing the bed at a higher temperature (e.g. 4 weeks at 15°C).

1.3. Routine Monitoring of Matured Aquaria

Plate 4A shows two of the 76 l aquaria tanks and Plate 4B, the inner view of one of these aquaria with the aeration spray (a), thermostatically controlled heater (b) and outflow tube (c) to the power filter pump (d) which can be seen at the far bottom right of the picture.

Each of the three aquaria, after their maturation period, was then put into routine use for supplies of fresh sea urchin coelomic fluid and the whole-animal infection experiments.

A programme of regular monitoring of each parameter was established. The results for three, two-year periods are shown in Appendices 5, 6 and 7.

PLATE 4A. Outer view of two 76 l recirculating artificial-seawater (RASWA).

PLATE 4B. Inner view of RASWA. Showing:

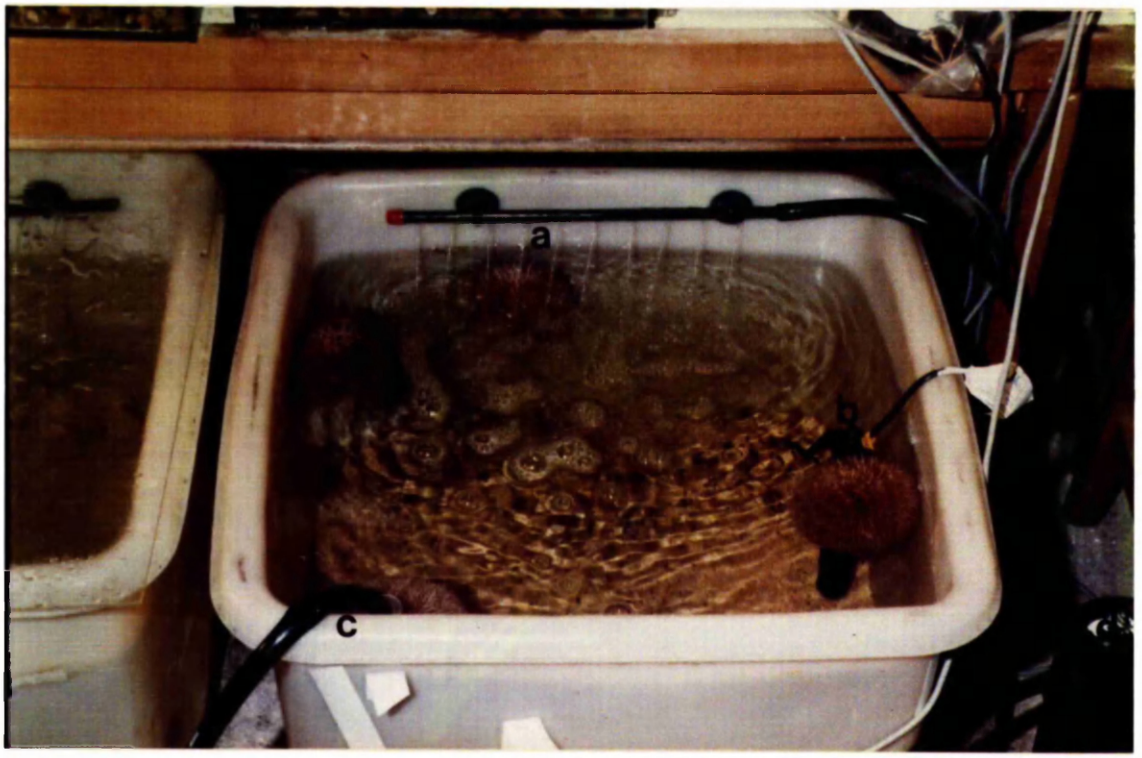
a. Aeration spray

b. Thermostatic heater

c. Outflow tube

d. Power filter—pump

A



B

1.3.1. Physical parameters : partial water changes, specific gravity and temperature

Partial water (ASW) changes of between 10-25% were done at approximately monthly periods and larger water changes, of up to 80% of the total volume, were carried out when animals died, (e.g. day 230, Appendix 5; day 240, Appendix 6) or on the occasion of spawning during the month of April (day 240, Appendix 7).

The SG of the water in each aquarium was maintained at about 1.0240 by the addition of fresh distilled water to replace that lost by evaporation, and the SG exceeded this value, or the additon of "Tropic Marin^{R}" when the SG fell below 1.0240 during partial water changes, for example.

The temperature was maintained at 10 $\pm$ 2°C by fine adjustment of the heater thermostat. The refrigerated room in which the apparatus was housed, was maintained at an average temperature of 8°C (range 4 to 12°C).

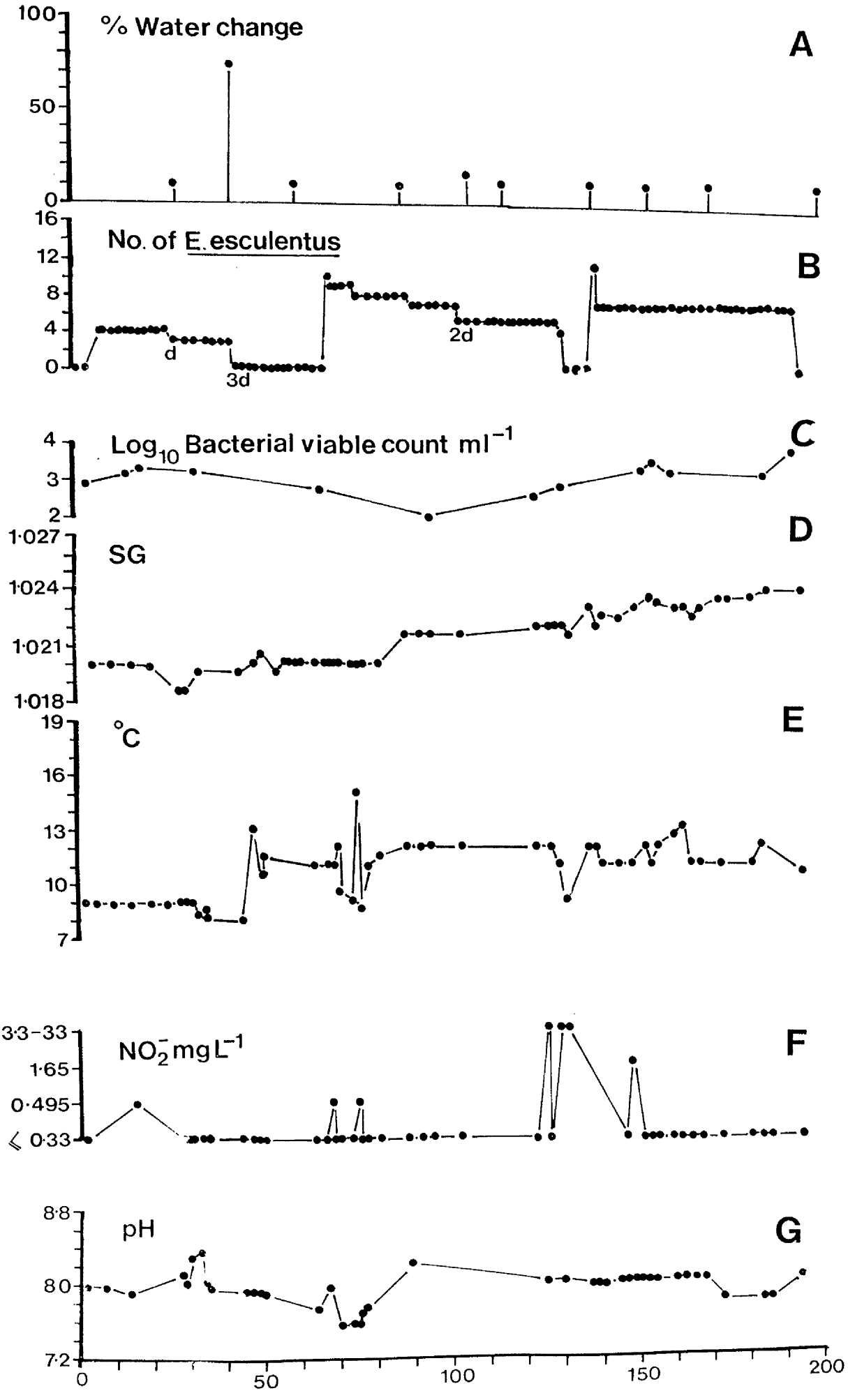
1.3.2. Chemical parameters : nitrite (mg l^{-1}) and pH

When one or more animals died or in the presence of moribund animals the nitrite level increased to greater than 0.495mg l^{-1}, and there was typically a fall in pH from 8.0 to 8.3 to 7.5 as shown in Figure 22 (B, F and G), day 80. (Appendix 5, day 230; Appendix 6, day 240; Appendix 7, day 230).

This pattern of rise in nitrite level and decline in pH was often masked due to several partial water changes immediately done on the detection of dead or moribund sea urchins and the additon of "Sea Buff^{R}" (see Materials and Methods, 2.3.1.3.) when the pH fell below 8.0.

FIGURE 22. Seven routinely-recorded measurements and observations over a 200-day period of an established RASWA aquarium tank C (76 l).

(d = Death of an animal)


% Water change
A
100
50
0
No. of E. esculentus
B
16
12
8
4
0
d
3d
2d
Log10 Bacterial viable count ml-1
C
4
3
2
SG
D
1·027
1·024
1·021
1·018
°C
E
19
16
13
10
7
NO2- mg L-1
F
3·3-33
1·65
0·495
< 0·33
pH
G
8·8
8·0
7·2
0
50
100
150
200

1.3.3. Biological : number of *E. esculentus* and bacterial viable count (ml^{-1}).

The number of sea urchins plated in each tank and mortalities were recorded (denoted d in Appendices 5, 6 and 7).

The bacterial viable count per ml of aquaria water was recorded on a regular basis to monitor possible changes as a result of animals present and sea urchin mortality. The change in the bacterial viable count (ml^{-1}) of the aquaria water was recorded over a 28-day period, during which time up to three sea urchins died within 12h as shown in Figure 23.

An increase of up to 50-fold was observed on the death of up to 3 sea urchins, although in some cases a net decrease of up to 10-fold was also observed.

1.4. Bacterial Viable Count of *E. esculentus* Coelomic Fluid

In the early stages of this investigation, difficulty was experienced in obtaining sterile-CF from freshly delivered *E. esculentus* for *in vitro* studies of antibacterial and antifungal activity. Consequently, the practice was established of routinely performing a sterility test on the CF of some of the sea urchins, by spreading 0.1ml of the CF on a 2216E marine agar plate.

The effect of maintaining sea urchins in recirculating artificial-seawater aquaria was examined by counting the number of colonies from 0.1ml of CF from a total of 147 *E. esculentus* (CF of which used for *in vitro* studies) which were kept for up to 84 days in RASWA. The data are shown in Figure 24 in the form of three histograms.

FIGURE 23. Effect of sea urchin mortality on the bacterial count of aquarium water. Viable counts were recorded over a 28-day period during which up to three sea urchins died within 12h periods. The solid line joining the median represents $\log_{10}$ change in bacterial viable count.

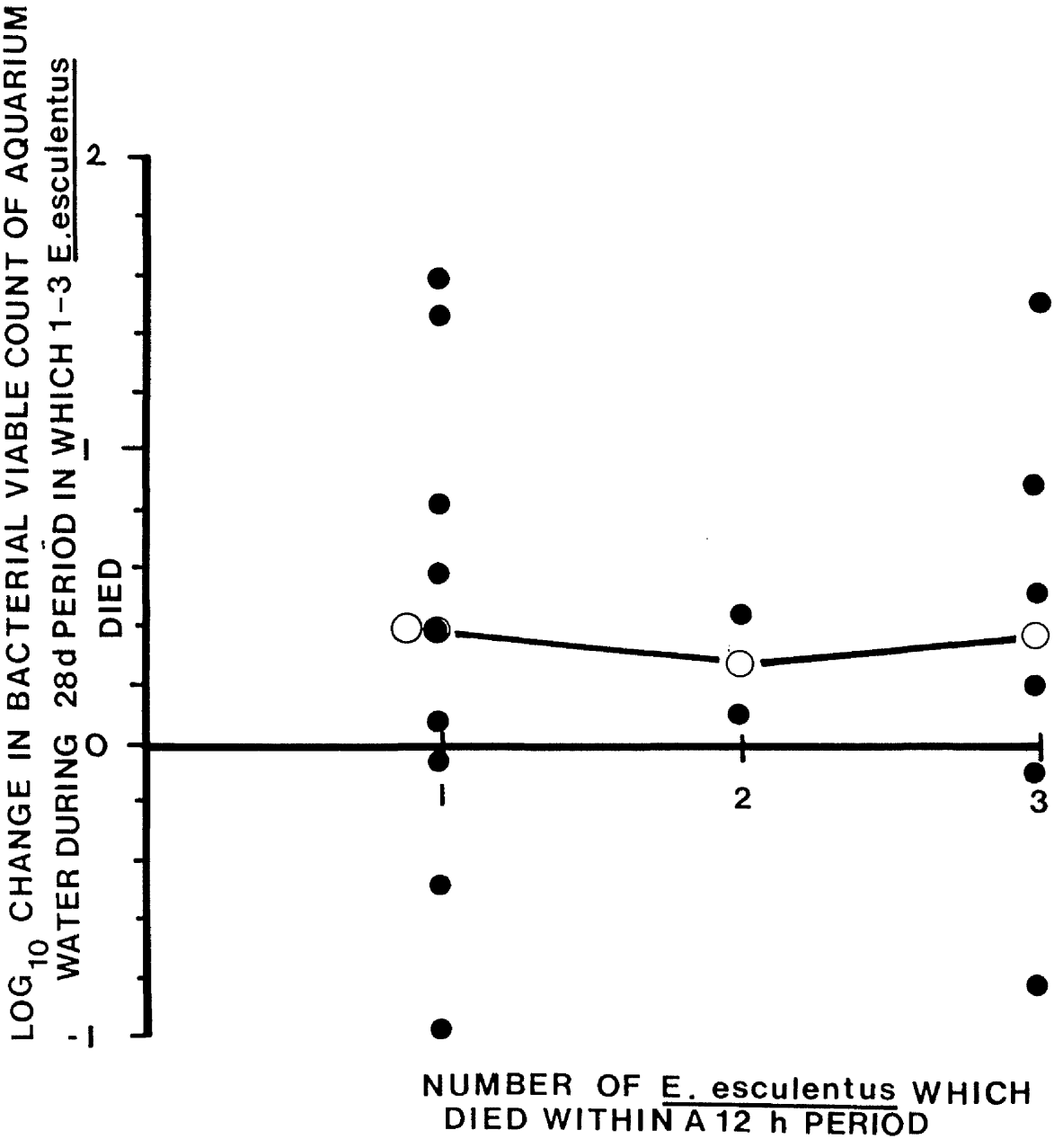
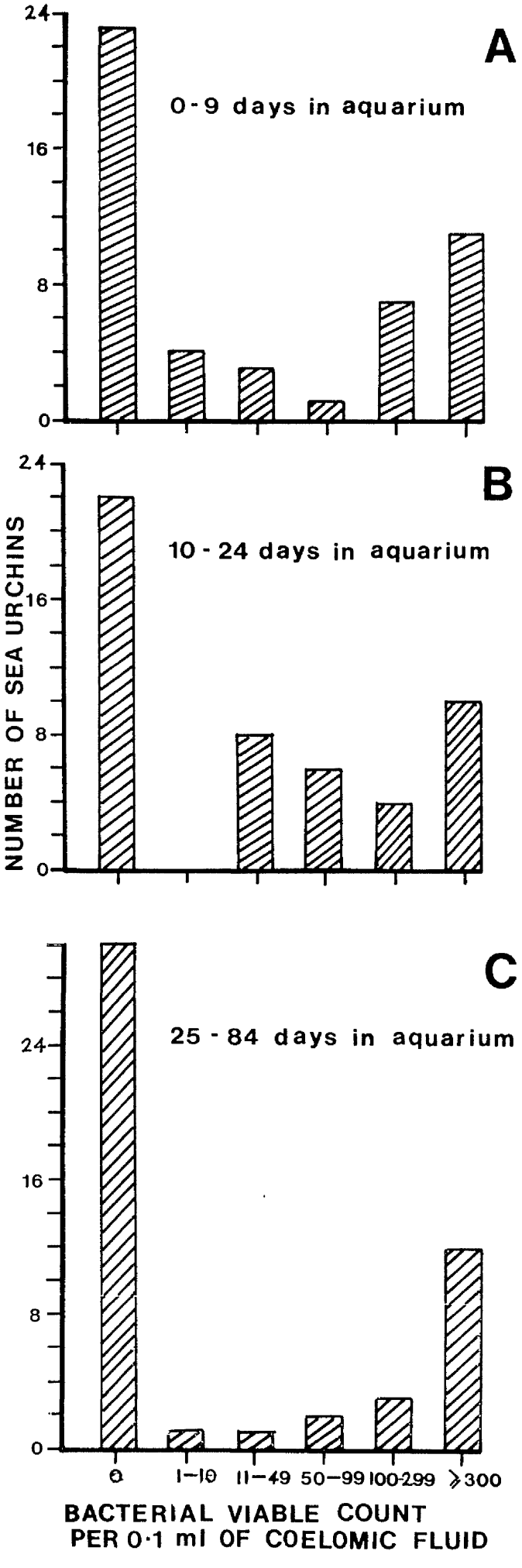

LOG10 CHANGE IN BACTERIAL VIABLE COUNT OF AQUARIUM WATER DURING 28d PERIOD IN WHICH 1-3 E.esculentus DIED
2
0
-1
1
2
3
NUMBER OF E. esculentus WHICH DIED WITHIN A 12 h PERIOD

FIGURE 24. Survey of background bacterial counts in coelomic fluid of *E. esculentus* (n = 147) maintained for different times in RASWA.


A
0-9 days in aquarium
B
10-24 days in aquarium
C
25-84 days in aquarium
NUMBER OF SEA URCHINS
24
16
8
0
0
1-10
11-49
50-99
100-299
≥300
BACTERIAL VIABLE COUNT
PER 0·1 ml OF COELOMIC FLUID

The bacterial viable counts per 0.1ml CF of sea urchins maintained in RASWA for between 0 to 9 days (Figure 24A) were observed to form a pattern of J-shaped distribution, 23 animals with zero colonies in 0.1ml CF, 11 sea urchins with between 11 and 299 colonies $0.1ml^{-1}$ and 11 animals with greater than 300 colonies $0.1ml^{-1}$ CF (confluent growth (c)).

This pattern of bacterial viable count did not change to a great extent with time of maintenance in RASWA. However, the number of *E. esculentus* with CF giving zero colonies $0.1ml^{-1}$ increased from 47% (Figure 24A) to 61% (Figure 24C) over a period inclusive of 0-84 days in RASWA, suggesting possible recovery with length of maintenance in the aquaria. Nevertheless, the number of CF's with greater than 300 colonies $0.1ml^{-1}$ remained between 20 and 24% of the total number of animals tested between 0 to 84 days in the RASWA.

Unsampled sea urchins maintained in RASWA for several months appeared physically healthy in external appearance with bright pink/purple colouration, uniform spine arrangement, firm attachment to tank wall and tube feet display (see Table 18).

2. *IN VITRO* ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *E. ESCULENTUS* COELOMIC FLUID

2.1. Preliminary Observations

2.1.1. Antibacterial activity

Previous studies on the bactericidal activity of *E. esculentus* coelomic fluid (CF) have all previously been done with freshly collected

specimens held in running-seawater aquaria at Millport (Unkles, 1976; Wardlaw and Unkles, 1978; Messer and Wardlaw, 1979; Service, 1982; Service and Wardlaw, 1985).

For the present investigation, it was therefore necessary to determine whether bactericidal activity would be demonstrable with CF from sea urchins kept at Glasgow in the recirculating artificial-seawater aquaria (RASWA) described in the Materials and Methods (section 2.1.).

Over a period of several months, sea urchins in batches of about 8 were placed in the RASWA and held at 10°C for at least three weeks and up to three months, before testing. The test consisted of withdrawing approx. 2ml CF, adding 1.8ml CF portion to 0.2ml *Ps.111* and plating 0.1ml of the remainder on marine agar (MA) as a check on whether there was initial background bacterial contamination of the CF. The bactericidal tests were incubated at 10°C and samples taken at 24, 48 and 72h for determination of residual viable microorganisms. Results were expressed as the Survival Index (SI):

$$SI = 100.\ Ct/Co$$

where, Ct = viable count at time t,

and Co = viable count at time O.

Thus, an index of O indicated complete killing of the bacteria, while an index of 100 or more represented full survival or net growth, respectively.

It may be noted that the 72h incubation period was longer than reported by previous investigators (Unkles, 1976; Service, 1982) who

recommended 4, 24 and 48h as standard sampling times.

The bactericidal activity of three *E. esculentus* CF's towards *Pseudomonas* strain 111 (*Ps.111*) are represented in the form of a "killing curve" in Figure 25. *Ps.111* grew well in the MBASW control fluid with SI's of greater than 200 (or confluent growth (c)) at 24, 48 and 72h. In the CF samples, the SI of *Ps.111* typically dropped to below 15 at 24h and to zero at 48h, with no regrowth of the bacteria at 72h. SI values with n = 26 specimens of *E. esculentus* CF's are presented as histograms in Figure 26, the actual experimental data being tabulated in Appendix 8. Figure 26A, which presents the 24h SI values, shows that eleven out of twenty-six of the CF's yielded SI's of zero. This means that there were no surviving *Ps. 111*, as measured by colony-forming ability, detected in the platings from CF plus bacteria at 24h. A further 7 CF's yielded SI's of 20 or less, indicating that 80%, or more, of the initial inoculum had been killed. There were then 6 samples of CF with SI's of between 20 and 100 and 2 with SI's of greater than 100. The latter 2 fluids were therefore, non-bactericidal.

Turning to Figure 26B, the 48h SI's are similar to those at 24h in showing a J-shaped distribution, with eighteen out of twenty of the CF's having SI's of 20 or less. At 72h (Figure 26C) the J-shape of the SI histogram is even more pronounced, with the CF's mainly separating into 2 categories; those that were obviously bactericidal (eighteen out of twenty-six) and those which by 72h had allowed regrowth of the inoculum (six out of twenty-six).

In regard to the background contamination of the CF's 62% were sterile - or at least contained too few contaminants to be detected in 0.1ml, five

FIGURE 25. The killing of *Ps.111* by *E. esculentus* CF (n = 3) and the growth of the bacteria in MBASW control fluid at 10°C. The animals had been kept in RASWA for up to 3 months.

Data are expressed as the SI of *Ps.111* at 24, 48 and 72h (Note SI = 100. Ct/Co) where Ct and Co are respectively, the final and initial counts.

Solid symbols : CF; open symbols : MBASW; dotted line : SI = 100.

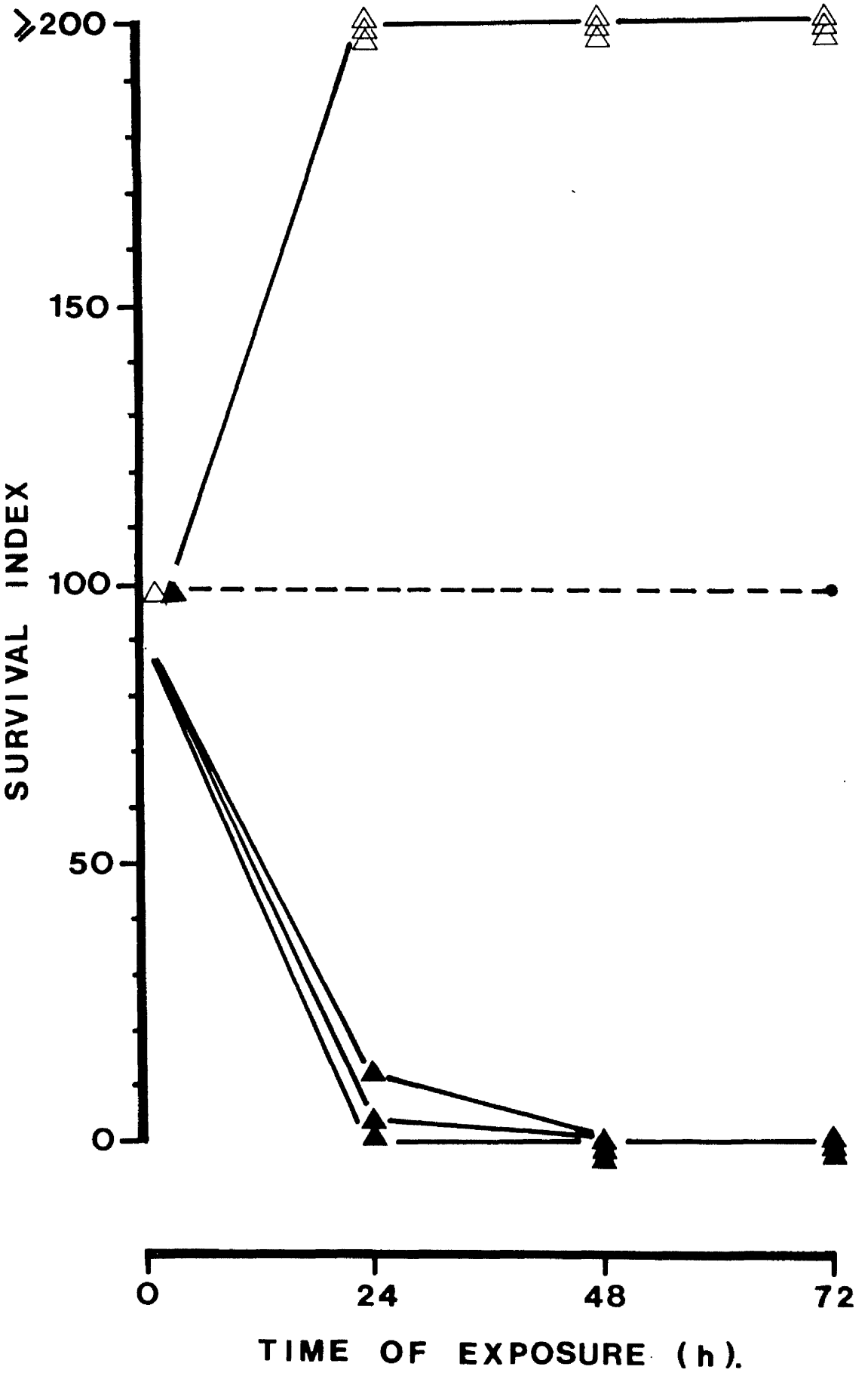
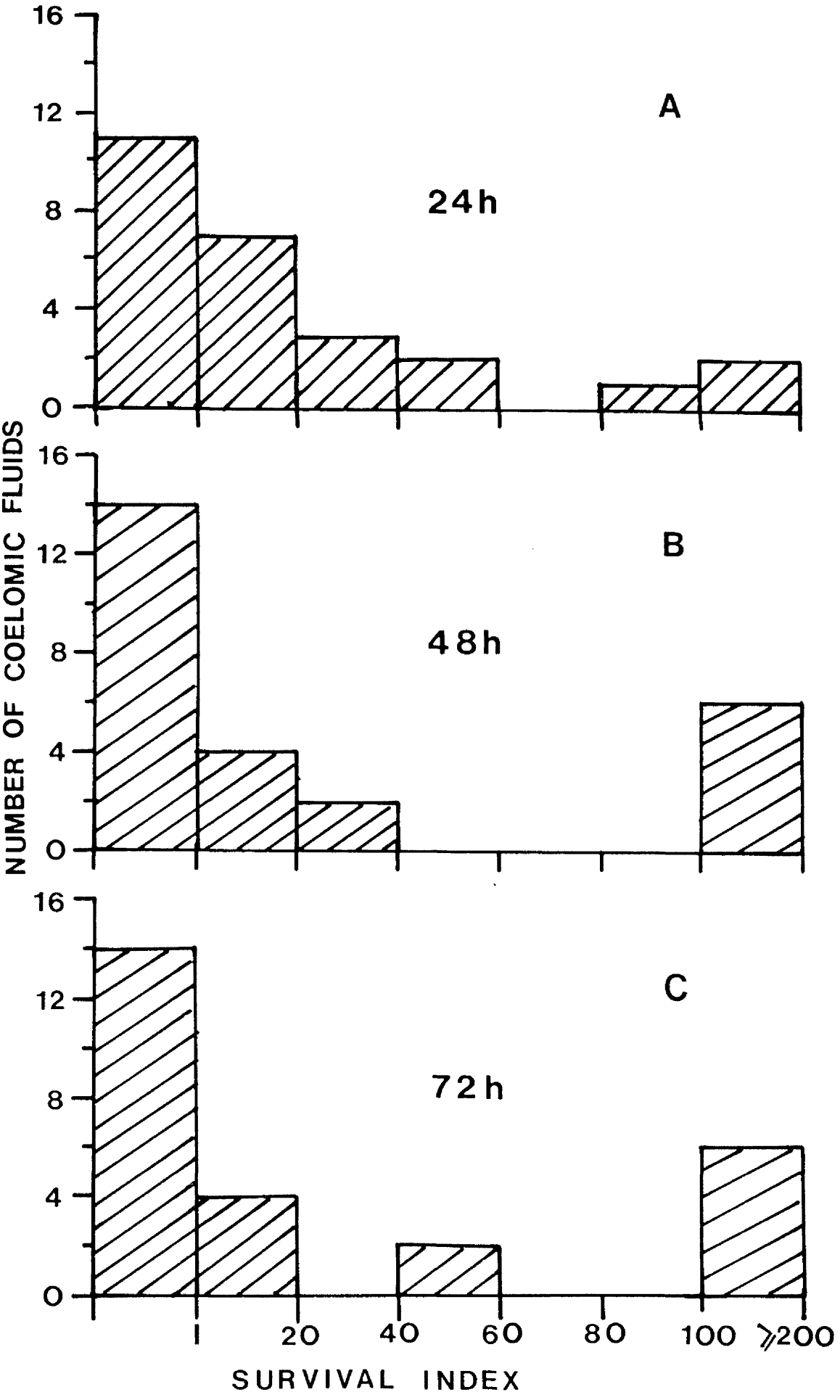

≥200
150
100
50
0
SURVIVAL INDEX
0
24
48
72
TIME OF EXPOSURE (h).

FIGURE 26 Histograms showing the bactericidal activity of *E. esculentus* CF (n = 26), kept in RASWA for 3 to 12 weeks, towards *Ps. 111* at 10°C.

Data are expressed as SI's at 24, 48 and 72h.


A
24h
B
48h
C
72h
16
12
8
4
0
NUMBER OF COELOMIC FLUIDS
20
40
60
80
100
⩾200
SURVIVAL INDEX

out of twenty-six yielded 1-10 colonies $0.1ml^{-1}$, two out of twenty-six yielded 11 $0.1ml^{-1}$ and 12 colonies $0.1ml^{-1}$ and the remainder yielded 42, 54 and greater than 200 colonies $0.1ml^{-1}$. Since *Ps.111* has black, agar-digesting colonies, the presence of a few contaminants (say, up to 20) was of little concern, since these, the latter, were never black-colonied. Only when the initial contamination of the CF was greater than about 200 $0.1ml^{-1}$ did a problem arise, and the animal was therefore rejected as a source of CF for *in vitro* antimicrobial studies.

2.1.2. Antifungal activity

Having demonstrated that *E. esculentus* kept in RASWA at 10°C had CF with bactericidal activity, the next step was to test for antifungal activity. First, however, it was necessary to choose suitable marine yeasts to act as target organisms. Initially four strains of marine yeast, obtained from the National Collection of Yeast Cultures (NCYC) were examined for suitability. The criteria of prime importance were :

a. distinctive colony-appearance so that test organisms could be easily distinguished from CF contaminants on MA,

b. ability to survive or grow in low-nutrient, seawater-based, control fluid (MBASW), at 10°C.

These criteria were fulfilled by the two strains, *Metschnikowia zobelli* (NCYC 783) and *Rhodotorula rubra* (NCYC 63) which had white, rough colonies and pink, smooth colonies (Appendix 1), respectively, and which both grew, albeit slowly, over a period of days at 10°C.

2.1.2.1. *Use of mixed inocula*

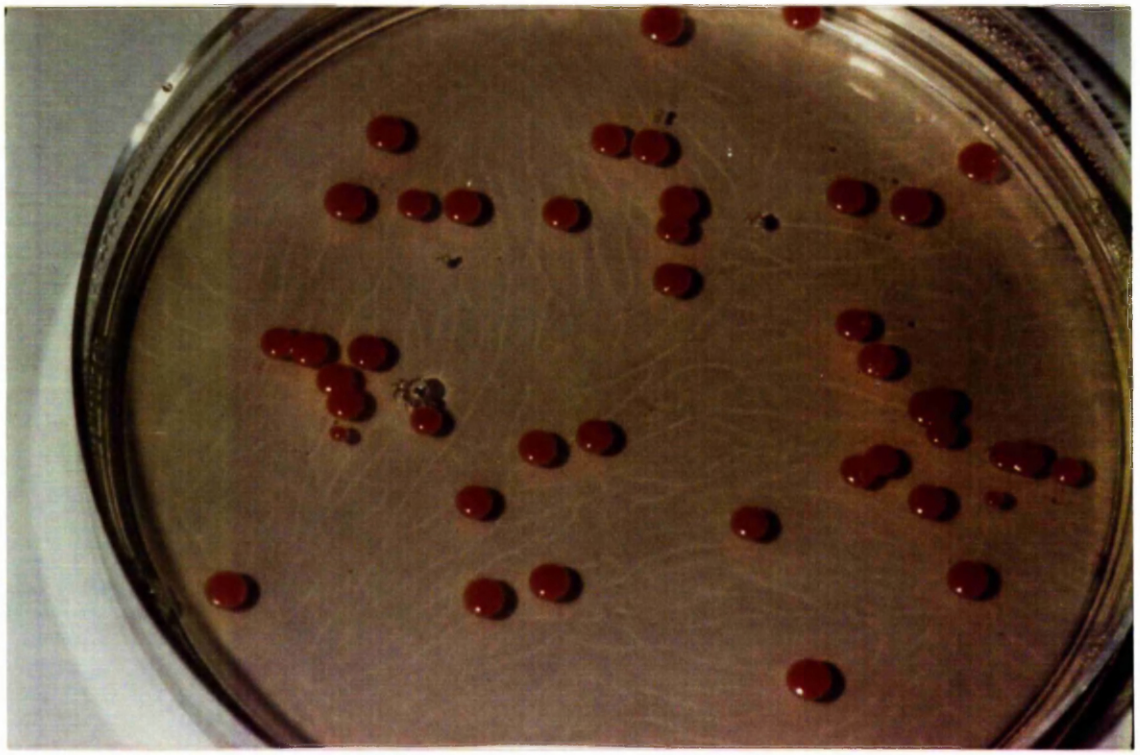
It was suspected that the antifungal activity of CF might be less apparent than the antibacterial activity of the CF. There was also the problem that not all CF's were antibacterial. It was therefore desirable to design the experiments to allow observation of this point, and the antifungal tests with CF were therefore done with a deliberately mixed bacterial/fungal inoculum.

Ps. 111 was used as the known CF - sensitive bacterium, and test-mixtures were plated on marine agar (MA), to grow *Ps.111*, and on yeast marine agar (YMA) which contained glucose and chloramphenicol, for the encouragement of yeast growth and suppression of bacterial growth (*Ps. 111* and CF bacterial contaminants respectively). The appearance of *R. rubra* (NCYC 63) on YMA is shown in Plate 5A, note the total exclusion of *Ps. 111* from the mixed inoculum sample and the easily countable yeast colonies. The same mixed inoculum plated onto MA is shown in Plate 5B, the black colonied *Ps.111* outgrowing the yeast probably due to lack of glucose in the MA. The colony-characteristics of *M. zobelli* (NCYC 783) also on YMA from a mixed inoculum of yeast and bacteria can be seen in Plate 6A and the growth of another mixed inoculum *Ps.111* and *M. zobelli* (NCYC 783) on MA (Plate 6B).

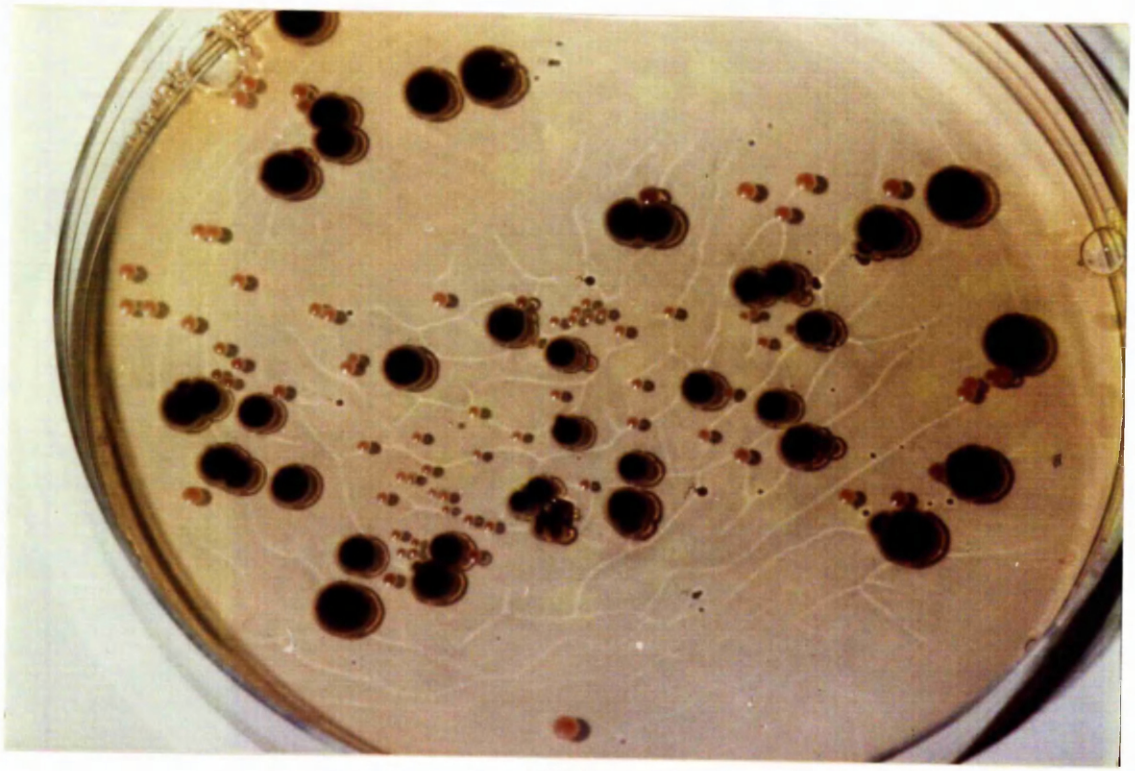
A typical test consisted of the addition of a mixed inoculum of 0.1ml *Ps.111* and 0.1ml of either *R. rubra* (NCYC 63) or *M. zobelli* (NCYC 783) to a 1.8ml volume of CF and gently mixed by swirling the tube. The fungicidal tests, with the bacterial control strain, were incubated at 10°C and samples taken at 24, 48 and 72h for determination of remaining viable

PLATE 5A. Pink colonies of *R. rubra* (NCYC 63) on YMA (containing glucose and antibiotic) from a mixed inoculum of yeast and *Ps.111*, after 72h incubation at approx. 22°C.

PLATE 5B. Black, agar-digesting colonies of *Ps.111* and smaller pink colonies of the yeast *R. rubra* (NCYC 63) on MA from a mixed inoculum of yeast and bacteria, after 72h incubation at approx. 22°C.



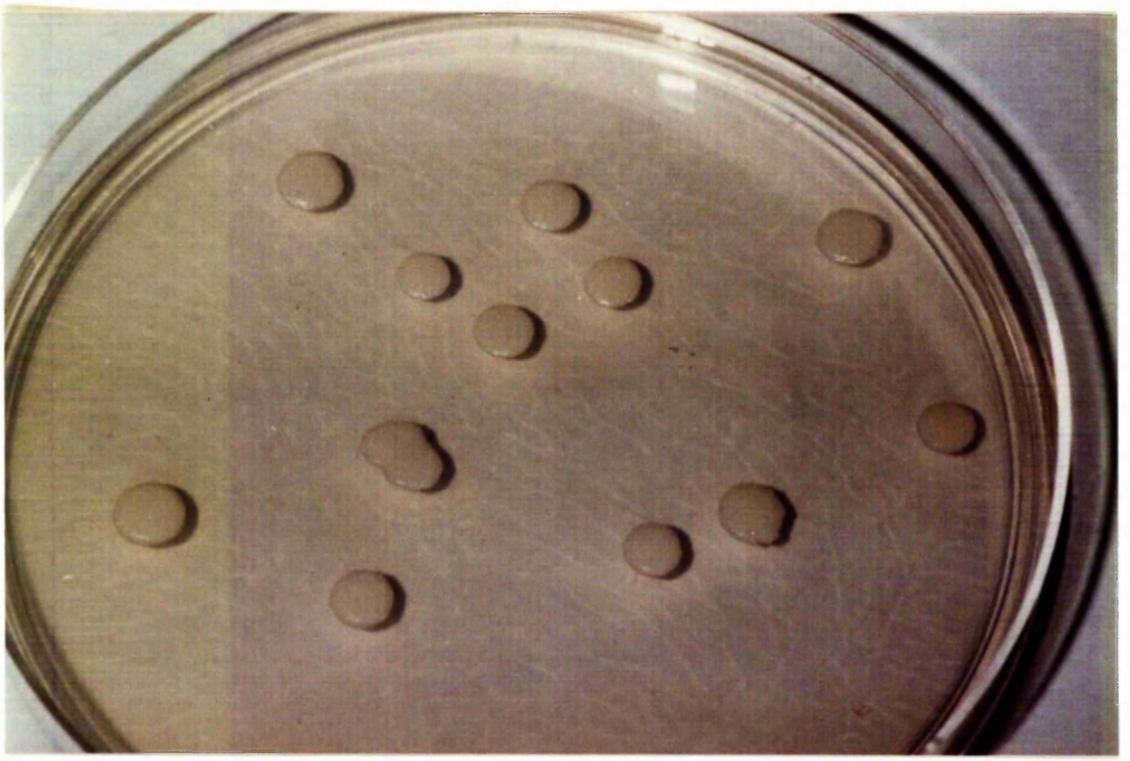
A



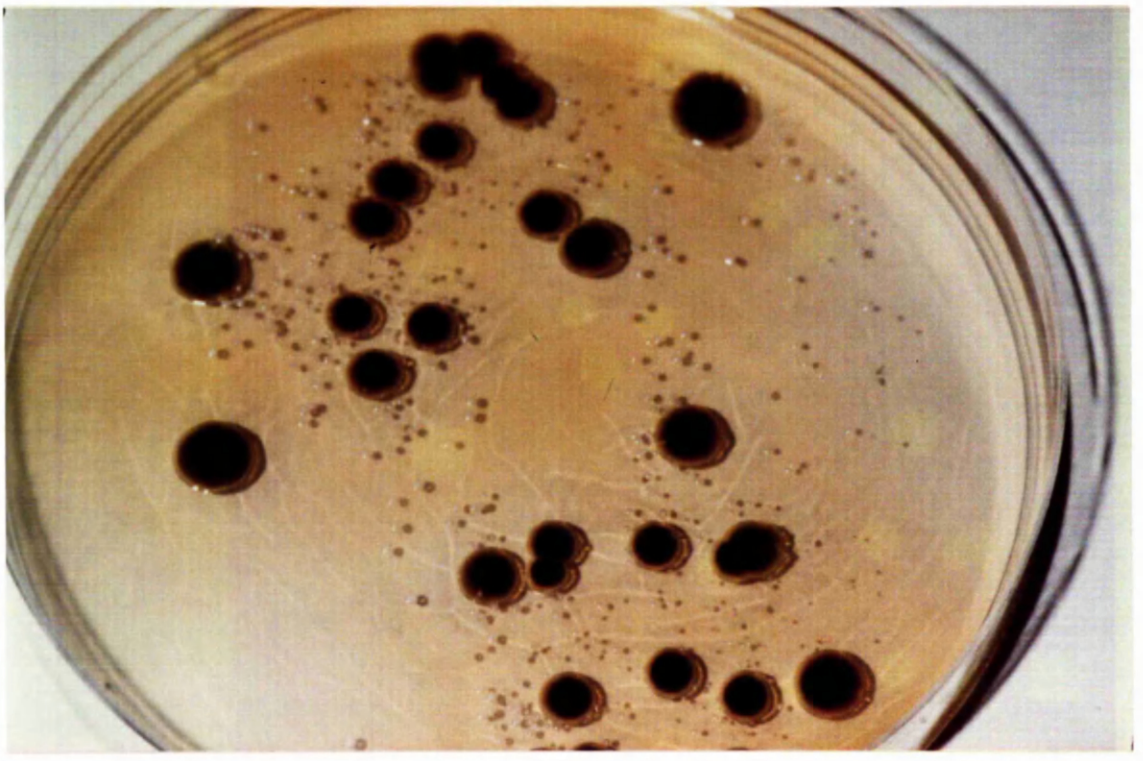
B

PLATE 6A. Characteristics of *M. zobelli* (NCYC 783) on YMA from a yeast/bacteria mixed suspension after 72h, at approx. 22°C incubation.

PLATE 6B. *Ps.111* and *M. zobelli* (NCYC 783) on MA from (another) mixed inoculum, after 72h incubation at approx. 22°C.



A



B

microorganisms. Again, the results were expressed as the SI.

The fungicidal and bactericidal activity of 3 CF's towards *R. rubra* (NCYC 63) and *Ps.111* are shown in Figure 27. Both *R. rubra* (NCYC 63) and *Ps. 111* grew well in the MBASW control fluid with SI's of greater than 200 at 24, 48 and 72h. In the CF samples the SI's of *Ps.111* dropped below 15 at 24h, at 48h the SI's of *Ps.111* remained below this level in 2 of the CF's and increased to less than 40 in the remaining CF. At 72h, only one CF reduced the SI to zero, but the remaining 2 CF's allowed regrowth of the bacteria. This indicated that only one out of three of the CF's were highly bactericidal, the 2 remaining CF's allowed regrowth of the bacteria at 72h.

By comparison, the SI values for *R. rubra* (NCYC 63) dropped only slightly within the first 24h, fell to about 25 at 48h and then regrew to about 40 at 72h. This indicated the yeast SI's remained static at 24h and a reduction in the SI's were most apparent at 48h. SI values of n = 11 specimens of *E. esculentus* are presented in Figure 28. At 24h (Figure 28A) none of the CF's yielded SI's of zero. The SI's of the yeast ranged between 1 and 80. At 48h (Figure 28B) the SI's were all below 60 and at 72h (Figure 28C) 3 out of the 11 fluids yielded SI's of zero and the remaining CF's between 20 and 40. Antifungal activity was evident therefore, particularly at 48 and 72h.

Figure 29 presents the results of the antifungal tests of *E. esculentus* CF on *M. zobelli* (NCYC 783) with *Ps.111* control. The results are essentially the same as those previously described (Figure 27) with *R. rubra* and *Ps.111*, except that *M. zobelli* (NCYC 783) showed more rapid killing than *R. rubra* (NCYC 63) within the first 24h. The SI values of *E.*

FIGURE 27. Comparison of the antifungal and antibacterial activity of *E. esculentus* CF (n = 3) towards a mixed inoculum of *R. rubra* (NCYC 63) and *Ps.111* at 10°C. The figure shows the contrasting growth of the organisms in MBASW control fluid.

Data are expressed as SI's at 24, 48 and 72h.

Open triangles : MBASW (*Ps.111*); open circles : MBASW (*R. rubra* (NCYC 63)); closed triangles : CF (*Ps.111*); closed circles : CF (*R. rubra* (NCYC 63)); dotted line : SI = 100.

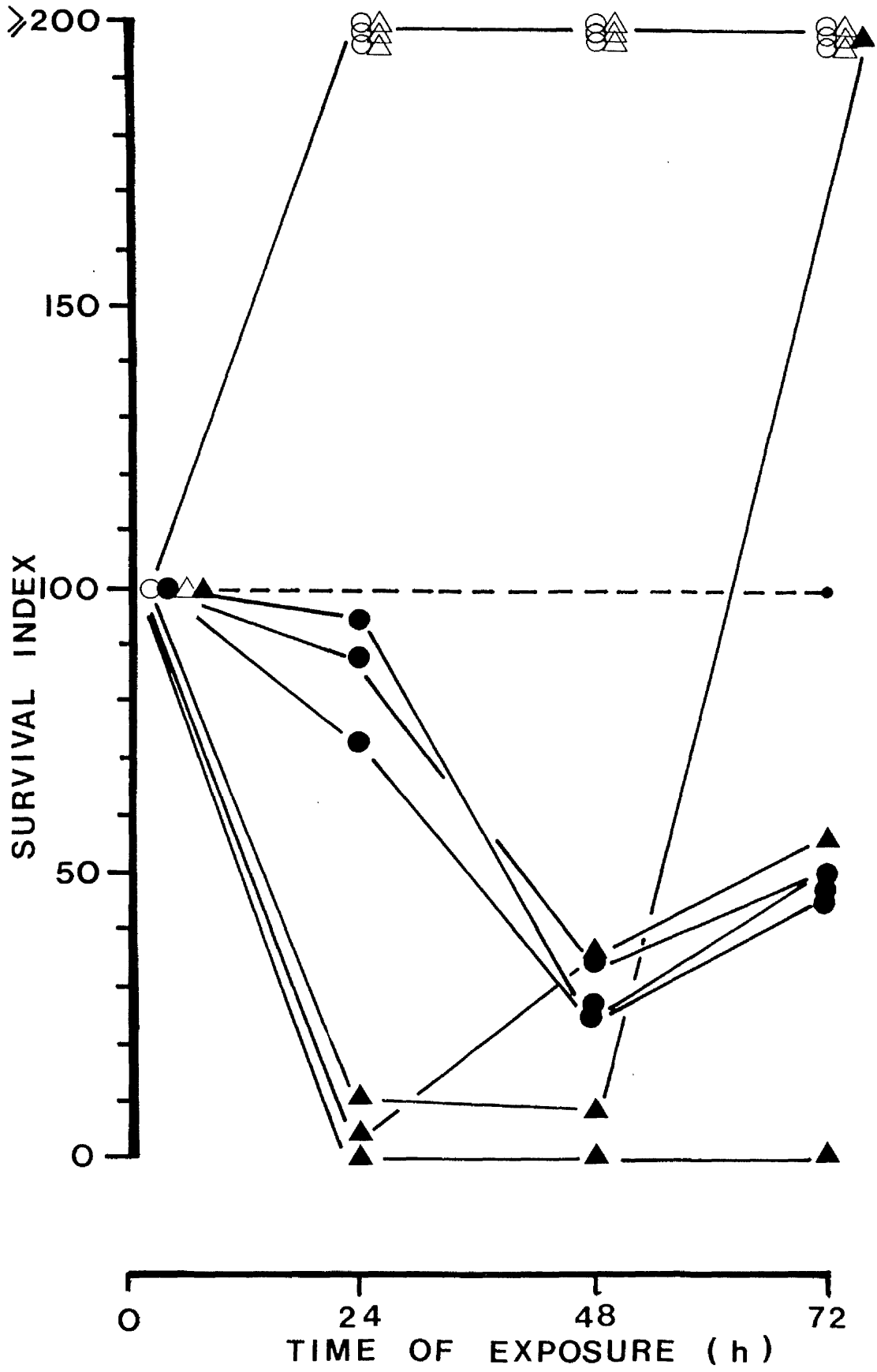

⩾200
150
100
50
0
SURVIVAL INDEX
0
24
48
72
TIME OF EXPOSURE (h)

FIGURE 28. Histograms showing the antifungal activity of *E. esculentus* CF (n = 11) towards *R. rubra* (NCYC 63) at 10°C.

The data are expressed as SI's at 24, 48 and 72h.

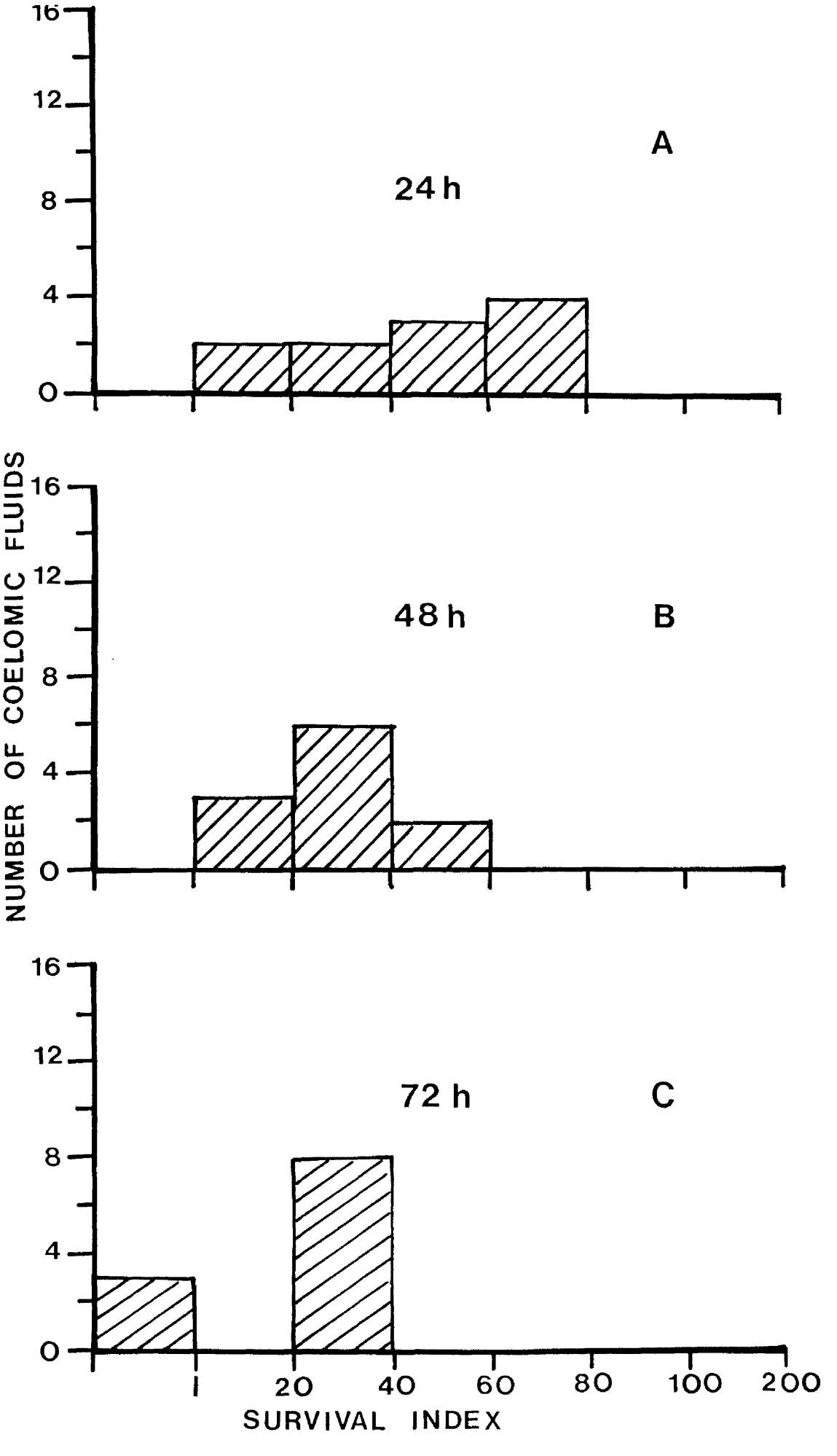

16
12
8
4
0
A
24 h
NUMBER OF COELOMIC FLUIDS
48 h
B
72 h
C
20
40
60
80
100
200
SURVIVAL INDEX

FIGURE 29. The relative antifungal and antibacterial activity of *E. esculentus* CF (n = 3) towards a mixed inoculum of *M. zobelli* (NCYC 783) and *Ps.111*, and contrasting growth of the microorganisms in MBASW control fluid at 10°C.

Data are expressed as SI's at 24, 48 and 72h.

Open triangles: MBASW *Ps.111*; open squares : MBASW (*M. zobelli* (NCYC 783)); closed triangles : CF (*Ps.111*); closed squares : CF (*M. zobelli* (NCYC 783)); dotted line : SI = 100

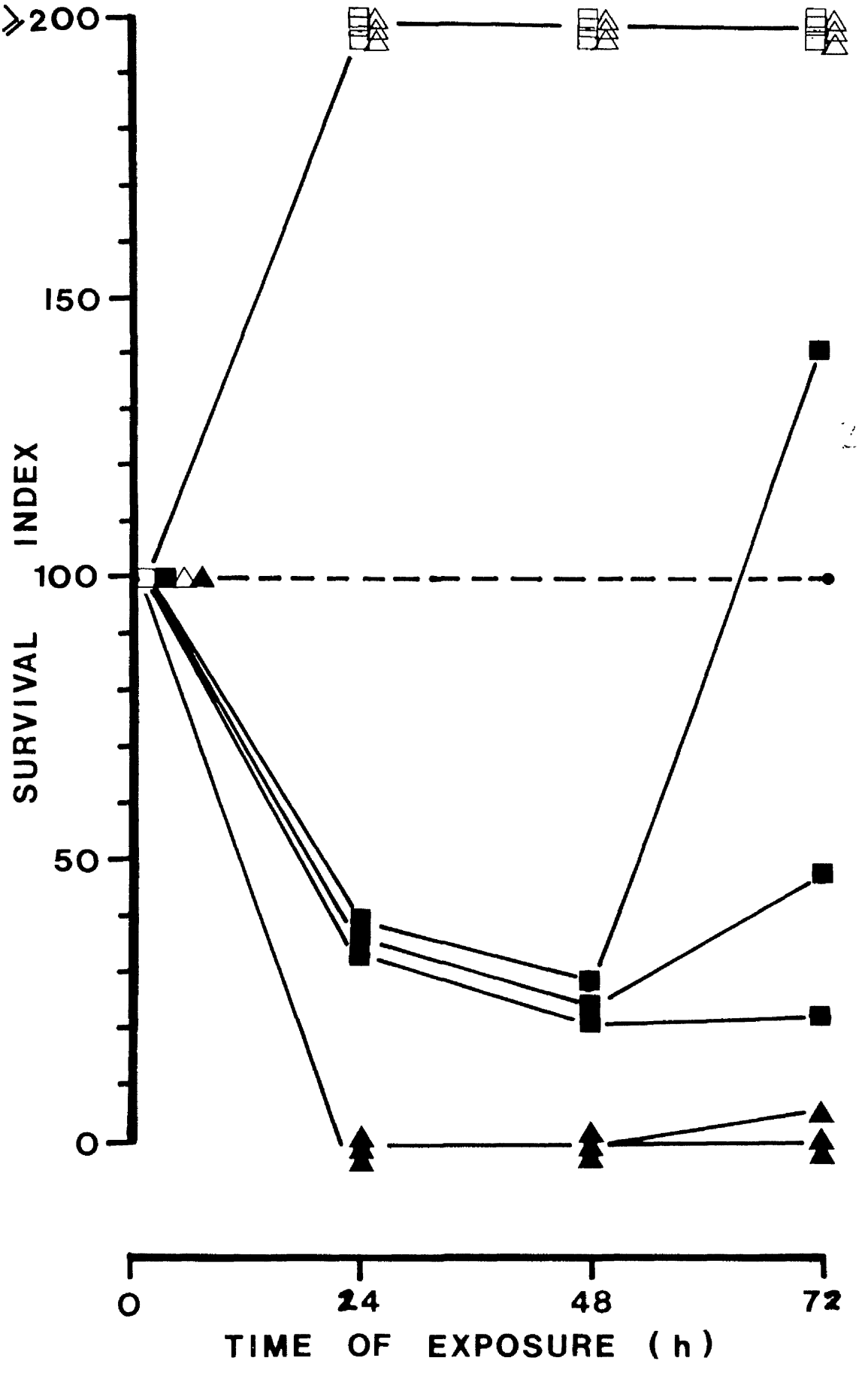
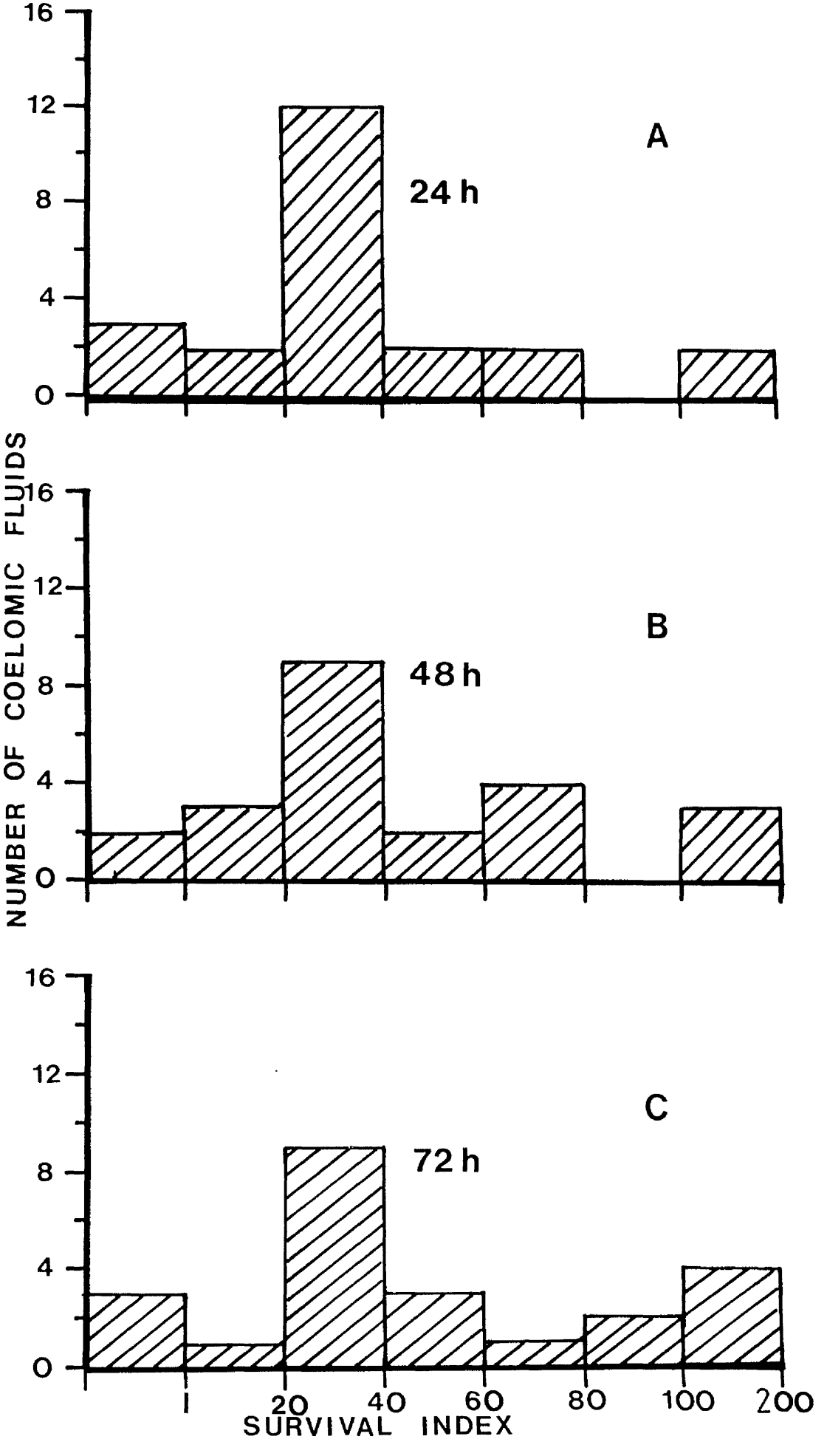

≥200
150
100
50
0
SURVIVAL INDEX
0
24
48
72
TIME OF EXPOSURE (h)

FIGURE 30. Histograms showing the antifungal activity of *E. esculentus* CF (n = 23) towards *M. zobelli* (NCYC 783) at 10°C.

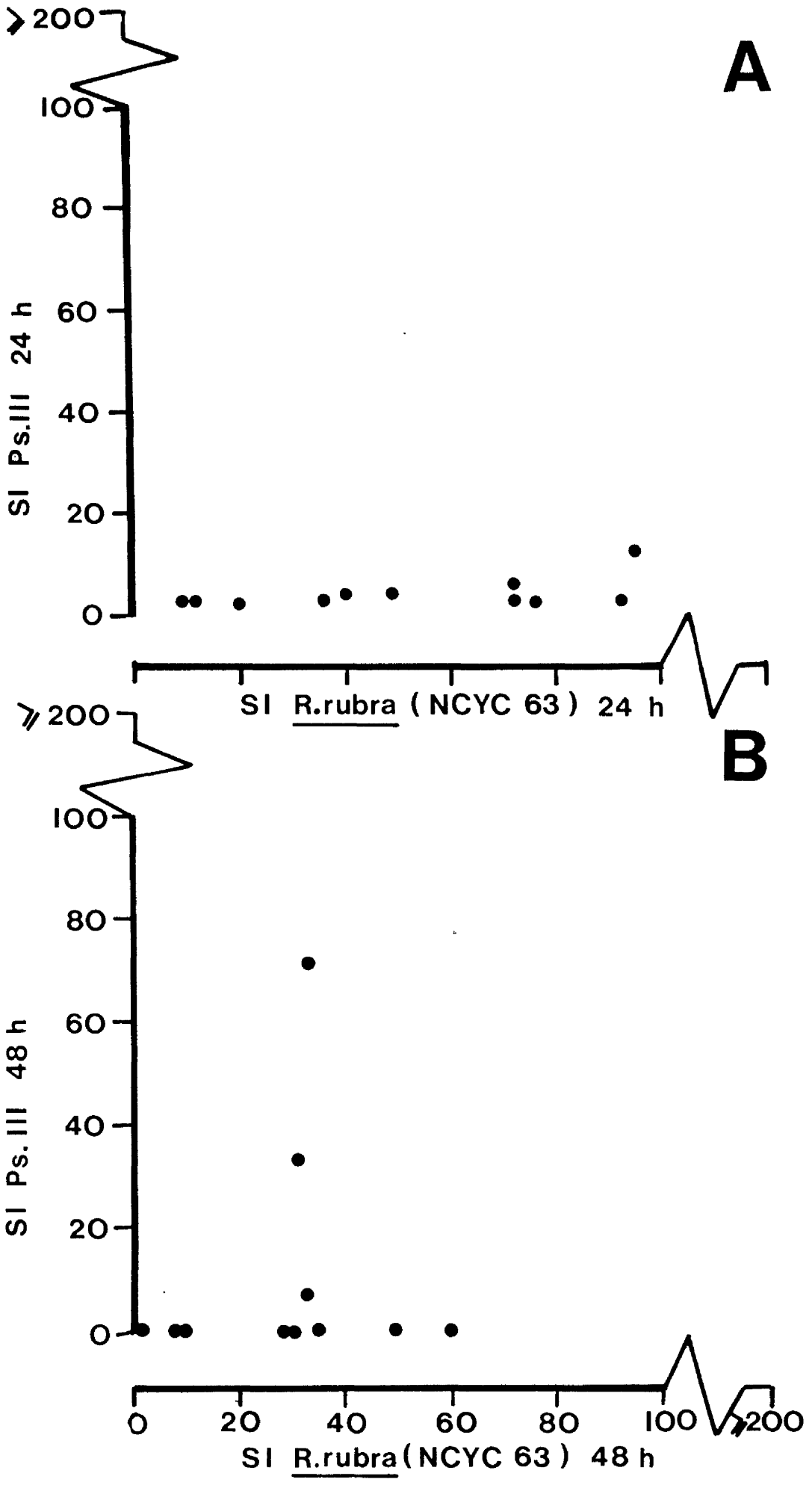
The data are expressed as SI's at 24, 48 and 72h.


16
12
8
4
0
A
24 h
B
48 h
C
72 h
NUMBER OF COELOMIC FLUIDS
1
20
40
60
80
100
200
SURVIVAL INDEX

esculentus CF's (n = 23) towards *M. zobelli* (NCYC 783) are presented in the form of three histograms in Figure 30. The first histogram (Figure 30A) shows a distribution of SI values between zero and greater than 200 at 24h. SI's of zero were yielded by three out of twenty-three CF's, two out of twenty-three less than 20, about one half of the twenty-three CF's gave SI's of between 20 and 40. A further 4 fluids reduced the SI's to between 40 and 80, the remaining 2 fluids supporting growth of the yeasts ($\geq$200). This means that the majority of the CF's at 24h (over 82%) reduced the SI's by more than 60% indicating antifungal activity, 9% of the fluids tested allowed growth, and were, therefore, non-antifungal. A similar pattern emerged at 48h (Figure 29B) with over 60% of the CF's reducing the SI's of *M. zobelli* (NCYC 783) by more than 60, 13 of the CF's were inactive towards the yeast. At 72h (Figure 30C) again more than 56% of the CF's reduced the SI's by greater than 60 and an increase in the number of CF's supporting actual growth from 9% at 24h to 17% at 72h.

It was therefore established that *E. esculentus* CF was antifungal towards the two selected strains of marine yeasts *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783). The incorporation of the bacterial control strain *Ps. 111* with the marine yeasts as a mixed inoculum allowed comparison of the antibacterial and antifungal activity of individual *E. esculentus* CF's. A comparison of the 24 and 48h SI's of *Ps.111* and *R. rubra* (NCYC 63) from mixed inocula exposed to *E. esculentus* CF's (n = 11) are presented in Figure 31. The data in Figure 31A shows that the majority of the CF's were highly bactericidal towards *Ps.111* reducing the SI's to less than 15 in every case at 24h. The SI's of the yeast *R. rubra* (NCYC 63) however, were seen to range from about 10 to 100 indicating there was apparently no

FIGURE 31. Comparison of 24 (A) and 48h (B) SI's of *R. rubra* (NCYC 63) and *Ps.111* exposed to *E. esculentus* CFs (n = 11) at 10°C.


A
B
SI Ps.III 24 h
SI R.rubra (NCYC 63) 24 h
SI Ps. III 48 h
SI R.rubra (NCYC 63) 48 h
0
20
40
60
80
100
200

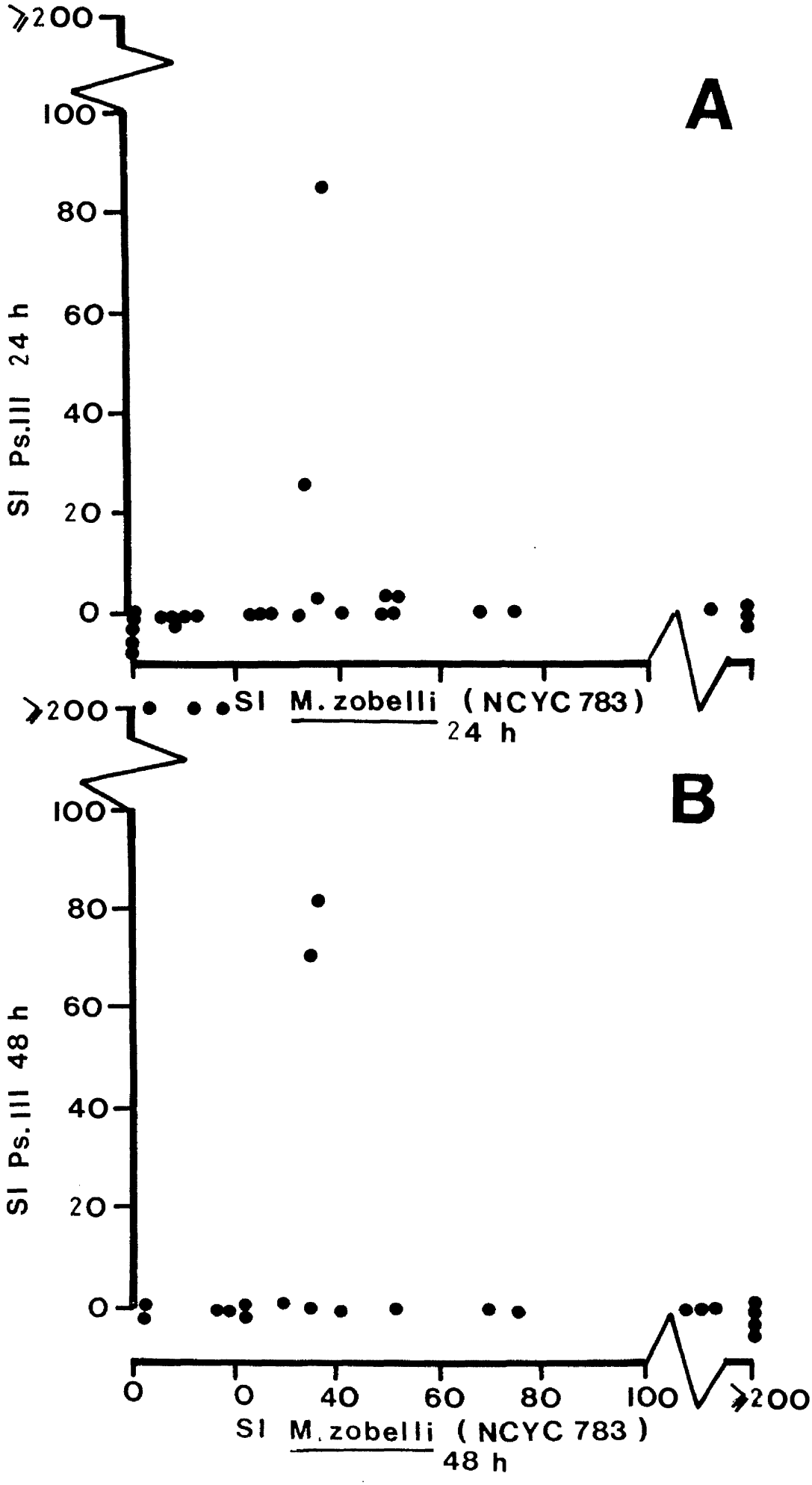
correlation here between antibacterial and antifungal activity. At 48h (31B), eight out of eleven of the CF's had SI's of 5, 35 and 70. A similar distribution of *R. rubra* (NCYC 63) SI's occurred with values ranging from 0 to 60, more of the fluids showing antifungal activity than at 24h, and only one CF with bactericidal activity also exhibiting fungicidal activity.

Again there was little evidence of the correlation of antibacterial and antifungal activity at 48h, since fluids which were highly bactericidal towards *Ps 111* showed a wide range of antifungal activity towards *R. rubra* (NCYC 63). This suggests antibacterial activity of undilute *E. esculentus* CF is not necessarily indicative of antifungal activity.

Correlation analysis similar to the above was done with the *M. zobelli* (NCYC 783) and *Ps.111* results (Figure 32) and yielded essentially the same outcome as was obtained in the *R. rubra* (NCYC 63)/*Ps.111* comparison (Figure 31).

It was established, therefore, that *E. esculentus* CF was antifungal towards *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) although it was not as pronounced as antibacterial activity demonstrable with *Ps.111* in undiluted CF. Additionally, as a general rule there was no correlation between the SI's of *Ps.111* and the SI's of *R. rubra* (NCYC 63) at 24 or 48h, and only one out of eleven of the CF's tested showing direct correlation at 48h. The comparison of the antifungal and antibacterial activities of CF towards *M. zobelli* (NCYC 783) and *Ps.111* resulted in a greater number (12%) of CF's showing direct correlation at 24 and 48h the remaining CF's indicating little or no correlation.

FIGURE 32. Comparison of 24 (A)and 48h (B) SI's of *M. zobelli* (NCYC 783) and *Ps.111* exposed to *E. esculentus* CFs (n = 28 and n = 24, respectively) at 10°C.


A
B
SI Ps.III 24 h
SI M. zobelli (NCYC 783) 24 h
SI Ps. III 48 h
SI M. zobelli (NCYC 783) 48 h
0
20
40
60
80
100
≥200

Since these preliminary investigations provided evidence for both antibacterial and antifungal activity of CF from *E. esculentus* maintained in RASWA at 10°C towards *Ps.111* and selected strains of marine yeast *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783), it was therefore of interest to extend these studies towards a wider spectrum of marine yeasts.

2.2. Spectrum of Antifungal Activity

A further panel of nine marine yeast strains were selected from the National Collection of Yeast Cultures (NCYC). They were chosen on the basis of their :

a. reported isolation from a marine environment,
b. ability to grow on YMA at room temperature (approx. 22°C)
c. survival or growth at 10°C in 1% marine broth in ASW (MBASW) or coelomic fluid supernate (CFSN).

The marine yeasts selected were representative of the three genera, *Candida, Debaryomyces* and *Rhodotorula.*

The fungicidal test was done as before with a mixed inoculum of *Ps.111* and marine yeast strain (0.2ml) added to 1.8ml volumes of *E. esculentus* CF. Samples (0.3ml) of the test mixture were taken at 24, 48, 72 and 96h; 0.1ml spread as undiluted CF onto MA and YMA, and 0.1ml used to prepare serial dilutions which were also spread (0.1ml) onto both media types. It may be noted that a further incubation period of 96h was included in some of the experiments to explore the incidence of regrowth. The control fluid MBASW was included in all experiments and an additional control fluid, coelomic fluid supernate (CFSN), was incorporated into the design of

the experiments. CFSN's were sterile preparations of individual CF's from which the coelomocytes had been removed by centrifugation. This fluid was tested for antibacterial and antifungal activity so as to determine whether coelomocytes were required for these activities.

2.2.1. Marine *Candida*

2.2.1.1. *Coelomic fluid*

Seven strains of *Candida*, *C. marina* (NCYC 784), *C. haemulonii* (NCYC 787), *C. maris* (NCYC 785), *C. guilliermondii* (NCYC 145), *C. famata* (NCYC 799), *C. famata* (NCYC 798), and *C. torresii* (NCYC 786) were tested with *E. esculentus* CF's (n = 3 to 11) and the data are represented in Figure 33. All of the seven marine *Candida* strains were susceptible (to some extent) to the antifungal activity of *E. esculentus* CF at 10°C. This is shown by the progressive reduction in the SI's from the initial value of 100. The marine *Candida* strains are arranged in approximate order of decreasing sensitivity to CF with *C. marina* (NCYC 784), (Figure 33A) as one of the most sensitive strains. The SI's were reduced to zero at 24h with incidence of regrowth in the following 48h to SI's of less than 20 at 96h. The most resistant strain *C. torresii* (NCYC 786) (Figure 33G) was reduced to SI's of approximately 50 with regrowth of the yeast to SI's of greater than 200 at 48, 72 and 96h.

2.2.1.2. *MBASW control fluid*

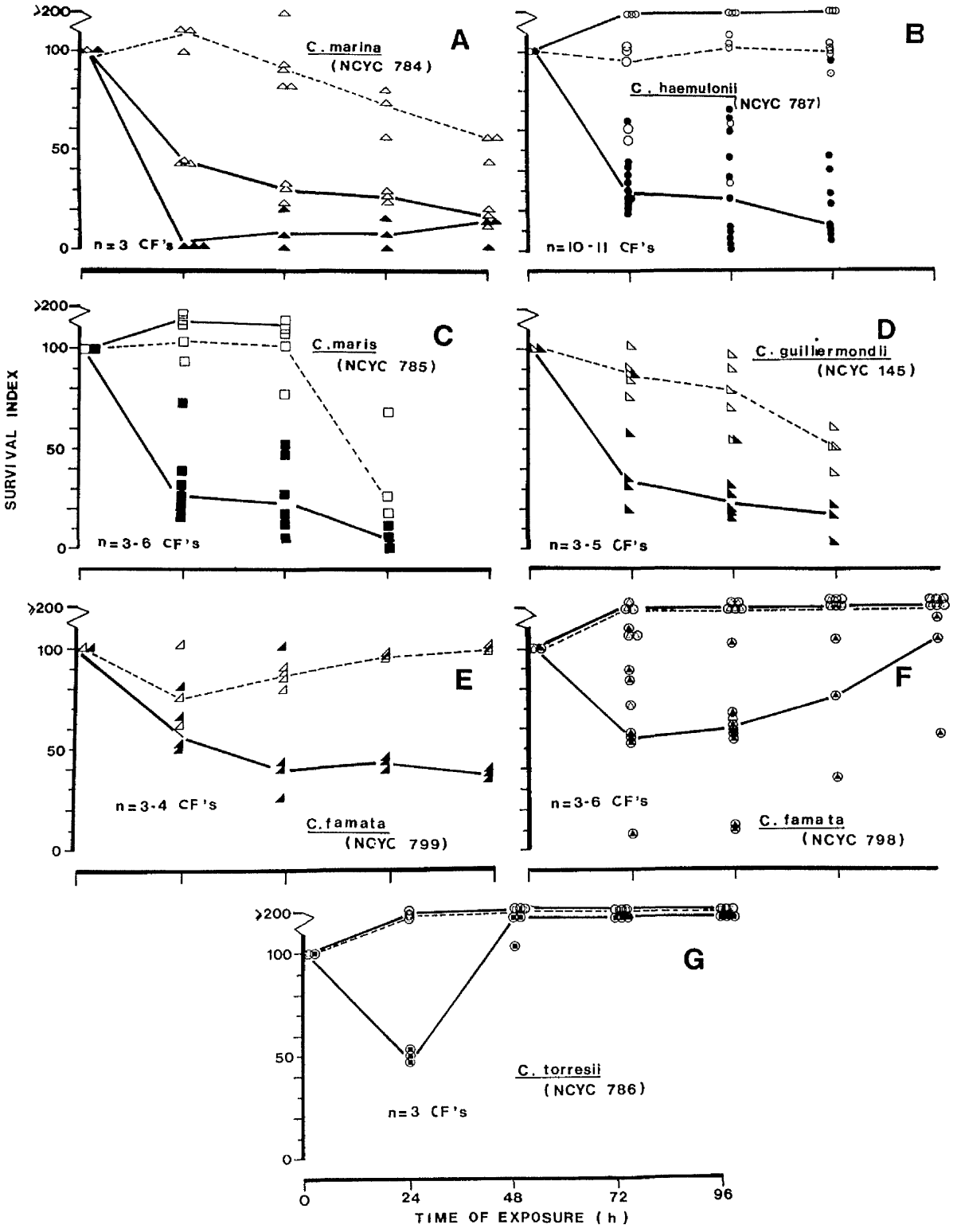
It will be noted that strains *C. marina* (NCYC 784) (Figure 33A), *C. maris* (NCYC 785) (Figure 33C) and *C. guilliermondii* (NCYC 145) (Figure 33D)

FIGURE 33. Antifungal activity of *E. esculentus* CF's (n = 3 to n = 11) towards seven strains of marine *Candida* and growth of the yeasts in CFSN and MBASW control fluids at 10°C. The data are expressed as SI's at 24, 48, 72 and 96h.

Solid points : CF ;

Open points with solid line : CFSN ;

Open points with dashed line : MBASW.


A
C. marina
(NCYC 784)
n=3 CF's
B
C. haemulonii
(NCYC 787)
n=10-11 CF's
C
C. maris
(NCYC 785)
n=3-6 CF's
D
C. guilliermondii
(NCYC 145)
n=3-5 CF's
E
n=3-4 CF's
C. famata
(NCYC 799)
F
n=3-6 CF's
C. famata
(NCYC 798)
G
C. torresii
(NCYC 786)
n=3 CF's
SURVIVAL INDEX
>200
100
50
0
0
24
48
72
96
TIME OF EXPOSURE (h)

did not retain viability in the MBASW over the 96h exposure period and strains *C. haemulonii* (NCYC 787) (Figure 33B) and *C. famata* (NCYC 799) (Figure 33E) retained viability but showed no net growth (i.e. the SI's remained about 100). The remaining two strains *C. famata* (NCYC 798) (Figure 33F) and *C. torresii* (NCYC 786) (Figure 33G) both grew to give uncountable colonies (from undiluted samples) during the 96h incubation period.

2.2.1.3. *CFSN control fluid*

Five of the seven *Candida* strains were exposed to CFSN. Four out of five of the yeasts grew well in the CFSN with SI's of greater than 200 at 24, 48, 72 and 96h (*C. haemulonii* (NCYC 787), Figure 33B; *C. famata* (NCYC 798), Figure 33F; and *C. torresii* (NCYC 786), Figure 33G). The fourth *Candida*, *C. maris* (NCYC 785), Figure 33C was tested at 24 and 48h only. The remaining yeast *C. marina* (NCYC 784), (Figure 33A) did not grow in CFSN after 24h, the SI's falling to 50 and a further decrease in SI's to approximately 20 at 96h.

Thus, when comparing the SI values of the seven *Candida* strains in the *E. esculentus* CF's relative to those in the control fluids, MBASW and CFSN, all of the strains were sensitive to some degree to whole *E. esculentus* CF *in vitro* at 10°C.

2.2.2. Marine Debaryomyces, *Metschnikowia* and *Rhodotorula*

2.2.2.1. *Coelomic fluid*

A further two marine yeast strains *Rhodotorula rubra* (NCYC 787), (Figure 34C) and *Debaryomyces hansenii* (NCYC 792), (Figure 34D) plus the two marine yeasts used in the previous investigations, *M. zobelli* (NCYC 783) (Figure 34B) and *R. rubra* (NCYC 63) (Figure 34A) were exposed to *E. esculentus* CF's (n = 3 to 34) and samples taken at 24, 48, 72 and 96h. The data for the bacterial control strain *Ps.111*, incorporated into all test mixtures are also presented in Figure 34E.

The SI's of both strains of *R. rubra* (NCYC 797 and 63) (Figures 34A and 34C) exposed to CF's, decreased to about 50 at 24h with a further fall of the SI's to about 25 at 72 and 96h. The SI's of *M. zobelli* (NCYC 783) (Figure 34B) were reduced to 25 at 24h and remained static at this level at 48h, the SI's increased to about 30 at 72h and further to 85 at 96h.

The SI's of *D. hansenii* (Figure 34D) were reduced to approximately 55 with regrowth occurring at a level of about 150 at 48, 72 and 96h.

2.2.2.2. *MBASW control fluid*

All four marine yeasts grew in the control fluid MBASW, albeit slowly at 10°C in all cases with an increase in SI's from 100 to greater than 200 at 48h to 72h incubation. *Ps.111* grew well in MBASW reaching SI's of greater than 200 at 24h.

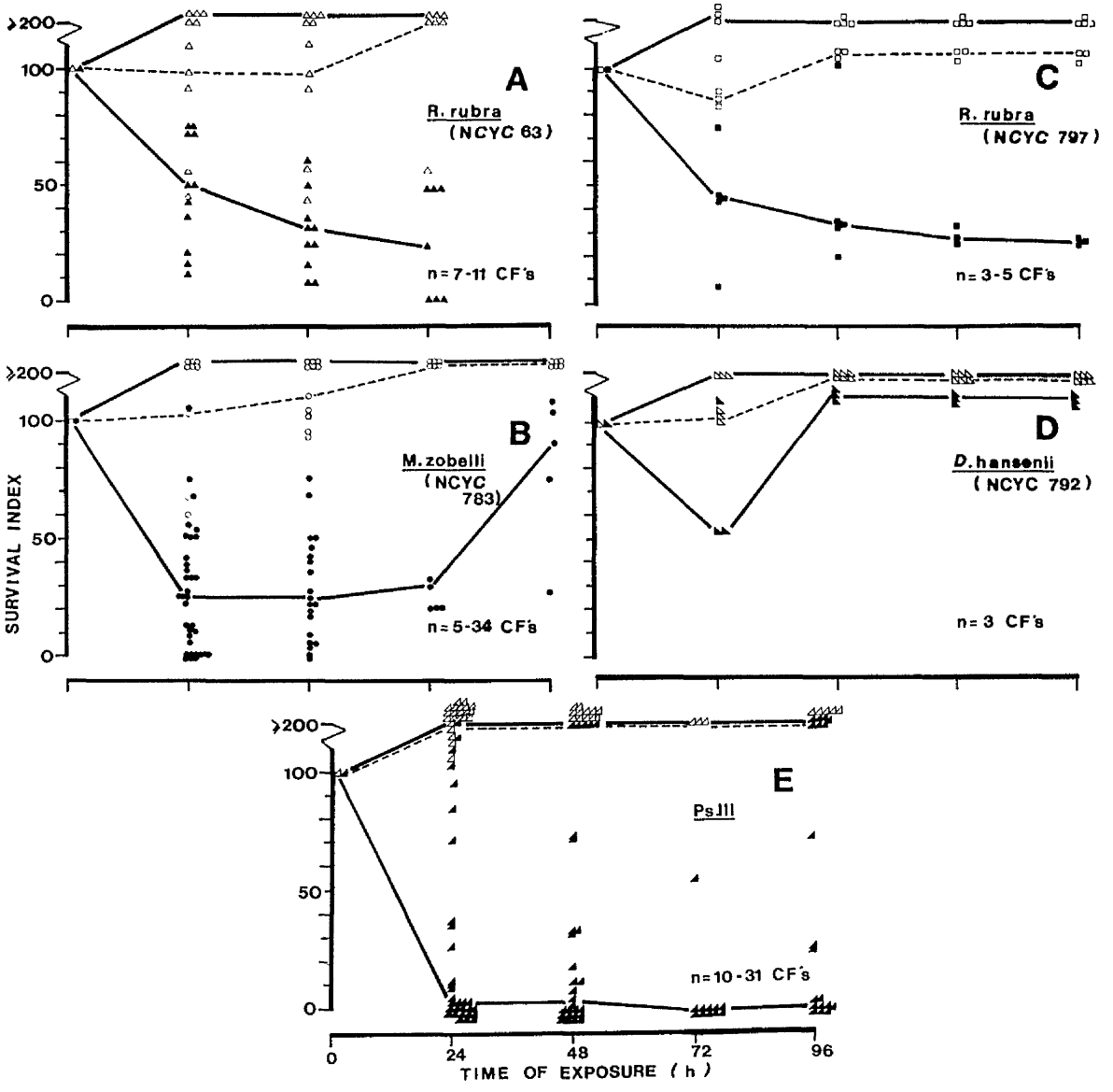
FIGURE 34. Antifungal and antibacterial activity of *E. esculentus* CFs (n = 3 to n = 4) towards strains of *Debaryomyces, Metschnikowia* and *Rhodotorula* and the bacterial control strain *Ps. 111* and growth of microorganisms in CFSN and MBASW control fluid at 10°C.

The data are expressed as SI's at 24, 48, 72 and 96h.

Solid points : CF ;

Open points with solid line : CFSN ;

Open points with dashed line : MBASW.


A
R. rubra
(NCYC 63)
n=7-11 CF's
B
M. zobelii
(NCYC
783)
n=5-34 CF's
C
R. rubra
(NCYC 797)
n=3-5 CF's
D
D. hansenii
(NCYC 792)
n=3 CF's
E
Ps III
n=10-31 CF's
SURVIVAL INDEX
TIME OF EXPOSURE (h)
>200
100
50
0
0
24
48
72
96

2.2.2.3. *CFSN control fluid*

All four marine yeast strains and *Ps.111* grew well in CFSN over the 96h, SI's increasing to greater than 200 at 24h.

Thus, with reference to the data in both Figures 33 and 34, the general trend was that all yeast strains tested were sensitive to the fungicidal activity of *E. esculentus* CF to some degree over the 96h exposure period, however, none of the eleven yeast strains were as sensitive to *E. esculentus* CF as was the highly sensitive bacterial control strain *Ps.111*.

In an attempt to compare the sensitivities of all eleven yeast strains they were arranged primarily on the basis of their SI values at 24h and then, when possible, the SI values at 48 and 72h. The 96h exposure period was not taken into consideration while arranging this list for two reasons:

a. not all yeast strains were tested at 96h (four out of eleven) and

b. marine yeasts four out of seven regrew at 96h and therefore difficulty arose in arrangement when the 96h SI's were included (Table 14).

Therefore, when the marine yeasts were arranged in order of sensitivity to *E. esculentus* CF (expressed as the median SI values), *C. marina* (NCYC 784) was found to be the most sensitive strain and *C. torresii* (NCYC 786) amongst the most resistant strains. From Table 14 a few trends are apparent. The two marine *Candida* strains (*C. marina* (NCYC 784) and *C. maris* (NCYC 785)) are seen to be amongst the most sensitive strains tested, however, it must also be taken into account that both of these yeasts did not retain viability over the 72h in the MBASW control fluid and the former

TABLE 14. Order of the sensitivity of marine yeasts and *Ps 111* to *E. esculentus* CF *in vitro* at 10°C.

	NCYC strain	Median SI at time of exposure of yeast strains to *E. esculentus* CF's* at			
		24	48	72	96 h
1	784 *C. marina*	0	5	5	10
2	785 *C. maris*	25	20	10	nt
3	783 *M. zobelli*	25	25	30	90
4	787 *C. haemulonii*	30	25	15	nt
5	145 *C. guilliermondii*	35	25	20	nt
6	63 *R. rubra*	50	30	25	nt
7	797 *R. rubra*	45	35	25	25
8	799 *C. famata*	60	40	45	40
9	798 *C. famata*	55	60	75	100
10	792 *D. hansenii*	55	150	150	150
11	786 *C. torresii*	50	>200	>200	>200
	Ps. 111	0	0	0	0

* (n = 3 - 34)

strain also did not survive in the second control fluid, CFSN. By contrast, all other yeast strains tested grew well in this CF, cell-free extract.

Both *R. rubra* strains (NCYC 63) and NCYC 797) were of a similar sensitivity to the CF's as were the two strains of *C. famata* (NCYC 798 and NCYC 799) and are grouped together in the Table (14) according to their median SI's.

All of the above observations were at an incubation temperature of 10°C.

2.3. Effect of Incubation Temperature

It was therefore of interest to demonstrate the antifungal and antibacterial activity of *E. esculentus* CF at incubation temperatures other than 10°C.

Since the effect of temperature on the antifungal and antibacterial activity of *E. esculentus* coelomic fluid was to be investigated, two extreme temperatures relative to 10°C were selected, 5 and 23°C. The former temperature was chosen since this was about the lowest temperature which may be found in the natural environment of *E. esculentus* during the Winter months. The second temperature, 23°C was chosen, since this may be of the order found in shallow waters during the Summer months.

Four marine yeast strains were selected, the main criterion for choice being that they must grow in MBASW control fluid at 10°C. The NCYC strains were also selected on the basis of their sensitivity to *E. esculentus* CF at

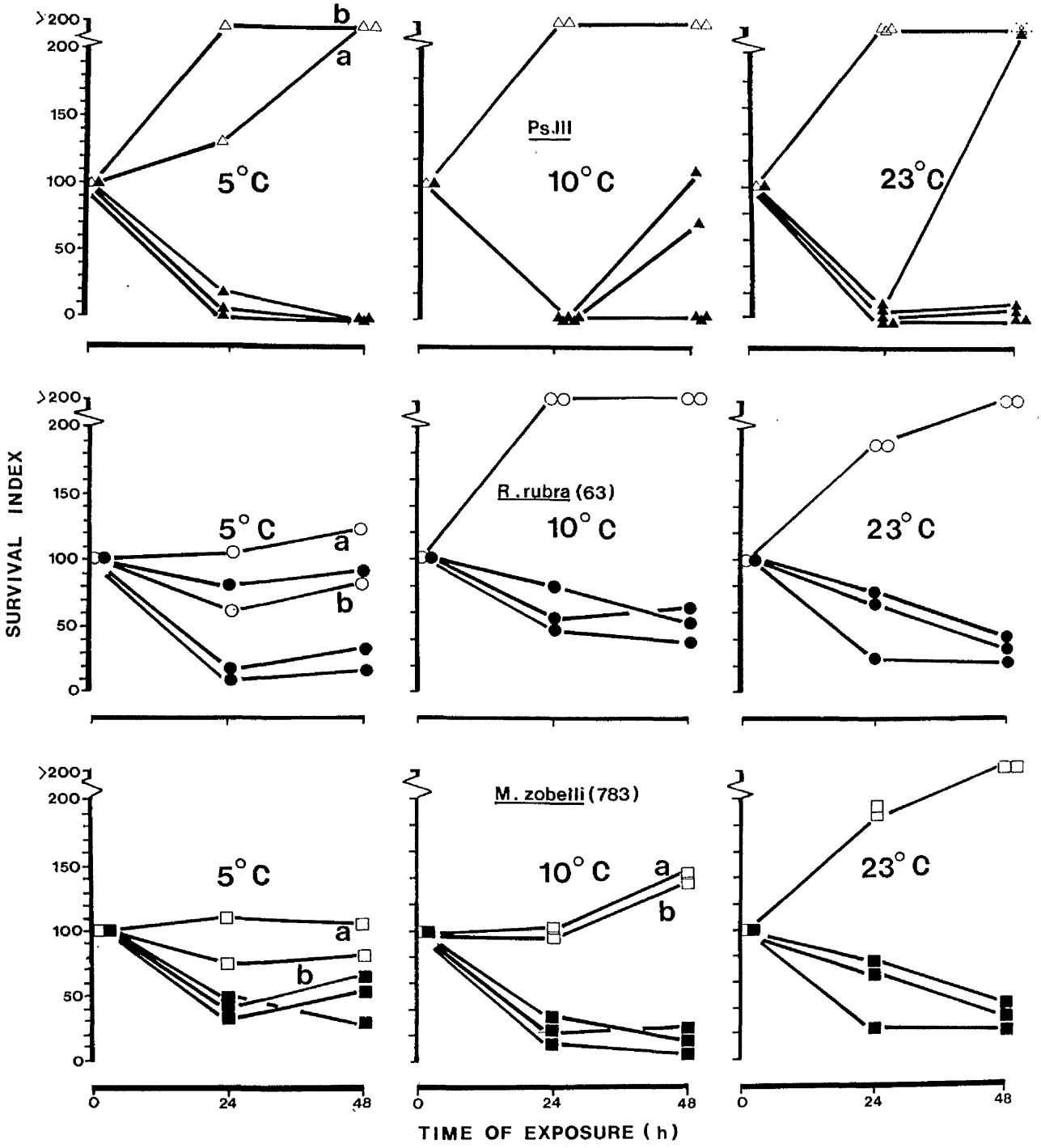
10°C, *C. marina* (NCYC 784) and *M. zobelli* (NCYC 783) two of the most sensitive strains tested, with median SI's of 0 and 25 respectively at 24h (Table 14). The marine strains *R. rubra* (NCYC 63) and *C. famata* (NCYC 798) with SI's of 45 and 55 respectively, at 24h were more resistant to the CF at 10°C. It may be noted here that the mean SI at 24h was found to be 40, the former therefore, of average sensitivity and the latter relatively resistant to *E. esculentus* CF.

The antimicrobial test was done as before, adopting a similar procedure of adding 1.8ml of *E. esculentus* CF to a 0.2ml mixed inoculum of marine yeast and control strain, *Ps.111.* The mixtures were incubated at room temperature, 23°C, in the aquaria at 10°C and in water at 5°C in a vacuum flask contained within a refrigerated room (8°C (range 4 to 12°C). Samples (0.1ml) were taken at 24 and 48h (*R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783)) and a further sampling time of 96h was included in the second instalment of the experiment to monitor the incidence of regrowth *C. marina* (NCYC 798) and *C. famata* (NCYC 784).

The effect of three incubation temperatures on the antifungal and antibacterial activity of CF's towards *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and *Ps.111* are presented in Figure 35. *Ps.111* was seen to grow well in both control fluids MBASW and CFSN at 24 and 48h (Figure 35) at all three temperatures. On exposure to *E. esculentus* CF, at 5°C the SI's were reduced to less than 20 at 24h, and completely killed at 48h with SI's of zero. At 10°C the *Ps.111* were reduced to SI's of zero at 24h, however, 2 of the fluids allowed regrowth of the bacteria at 48h. At 23°C a similar pattern emerged, SI's of zero at 24h, and one CF allowed regrowth at 48h.

FIGURE 35. Effect of incubation temperature on the antifungal activity of *E. esculentus* CF (n = 3 to n = 5) towards *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and *Ps. 111* and growth of the microorganisms in CFSN (a) and MBASW (b) control fluids.
The data are expressed as SI's at 24 and 48h.

Solid symbols : CF ;
Open symbols : control fluids CFSN (a) and MBASW (b).


SURVIVAL INDEX
>200
200
150
100
50
0
5°C
10°C
23°C
a
b
Ps.III
R.rubra (63)
M. zobelli (783)
0
24
48
TIME OF EXPOSURE (h)

Turning to one of the yeast strains, *R. rubra* (NCYC 63) (Figure 35B), it grew slowly in the control fluid CFSN (a) and did not grow in MBASW (b) at 5°C, but SI's of 200 were reached at 10°C at 24 and 48h. *R. rubra* (NCYC 63) also grew well in both of the control fluids at 23°C. On exposure to *E. esculentus* CF's (n = 3) *R. rubra* (NCYC 63) was most sensitive at 5°C; the SI's to below 30 over the 48h incubation period. At 10 and 23°C the SI's ranged between 25 and 80 over 48h.

M. zobelli (NCYC 783) (Figure 35C) was observed to grow in the control fluids CFSN (a) and MBASW (b) at all 3 incubation temperatures, with better growth at 10 and 23°C. *M. zobelli* (NCYC 783) was most sensitive to *E. esculentus* CF (n = 3) at 10°C the SI's reduced to about 20 over 48h exposure. At 5°C the SI's were reduced to about 50 over 48h and at 23°C the yeast was less sensitive at 24h (two out of three with SI's at 80) and at 48h SI's about 25.

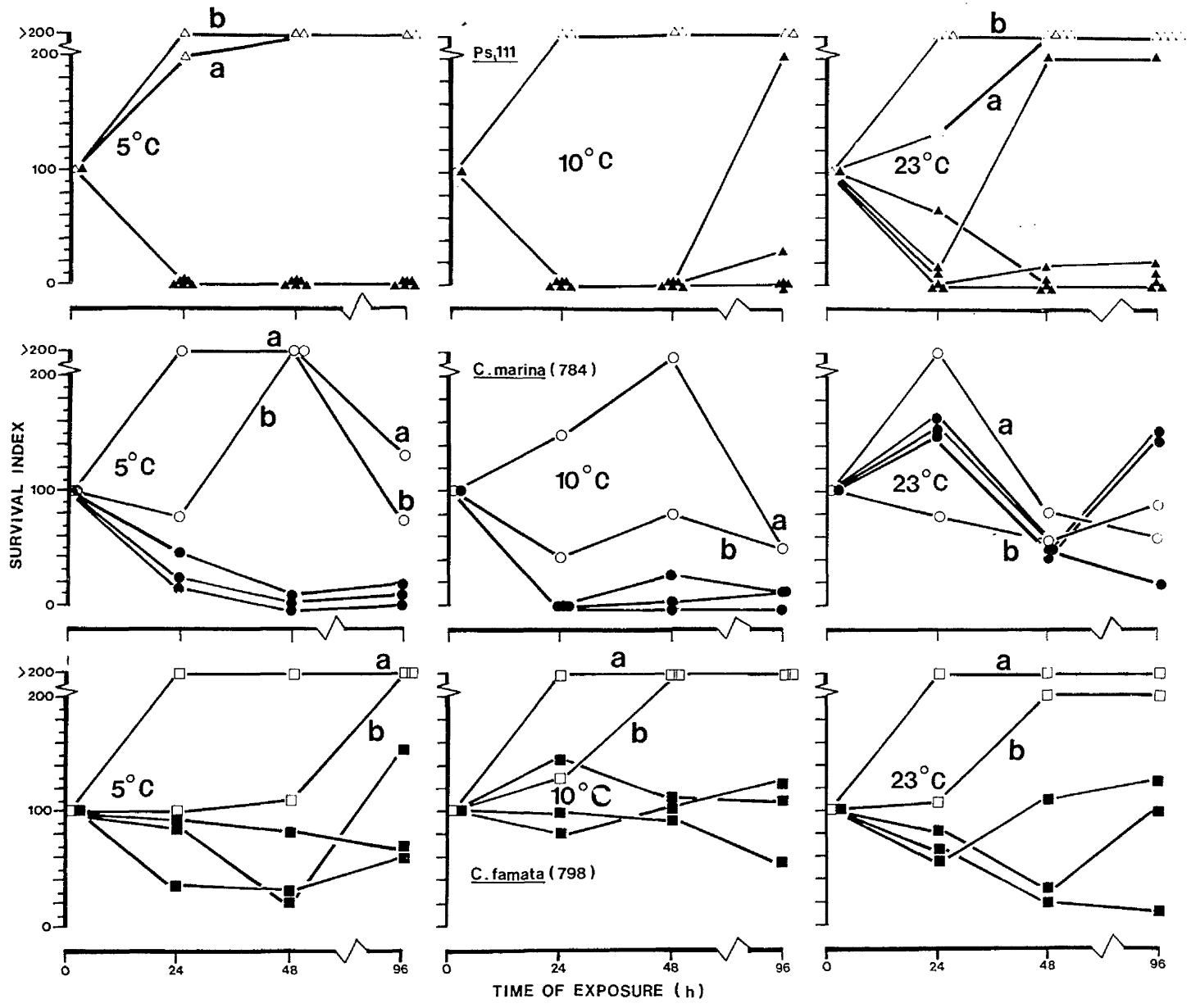
The effect of incubation temperature on the antibacterial and antifungal activity of *E. esculentus* CF towards *Ps.111*, *C. marina* (NCYC 784) and *C. famata* (NCYC 798) are presented in Figure 36. The bacterial control strain *Ps.111* (Figure 36) followed a similar pattern as before, with growth in the control fluids MBASW and CFSN (superior growth in the latter fluid) at all three temperatures of incubation, 5°C, 10°C and 23°C. On exposure to *E. esculentus* CF the bacteria were completely killed within 24h at 5°C with no regrowth on further incubation at 72h. At 10°C all 6 CF's reduced the SI's of *Ps.111* below zero over 48h.

At 23°C the majority of the CF's (five out of six) reduced the SI's to less than 20, remaining at this level over the following 72h. *C. marina* (NCYC 784) grew consistently better in the CFSN than in the MBASW control

FIGURE 36. Effect of incubation temperature on the antifungal and antibacterial activity of *E. esculentus* CF (n = 3 to n = 5) towards *C. marina* (NCYC 784), *C. famata* (NCYC 798) and *Ps.111*, respectively and growth of the microorganisms in CFSN (a) and MBASW (b) control fluids.

The data are expressed as SI's at 24, 48 and 72h.

Solid symbols : CF ; open symbols : control fluids CFSN (a) and MBASW (b).


SURVIVAL INDEX
>200
200
100
0
5°C
10°C
23°C
Ps.111
C. marina (784)
C. famata (798)
a
b
0
24
48
96
TIME OF EXPOSURE (h)

fluid at all three temperatures. On exposure to *E. esculentus* CF the yeast was more sensitive at the lower temperatures with the majority of the CF's reducing SI's to less than 20 over the 96h. At 23°C, however, the yeast appeared to grow (SI's about 150) with a consequent fall in the SI's to about 25 at 48h. At 96h regrowth occurred in two out of three fluids the remaining CF reducing the SI further to about 25.

C. famata (NCYC 798) also grew consistently better in CFSN compared to MBASW at all three temperatures. On exposure to *E. esculentus* CF the SI's of this yeast remained at about 100 at 10°C over 96h, with similar evidence of antifungal activity at 5 and 23°C.

2.4. Sonicated Coelomic Fluid

Having detected fungicidal activity in *E. esculentus* coelomic fluid, it was clearly of interest to determine whether intact coelomocytes were required for this process. Therefore, a coelomocyte lysate (CFL) was prepared by sonication and a clear extract obtained by sterile-filtration. Information on the antibacterial properties of this type of preparation towards *Ps.111* was available from Unkles (1976), and for this reason this bacterial strain was again chosen as a control.

The yeast strain *Candida haemulonii* (NCYC 787) was chosen because :

a. it grew in both MBASW and CFSN control fluids, and

b. it was amongst the most sensitive yeast strains to *E. esculentus* CF.

The antimicrobial test was set up by adding a mixed inoculum of (0.2ml) *Ps.111* and *C. haemulonii* (NCYC 787) to a 1.8ml volume of coelomic fluid lysate (CFL). Samples were taken at 24 and 48h intervals and 0.1ml volumes (undiluted and serial dilutions) spread on MA and YMA and incubated at 48-72h at room temperature (approx. 22°C).

The SI's of *Ps.111* in MBASW and CFSN control fluids and five CFL's are presented in Figure 37. It will be noted here that the SI's are presented as Log_{10} values in place of the arithmmetic scale, since the use of serially diluted samples allowed enumeration of the growth of *Ps.111* in the control fluids beyond 200 to values exceeding 1×10^5. Since the SI's of the bacteria were below 100 in the test fluid the use of a logarithmic scale was more convenient to incorporate and offset the contrasting data of growth in control and test fluids. *Ps.111* grew well in both control fluids MBASW and CFSN although significantly better in the former.

On exposure to CFL the bacteria were completely killed within 24h, all 5 CFL's yielding zero SI's and no regrowth of the bacteria after a further 24h incubation.

The data of *C. haemulonii* (NCYC 787) in both control (MBASW and CFSN) and test fluids (CFL) are presented in Figure 38. The yeast grew well in both control fluids MBASW and CFSN, particularly in the latter. The

FIGURE 37. Bactericidal activity of *E. esculentus* coelomic fluid lysate (CFL) (n = 5) towards *Ps.111* and growth of the bacteria in CFSN and MBASW control fluids at 10°C.

Data are expressed as SI's at 24 and 48h.

Closed symbols : CFL ; open symbols with solid line : MBASW ; open symbols with dotted line : CFSN ; dotted line only : SI = 100.

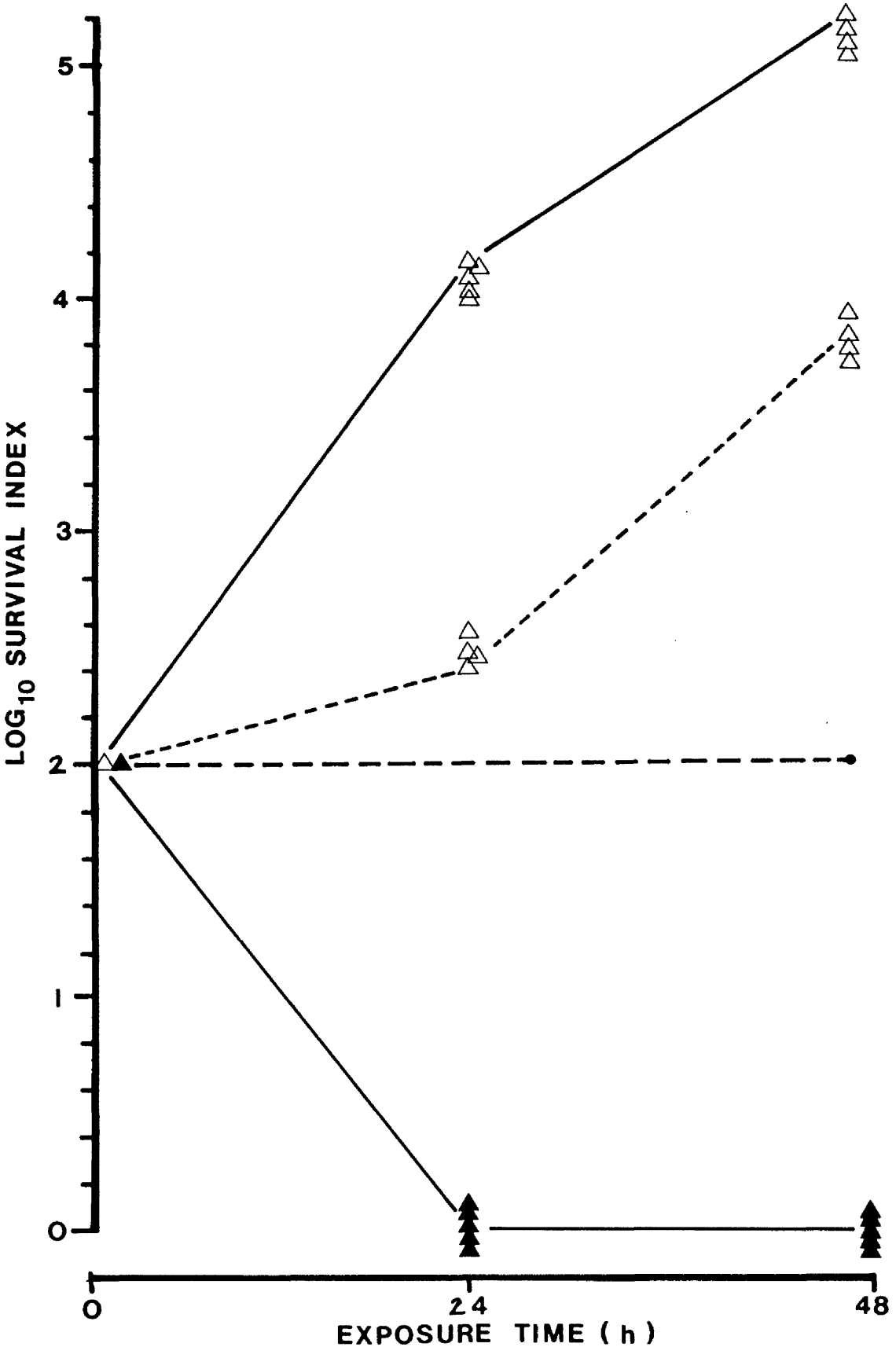
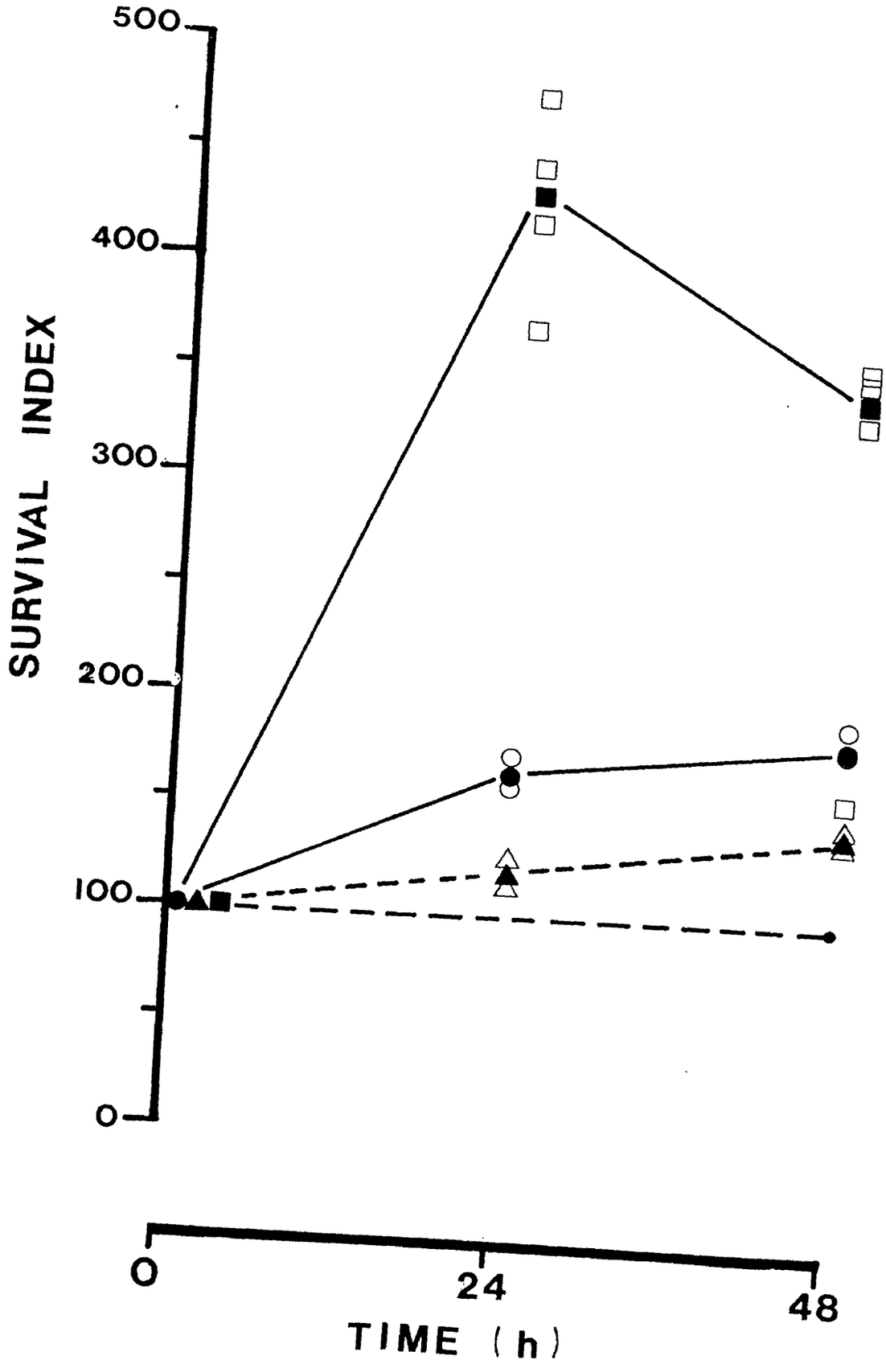

5
4
3
2
1
0
LOG10 SURVIVAL INDEX
0
24
48
EXPOSURE TIME (h)

FIGURE 38. Antifungal activity of *E. esculentus* coelomic fluid lysate (CFL) (n = 5) towards *Candida haemulonii* (NCYC 787) and growth of the yeast in CFSN and MBASW control fluids at 10°C.

Data are expressed as SI's at 24 and 48h.

Open squares : CFL ; closed squares : CFL (mean) ; open circles : CFSN ; closed circles : CFSN (mean); open triangles : MBASW ; closed triangles : MBASW (mean) ; dotted line : SI = 100.


500
400
300
200
100
0
SURVIVAL INDEX
0
24
48
TIME (h)

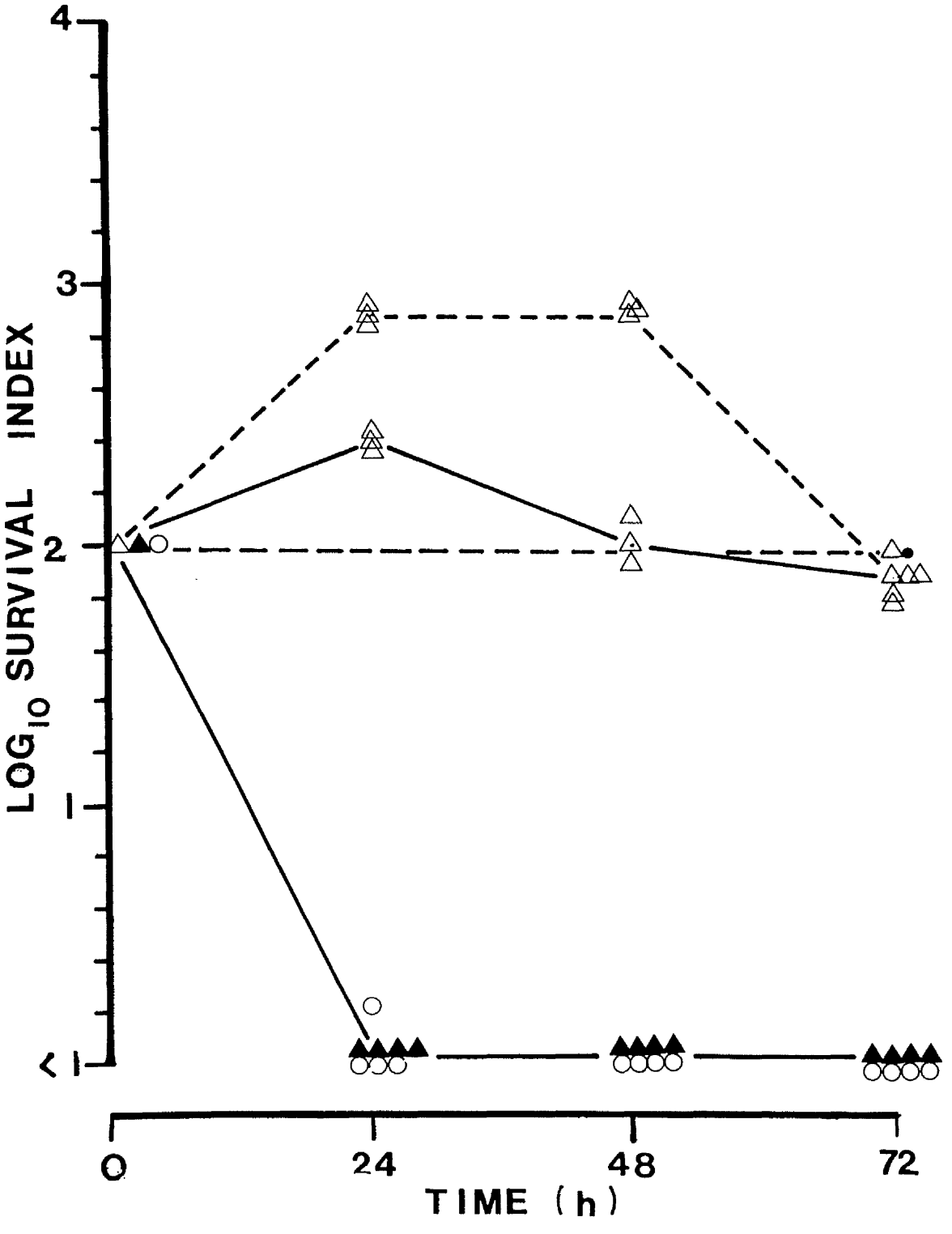
reverse was true for *Ps.111* with superior growth in the MBASW control fluid. CFL allowed growth of *C. haemulonii* (NCYC 787) and there was no evidence of antifungal activity. Growth in this fluid was greater than that in either control fluids MBASW or CFSN. This means there was potent bactericidal activity of the CFL towards *Ps.111*, but the same preparation of sonicated CF behaved as a good growth medium for the marine yeast, *C. haemulonii* (NCYC 787).

These experiments were done with MBASW and CFSN control fluids and CFL's. To compare the antimicrobial activity of the *E. esculentus* whole coelomic fluids with the corresponding CFL's, these were incorporated into the design of further experiments. The MBASW control fluid was also replaced with ASW (without the trace of nutrient) as one of the control fluids, to compare growth of the yeast and bacteria in the absence of added 1% (w/v) MB. The experiment was extended to 72h exposure to observe the possibility of regrowth of remaining viable cells after exposure to CF and CFL.

The data obtained for the antibacterial activity of CF and CFL towards *Ps. 111* are presented in Figure 39. The bacteria grew in both control fluids ASW and CFSN, again growth was superior in the ASW even in the absence of the trace of nutrient (1% (w/v) MB). The control strain *Ps.111* was completely killed in both the whole CF and CFL within 24h, and the SI's remained at zero over 48 and 72h and no regrowth of the bacteria occurred.

FIGURE 39. Comparison of the antibacterial activities of *E. esculentus* CF and CFL (n = 4) towards *Ps.111* and growth of the bacteria in CFSN and ASW control fluids at 10°C. Presented as SI's at 24, 48 and 72h.

Closed triangle, solid line : CF ; open triangles, dotted line : ASW ; open triangles, solid line : CFSN ; open circles, solid line : CFL ; dotted line : SI = 100.


4
3
2
1
< 1
LOG10 SURVIVAL INDEX
0
24
48
72
TIME (h)

Comparison of the antifungal activity of both *E. esculentus* whole CF and CFL is presented as SI's at 24, 48 and 72h in Figure 40. The yeast did not grow in either the ASW or CFSN control fluids, however, the yeast did survive to some extent, particularly in the latter fluid. Whole *E. esculentus* CF reduced the *C. haemulonii* (NCYC 787) by about 55% of the original inoculum at 24h, remaining static at this level over 72h. Conversely, the CFL was observed to behave as a growth medium of the yeast maintaining the SI's of *C. haemulonii* (NCYC 787) at 100 over 24 and 48h, the SI's increasing to about 160 at 72h.

Summarizing therefore, it was established that CFSN supports growth of *Ps.111* and survival of *C. haemulonii* (NCYC 787). CFL and CF was found to be highly bactericidal towards *Ps.111*, however CFL was not antifungal allowing growth of *C. haemulonii* (NCYC 787), the corresponding CF showing some antifungal activity. The implication being that intact cells are required for antifungal activity, therefore, the next step was to separate the CF into fractions and examine the relative antifungal and antibacterial activity.

2.5. Fractionated Coelomic Fluid

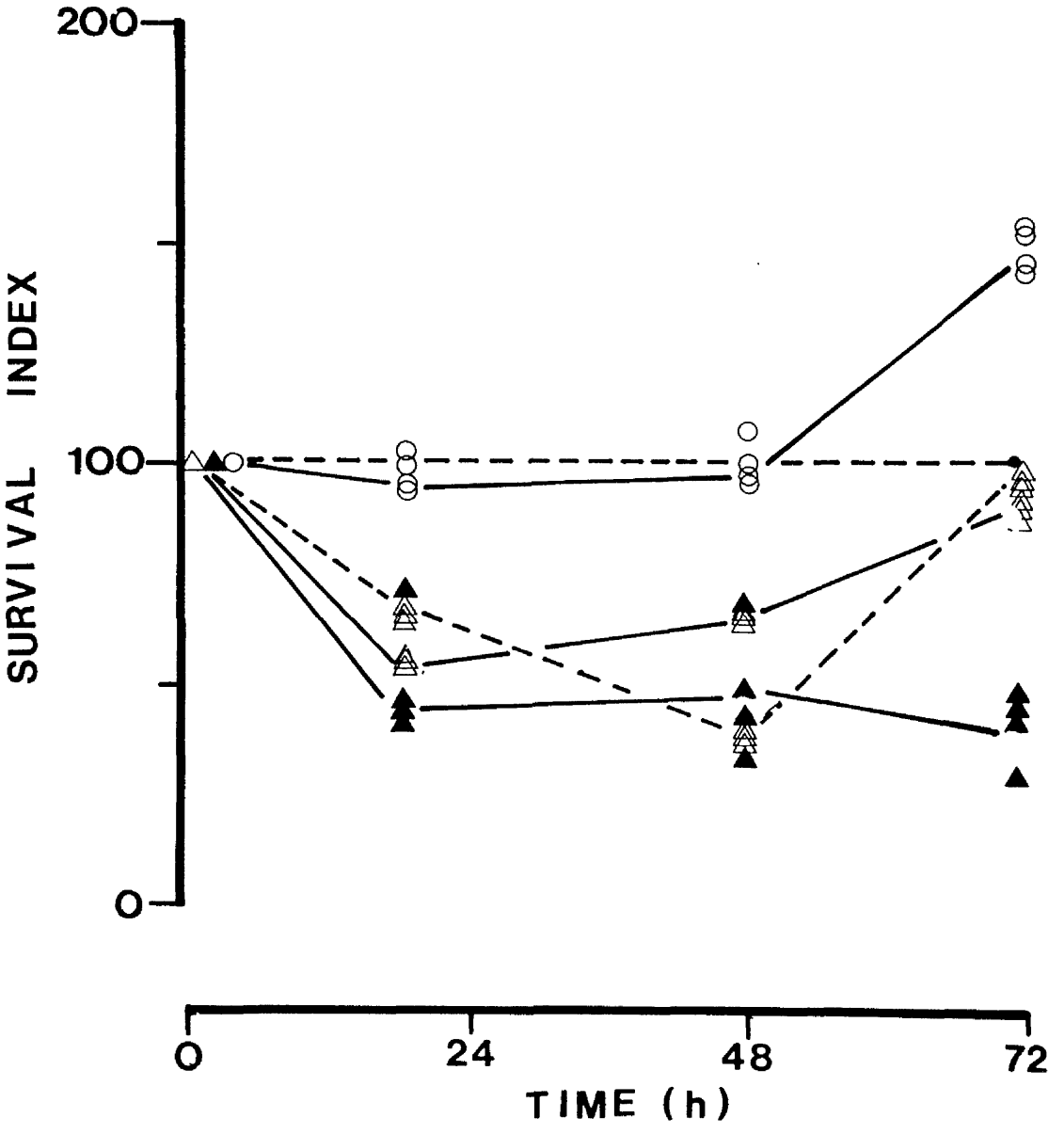
2.5.1. Density gradient separation of coelomocytes

To examine the antifungal and antibacterial activities of separated coelomocytes *E.esculentus* CF was fractionated on a step-wise,discontinuous

FIGURE 40. Comparison of the antifungal activities of *E. esculentus* CF and CFL (n = 4) towards *C. haemulonii* (NCYC 787) and growth of the yeast in CFSN and ASW control fluids at 10°C.

The data are presented as SI's at 24, 48 and 72h.

Closed triangle, solid line : CF ; open triangle, dotted line : ASW ; open triangle, solid line : CFSN ; open circles, solid line : CFL ; dotted line : SI = 100.


200
100
0
SURVIVAL INDEX
0
24
48
72
TIME (h)

"Ficoll^{R}/Isopaque^{R}" density gradient. However, since the calcium-chelator EGTA (14mM) was to be used to prevent clotting of the CF, the effect of this concentration of anticoagulant towards the two marine yeast strains *C. haemulonii* (NCYC 787), *D. hansenii* (NCYC 792) and the bacterial control strain *Ps.111* were examined. This concentration of EGTA was incorporated to prevent clotting in the CF and throughout the density gradient as described in the Materials and Methods (1.3.6.).

The effect of exposure of *Ps.111* to 14mM EGTA in MBASW is presented in Figure 41. The SI's of *Ps.111* was observed to decrease to zero at 48h with regrowth exceeding SI's of 100 at 72h, the EGTA exerting a deleterious effect toward *Ps.111*. The bacteria grew well in both control fluids MBASW and CFSN.

The marine yeast *D. hansenii* (NCYC 792) was tested for its growth in the presence of EGTA alongside *C. haemulonii* (NCYC 787) because it was also amongst the most sensitive yeasts to *E. esculentus* CF and therefore if *C. haemulonii* (NCYC 787) reacted adversely to the anticoagulant it could have been used as an alternative test strain. The effect of EGTA towards *D. hansenii* (NCYC 792) is shown in Figure 42. The anticoagulant did not affect growth of the yeast supporting growth comparable to the CFSN control, better growth occurred in the second control MBASW. A similar pattern emerged on exposure of the yeast *C. haemulonii* (NCYC 787) to the same concentration of EGTA (Figure 43) good growth was evident comparable to growth in the MBASW control fluid and better growth than in CFSN.

However, since it was of a distinct advantage to compare the antifungal and antibacterial activities of each CF fraction, the EGTA was washed from the CF fractions before adding them to the antimicrobial test

FIGURE 41. Effect of 14mM EGTA, CF anticoagulant, on the growth of *Ps.111* in MBASW compared with growth of the bacteria in CFSN and MBASW at 10°C.

Data are expressed as SI's at 24, 48, 72 and 96h. (n = 2)

Solid symbols : EGTA in MBASW ; open symbols, solid line : CFSN ; open symbols, broken line : MBASW ; broken line only : SI = 100.

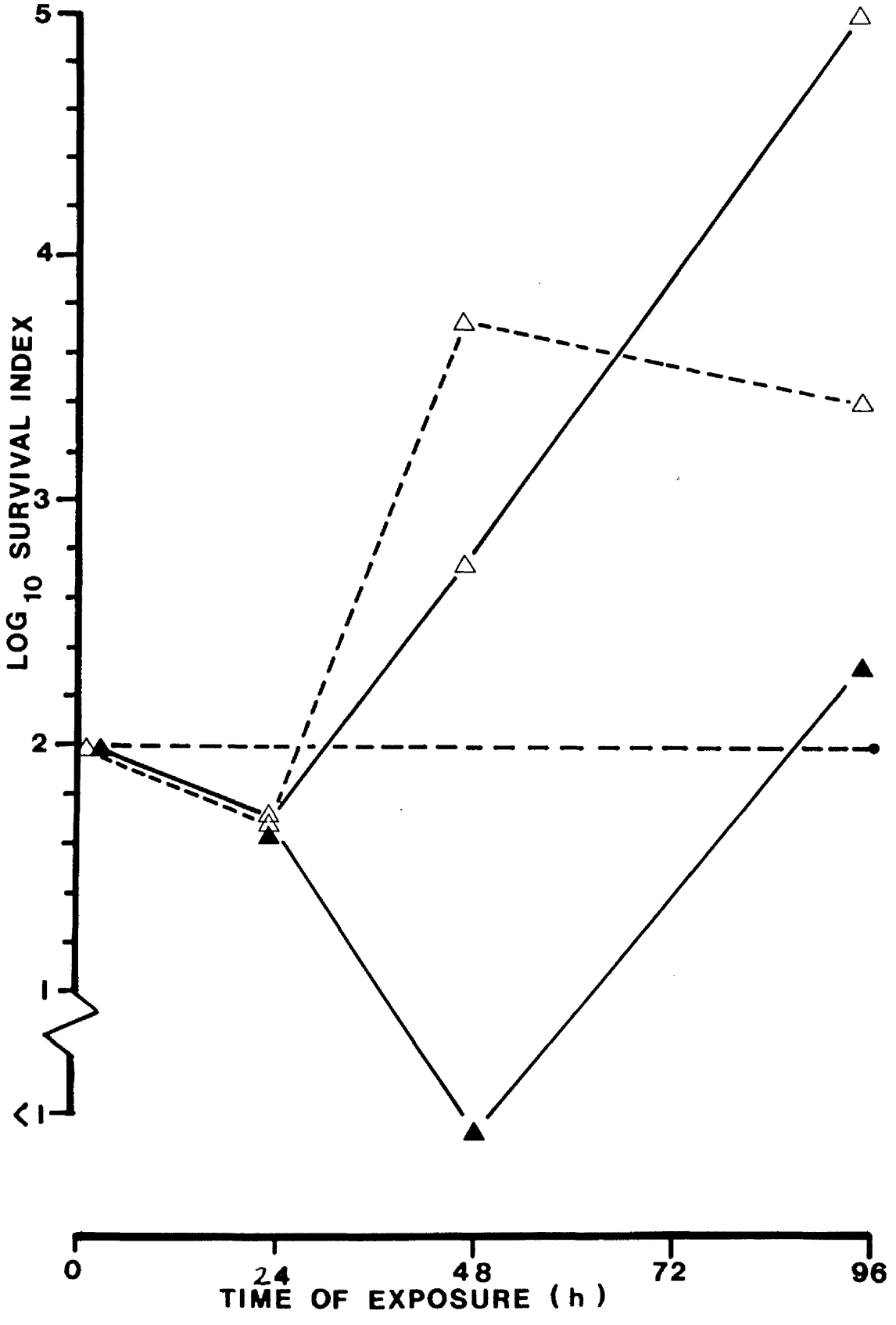

5
4
3
2
1
<1
LOG 10 SURVIVAL INDEX
0
24
48
72
96
TIME OF EXPOSURE (h)

FIGURE 42. Effect of 14mM EGTA, CF-anticoagulant, on the growth of *D. hansenii* (NCYC 792) in MBASW compared with growth of the yeast in CFSN and MBASW control fluid at 10°C.

The data are expressed as SI's at 24, 48, 72 and 96h.

(n = 2)

Closed symbols : EGTA in MBASW ; open symbols, solid line : CFSN ; open symbols, broken line : MBASW ; broken line only : SI = 100.

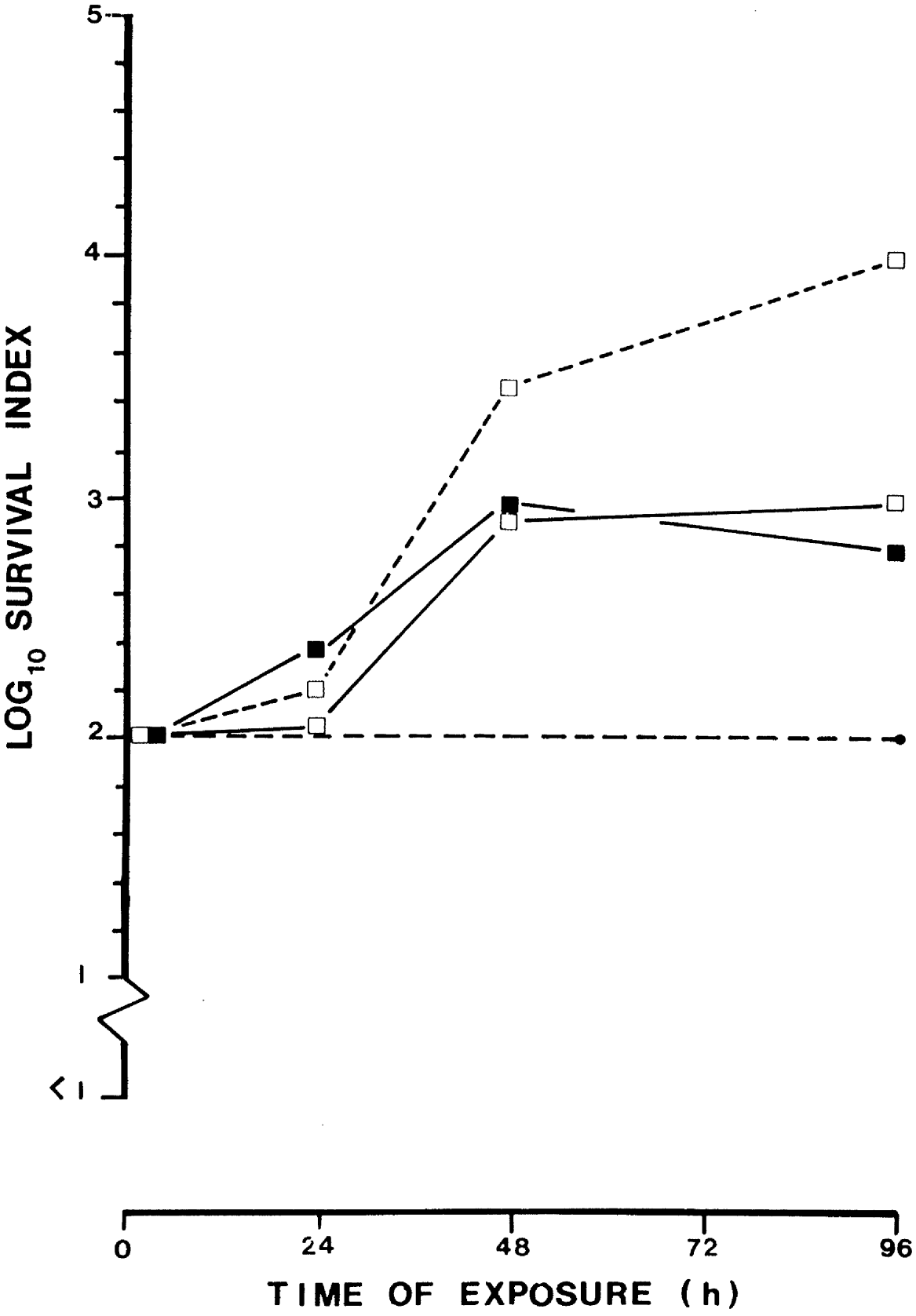
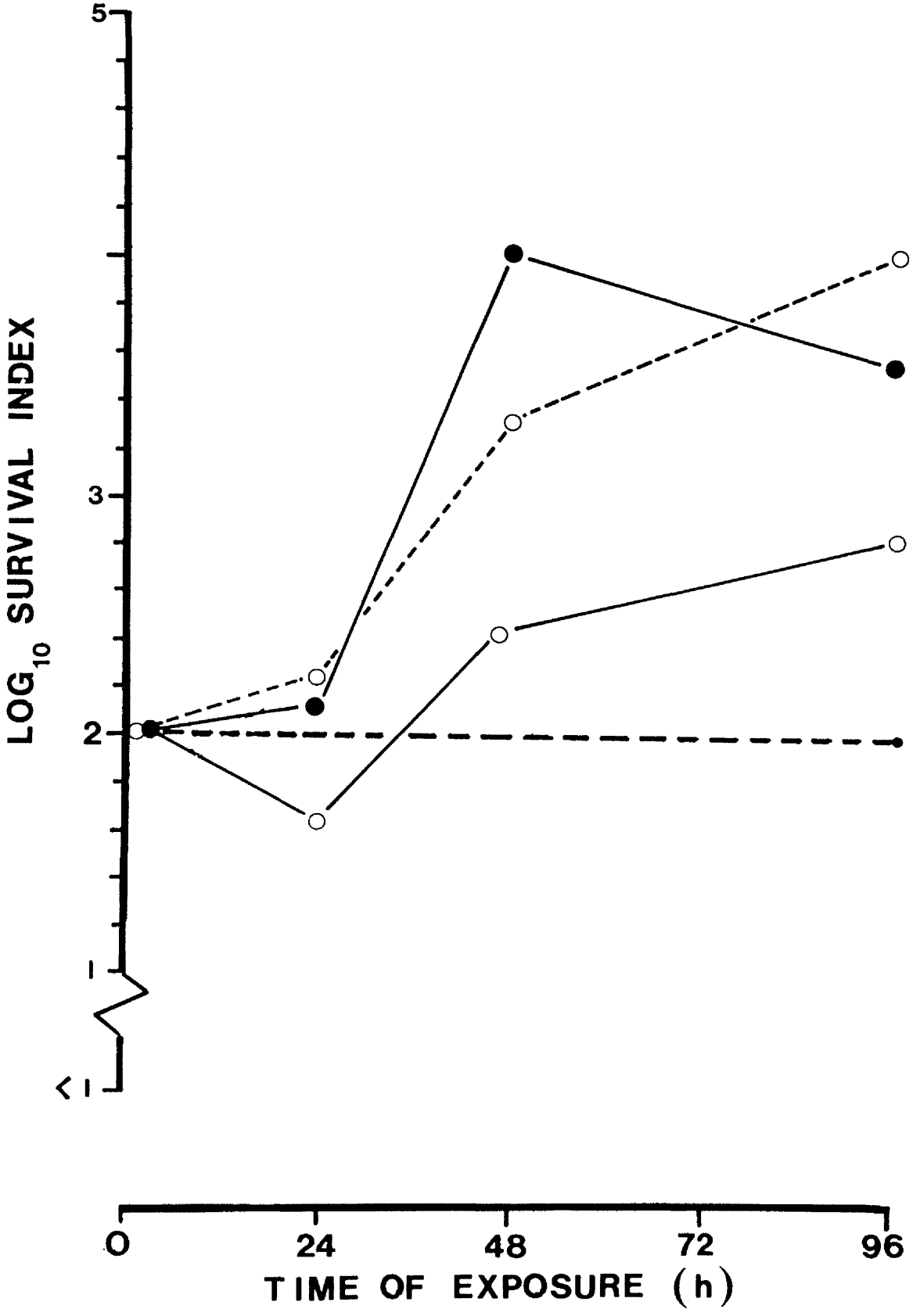

5
4
3
2
1
<1
LOG10 SURVIVAL INDEX
0
24
48
72
96
TIME OF EXPOSURE (h)

FIGURE 43. Effect of 14mM, CF-anticoagulant, EGTA on the growth of *C. haemulonii* (NCYC 787) in MBASW compared with growth of the yeast in CFSN and and MBASW control fluids at 10°C.

The data are expressed as SI's at 24, 48, 72 and 96h.
(n = 2)

Solid symbols : EGTA in MBASW ; open symbols, solid line : CFSN ; open symbols, broken line : MBASW ; broken line only : SI = 100.


5
3
2
1
<1
LOG10 SURVIVAL INDEX
0
24
48
72
96
TIME OF EXPOSURE (h)

mixture of marine yeast and bacteria (this was because 14mM EGTA was found to be deleterious to the growth of the control strain *Ps.111*).

The prepared gradient was loaded with 1.8ml of freshly drawn *E. esculentus* CF containing 0.2ml 140mM EGTA (Figure 44A). The loaded gradients were then centrifuged at 600g for 6 min. Three distinct bands were observed on the gradient (Figure 44B). Microscopical examination of these bands showed that they corresponded to the major types of coelomocytes previously described by Boolootian and Giese (1958), Johnson (1969a, b and c) and Bertheussen and Seljelid (1978) with other species of sea urchin. Thus from the top of the gradient downwards were the bladder amoebocytes or phagocytic leucocytes (PL) (Figure 44B, 1), the vibratile cells (VC) and colourless spherule (morula) cells (CSC) (Figure 44B, 2) and red spherule (morula) cells (RSC) (Figure 44B, 3). Microscopical examination revealed that the layers were not completely pure e.g. vibratile cells were also present in the phagocytic leucocyte layer. The microscopical appearance of each cell type are shown in Figure 45. The VC's possess a long flagellum and were observed to swim rapidly through the fluid (Figure 45A). The PL's were observed to be very motile and spread rapidly on glass, accompanied by a complete change of their morphology to flattened cells with peripheral ruffling (Figure 45B and C).

The most prominent cell band was that containing the phagocytic leucocytes (PL's). These were of two types : rounded bladder amoebocytes and filiform amoebocytes with petaloid ectoplasmic extensions which clumped together to form an interweaving mesh. Other types of coelomocyte were frequently trapped in this mesh, notably the colourless and red spherule cells. Inclusion of the EGTA anticoagulant helped minimize this

FIGURE 44. Fractionation of *E. esculentus* CF. On a stepwise, discontinuous density gradient loaded with 1.8ml CF and 0.2ml 140mM EGTA, before (A) and after centrifugation (B). The coelomocytes were separated into three distinct bands (1, 2 and 3B).

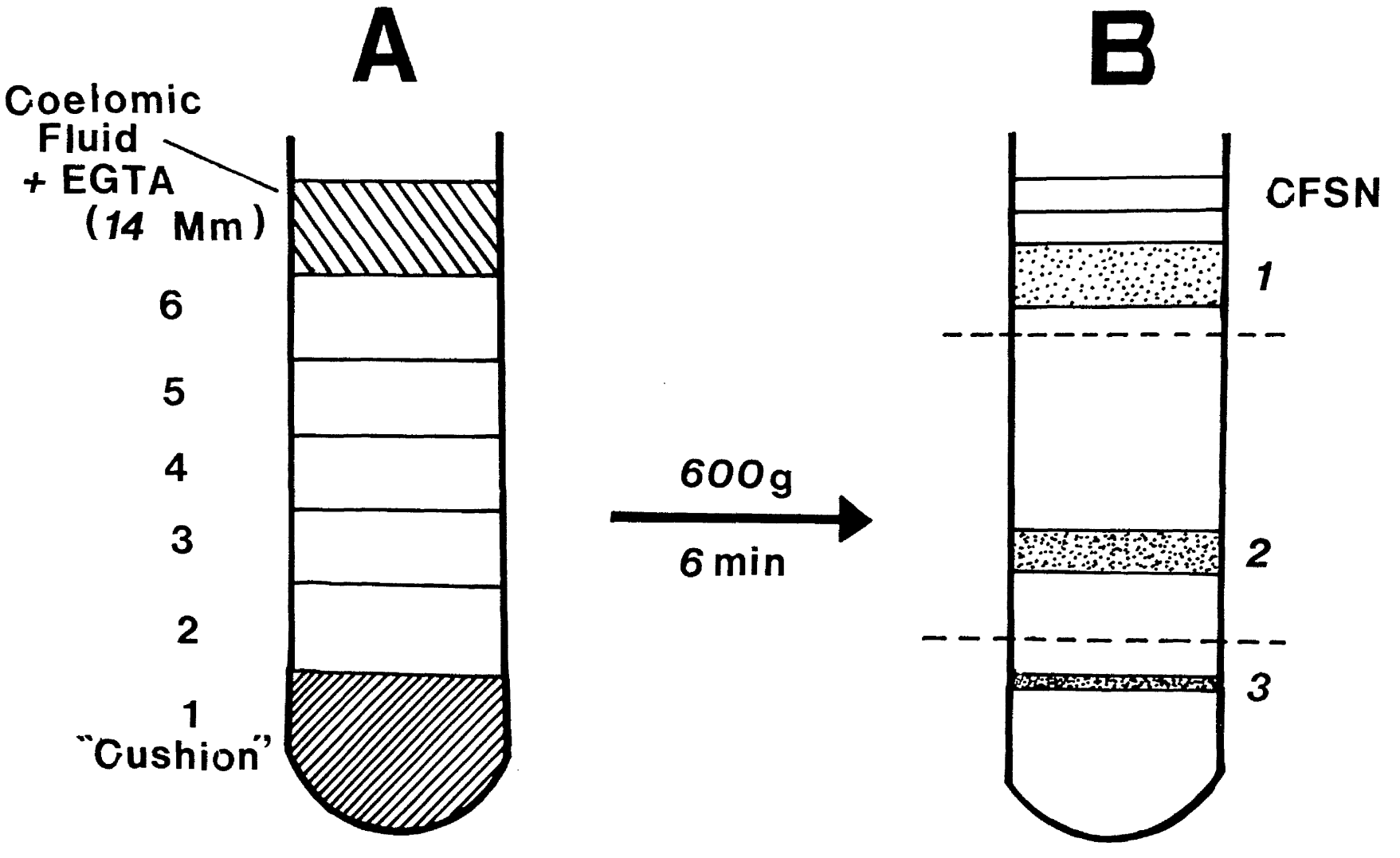

A
B
Coelomic
Fluid
+ EGTA
(14 Mm)
6
5
4
3
2
1
"Cushion"
600g
6 min
CFSN
1
2
3

FIGURE 45. Sketches of the microscopical appearance of wet films of the four coelomocyte types (x 1000) of *E. esculentus* coelomic fluid after density gradient separation.

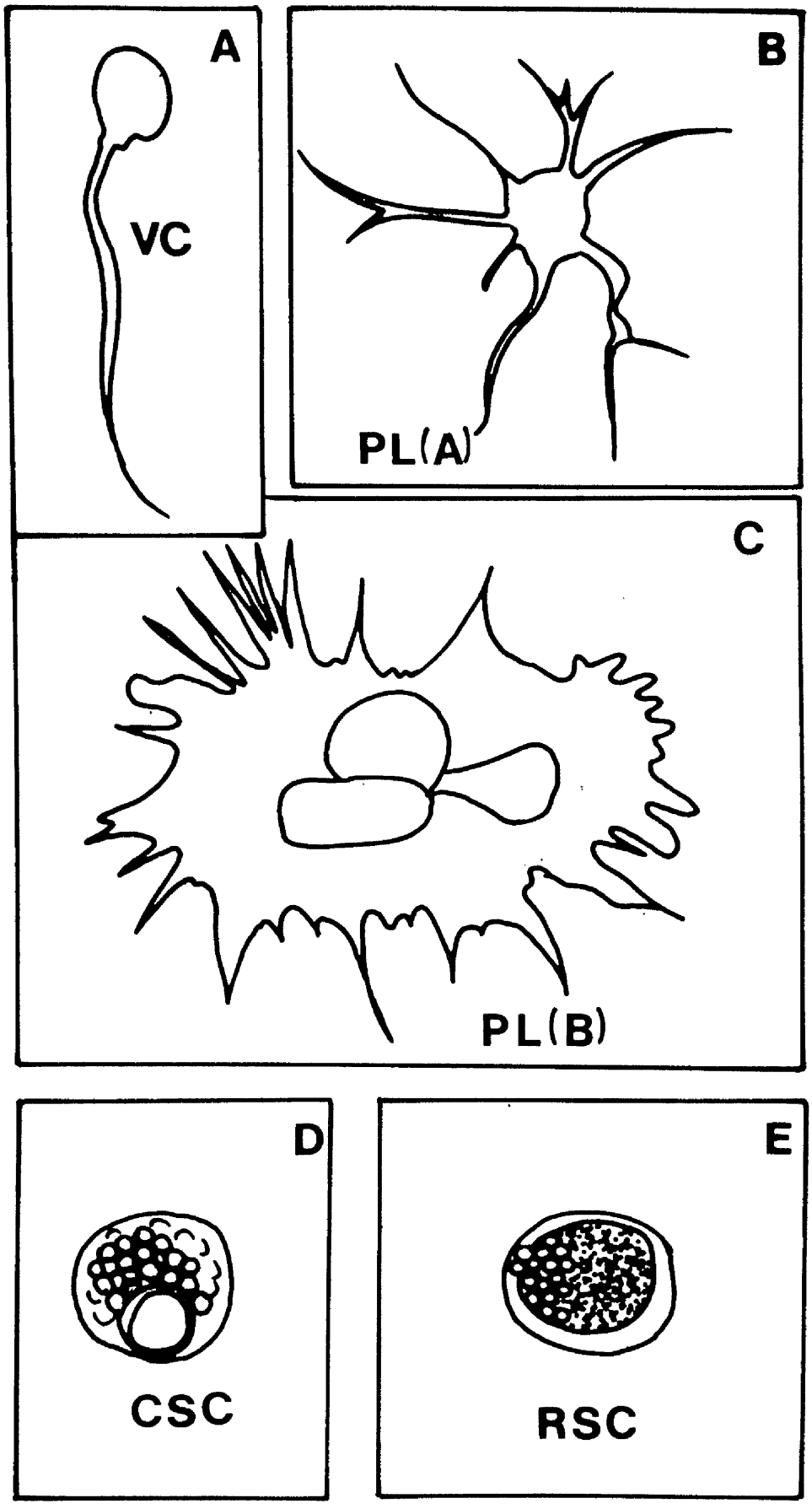
VC = Vibratile cells

PL = Phagocytic leucocytes :

(A) = suspended,
(B) = on glass

CSC = Colourless spherule (morula) cells

RSC = Red spherule (morula) cells


A
VC
B
PL(A)
C
PL(B)
D
CSC
E
RSC

contamination. When the centrifuged coelomocyte gradients were allowed to stand in an ice bath, the band of phagocytic leucocytes formed a coherent disc of aggregated cells which retracted from the walls of the centrifuge tube. This cell-aggregation resembled what took place when whole coelomic fluid was drawn without anticoagulants and allowed to stand at 0 - 4°C. The PL's are therefore believed to be the main aggregating cells in CF *in vitro*. A colour change of the coelomocyte clot was also observed on exposure to air. The clot when first formed was pink, rapidly turning red and then to brown/black. The last process was slow and required between 12 and 24h at room temperature (approx. 22°C). The colourless spherule cells and vibratile cells formed a faint layer below the phagocytic leucocytes. When the gradient was left to stand for a few minutes the band separated into large and small spherule cells and vibratile cells.

The red spherule cells were the densest coelomocytes and were found at the interface between the "cushion" made up of "Ficoll^{R}" in ASW and "Isopaque^{R}". The cells form an easily identifiable red band in the gradient, although on standing at room temperature, the colour faded, changing firstly to orange and then to yellow, gradually diffusing into the "cushion" as the cells lysed.

Each band was gently removed with a Pasteur pipette and washed by suspending the separated bands of coelomocytes in 20ml ASW, centrifuging at 1200g for 15 min to sediment the cells, and the supernates decanted. Each coelomocyte pellet was then resuspended in 1.8ml MBASW (the original volume of CF minus the EGTA).

2.5.2. Antibacterial and antifungal activity of separated coelomocytes.

The antibacterial and antifungal activity of each CF fraction (PL; CSC and VC; RSC) was tested towards *Ps.111* and *C. haemulonii* (NCYC 787). The test consisted of the addition of a 0.2ml mixed inoculum of the yeast and bacteria to a 1.8ml CF fraction. Samples (0.1ml dilute and serially diluted samples) were taken at 24, 48 and 72h and spread on to MA and YMA and incubated 48-72h at room temperature (approx. 22°C). The antibacterial activity of each fraction with MBASW and CFSN as control fluids are shown in Figure 46.

The bacterial control strain, *Ps.111* grew well in both control fluids MBASW and CFSN (Figure 46A) with superior growth in the former fluid. SI's exceeded 10^5 over 48h in both fluids. A decline in growth of *Ps.111* in the *E. esculentus* CF fraction composed mainly of phagocytic leucocytes (PL) (Figure 46B) occurred. The SI's remained at about 100 over 24h with growth of the bacteria exceeding SI's of over 10^6 the following 48h. This means there was a bacteriostatic effect towards *Ps.111* when comparing with growth in the control fluids. Bactericidal activity was present in the red spherule cells (RSC) fraction (Figure 46C) the SI's of *Ps.111* falling to about 50 at 24h, and regrowing to about 50 on a further 24h incubation. Finally, the CF fraction containing a mixture of both colourless spherule cells (CSC) and vibratile cells (VC), as shown in Figure 46D supported growth of *Ps.111* over the 72h exposure period reaching SI's of 10^5.

The antifungal activity of *E. esculentus* CF fractions towards the marine yeast strain *C. haemulonii* (NCYC 787) is presented in Figure 47. Growth of the yeast occurred in both control fluids MBASW and CFSN (Figure

FIGURE 46. Antibacterial activity of density gradient separated *E. esculentus* coelomocytes (B, C and D) (n = 4) towards *Ps.111* and growth of the bacteria in CFSN and MBASW control fluids (A) at 10°C.

The data are presented as SI's at 24, 48 and 72h.

Solid symbols : median plot ; open symbols : actual data ; open and closed triangles (A) : CFSN ; open and closed circles (A) : MBASW.

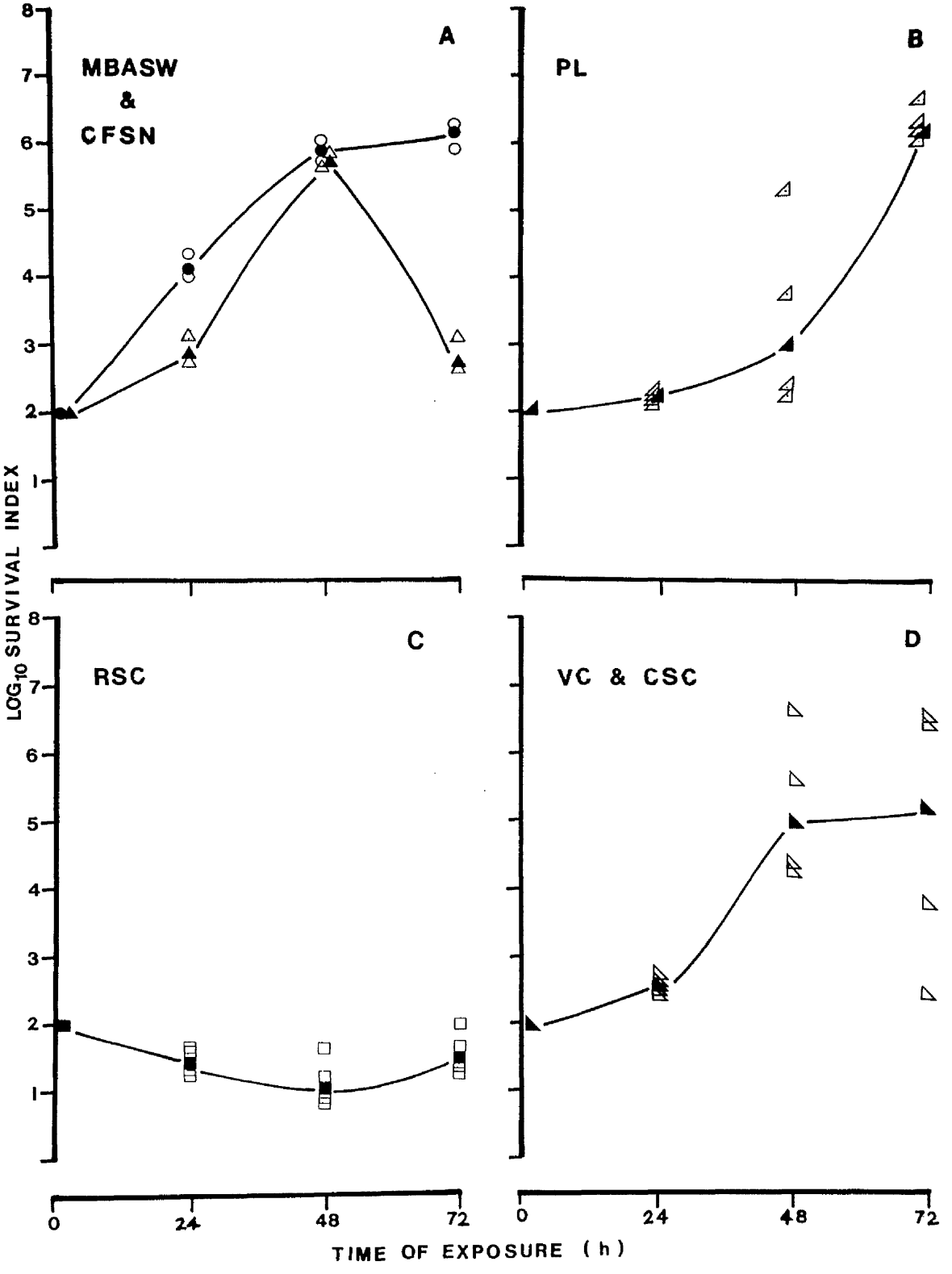
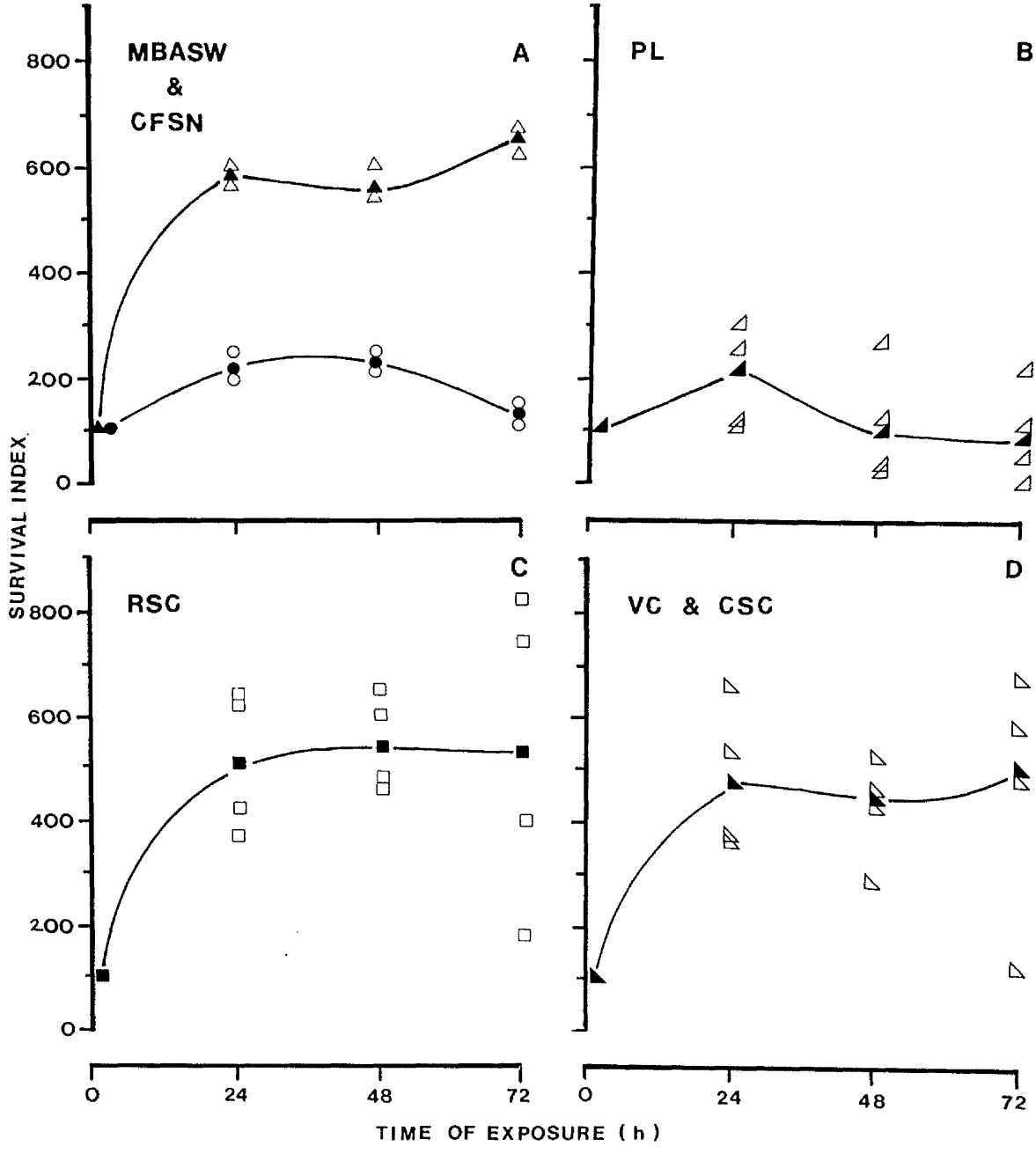

A
MBASW
&
CFSN
B
PL
C
RSC
D
VC & CSC
LOG10 SURVIVAL INDEX
TIME OF EXPOSURE (h)
0
24
48
72
1
2
3
4
5
6
7
8

FIGURE 47. Antifungal activity of density gradient separated *E. esculentus* coelomocytes (B, C and D) (n = 4) towards *C. haemulonii* (NCYC 787) and growth of the yeast in CFSN and MBASW control fluids at 10°C.

The data are expressed as (A) SI's at 24, 48 and 72h.

Solid symbols : median plot ; open symbols : actual data ; open and closed triangles (A) : CFSN; open and closed circles (A) : MBASW.


MBASW
&
CFSN
A
PL
B
RSC
C
VC & CSC
D
SURVIVAL INDEX
800
600
400
200
0
0
24
48
72
TIME OF EXPOSURE (h)

47A). The SI's of *C. haemulonii* (NCYC 787) exceeded 200 at 24 and 48h, with a decline at 72h to SI's of approximately 100. The yeast grew better in the CFSN. On exposure of the *C. haemulonii* (NCYC 787) to the CF fraction containing mainly PL's (Figure 47B), the SI's of the yeast increased to 200 at 24h, however, after a further 24h incubation the SI's were reduced to 50% of the initial inoculum and remained at this level after 72h.

The SI's of *C. haemulonii* (NCYC 787) in the fraction containing RSC's are presented in (Figure 47C). Growth of the yeast occurred which was comparable to that in the control fluid CFSN (Figure 47A), SI's increasing to about 500 at 24, 48 and 72h. A similar situation was observed in the fraction containing CSC and VC's SI's of the yeast exceeded 500 over the 72h exposure period.

Comparing the antifungal and antibacterial activities therefore, of each *E. esculentus* CF fraction towards *C. haemulonii* (NCYC 787) and *Ps.111*, there was evidence of antifungal activity by the PL's. The same fraction exerted a temporary bacteriostatic effect towards *Ps. 111*. The fraction containing the RSC's supported growth of the yeast comparable to that of the control fluid CFSN. Conversely, *Ps.111* was reduced by about 80% over the 72h incubation period, indicating antibacterial activity. Finally, the third fraction consisting of a mixture of CSC and VC's displayed neither antibacterial not antifungal activity, apparently supporting growth of both organisms.

Having established the main locus of bactericidal activity was confined to the CF fraction containing the RSC, the same fraction apparently did not exert any antifungal activity towards *C. haemulonii*

(NCYC 787). It was therefore of interest to test the activity of purified extract from the RSC's, namely echinochrome-A, towards a mixed inoculum of yeast and bacteria.

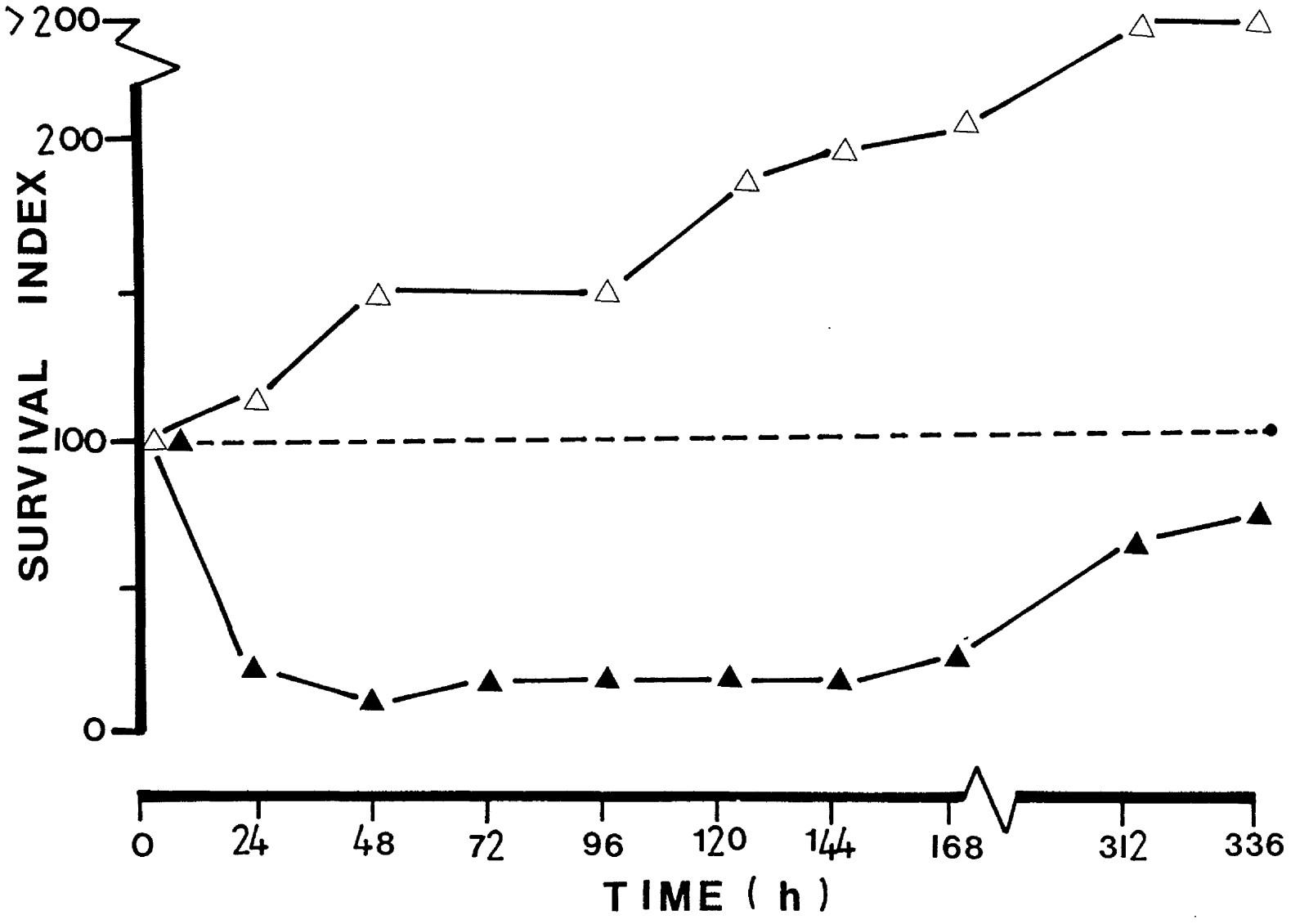
2.5.3. Purified echinochrome-A

Previous work by Service and Wardlaw (1984) showed that purified, crystalline, echinochrome-A (Ech-A), dissolved in MBASW with mammalian proteins , bovine serum albumin (BSA), human serum albumin (HSA), bovine gamma globulin (BGG) and human gamma globulin (HGG) as dispersants, (Ech-A was found to be only sparingly soluble in seawater (Johnson, 1970)) were bactericidal towards *Ps.111* and a range of other Gram-negative and Gram-positive bacteria. In comparing the four different mammalian proteins in dissolving the Ech-A (Service, 1982), minor quantitative differences in activity emerged, therefore, BGG was employed in further studies.

Previous studies showed that bactericidal activity was evident at a concentration of 50µg ml^{-1} Ech-A which was dissolved in a 2mg ml^{-1} BGG in MBASW. In this investigation a single strength 0.2ml inoculum of *Ps.111* was added to 1.8ml of the dissolved Ech-A and samples taken at 24h intervals over 14 days. The control fluid consisted of MBASW with 2mg l^{-1} dissolved BGG. The data are presented in Figure 48. The experiments were all conducted at 10°C. The bacteria grew well in the control fluid exceeding SI's of 100 at 24h and the SI's did not fall below this level over the 336h (14 days) incubation period. After 24h the SI's were reduced to 20. This means 80% of the original inoculum were killed. The SI's of *Ps.111* remained about this level during the next 6 day period, and

FIGURE 48. Bactericidal activity of approximately 50µg ml^{-1} Ech-A towards *Ps.111* over a 336h incubation period and growth of the bacteria in 2mg ml^{-1} BGG in MBASW control fluid at 10°C.
(n = 2)

Solid symbols : Ech-A ; open symbols : control fluid.


>200
200
100
0
SURVIVAL INDEX
0
24
48
72
96
120
144
168
312
336
TIME (h)

then increased to about 70 at 336h.

The concentration of Ech-A was then increased to 100µg ml^{-1} in the test solution. The SI's of *Ps.111* over a 168h (7 day) period are presented in Figure 49. Again there was growth of the bacteria in the control fluid (MBASW containing BGG (2mg ml^{-1})) over the 7 day period. The SI's reached 200 over 48h and SI's of greater than 200 occurred over 96-168h. On exposure of the *Ps.111* to Ech-A the SI's fell below 30 at 24h, and further to 20 at 48h. Thereafter the *Ps.111* remained at about this level until 144h when there was again regrowth of the bacteria.

Increasing the Ech-A concentration yet further to approximately 150µg ml^{-1} (Figure 50) the *Ps.111* SI's fell to less than 20 at 24h and were stable at this level until 96h when there was an increase in the SI's of the bacteria to greater than 200.

Therefore incomplete killing of *Ps.111* was observed on exposure to the three concentrations of Ech-A 50, 100 and 150µg ml^{-1}. Hence, bactericidal activity appeared not to be concentration-dependent. However, considerable difficulty arose when attempting to dissolve the crystalline Ech-A in the BGG-MBASW diluent and it was noticeable that some of the undissolved Ech-A was blocking the filter assembly during sterile filtration (0.45µm) and the preceding concentrations were, as a result, over-estimated. Therefore, in further experiments the solution of Ech-A in MBASW-BGG was analysed spectrophotometrically before and after sterile-filtration to estimate the amount lost during the procedure (see Materials and Methods 1.3.7.). Typically, 8-10% of the Ech-A was undissolved and therefore, retained in the "Millipore^{R}" filter-unit.

FIGURE 49. Bactericidal activity of approximately 100µg ml^{-1} Ech-A towards *Ps.111* over a 168h incubation period and growth of the bacteria in 2mg ml^{-1} BGG in MBASW control fluid at 10°C.
(n = 2)

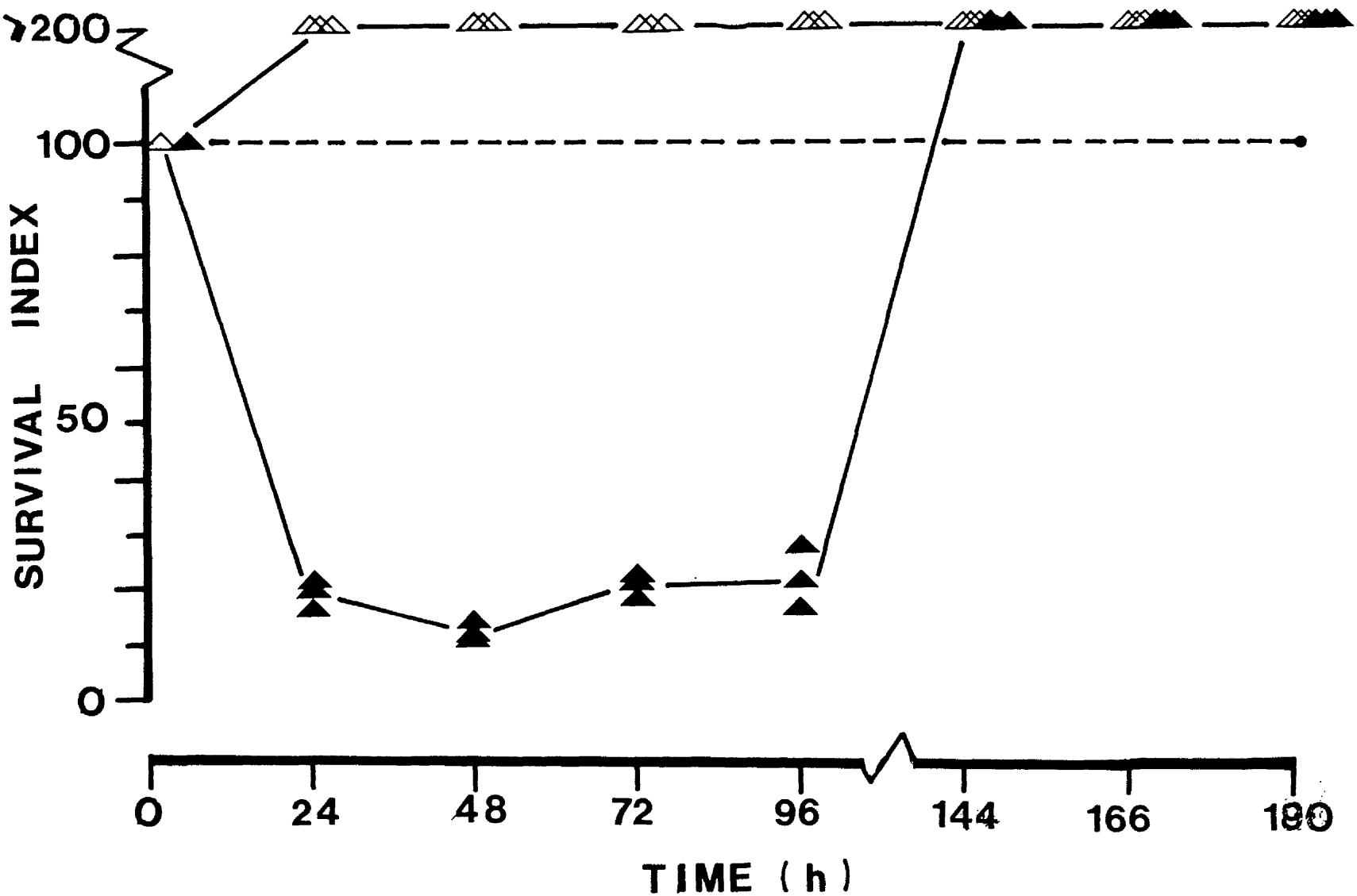
Solid symbols : Ech-A ; open symbols : control fluid.

SURVIVAL INDEX
0
100
200
>200
TIME (h)
0
24
48
72
96
120
144
168

FIGURE 50. Bactericidal activity of approximately 150µg ml^{-1} Ech-A towards *Ps. 111* over a 190h incubation period and growth of the bacteria in 2mg ml^{-1} BGG in MBASW control fluid at 10°C.

(n = 2)

Solid symbols : Ech-A ; open symbols : control fluid.


>200
100
50
0
SURVIVAL INDEX
0
24
48
72
96
144
166
190
TIME (h)

Having established there was some antibacterial activity of the dissolved crystalline Ech-A between the estimated concentration of 50 to 150µg ml^{-1} (although incomplete) it was of interest to test the maximum concentration (150µg ml^{-1}) therefore, towards two selected marine yeast strains *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783). The concentration of 150µg ml^{-1} Ech-A was also chosen because in previous experiments it was found that the marine yeast *C. haemulonii* (NCYC 787) grew in CFL which contained Ech-A released from the RSC's during lysis. Since it is known that concentrations of Ech-A found in healthy specimens of *E. esculentus* ranged from 3 to 60µg ml^{-1} Ech-A with a geometric mean of 14µg ml^{-1} (Service, 1982), it was decided to use a higher concentration of Ech-A to establish whether the substance exerted antifungal activity towards marine yeasts.

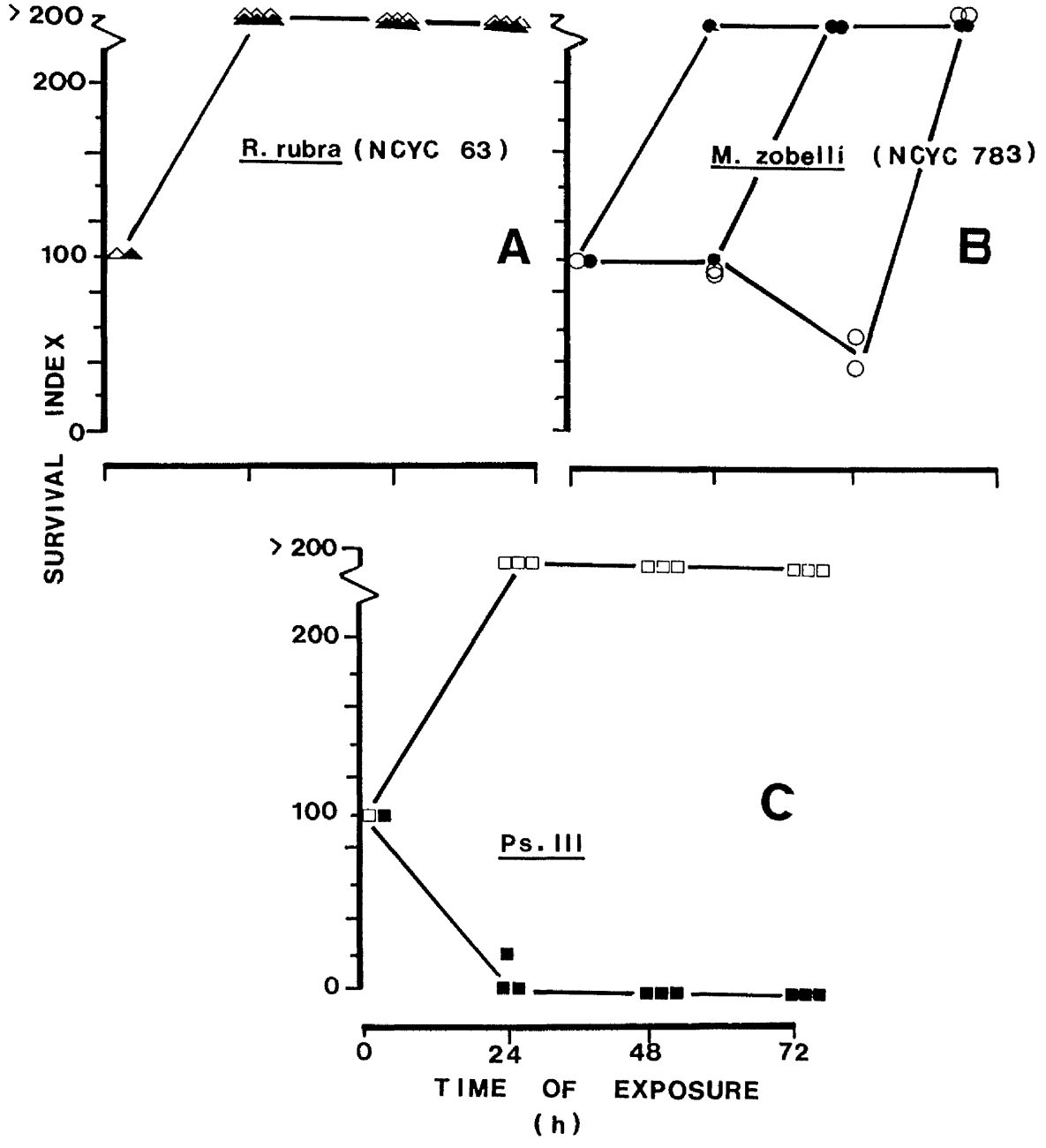
The data obtained for the antifungal and antibacterial activity of Ech-A towards the two marine yeasts *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) and *Ps.111* are presented in Figure 51. Allowing for an estimated 8-10% loss during sterile filtration of the incompletely dissolved Ech-A, a concentration of 135µg ml^{-1} was tested. The control fluid consisted of MBASW with 2mg ml^{-1} BGG supported growth of all 3 organisms with SI's of greater than 200 at 24h.

R. rubra (NCYC 63) (Figure 51A) was observed to grow to the same extent in Ech-A as in the control fluid, the SI's remained above 200 over the 72h incubation period. The SI's of *M. zobelli* (NCYC 783) (Figure 51B) remained at about 100 at 24h increasing to SI values of greater than 200 in the next 48h. Therefore, from the results, this concentration of Ech-A (approx. 135µg ml^{-1}) was found to be highly bactericidal towards *Ps. 111.*

FIGURE 51. Antifungal and antibacterial activity of 135µg ml^{-1} Ech-A towards *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and *Ps.111* and growth of the organisms in 2mg ml^{-1} BGG in MBASW control fluid at 10°C.
(n = 3)

The data are presented as SI's at 24, 48 and 72h

Solid symbols : Ech-A ; open symbols : control fluid.


> 200
200
100
0
SURVIVAL INDEX
R. rubra (NCYC 63)
A
M. zobelli (NCYC 783)
B
> 200
200
100
0
Ps. III
C
0
24
48
72
TIME OF EXPOSURE
(h)

However, there was little antifungal activity towards the yeast *R. rubra* (NCYC 63) and only a temporary fungistatic effect towards *M. zobelli* (NCYC 783). The concentration of Ech-A in further test solutions was therefore increased to approximately 225µg ml^{-1} to assess whether higher concentrations of the pigment may exert antifungal activity. This concentration was tested against seven yeast strains with *Ps.111* as the bacterial control strain. The data for the effect of 225ug ml^{-1} Ech-A towards the yeast strains *R. rubra* (NCYC 63 and NCYC 797), *M. zobelli* (NCYC 783) and *Ps 111* are shown in Figure 52. Three of the test organisms, *R. rubra* (NCYC 797), *M. zobelli* (NCYC 783) and *Ps.111* grew over the 72h incubation period in the MBASW-protein control fluid. *R. rubra* (NCYC 63) however, (Figure 52A) survived with SI's of about 80 over 48h and a decline in the SI's of 50 at 72h.

On exposure of *R. rubra* (NCYC 63) (Figure 52A) to the Ech-A it was reduced by more than 90% of the initial inoculum, at 12h, the SI's remained at less than 20% during the following 60h. The SI's of *R. rubra* (NCYC 797) (Figure 52B) were reduced to SI's of 40% at 12h, the remaining viable organisms regrew to SI values exceeding 50% at 24, 48 and 72h. *M. zobelli* (NCYC 783) (Figure 52C) was reduced by 80% of the initial inoculum at 12h, remaining at this level throughout the 72h incubation period. Figure 52D shows the complete killing of the bacterial control strain *Ps.111* within 24h the SI's remaining at SI's of zero throughout the 72h incubation period.

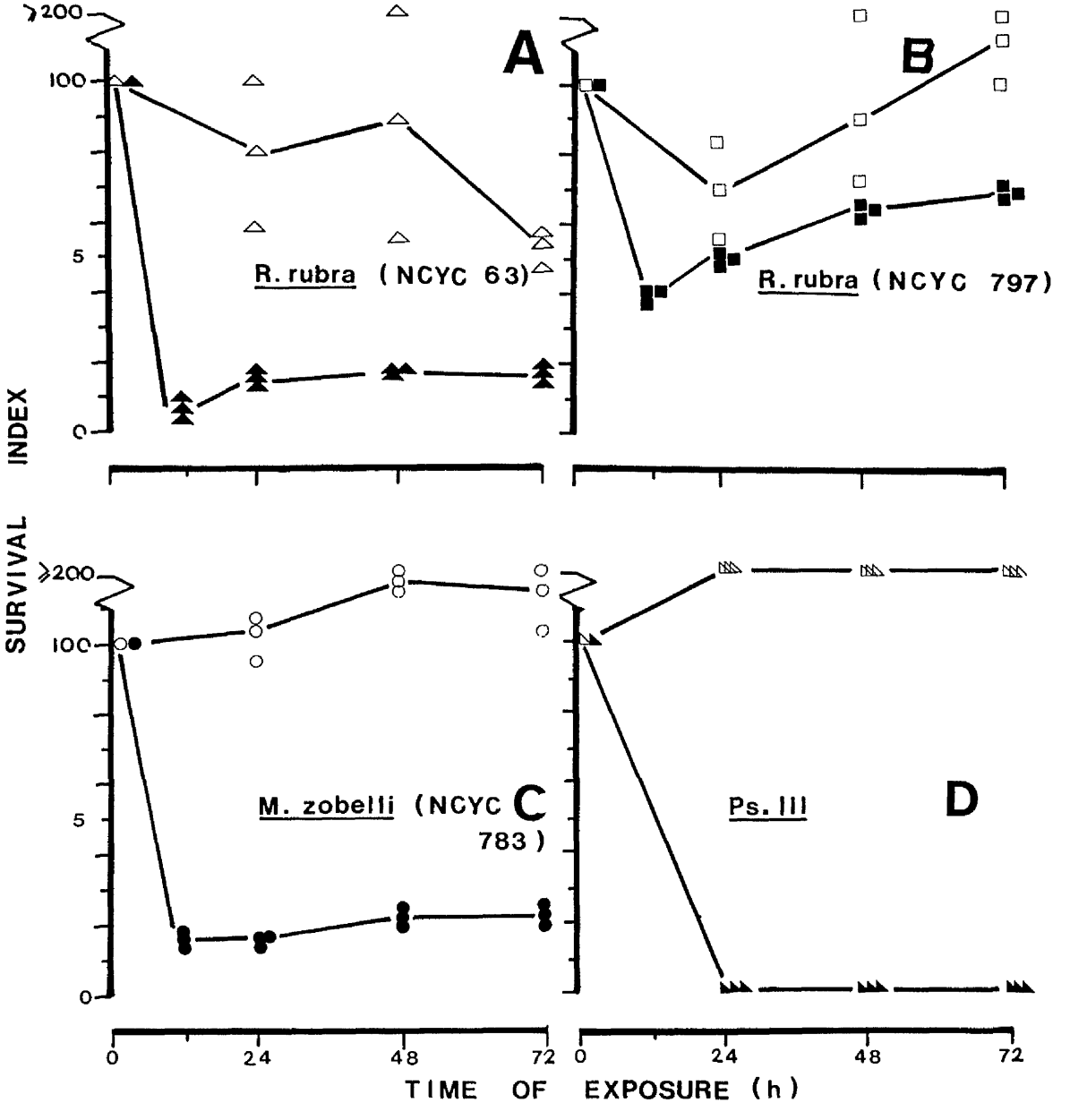
This concentration of Ech-A (approx. 225µg ml^{-1}) which was nearly four times the maximum physiological concentration found in the coelomic fluid of healthy specimens of *E. esculentus* (range 3-60µg ml^{-1}) was found to be antifungal to some extent towards the three yeast strains tested.

FIGURE 52. Antifungal and antibacterial activity of 225µg ml^{-1} Ech-A towards *R. rubra* (NCYC 63 and NCYC 797), *M. zobelli* (NCYC 783) and *Ps.111* and growth of the organisms in 2mg ml^{-1} BGG in MBASW control fluid at 10°C.

The data are presented as SI's at 12, 24, 48 and 72h.

(n = 3)

Solid symbols : Ech-A ; open symbols : control fluid.


A
B
C
D
R. rubra (NCYC 63)
R. rubra (NCYC 797)
M. zobelli (NCYC 783)
Ps. III
SURVIVAL INDEX
>200
100
5
0
0
24
48
72
TIME OF EXPOSURE (h)

Since antifungal activity was evident at this concentration of Ech-A, the same preparation was tested against four other strains of yeasts, the marine *Candida* spp., *C. maris* (NCYC 785), *C. guilliermondii* (NCYC 145), *C. famata* (NCYC 799) and *C. famata* (NCYC 798), the data are presented in Figure 53.

Two of the marine *Candida* strains, *C. maris* (NCYC 785) (Figure 53A) and *C. famata* (NCYC 799) (Figure 53C) did not grow in the MBASW-protein control fluid, the SI's falling from 100 to about 50 in 48h. However, the SI's of these two strains on exposure to Ech-A were markedly less than those in the control fluids, particularly at 24h when they were reduced by greater than 40% relative to the SI's in the control fluid.

C. guilliermondii (NCYC 145) and *C. famata* (NCYC 798) did not grow in the control fluid either, SI's of the former fell to 90 at 24h, 70 at 48h and 60 at 72h. The latter yeast strain survived at SI's of about 90 throughout the 72h incubation period. However, there was a significant difference in the SI's of these two yeasts in the control fluid and on exposure to the Ech-A. The SI's of *C. guilliermondii* (NCYC 145) (Figure 53B) were reduced by about 40% (relative to the control fluid) at 24h, 50% at 48h and 30% at 72h. The yeast *C. famata* (NCYC 798) (Figure 53D) was also reduced to SI's of 80% of the initial inoculum at 12 and 24h, 70% at 48h and 60% at 72h.

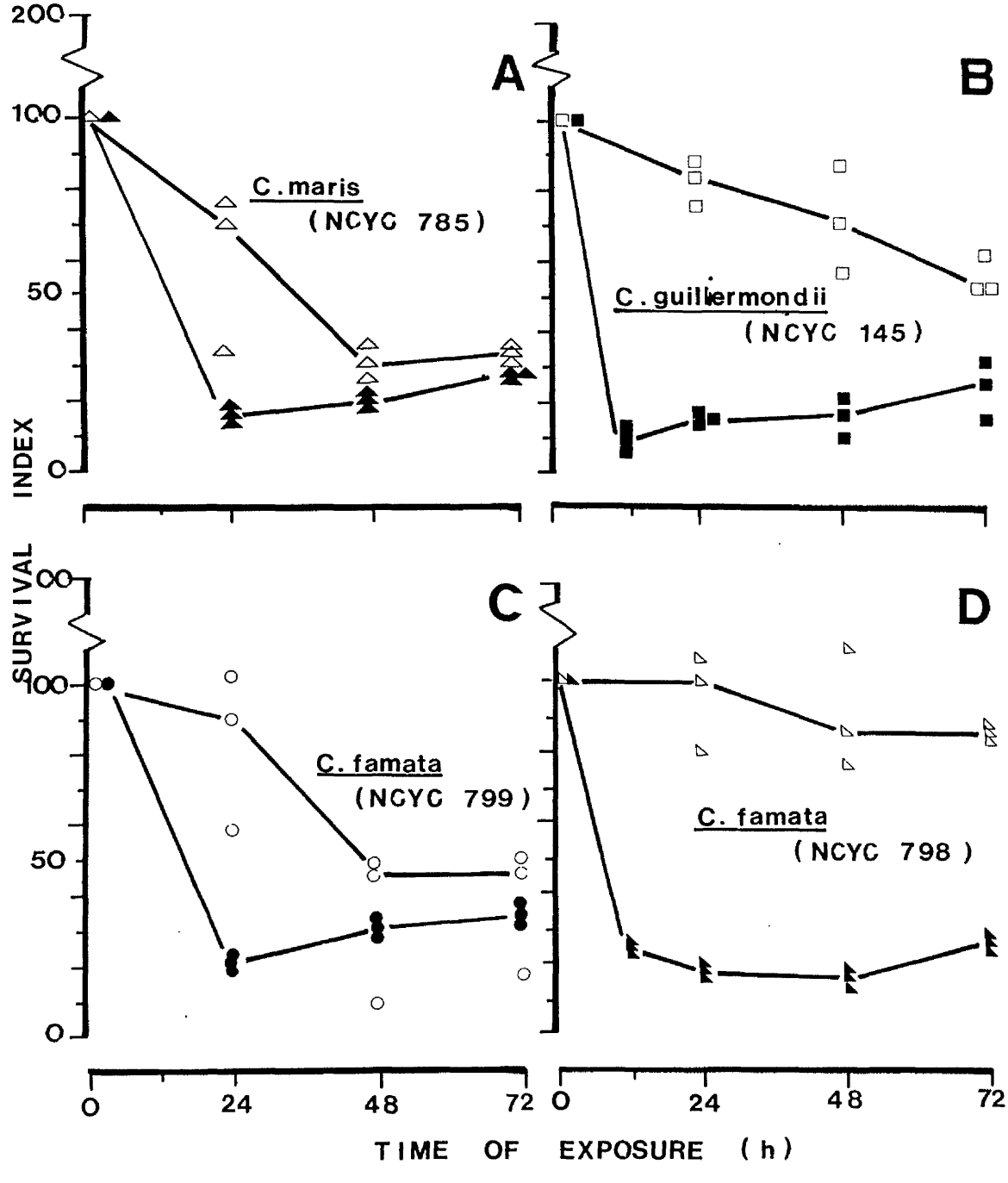
Summarizing, therefore, although there was no growth of the yeasts in the control fluid, there was a decrease in the SI's of all four marine *Candida* spp. exposed to Ech-A during some stage of the 72h incubation relative to the SI's in the control fluid. This means there was some antifungal activity of Ech-A at this concentration (approx. 225μg ml^{-1}).

FIGURE 53. Antifungal activity of 225µg ml^{-1} Ech-A towards the four marine *Candida* strains, *C. maris* (NCYC 785), *C. guilliermondii* (NCYC 145), *C. famata* (NCYC 799) and *C. famata* (NCYC 798) and growth of the organisms in 2mg ml^{-1} BGG in MBASW control fluid at 10°C.

The results are presented as SI's at 12, 24, 48 and 72h.

(n = 3)

Solid symbols : Ech-A ; open symbols : control fluid.


200
100
50
0
INDEX
A
B
C. maris
(NCYC 785)
C. guilliermondii
(NCYC 145)
SURVIVAL
100
50
0
C
D
C. famata
(NCYC 799)
C. famata
(NCYC 798)
0
24
48
72
TIME OF EXPOSURE (h)

None of the seven strains tested were completely killed i.e. reduced to SI's of zero as was the case for the bacterial control strain.

2.6. Agglutination Studies on Marine Yeasts and Bacteria

In all experiments with whole coelomic fluid *in vitro* there was a decrease in the viable count of marine yeasts, with time of exposure, although in some cases there was incidence of regrowth of the organisms.

In an attempt to answer the question, "was there a genuine antifungal effect decreasing the viable count, or was this merely agglutination of the yeast cells?" studies on the agglutination of the bacterial control strain *Ps 111* and *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) were done.

The three strains were exposed to three fluids, coelomic fluid lysate (CFL), coelomic fluid supernate (CFSN) and 1% marine broth in artificial seawater ("Tropic Marin^{R}") (MBASW). The fluids were titrated against a suitable concentration bacteria or yeasts (previously determined) in microtitre trays, by serial dilution with ASW ("Tropic Marin^{R}") (details in Materials and Methods 1.3.9.). The trays were incubated for 2h at room temperature (22°C) initially and examined, and in the second instance after overnight refrigeration.

The results are presented in Table 15. The bacterial control strain *Ps 111* did not agglutinate in either control fluids MBASW or CFSN, or test fluid CFL. The marine yeast strains *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) however, were observed to agglutinate in all three fluids tested, MBASW, CFSN and CFL.

TABLE 15. Agglutination titrations of *Ps.111*, *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) and control fluids in coelomic fluid lysate (CFL) and control fluids MBASW and CFSN.

Test/Control Fluid	Yeast/Bacterial Suspension	Agglutination + or -
Coelomic fluid lysate (CFL)	*Ps.111*	-
1% marine broth in artificial seawater (MBASW)	*Ps.111*	-
Coelomic fluid supernate (CFSN)	*Ps.111*	-
CFL	*R. rubra* (NCYC 63)	+
MBASW	(NCYC 63)	+
CFSN	(NCYC 63)	+
CFL	*M. zobelli* (NCYC 783)	+
MBASW	(NCYC 783)	+
CFSN	(NCYC 783)	+

2.7. Growth Characteristics of Marine Yeasts and Bacteria

2.7.1. Marine broth

R. rubra (NCYC 63), *M. zobelli* (NCYC 783) and *Ps.111* all grew in MBASW (1% (w/v) marine broth in artificial seawater) over 72h at 10°C. It was therefore of interest to monitor the growth characteristics of these three organisms in nutrient-rich marine broth (MB) to allow calculation of the mean generation time (MGT) at 10°C. Glucose was added to the yeast broth to a final concentration of 50mM (YMB). Volumes of 100ml MB and YMB were inoculated with 0.2ml of mid-exponential phase MB and YMB cultures of *Ps 111* and marine yeasts respectively. Samples were taken from each flask (about 2.9ml) and the OD, at 610nm, read at convenient intervals along the growth curve.

A growth curve of *Ps.111* in MB at 10°C is presented in Figure 54. The mean generation time (MGT) or mean doubling time of *Ps. 111* was estimated to be 7h. The MGT's of *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) (Figures 55 and 56) in YMB at 10°C were 20h and 18h respectively.

2.7.2. Artificial and natural seawaters

Since artificial seawaters (ASW) were used routinely throughout the investigations it was of interest to compare growth of the key target organisms. *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and *Ps. 111* in natural seawater (NSW) and two different ASW's "Tropic Marin^{R}" and "Sea Salt^{R}" in the absence of added trace nutrient.

A mixed inoculum of marine yeast and *Ps.111* (0.2ml) was added to a

FIGURE 54. Growth curve of *Pseudomonas* strain number 111 in nutrient rich marine broth (MB) at 10°C.
(n = 2)

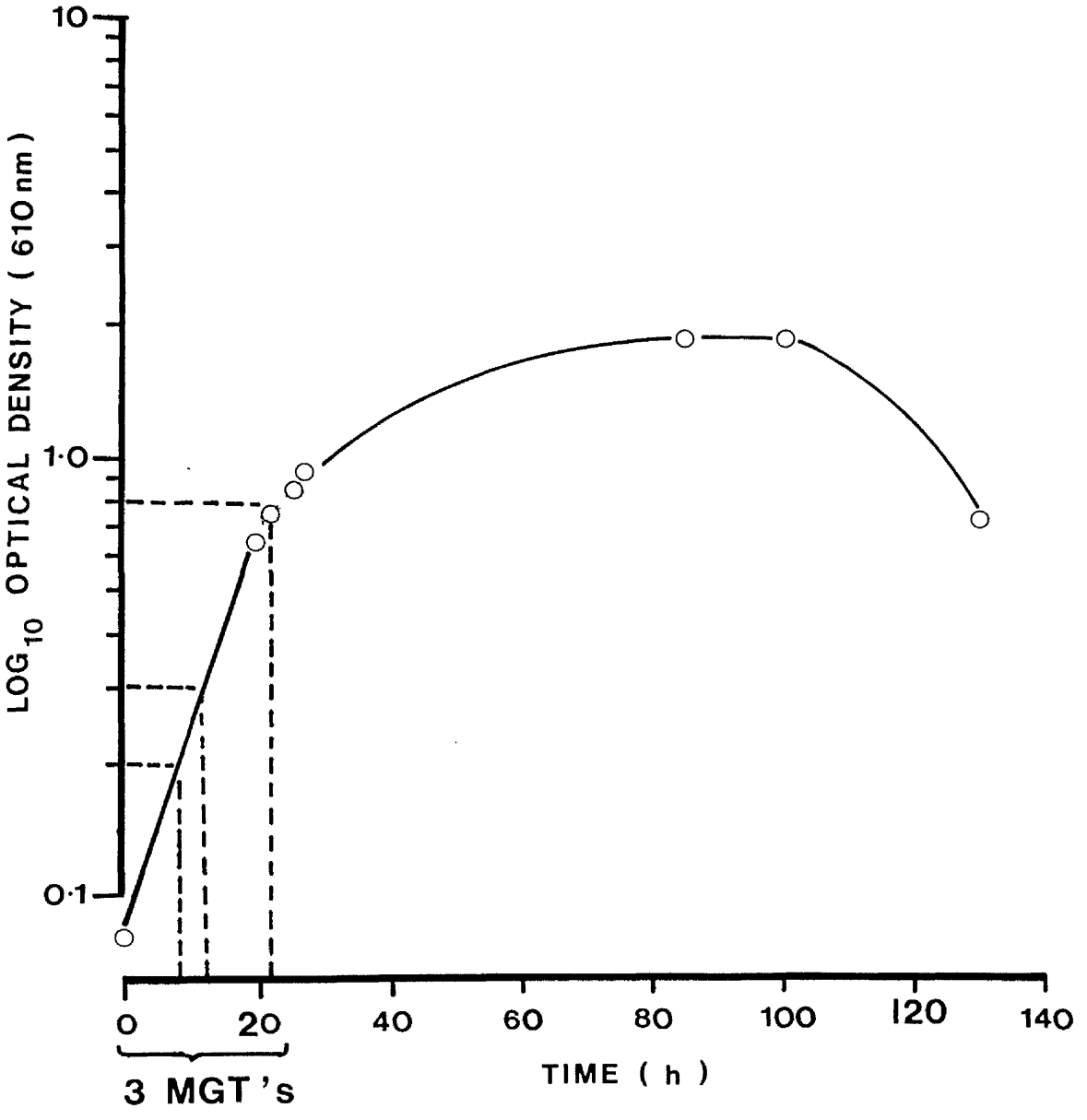

10
1·0
0·1
LOG10 OPTICAL DENSITY (610 nm)
0
20
40
60
80
100
120
140
TIME (h)
3 MGT 's

FIGURE 55. Growth curve of *R. rubra* (NCYC 63) in nutrient rich broth with added glucose (YMB) at 10°C.
(n = 2)

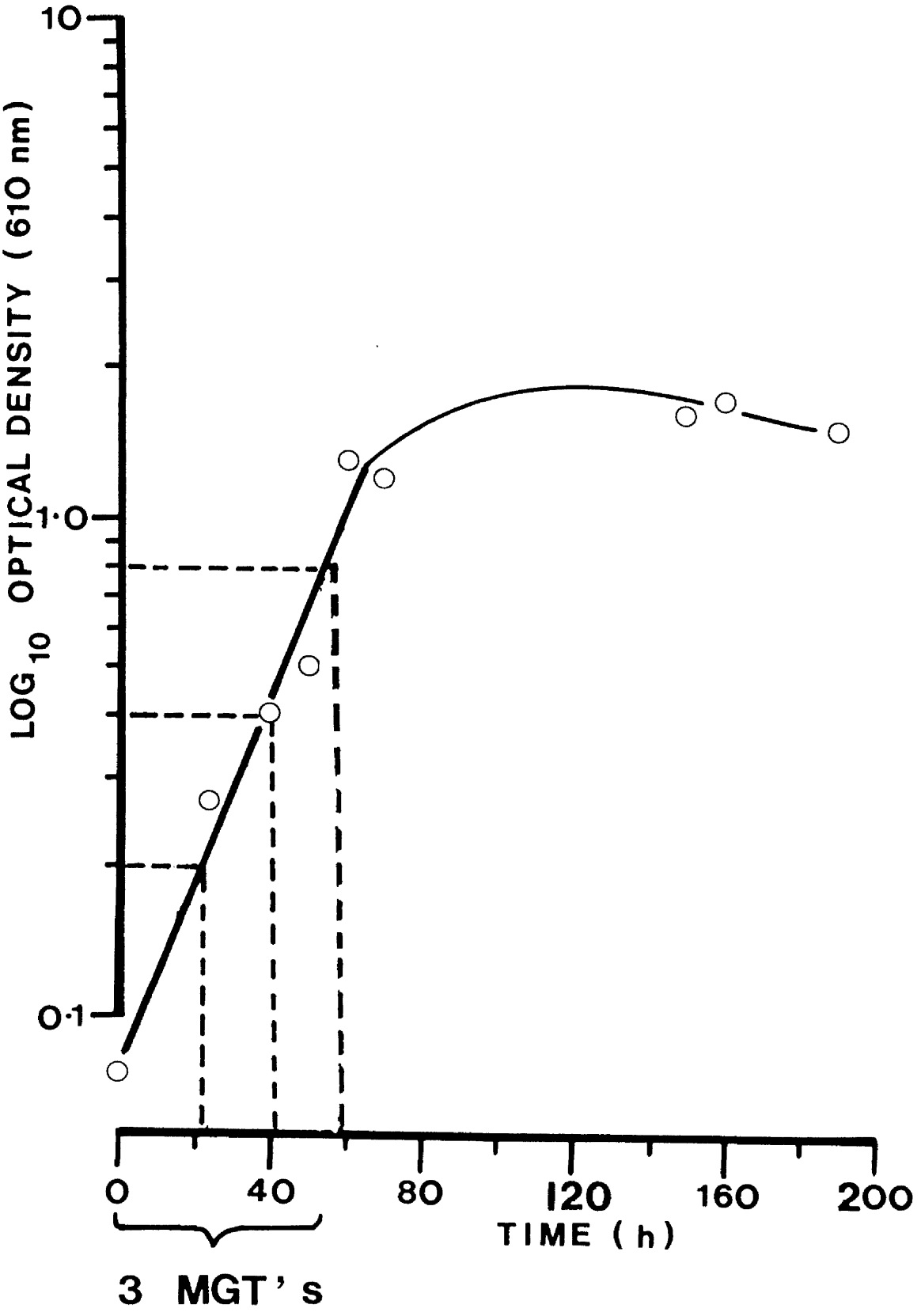
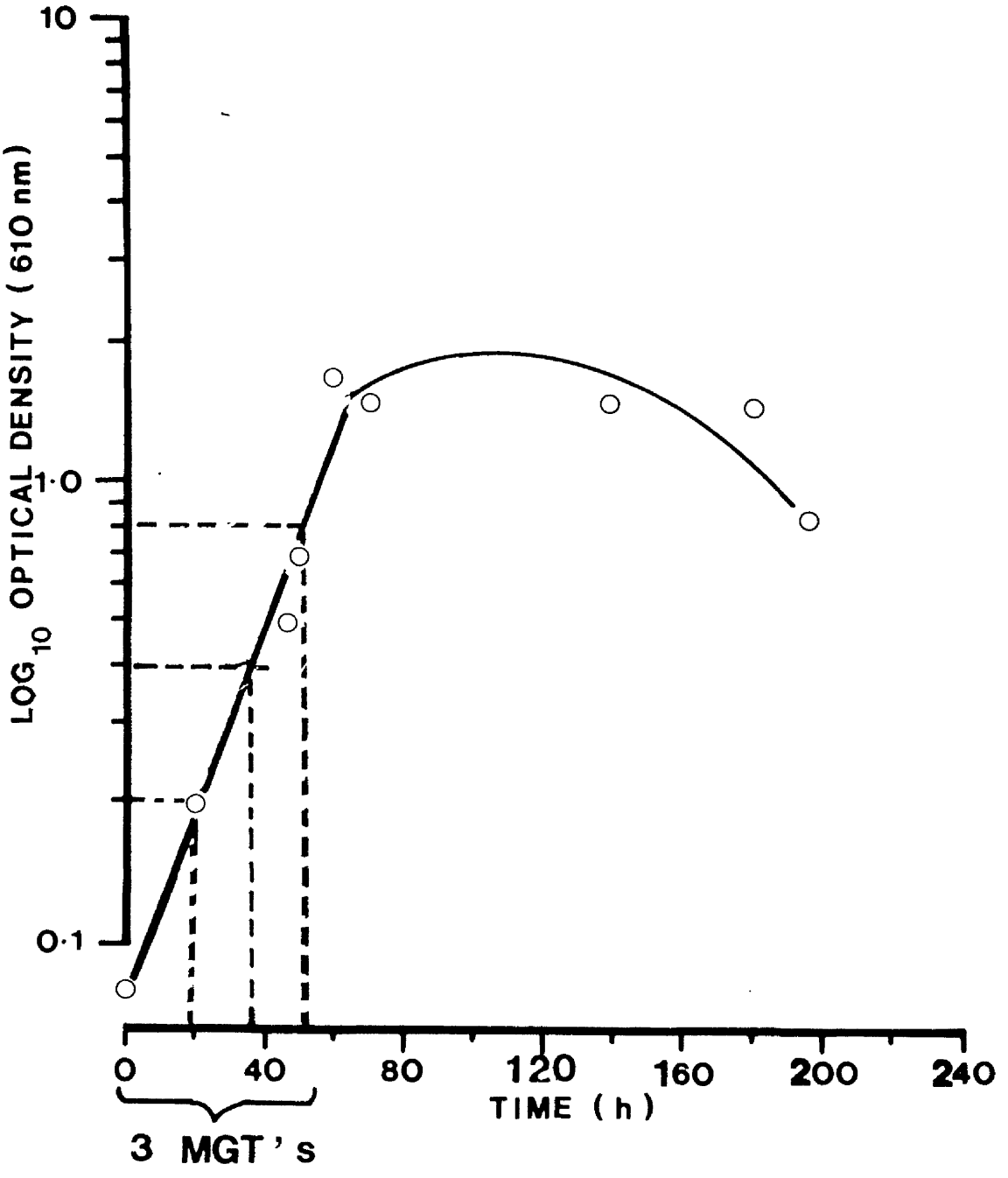

10
1·0
0·1
LOG10 OPTICAL DENSITY (610 nm)
0
40
80
120
160
200
TIME (h)
3 MGT's

FIGURE 56. Growth curve of *M. zobelli* (NCYC 783) in nutrient rich broth with added glucose (YMB) at 10°C.
(n = 2)


10
1·0
0·1
LOG10 OPTICAL DENSITY (610 nm)
0
40
80
120
160
200
240
TIME (h)
3 MGT's

1.8ml volume of each seawater type and incubated at room temperature (approx. 22°C), samples were taken at 24h intervals up to 72h. The data are presented in Figure 57. In each case better growth of the marine yeasts was observed in NSW (obtained from the Clyde Estuary, Millport). In the ASW's superior growth was evident in "Tropic Marin^{R}" compared with "Sea Salt^{R}". *Ps.111*, however, appeared to grow well in all three seawater types. ASW, "Tropic Marin^{R}" was therefore used routinely as control diluent in all microbiocidal tests since NSW would introduce an element of biological variability between individual samples.

3. CLEARANCE AND PATHOGENICITY OF MARINE YEASTS FOR *ECHINUS ESCULENTUS*

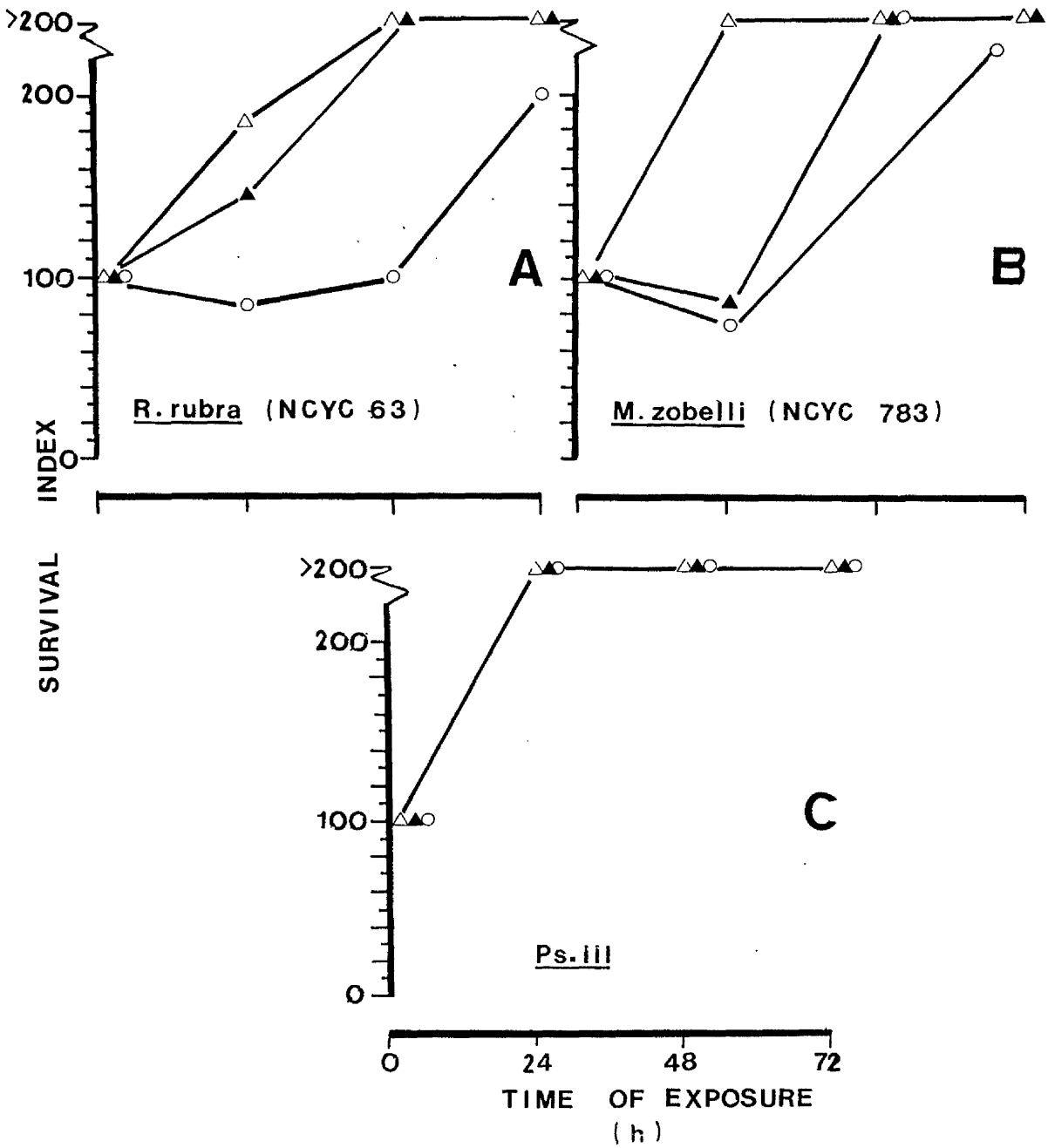
The investigations so far were involved with the antifungal and antibacterial activity of *E. esculentus* coelomic fluid *in vitro*. The following section describes experiments *in vivo* to study the fate of injected marine yeasts into the coelom of the whole animal, to determine whether the yeasts were capable of causing a fatal infection and to define cellular response to infection with marine yeasts in terms of the total numbers and relative types of coelomocytes. The marine bacteria *Ps. 111* was again incorporated into the design of the experiments as a reference strain of known high sensitivity to killing by CF *in vitro*.

FIGURE 57. Growth of *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and *Ps. 111* in natural seawater (NSW) and artificial seawaters "Tropic Marin^{R}" and "Sea Salt^{R}" at 22°C.

The results are expressed as SI's at 24, 48 and 72h.

(n = 3)

Open triangles : NSW ; closed triangles : "Tropic Marin^{R}" ; open circles : "Sea Salt^{R}".


>200
200
100
0
SURVIVAL INDEX
A
R. rubra (NCYC 63)
B
M. zobelli (NCYC 783)
C
Ps. III
0
24
48
72
TIME OF EXPOSURE
(h)

3.1. Fate of Injected Marine Yeasts and Bacteria in the Coelom of *E. esculentus*

3.1.1. Measurement of clearance

Previous studies on the clearance of *Ps. 111* from the coelomic cavity of *E. esculentus* showed that large doses of injected bacteria (10^9, which were estimated to undergo an approximate hundred fold dilution by the coelomic fluid of the whole animal, to a notional zero-time concentration of 10^7 organisms ml^{-1} CF) were rapidly reduced over 24h to a viable count of less than $10ml^{-1}$ CF (Unkles, 1976; Wardlaw and Unkles, 1978; Service, 1982).

In the preliminary stage of the investigations it was of interest therefore, to investigate whether there would be clearance of suspensions of marine yeasts and *Ps.111* injected into the coelomic cavity of *E. esculentus* held in RASWA at 10°C.

The two marine yeast strains *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) were selected on the basis of several criteria :

a. both yeast strains were sensitive to *E. esculentus* whole coelomic fluid *in vitro* at 10°C.

b. they both had highly distinctive colony-characteristics and were readily distinguishable from each other and bacterial contaminants present in the CF.

c. the yeast colonies were easily counted on YMA after 72h incubation at room temperature (approx. 22°C).

Pseudomonas strain number 111 was chosen as a bacterial reference strain because :

a. it was highly sensitive to *E. esculentus* CF *in vitro* at 10°C.

b. it was previously found to be rapidly cleared from the coelom of *E. esculentus* within 24h after injection, and

c. it was readily distinguishable from CF background bacterial contaminants.

Suspensions of the marine yeasts and bacteria (Materials and Methods, 1.4.2.) were injected into the coelom of *E. esculentus* via the peristomial membrane (Figure 7). In the preliminary experiments a sample of the CF of each sea urchin was taken before injection (typically 0.1ml) as a sterility check, but this was not done in later experiments so as to minimize the number of times the peristomial membrane was punctured and therefore reduce the risk of contamination by this route. The *E. esculentus* injected with the suspensions of yeast and bacteria were placed in separate compartments in the RASWA for ease of identification (Plate 2). A sample volume of approximately 0.3ml of CF was taken from each animal after 12h, daily for the first few days and then weekly, (again to minimize puncturing of the peristomial membrane), until death of the test animals. Samples (0.1ml) of CF from animals injected with marine yeasts were plated onto both YMA (to detect recovered injected organisms) and MA (to count background bacterial contaminants) and the remaining 0.1ml was used to make serial dilutions of the CF in MBASW, which were spread (0.1ml) onto both media types. CF from animals injected with *Ps. 111.* (0.1ml) were plated (undiluted and serial dilutions) onto MA only, for enumeration of recovered

viable bacteria and background bacterial contamination.

In the first instance, inocula of approximately 10^9 bacteria and 10^7 yeasts were injected (1.0ml) into the coelom of 5 animals; allowing for an approximate one hundred fold dilution by the CF in the coelomic cavity, giving a notional zero-time concentration of 10^7 bacteria and 10^5 yeasts ml^{-1} CF.

The data for *E. esculentus* injected with approximately 10^9 bacteria (notional zero-time concentration of 10^7 ml^{-1} CF) are presented in Figure 58. Interpreting the data, an estimated zero-time viable count of 10^7 *Ps. 111* ml^{-1} CF was rapidly reduced to 10^3 after 4h (99.9% clearance) and a further reduction to 10 or fewer organisms per millilitre CF within one week. In sampling the CF from the same animals over a period of 3 weeks, no remaining viable *Ps. 111* were detected, indicating complete clearance of the bacteria in less than one week. There was no regrowth of the *Ps. 111* up to 10 weeks at which time the experiment was terminated (T).

Data for the clearance of injected marine yeasts *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) are presented in Figure 59. After 4h over 98% of the original inoculum of *R. rubra* (NCYC 63) (Figure 59A) was cleared from the coelom, 2000 yeasts ml^{-1} or less persisting in the CF until the death (d) of the animals 4-5 weeks after injection.

M. zobelli (NCYC 783) (Figure 59B) was reduced by about 80% of its original inoculum within 1 week post injection, persisting at values of about 5×10^3 ml^{-1} CF until death of the animals at about 4 weeks.

Therefore, summarizing the preliminary results, the bacterial inoculum

FIGURE 58. Recovery of viable *Ps. 111* from the coelom of *E. esculentus* (n = 3)at different times after injection of 10^9 bacteria (giving a notional zero-time concentration in the CF of 10^7 *Ps. 111* ml^{-1})

T = Experiment terminated

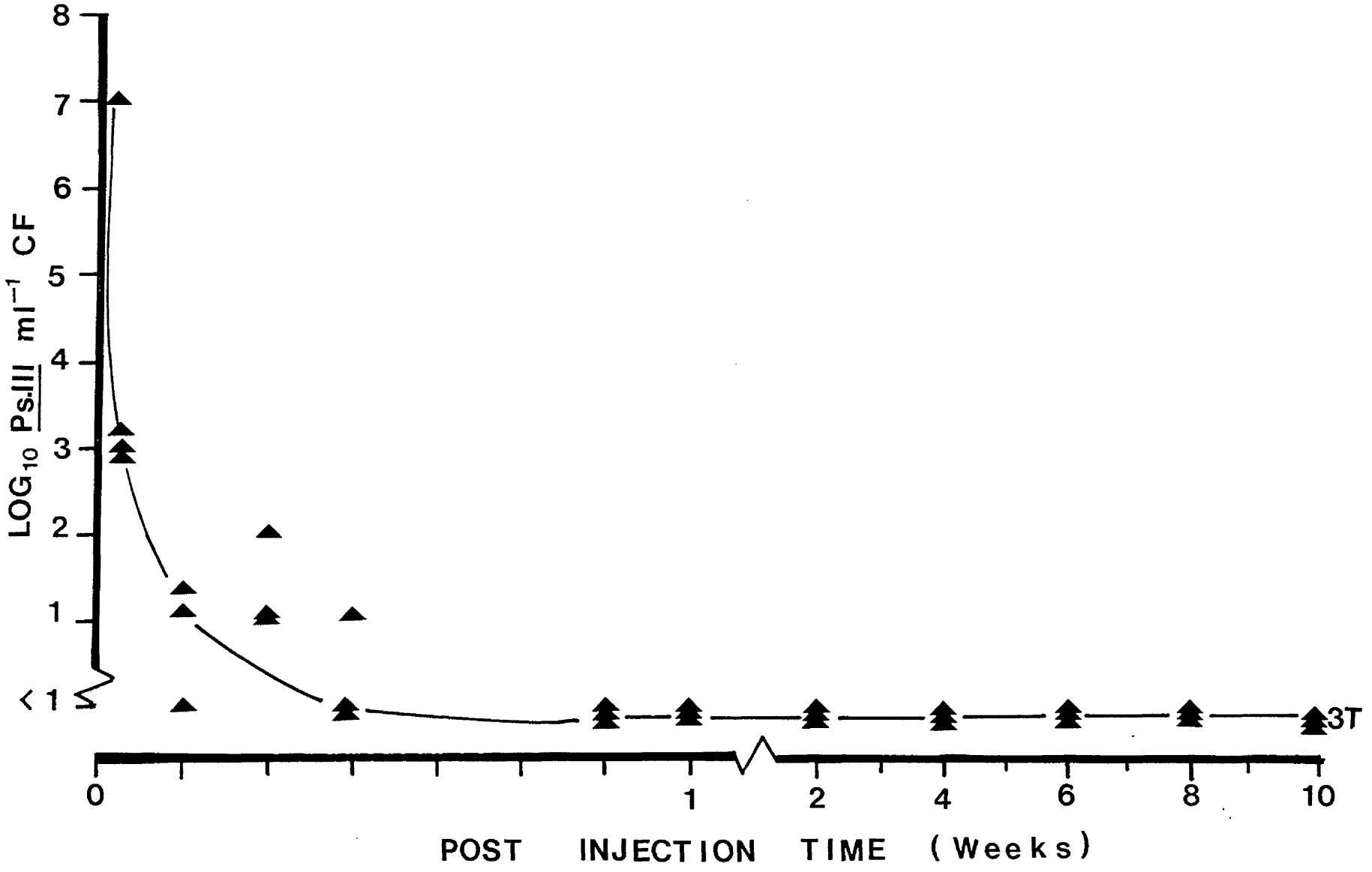

8
7
6
5
4
3
2
1
< 1
LOG10 Ps.III ml-1 CF
3T
0
1
2
4
6
8
10
POST INJECTION TIME (Weeks)

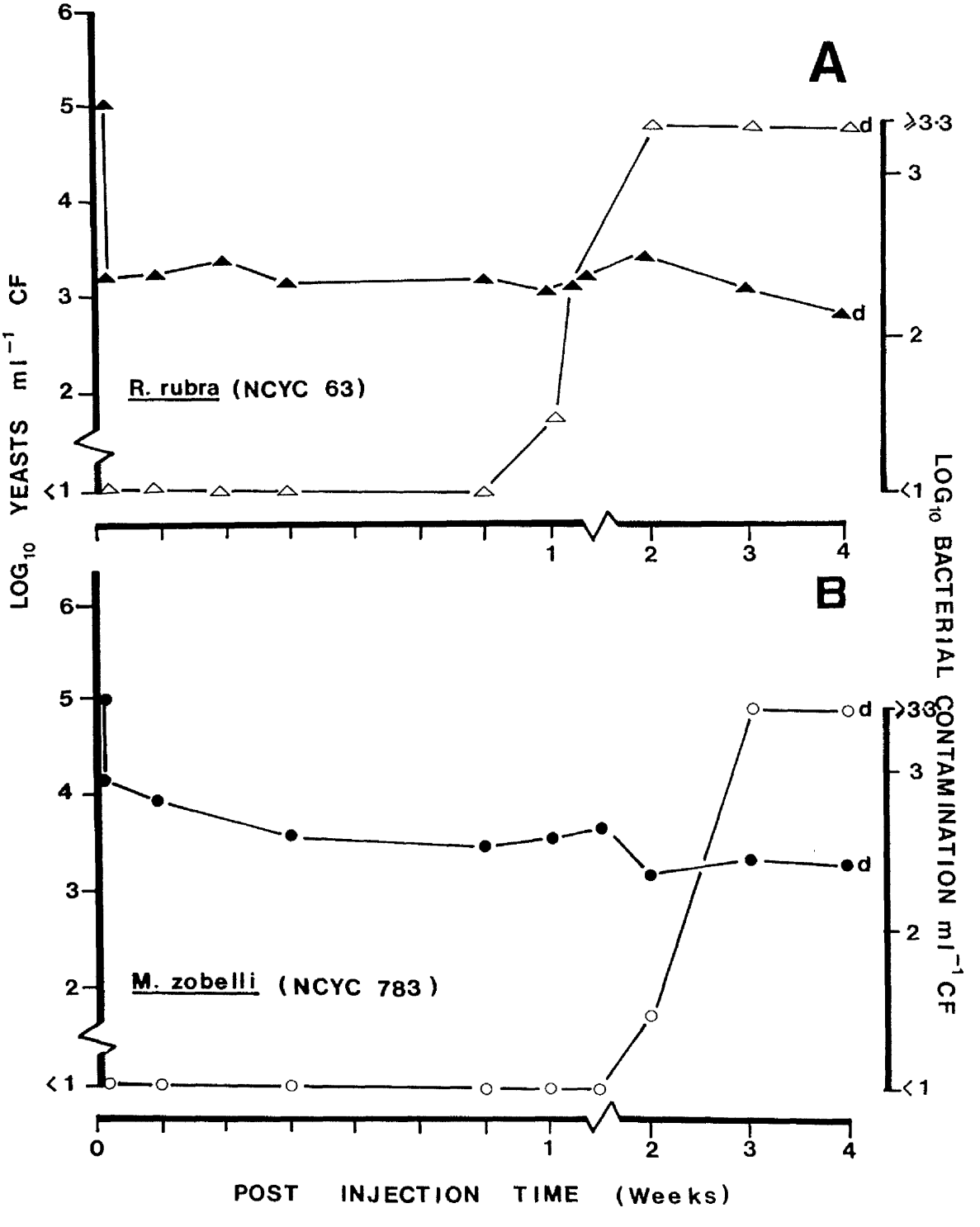
FIGURE 59. Recovery of viable *R. rubra* (NCYC 63) (A) and *M. zobelli* (NCYC 783) (B) from the coelom of *E. esculentus* (n = 1) at different times after injection of yeasts (giving a notional zero-time concentration in the CF of 10^5 ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered yeasts, log_{10} ml^{-1} CF;

open symbols : bacterial contamination, log_{10} ml^{-1} CF.


A
B
R. rubra (NCYC 63)
M. zobelli (NCYC 783)
LOG10 YEASTS ml-1 CF
LOG10 BACTERIAL CONTAMINATION ml-1 CF
POST INJECTION TIME (Weeks)
6
5
4
3
2
<1
0
1
2
3
4
≥3·3
d

of notional zero-time concentration of 10^7 ml^{-1} CF was cleared completely (none detectable in 0.1ml) from the coelom within 6 days and with no regrowth of the injected organisms during the following 10 weeks. Similarly, between 80-98% of the marine yeasts were cleared from the coelom within 24h but in contrast the remaining 2-10% persisted in the coelom of the animals until death of the animals at 4 weeks. An accompanying bacterial contamination exceeding 200 organisms $0.1ml^{-1}$ (undiluted CF) after about one week post-injection until death of the animals was recorded in each case.

3.1.2. Quantitative studies

Since the above preliminary experiments were done with only one animal injected with each marine yeast and three with *Ps.111*, in the absence of other control animals, a similar procedure was repeated with three *E. esculentus* per injected test organism. An additional set of three control animals were injected with sterile diluent (1.0ml) MBASW and were placed in the same RASWA ("infection" tank) as the animals that had been injected with yeast or *Ps.111*.

In these experiments the post-injection sampling times of *E. esculentus* were less frequent (weekly) to minimize bacterial contamination of the CF by successive puncturing of the peristomial membrane.

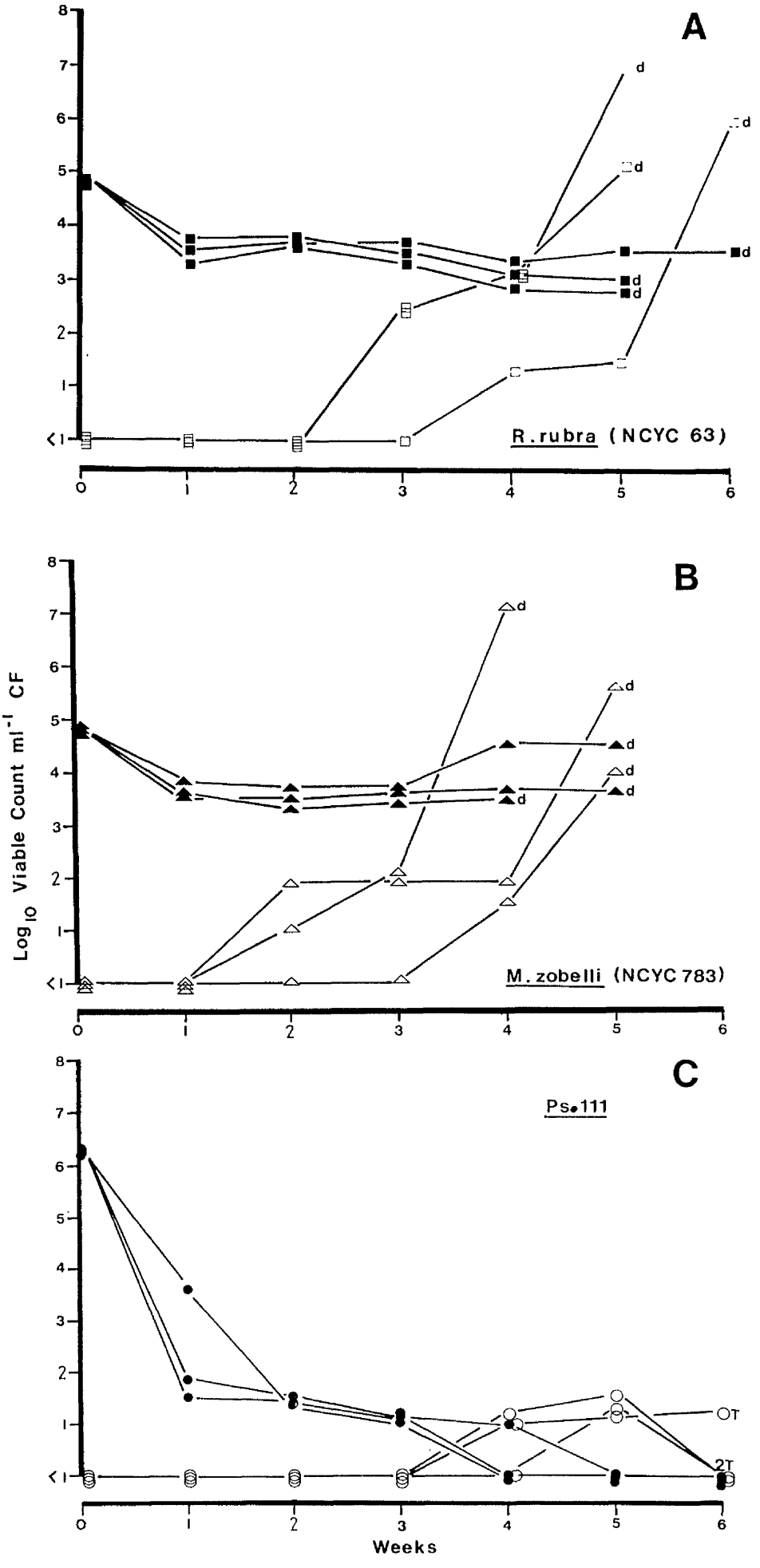
The data obtained from the clearance of *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and *Ps.111* and the appearance of bacterial contamination in the CF are presented in Figure 60. The group of animals in this particular experiment only were found to have an average CF volume of 150ml (range 75-225ml) and were injected with 10^7 yeasts and 3×10^8 *Ps.111*. This gave a

FIGURE 60. Recovery of viable *R. rubra* (NCYC 63) (A), *M. zobelli* (NCYC 783) (B) and bacterial reference strain *Ps. 111* (C) from the coelom of *E. esculentus* (n = 3) at different times after injection of 10^7 yeasts and 3×10^8 *Ps. 111* (giving a notional zero-time concentration in the CF of 7×10^4 yeasts and 2×10^6 bacteria per millilitre, respectively).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered yeasts or bacteria, $\log_{10}$ ml^{-1} CF ; open symbols : bacterial contamination, $\log_{10}$ ml^{-1} CF.


A
R.rubra (NCYC 63)
B
M.zobelli (NCYC 783)
C
Ps.111
Log10 Viable Count ml-1 CF
Weeks
OT
2T
d

notional zero-time concentration of yeasts in the CF which was therefore calculated by dividing the injected dose by the average volume of CF.

The zero-time concentration of 6.7×10^4 ml^{-1} CF *R. rubra* (NCYC 63) (Figure 60A) was reduced by over 90% within one week post-injection, the remaining yeasts persisted in the coelom until death of the animals between 4 and 5 weeks. The bacterial viable count of the CF was observed to increase from sterility (none detected in 0.1ml CF at zero-time) after about 2 weeks to between 10^5 and 10^7 ml^{-1} CF just before death.

A similar pattern of results was found after injection of *M. zobelli* (NCYC 783) (Figure 60B), approximately 90% of the initial inoculum was cleared from the coelom within one week after injection. Again, there was persistence of the remaining yeasts in the coelom until death of the animals between 5 and 6 weeks, the accompanying bacterial contamination having risen from zero (none detectable in 0.1ml) initially between 10^3-10^7 ml^{-1} CF before death.

By contrast, *E. esculentus* injected with a 3×10^8 dose *Ps.111* (Figure 60C) (therefore, a zero-time concentration of 2×10^6 ml^{-1} CF) was reduced by greater than 98% within one week after injection, the remaining 2% were completely cleared within 5-6 weeks (less than $10ml^{-1}$ CF at 3-4 weeks). Bacterial contamination remained low at about $20ml^{-1}$ CF up to 6 weeks at which time the experiment was discontinued (T).

Animals injected with sterile diluent only (MBASW) remained healthy with little bacterial contamination 6 weeks post-injection (less than $100ml^{-1}$ CF) when the experiment was ended (Table 16).

TABLE 16. Bacterial contamination in control (MBASW - injected) *E. esculentus*, present in the same "infection" tanks as microbial-injected animals.

Sea urchin number	cfu* ml^{-1} at week number						
	1	2	3	4	5	6	
1	0	30	30	<10	0	0	T**
2	0	0	<10	<10	<10	60	T
3	0	30	<10	<10	0	20	T

* Based on plating 0.1 ml
** T = Experiment terminated

Since a notional zero-time concentration of 10^5 yeasts ml^{-1} CF were reduced by greater than 90% from the coelom of *E. esculentus* 1 week post-injection and *Ps.111* (approx. 2 x 10^6 ml^{-1} CF) completely cleared within 1-3 weeks after injection, higher and lower graded-doses of each microorganism were used in further quantitative studies in the form of virulence titrations.

3.2. Virulence Titrations and Clearance Curves of Marine Yeasts and Bacteria in *E. esculentus*

3.2.1. Clearance of *Ps.111*

In preliminary observations, inocula of 2 x 10^6 to 10^7 ml^{-1} CF were cleared to less than $10ml^{-1}$ CF within 1-4 weeks respectively, and the animals lived for over 6 weeks after which time they were discarded. As a secondary observation secondary bacterial infection increased from zero (as detectable in 0.1ml CF) to greater than 30 bacterial contaminants ml^{-1} CF between 3 to 6 weeks post-injection.

To assess the quantitative nature of the clearance of *Ps.111* from the coelom of *E. esculentus* in terms of rate of clearance, median survival time, appearance of secondary bacterial contamination and physical symptoms of infection, graded doses of the bacteria were injected into healthy specimens.

E. esculentus of suitable size, healthy appearance and less than 200 bacterial contaminants ml^{-1} CF (greater contamination than this and the animal was rejected) were injected with very low, low, medium and high doses of 6 x 10^4, 5 x 10^5, 5 x 10^{7-9} and 1-2 x 10^{10} *Ps 111*, respectively.

The initial concentration of bacteria at zero-time was estimated by allowing for a 1:100 dilution by the CF (range 50 to 150 ml CF). It was also assumed that the injected microorganisms did not localize at the point of inoculation but were spread evenly throughout the CF in the coelomic cavity of *E. esculentus*. Yui and Bayne (1983) reported that 40 to 90 min were required for even dispersal of injected bacteria in the coelom of *S. purpuratus*. The rate of clearance of the injected bacteria was observed by sampling the CF (undiluted and serial dilutions) at weekly intervals on MA. The undiluted and serial diluted CF was also plated on this media to monitor the incidence of post-injection bacterial contamination.

The physical appearance of each specimen was recorded before, during and after injection, this is discussed in detail later (3.2.4.). Two groups of control animals alongside each group of infected animals were injected with 1.0ml sterile diluent (MBASW). One group were placed in the "infection" tank, that is the aquarium in which the bacterial and yeast-injected animals were kept. The second group were placed in a separate "storage" tank in which only healthy, uninjected specimens of *E. esculentus* were present.

Data showing the clearance of four doses of *Ps. 111* very low (VL), low (L), medium (M) and high (H) from the coelom of *E. esculentus* and the appearance of secondary bacterial contamination of the CF are presented in Figure 61 and 62. A notional zero-time concentration of 6×10^2 *Ps. 111* ml^{-1} CF (Figure 61A) and 5×10^3 *Ps. 111* ml^{-1}CF (Figure 61C) were completely cleared from the coelom of the animals within one week after injection with no evidence of regrowth of the *Ps. 111*. Animals injected with the lower dose (Figure 61A) remained alive until the experiment was stopped at 7

FIGURE 61. Clearance of injected doses of 6×10^4 (A) and 5×10^5 (C) *Ps.111* in the CF (giving a notional zero-time concentration of 6×10^2 (A) and 5×10^3 (B) ml^{-1}, respectively) from the coelom of *E. esculentus* (n = 3 to n = 4) at different times after injection and the appearance of secondary bacterial contamination (B and D) of the CF (ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered *Ps.111*, $\log_{10}$ ml^{-1} CF ;

open symbols : bacterial contamination, $\log_{10}$ ml^{-1} CF.

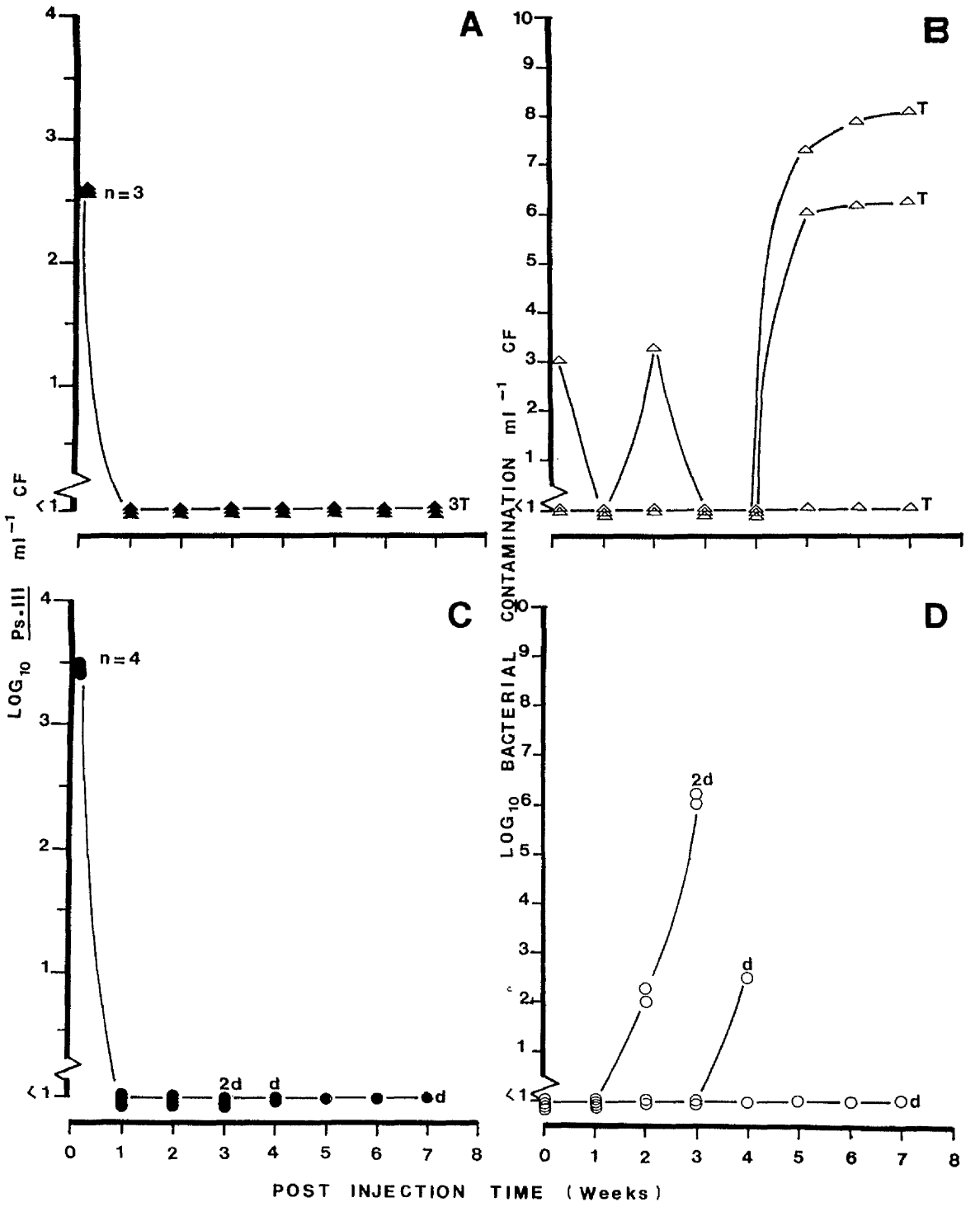

A
B
C
D
n=3
3T
T
T
n=4
2d
d
d
2d
d
d
ml^{-1} CF
LOG_{10} Ps.III
LOG_{10} BACTERIAL CONTAMINATION ml^{-1} CF
POST INJECTION TIME (Weeks)

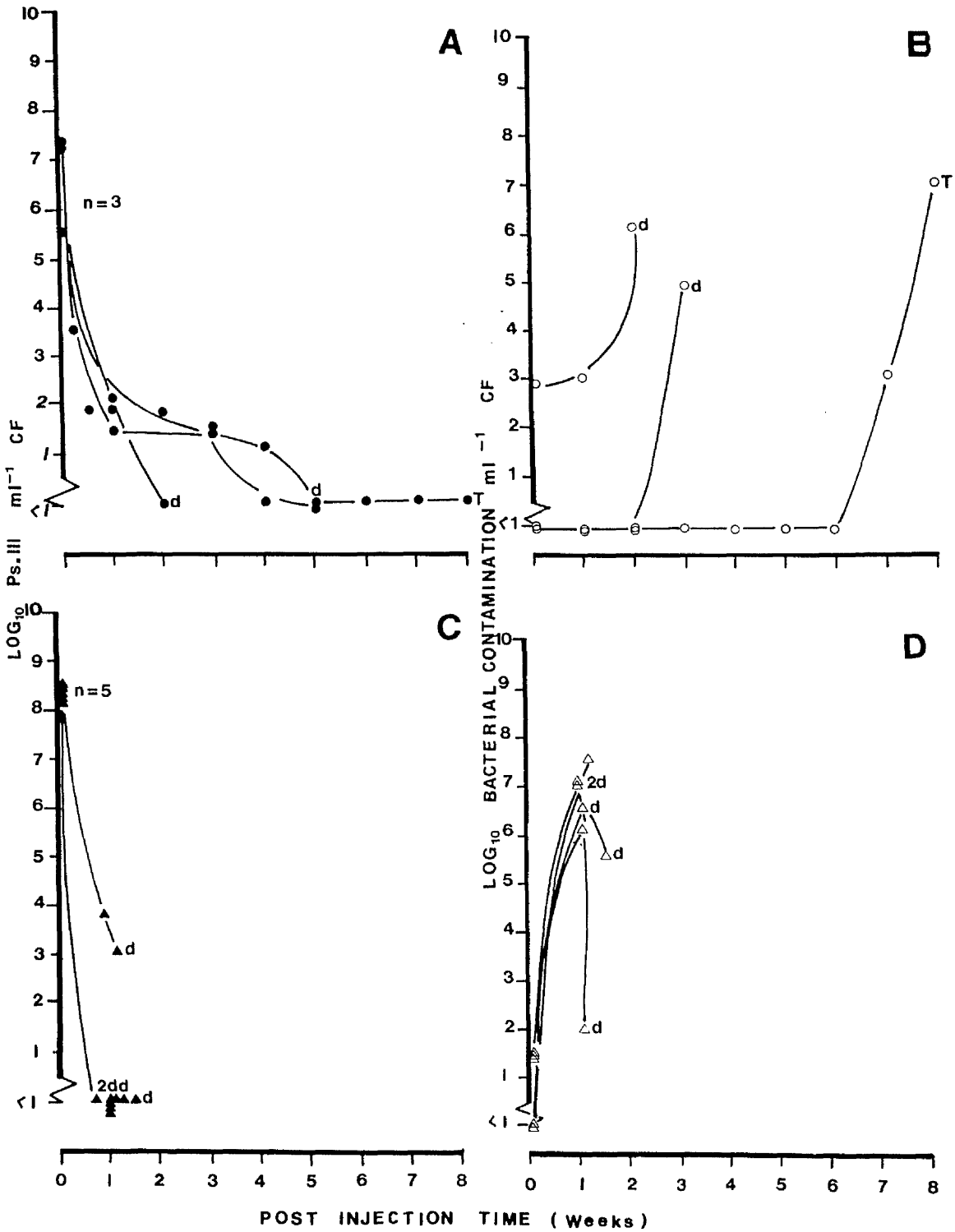
FIGURE 62. Clearance of injected doses of 5 x 10^7 - 10^9 (A) and 1 - 2 x 10^{10} (C) *Ps.111* (giving a notional zero-time concentration in the CF of 5 x 10^5 - 10^7 (A) and 1 - 2 x 10^8 (C) ml^{-1}, respectively) from the coelom of *E. esculentus* (n = 3 to n = 5) at different times after injection and the appearance of secondary bacterial contamination (B and D) of the CF (ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered *Ps.111*, log_{10} ml^{-1} CF;

open symbols : bacterial contamination, log_{10} ml^{-1} CF.


A
B
C
D
n=3
n=5
d
T
2d
2dd
Ps. III
CF
ml⁻¹
LOG10
BACTERIAL CONTAMINATION
POST INJECTION TIME (Weeks)

weeks. Animals injected with the higher dose (Figure 61C) died within 7 weeks post-injection; 2 at 3 weeks, 1 at 4 weeks and the remaining animals at 7 weeks.

Bacterial contamination of the CF fluctuated after injection reaching 10^6-10^8 ml^{-1} CF at 4-7 weeks in two of the animals injected with 6 x 10^4 *Ps. 111* (Figure 61B), the CF of the third animal remained sterile throughout. It should be noted however, that one animal had an unusually high zero-time background bacterial contamination of 10^3 ml^{-1} CF, this fluctuated around zero until it reached overwhelming proportions at 7 weeks (10^8 ml^{-1} CF). Bacterial contamination of the second group of animals injected with 5 x 10^5 *Ps.111* ml^{-1} CF at death. The CF of the third animal remained sterile until time of death at 4 weeks when the background contamination increased to 500 ml^{-1} CF. The CF of the fourth remained sterile throughout until death at 7 weeks.

Higher doses of *Ps.111* (5 x 10^7 - 2 x 10^{10}, giving a notional zero-time concentration in the CF of 5 x 10^5 - 2 x 10^8 ml^{-1}) were also rapidly cleared to less than $20ml^{-1}$ CF a week after injection (Figure 62A and C). However, there were many mortalities, particularly animals injected with the higher dose which all died within one week (Figure 62C) and were accompanied by high background bacterial contamination (up to 10^7) of the CF (Figure 62B and D).

The data for the two groups of *E. esculentus* injected with sterile diluent (MBASW) are presented in Table 17. The controls were split into two groups, one group placed in the "infection" tank with the microbial-injected animals and the other in a "storage" tank with uninjected animals. The bacterial contamination of the CF of the animals placed in the

TABLE 17. Bacterial contamination in the CF of control/(MBASW-*injected)* E. *esculentus* and the influence of having deliberately-infected animals in the same tank.

Size of infecting *Psl11* or yeasts	Tank location of MBASW-injected *E. esculentus*	Urchin Number	Bacterial contamination of CF (ml^{-1}) in MBASW-injected animals at week number 0	1	2	3	4	5	6	7	8	
A	Infection	1	90	90	<10	<10	<10	50	$1x10^4$	$3x10^7$	$8x10^7$	T*
		2	<10	<10	<10	<10	<10	<10	<10	80	500	T
		3	<10	<10	<10	<10	<10	<10	<10	10	50	T
Very Low *** (VL)												
	Storage	4	<10	<10	<10	<10	<10	<10	30	50	80	T
		5	<10	<10	<10	<10	<10	<10	<10	<10	<10	T
		6	<10	<10	<10	<10	<10	<10	<10	<10	<10	T
B	Infection	7	<10	<10	$3x10^4$d**	-	-	-	-	-	-	
		8	<10	<10	<10	<10	<10	<10	<10	<10	<10	T
		9	<10	<10	<10	<10	<10	20	200	250	460	T
Low *** (L)												
	Storage	10	<10	<10	<10	<10	<10	<10	20	20	30	T
		11	<10	<10	<10	<10	<10	<10	<10	<10	<10	T
		12	<10	<10	<10	<10	<10	<10	<10	<10	<10	T

* T = Experiment terminated
** d = Death of an animal
*** = See Table 11.

TABLE 17. (continued).

Size of infecting dose of *Ps. III* or yeasts	Tank location of MBASW-injected *E. esculentus*	Urchin Number	Bacterial contamination of CF (ml^{-1}) in MBASW-injected animals at week number								
			0	1	2	3	4	5	6	7	8
C	Infection	13	<10	80	500	1×10^4 d**	-	-	-	-	-
		14	<10	<10	<10	<10	<10	<10	<10	<10	0 T*
		15	<10	<10	<10	<10	<10	<10	<10	<10	0 T
Medium *** (M)											
	Storage	16	<10	<10	<10	<10	<10	<10	<10	<10	<10 T
		17	<10	<10	<10	<10	<10	<10	<10	<10	<10 T
		18	<10	<10	<10	<10	<10	<10	<10	<10	<10 T
D	Infection	19	<10	5×10^7 d	-	-	-	-	-	-	-
		20	100	2×10^6 d	-	-	-	-	-	-	-
		21	<10	2×10^7 d	-	-	-	-	-	-	-
		22	500	3×10^3 d	-	-	-	-	-	-	-
		23	510	30 d	-	-	-	-	-	-	-
High *** (H)											
	Storage	24	<10	<10	<10 T	-	-	-	-	-	-
		25	<10	<10	<10 T	-	-	-	-	-	-
		26	<10	<10	<10 T	-	-	-	-	-	-

* T = Experiment terminated
** d = Death of an animal
*** = See Table 11

"storage" tank typically remained below 10 colonies ml^{-1} CF over the 2-8 weeks sampling time with the CF of only two of the animals reaching about 80 bacterial contaminants ml^{-1} CF. In contrast, MBASW-injected animals, placed in the "infection" tank alongside yeast and *Ps.111* injected animals (Table 17), exhibited high bacterial contamination of the CF (range, 10^4-8 x 10^7 ml^{-1}) in one out of three sea urchins in the VL, L and M dose groups (see Table 11). It was particularly evident in controls alongside the H dose-injected animals with a resultant 100% mortality of these animals (in parallel with infected animals) within a week (Table 17D).

3.2.2. Virulence Titrations of Marine Yeasts

To determine the relative virulence of the two strains of marine yeasts, *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) towards *E. esculentus*, several doses of the yeasts were injected into the coelom of selected healthy animals with minimal bacterial contamination (usually about 200 $0.1ml^{-1}$ CF).

Four doses of marine yeasts were injected into the coelom of 4 groups of animals, 10^4 (VL), 4 x 10^5 - 2 x 10^6 (L), 1-5 x 10^7 (M) and 5 x 10^8-10^9 (H) yeasts. Allowing for an approximate dilution factor of 1:100 by the CF of each animal (Materials and Methods 1.4.4.) the resulting notional zero-time concentrations in the CF were therefore estimated to be 10^2, 4 x 10^3 - 2 x 10^4, 1-5 x 10^5 and 5 x 10^6 - 10^7 yeasts ml^{-1}.

3.2.2.1. *Rhodotorula rubra (NCYC 63)*

The data showing the clearance curves of *R. rubra* (NCYC 63) and

appearance of secondary bacterial contamination are presented in Figures 63 and 64.

There was an initial clearance of all four dose sizes of (ranging from 10^4 - 10^9 yeasts) injected yeasts from the coelom of 100-95% respectively, within one week after injection. The remaining percentage of viable yeasts in the coelom persisted until death (d) of the animals, or until the end of the experiment (T).

Animals injected with the lowest dose of 10^4 yeasts (Figure 63A) were cleared completely from the coelom within one week remaining at this level until the experiment was stopped at 12 weeks, with one exception, which died at 5 weeks. The higher doses of yeasts 4 x 10^3 - 10^7 (Figure 63C and 64A and C) were cleared initially (with 1 week) by greater than 95% the remaining yeasts persisted in the coelom resulting in a high incidence of mortality, between 1 and 7 weeks, with an apparent relationship between the dose size and mortality rate (discussed later).

Background bacterial contamination (Figures 63 and 64 B and D) increased rapidly after injection of the 4 x 10^5 - 10^9 yeasts, to 10^2 - 5 x 10^7 bacteria ml^{-1} CF at time of death. The secondary bacterial contamination of the group of animals injected with 10^4 yeasts typically remained at a level of less than 1000ml^{-1} CF until the experiment ended at 11 weeks (Figure 63C).

3.2.2.2. *Metschnikowia zobelli (NCYC 783)*

The data presented in Figures 65 and 66 show clearance curves of *M.*

FIGURE 63. Recovery of injected doses 10^4 (A) and 4×10^5 - 2×10^6 *R. rubra* (NCYC 63) (giving a notional zero-time concentration in the CF of 10^2 (A) and 4×10^3 - 2×10^4 (C) ml^{-1}, respectively) from the coelom of *E. esculentus* (n = 3 to n = 5) at different times after injection and the appearance of secondary bacterial contamination (B and D) of the CF (ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered yeasts, log_{10} ml^{-1} CF ; open symbols : bacterial contamination, log_{10} ml^{-1} CF.

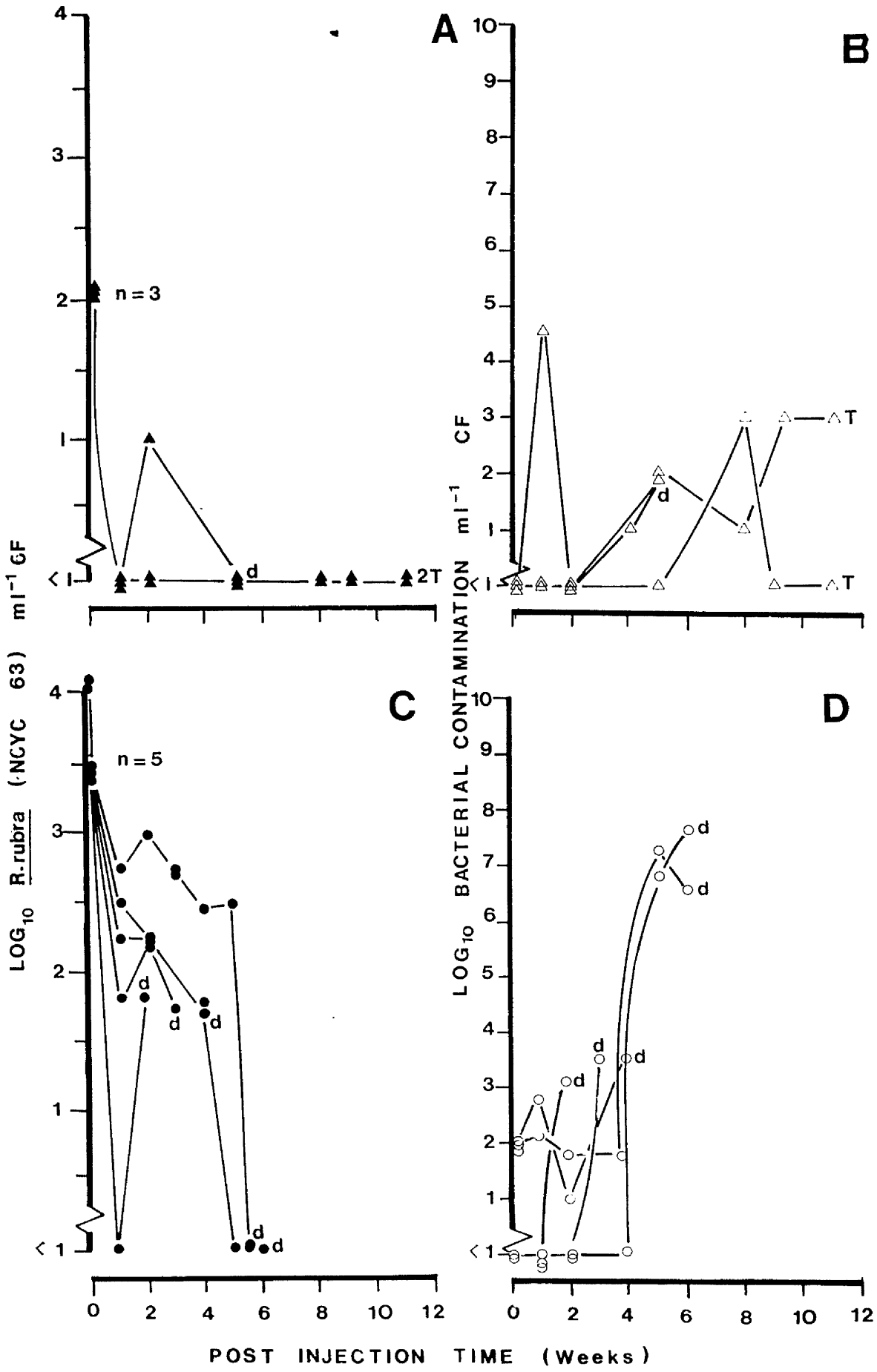

A
B
C
D
n = 3
n = 5
d
T
2T
< 1
GF ml⁻¹
CF ml⁻¹
LOG10 R.rubra (NCYC 63) ml⁻¹
LOG10 BACTERIAL CONTAMINATION ml⁻¹
POST INJECTION TIME (Weeks)

FIGURE 64. Recovery of injected doses 1 - 5 x 10^7 (A) and 10^9 (C) *R. rubra* (NCYC 63) (giving a notional zero-time concentration in the CF of 1 - 5 x 10^5 (A) and 10^7 (C) ml^{-1}, respectively) from the coelom of *E. esculentus* (n = 4 to n = 6) at different times after injection and the appearance of bacterial contamination (B and D) of the CF (ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered yeasts, log_{10} ml^{-1} CF ;

open symbols : bacterial contamination, log_{10} ml^{-1} CF.

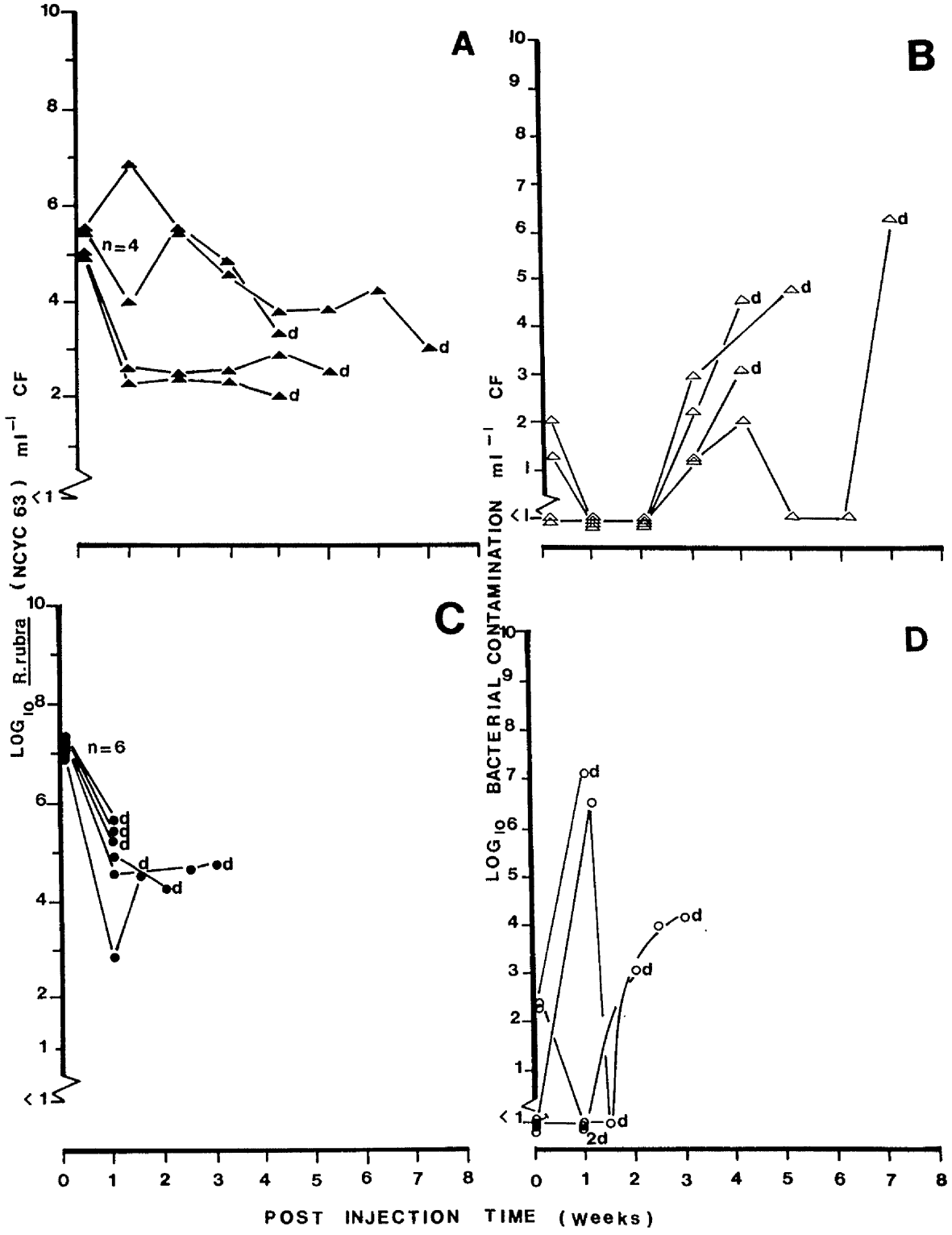

A
B
C
D
n=4
n=6
(NCYC 63) ml⁻¹ CF
BACTERIAL CONTAMINATION ml⁻¹ CF
LOG10 R.rubra
LOG10
POST INJECTION TIME (Weeks)

FIGURE 65. Recovery of injected doses 10^4 (A) and $5 - 8 \times 10^5$ (C) *M. zobelli* (NCYC 783) (giving a notional zero-time concentration in the CF of 10^2 (A) and $5 - 8 \times 10^3$ (C) ml^{-1}, respectively) from the coelom of *E. esculentus* (n = 3 to n = 5) at different times after injection and the appearance of secondary bacterial contamination (B and D) of the CF (ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered yeasts, $\log_{10}$ ml^{-1} CF ;

open symbols : bacterial contamination, $\log_{10}$ ml^{-1} CF.

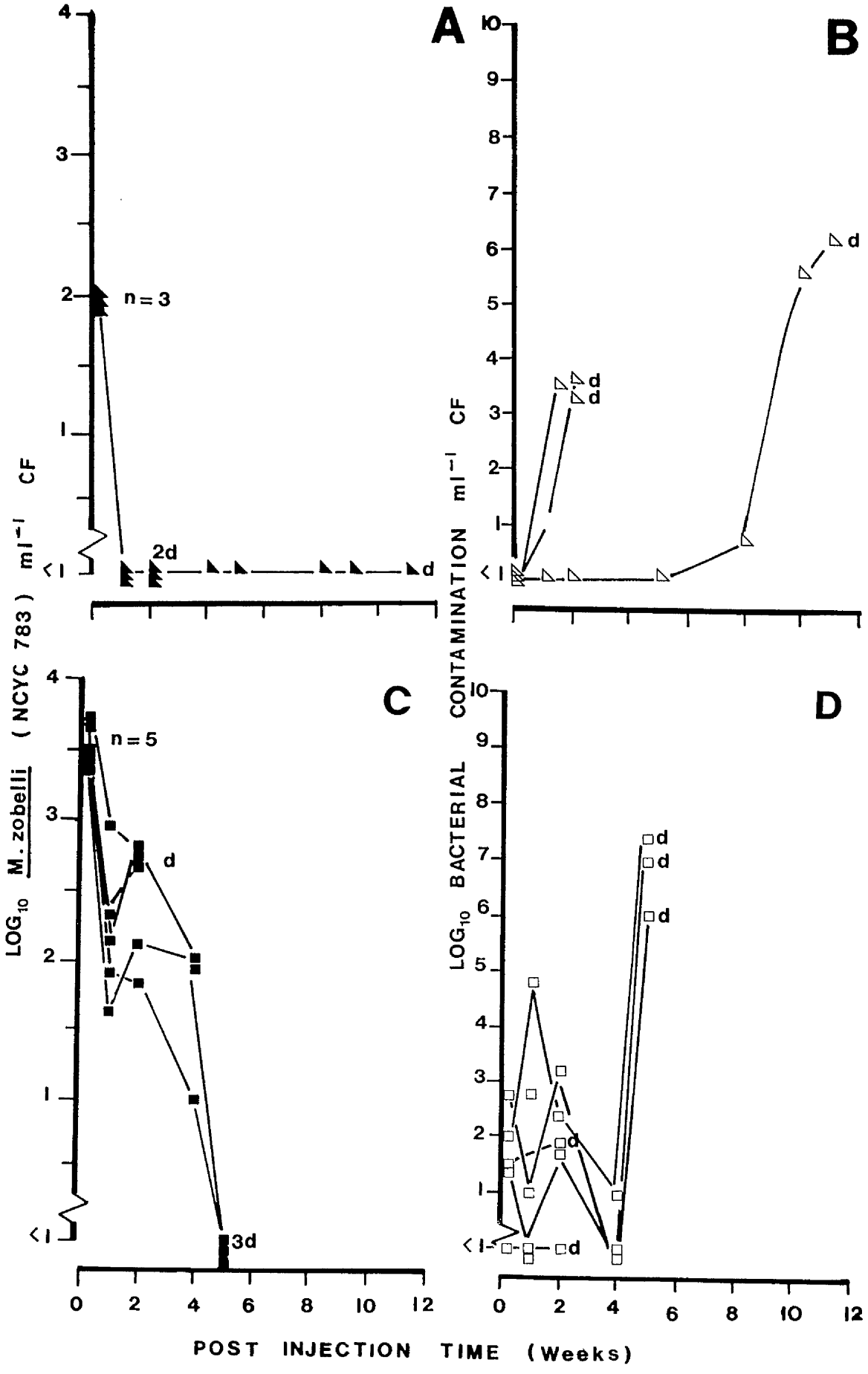

A
B
C
D
n = 3
2d
d
n = 5
3d
LOG10 M. zobelli (NCYC 783) ml-1 CF
LOG10 BACTERIAL CONTAMINATION ml-1 CF
POST INJECTION TIME (Weeks)

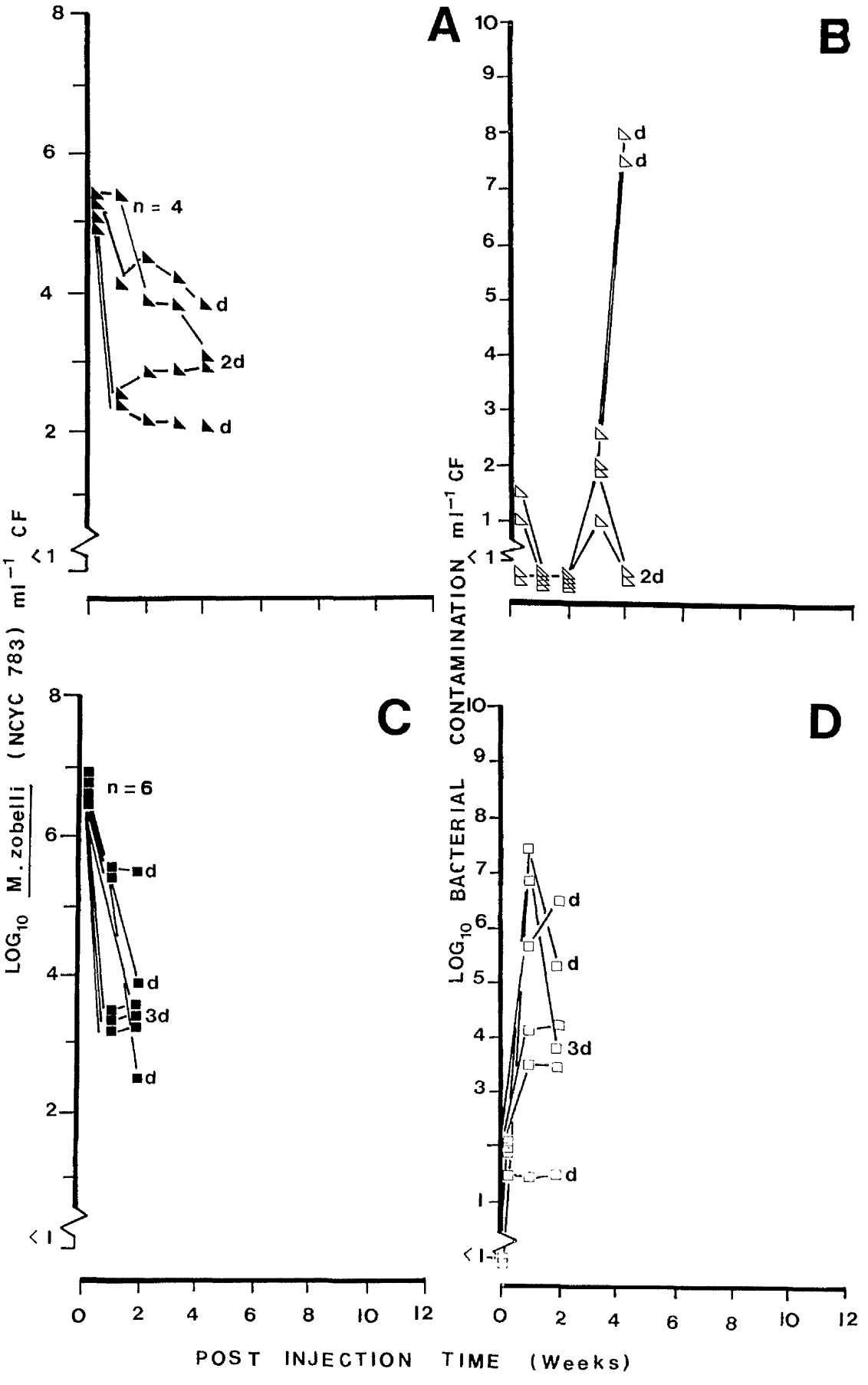
FIGURE 66. Recovery of injected doses 1 - 3 x 10^7 (A) and 5 - 9 x 10^6 (C) *M. zobelli* (NCYC 783) (giving a notional zero-time concentration in the CF of 1 - 3 x 10^5 (A) and 5 - 9 x 10^6 (C) ml^{-1}, respectively) from the coelom of *E. esculentus* (n = 4 to n = 6) at different times after injection and the appearance of secondary bacterial contamination (B and D) of the CF (ml^{-1}).

T = Experiment terminated

d = Death of an animal

Solid symbols : recovered yeasts, log_{10} ml^{-1} CF ;

open symbols : bacterial contamination, log_{10} ml^{-1} CF.


A
B
C
D
n = 4
d
2d
d
d
d
2d
n = 6
d
d
3d
d
d
d
3d
d
LOG10 M. zobelli (NCYC 783) ml-1 CF
LOG10 BACTERIAL CONTAMINATION ml-1 CF
POST INJECTION TIME (Weeks)

zobelli (NCYC 783) from the coelom of *E. esculentus*. A similar pattern emerged as for animals injected with *R. rubra* (NCYC 63). All four doses 10^4 - 9 x 10^6 were cleared by 90-100% from the coelom in the majority of animals within one week after injection. Very low dose inocula (VL) (10^4) were completely cleared from the coelom, although two of the three animals died within 2 weeks, the third lived until the experiment terminated at 12 weeks. A high incidence of mortality was also observed in low (L), medium (M) and high (H) dose injected animals 5 x 10^5 - 9 x 10^6 yeasts at between 2 and 5 weeks post-injection. There was accompanying bacterial contamination just before death ranging between 10 - 5 x 10^7 ml^{-1} CF.

3.2.2.3. *Heat-killed yeast inocula*

The bacterial contamination of the CF of sea urchins injected with heat-killed (HK) yeasts (10^7) (Figure 67) fluctuated between zero and about 3 x 10^4 ml^{-1} CF until the experiment was terminated at 16 weeks. However, the bacterial contamination of the CF of animals injected with HK *M. zobelli* (NCYC 783) (Figure 67A) reached higher levels than those injected with HK *R. rubra* (NCYC 63) and one animal died at 6 weeks post-injection. Animals injected with sterile diluent (MBASW) (Figure 67C) were placed in the same tank as the HK yeast-injected animals. They had bacterial contamination of $100ml^{-1}$ CF or less and remained healthy until the experiment was terminated at 16 weeks.

3.2.3. Quantitative Assessment of Virulence

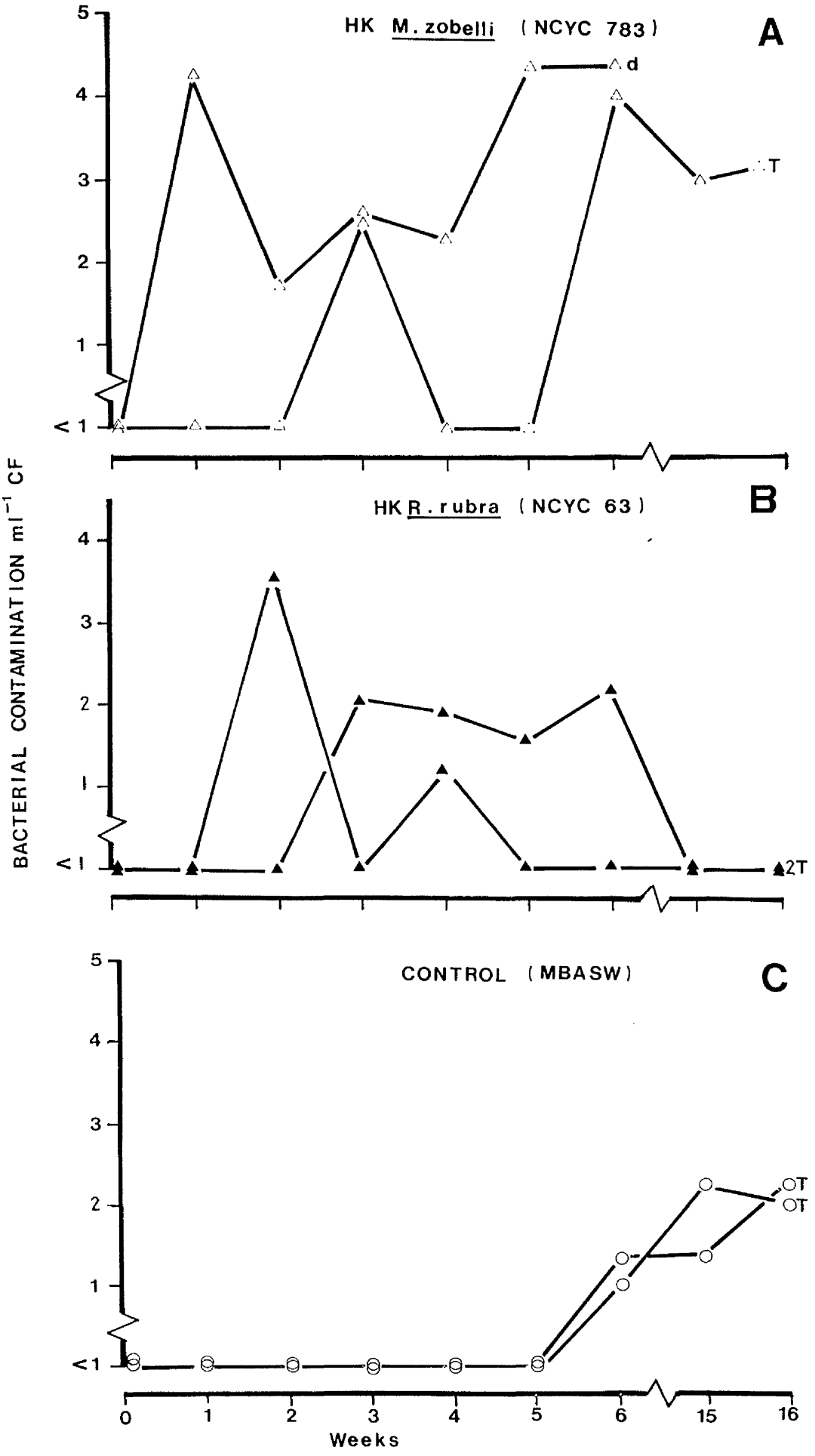
3.2.3.1. *Cumulative percentage mortality*

Quantitative analyses of the percentage mortality of *E. esculentus* injected

FIGURE 67. Background bacterial contamination of *E. esculentus* CF (n = 2) at different times after injection of heat-killed *M. zobelli* (NCYC 783) (A), *R. rubra* (NCYC 63) (B) and sterile diluent (MBASW) (C).

T = Experiment terminated

d = Death of an animal


HK M. zobelli (NCYC 783)
A
d
T
HK R. rubra (NCYC 63)
B
2T
CONTROL (MBASW)
C
OT
OT
BACTERIAL CONTAMINATION ml⁻¹ CF
5
4
3
2
1
<1
0
1
2
3
4
5
6
15
16
Weeks

with four dose sizes of *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) (expressed as notional zero-time concentration of yeasts ml^{-1} CF) are presented in Figure 68.

Initial concentrations of 10^7 (H) *R. rubra* (NCYC 63) ml^{-1} CF (Figure 68A) resulted in 100% mortality within 3 weeks. 10^{3-5} (L and M) yeasts ml^{-1} CF 100% mortality within 7 weeks and 10^4 yeasts 30% mortality within 12 weeks.

A similar pattern of dose-dependent mortality emerged in *E. esculentus* injected with *M. zobelli* (NCYC 783) (Figure 68B) however the mortalities occurred consistently earlier by about one week.

Cumulative percentage mortality of *E. esculentus* injected with *Ps.111* and sterile diluent are presented in Figure 69. There were similarities in the pattern of dose-dependent mortality of animals injected with *Ps. 111* (Figure 69A) and yeasts.

The cumulative percentage mortality of MBASW-injected control *E. esculentus* (Figure 69B) present in the "infection" tank followed a similar trend to the microbial-injected animals with an increase in mortality rate in the presence of high dose microbial injected animals. It should be noted here that there were no organisms of experimental infection present in the CF of the control animals detected during routine sampling. However, the control animals present in a separate "storage" tank remained healthy and alive indefinitely (12 weeks) (Figure 69C).

FIGURE 68. Cumulative percentage mortality of *E. esculentus* injected with four graded doses of *R. rubra* (NCYC 63) (A) and *M. zobelli* (NCYC 783) (B). The individual lines are labelled with the different doses of yeasts expressed as high (H), medium (M), low (L) and very low (VL) (see Table 11).

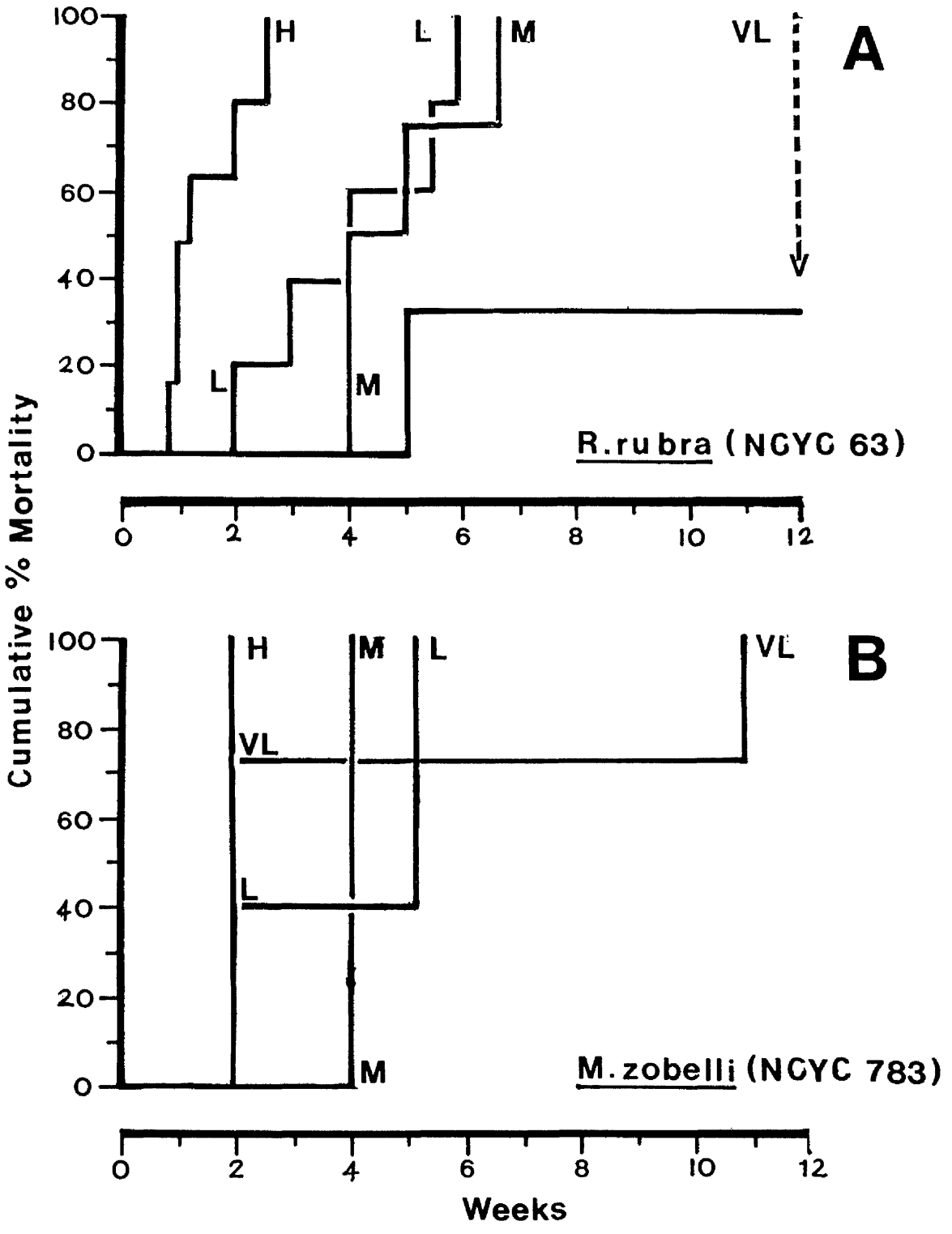
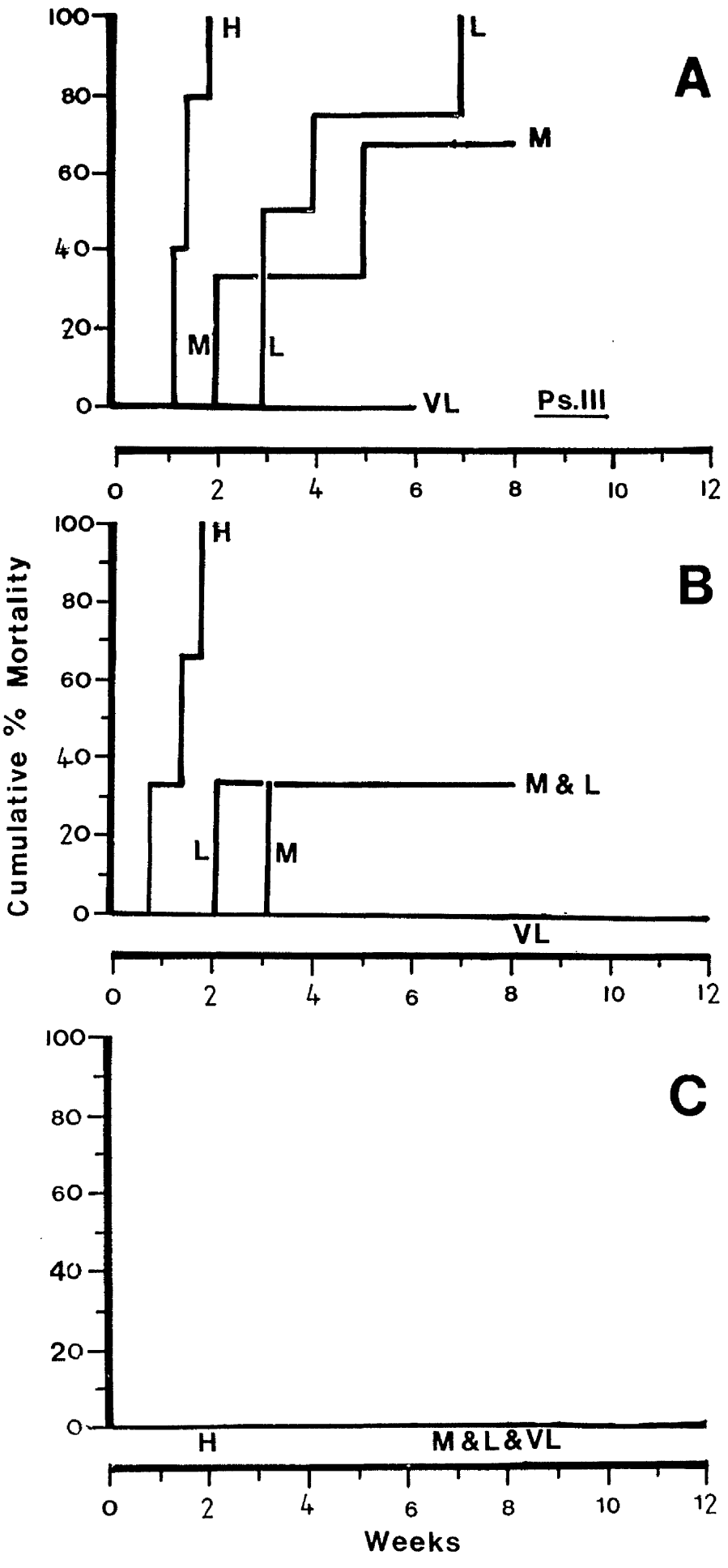

A
H
L
M
VL
L
M
R.rubra (NCYC 63)
B
H
M
L
VL
VL
L
M
M.zobelli (NCYC 783)
Cumulative % Mortality
Weeks
0
20
40
60
80
100
0
2
4
6
8
10
12

FIGURE 69. Cumulative percentage mortality of *E. esculentus* injected with different doses of bacterial reference strain *Ps.111* (A) and of control animals (injected with sterile diluent). They were kept (B) in either the same tank as the infected animals or (C) in a separate storage tank. The individual lines are labelled with the different doses of *Ps.111* (A) and both yeast- and bacterial- injected animals (B and C) present alongside the control animals expressed as high (H), medium (M), low (L) and very low (VL). (see Table 11).


A
100
80
60
40
20
0
H
L
M
M
L
VL
Ps.III
0
2
4
6
8
10
12
B
Cumulative % Mortality
100
80
60
40
20
0
H
M & L
L
M
VL
0
2
4
6
8
10
12
C
100
80
60
40
20
0
H
M&L&VL
0
2
4
6
8
10
12
Weeks

3.2.4. Qualitative Assessment of Infection

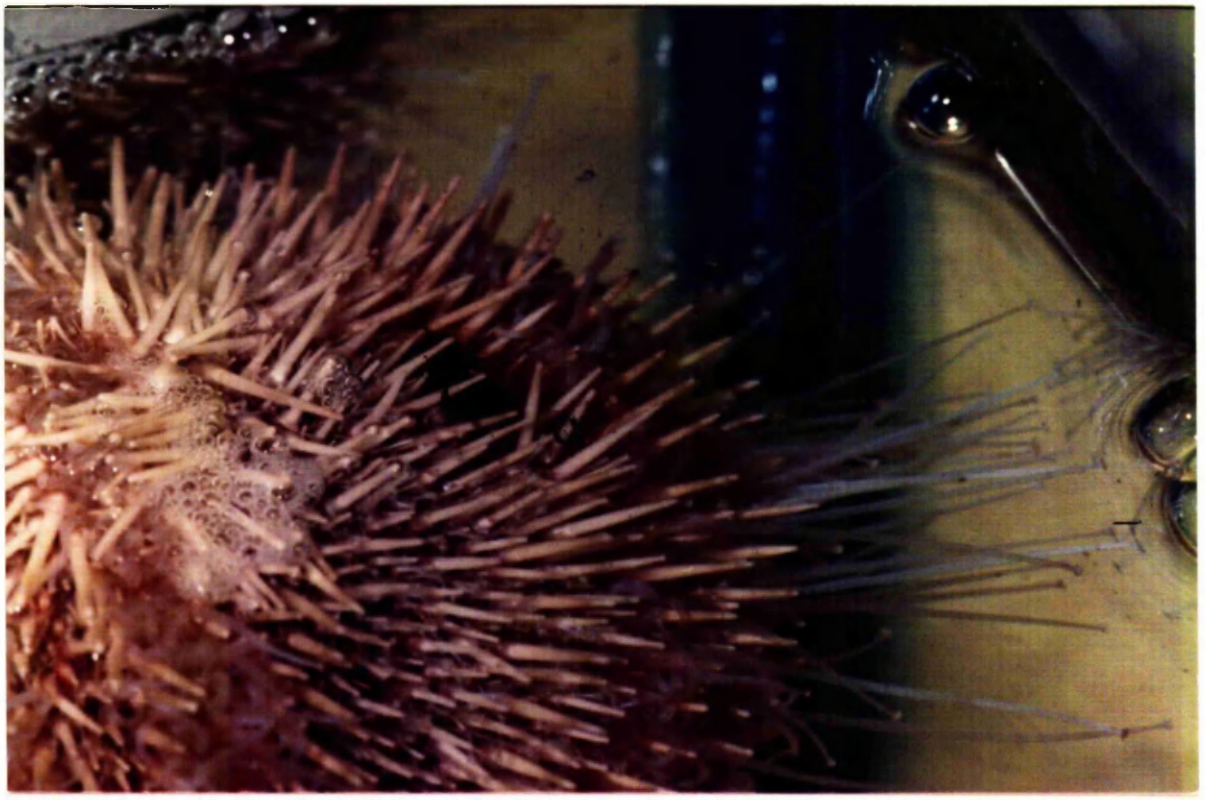
3.2.4.1. *Development of lesions*

Physical changes in the external appearance of *E. esculentus* injected with marine yeasts and *Ps. 111* were evident. In particular, were the appearance of black/red gelatinous lesions, of approximately 1cm in diameter, on the upper hemisphere of the test of otherwise healthy appearing animals (e.g. bright test colouration, attachment to the tank wall, extension of tube feet, erect spine etc.) (Plates 7A and B). These lesions were typically formed between 1 and 3 weeks after injection. During later stages of lesion development, with the onset of infection and time of incubation, the lesions were often observed to increase in diameter (typically up to 3cm in diameter) (Plate 9B) and/or in number (often to 3 in number).

Dissection of the affected area showed that the lesion was also present immediately below the affected area on the outer surface of the test, in the inner coelomic lining of the animal (Figure 70). Swabs taken from the inner and outer lesion area revealed high concentrations of injected yeasts, but not *Ps. 111* and accompanying high bacterial contamination. However, this was not quantitated. A diagram of the exterior surface of the lesions is presented in Figure 71. A denuded area was commonly formed around the lesion. Surrounding the gelatinous red/black lesion there was typically an area where the spines had dropped, the lesion was found to consist of red spherule cells, phagocytic leucocytes, colourless spherule cells and vibratile cells in that order of decreasing abundance. The lesion also was infected with algae, bacterial

PLATE 7A. Black/red gelatinous lesion on the exterior surface of the test of *E. esculentus* after injection with marine yeasts or *Ps. 111*. The animal appeared to be in an otherwise healthy physical condition, showing attachment, tube feet extension, uniform spine arrangement and bright test colouration.

PLATE 7B. Close-up view of the lesion of approximately 1cm in diameter.



A

B

FIGURE 70. Diagram of lesion cross-section on the test surface of experimentally-infected *E. esculentus* with marine yeasts *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) or *Ps. 111.*

The test is composed of three layers :

1. External epidermis,
2. Middle dermis (connective tissue and calcaereous exoskeleton),
3. Inner coelomic lining.

l. lesion

s. spine

t. tube foot

T. test

3
2
1
S
T
t

FIGURE 71. Diagram of lesion on exterior test surface of *E. esculentus* experimentally-infected with marine yeasts *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) or *Ps. 111.*

a. Spine

b. Denuded area of test

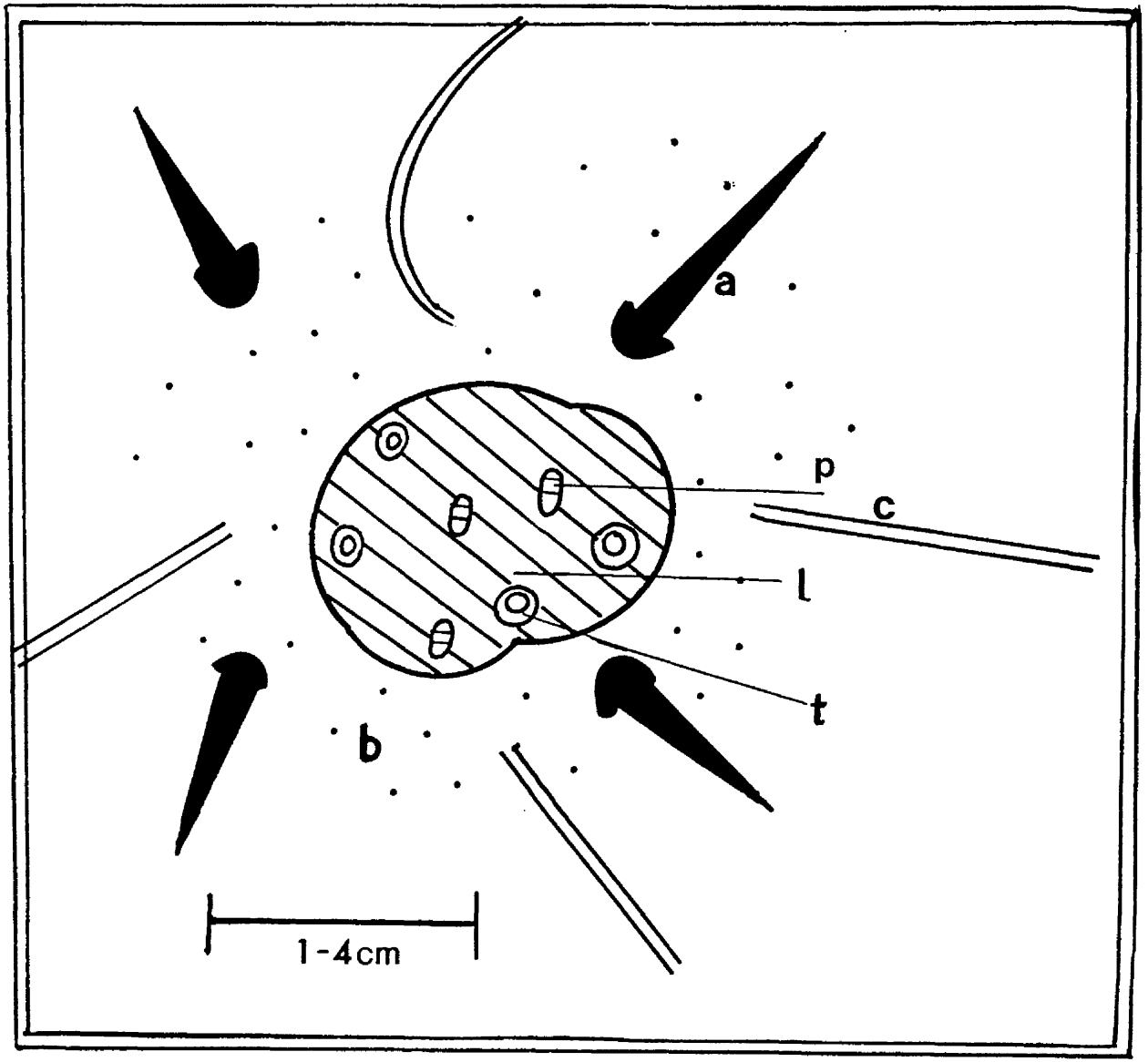
c. Extended tube feet

p. Tube foot pore

t. Spine socket

l. - lesion area - (1 - 4cm diameter)

(see Plate 7A and B)


a
p
c
l
t
b
1-4cm

contamination and organisms of experimental infection. The lesion was seen to occur inside the test immediately below the externally visible lesion. The entire test was also seen to be reddened probably due to the influx of red spherule cells.

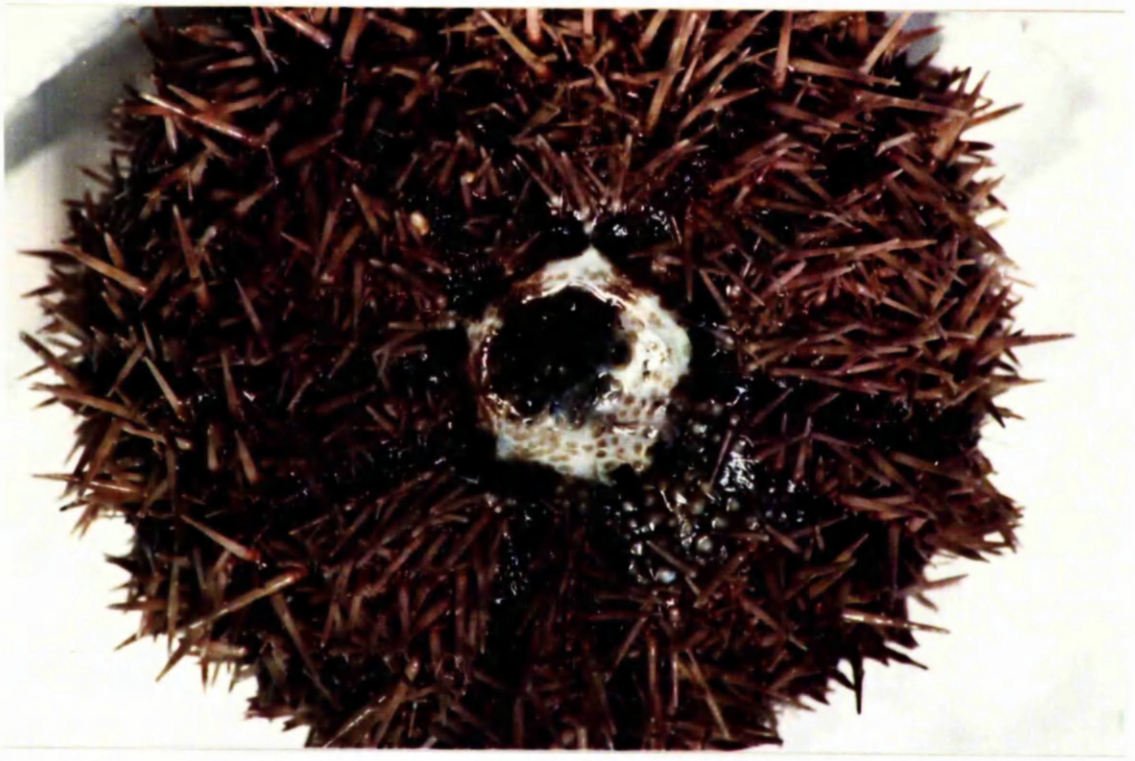
Animals injected with very low dose inocula of marine yeasts *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) (10^4) rarely displayed exterior test lesions as previously described. Instead, small lesions of about 3mm diameter were often formed on and around the peristomial membrane as shown in Plates 8A and B.

In the absence or presence of either types of lesion, large denuded areas of the test were often observed up to 50-60% of the entire *E. esculentus* test surface which was often accompanied by green necrotic tissue and algal colonization (Plate 9A).

3.2.4.2. *Other physical changes*

In addition to the formation of red/black gelatinous lesions, several other physical changes in the external appearance of *E. esculentus* experimentally-infected with marine yeasts and *Ps. 111* were also observed (Table 18). Nine features (a - i) of the physical appearance of *E. esculentus* were recorded before, during and after (at death (d) or termination (T)) experimental infection. The number of animals with abnormal features were recorded between three and seven days before death, and are represented as a percentage of the total group number as shown in Figure 72. Abnormalities in physical features which were seen to increase with an increase in the time of death were : the formation of lesions, darkening of the test colouration, change in colour and consistency of the

PLATE 8 A and B. Small red/black gelatinous lesions on and around the peristomial membrane and partially denuded area of experimentally-infected *E. esculentus* with low doses of (10^4-10^6) marine yeasts or *Ps. 111*.

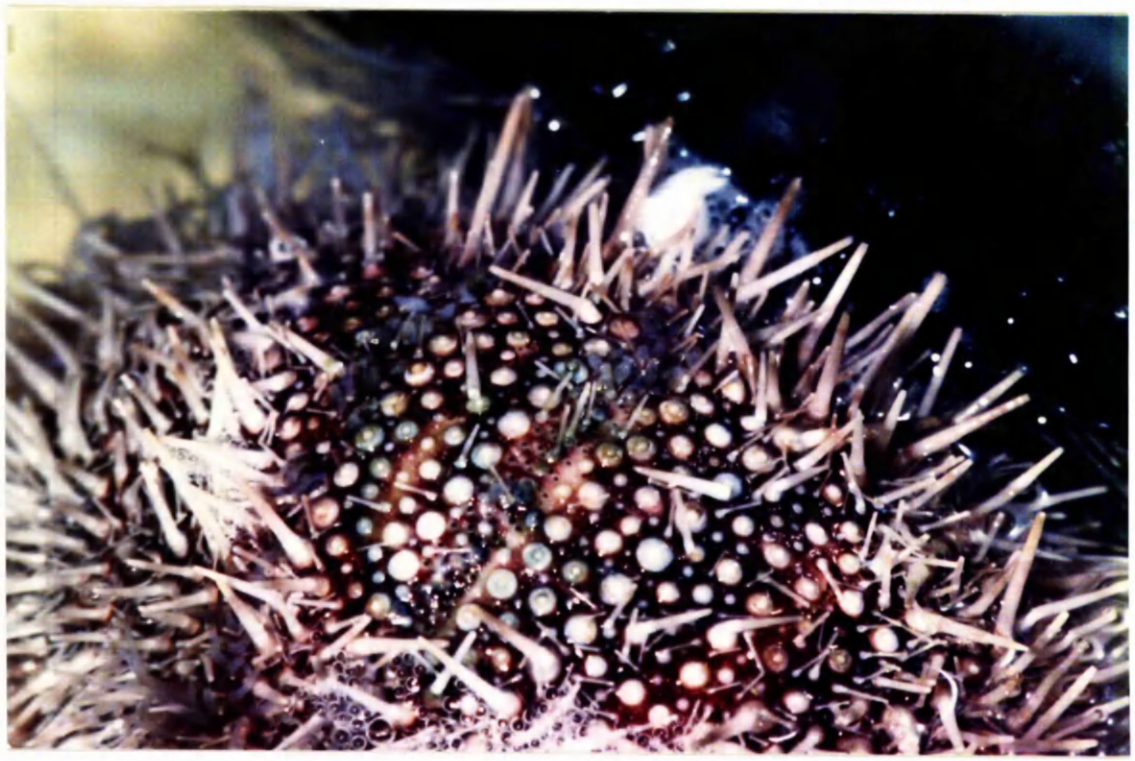


A

B

PLATE 9. Denuded area of *E. esculentus* test (due to spine loss) injected with marine yeast. *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) or *Ps.111* during the later stages of an experimental infection. Note approximately 20% of the test surface denuded and small patches of algal colonization and curled tube feet.

PLATE 9B. Increase in size of red/black gelatinous lesion after about 4 - 5 weeks post-injection with marine yeasts or *Ps.111*.



A

B

TABLE 18. List of the normal and abnormal physical characteristics of healthy and experimentally-infected *E. esculentus*, respectively.

	Physical characteristics	Features Normal	Abnormal
a	**Colouration**		
	Calcaerous test	Pink, purple, violet.	Dark red, purple.
	Spines	Pale with pink, violet tips.	As healthy.
b	**Algae (colonization)**		
	Test	-	+ or -
	Spine	-	+ or -
	Mouth	-	+ or -
c	Spines	Erect, uniform arrangement, movement.	Often broken spine tips, spine shedding, lying flat, or non-uniform arrangement.
d	Denudation	-	+ or -; 5 - 90% of the test area.
e	Lesions	-	+ or -; 1 - > 3 cm diameter,
	Test	-	1 - 3 per animal surface/
	Peristomial membrane	-	+ or -; several on or/and around the membrane
f	Mouth	Closed	Gaping

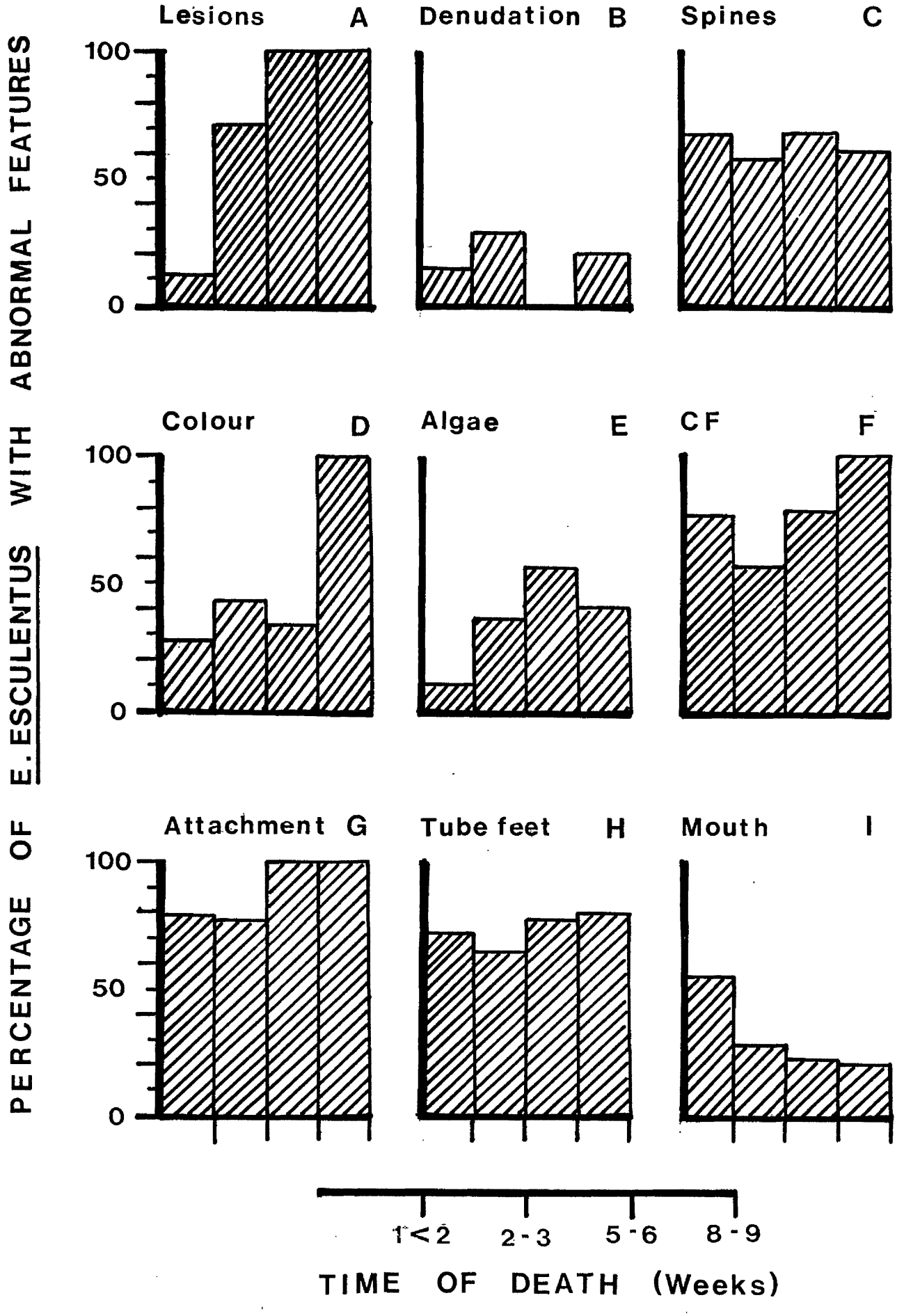
TABLE 18. (continued).

	Physical Characteristics	Features: Normal	Features: Abnormal
g	Attachment	Firm attachment.	Loose attachment/unattached.
h	Tube feet	Extended tube feet.	Curled tube feet, or no tube feet display.
i	Coelomic fluid (CF)	Pink/clear, viscous, clots within minutes after withdrawal from *E. esculentus*.	Clear, yellow/green, watery fluid, with a few suspended clots.

FIGURE 72. Histograms of the occurrence of nine *abnormal external physical features of *E. esculentus* (a to i) as a result of experimental-infection with marine yeasts and *Ps. 111*. This data was recorded between three and seven days before death.

At time of death, group numbers in brackets as follows : 1 2 (n = 29) ; 2 - 3 (n = 14) ; 5 - 6 (n = 9) ; 8 - 9 (n = 5).

* See Table 18.


Lesions A
Denudation B
Spines C
Colour D
Algae E
CF F
Attachment G
Tube feet H
Mouth I
100
50
0
PERCENTAGE OF E. ESCULENTUS WITH ABNORMAL FEATURES
1<2
2-3
5-6
8-9
TIME OF DEATH (Weeks)

CF and loss of attachment of the animals from the side of the aquarium. Abnormal features which occurred at a static level with time of death included : denudation or spine loss, non-uniform spine arrangement or tip breakage and absence of tube feet display. Finally, features which appeared erratically or declined were, the incidence of algal colonization or gaping of the mouth.

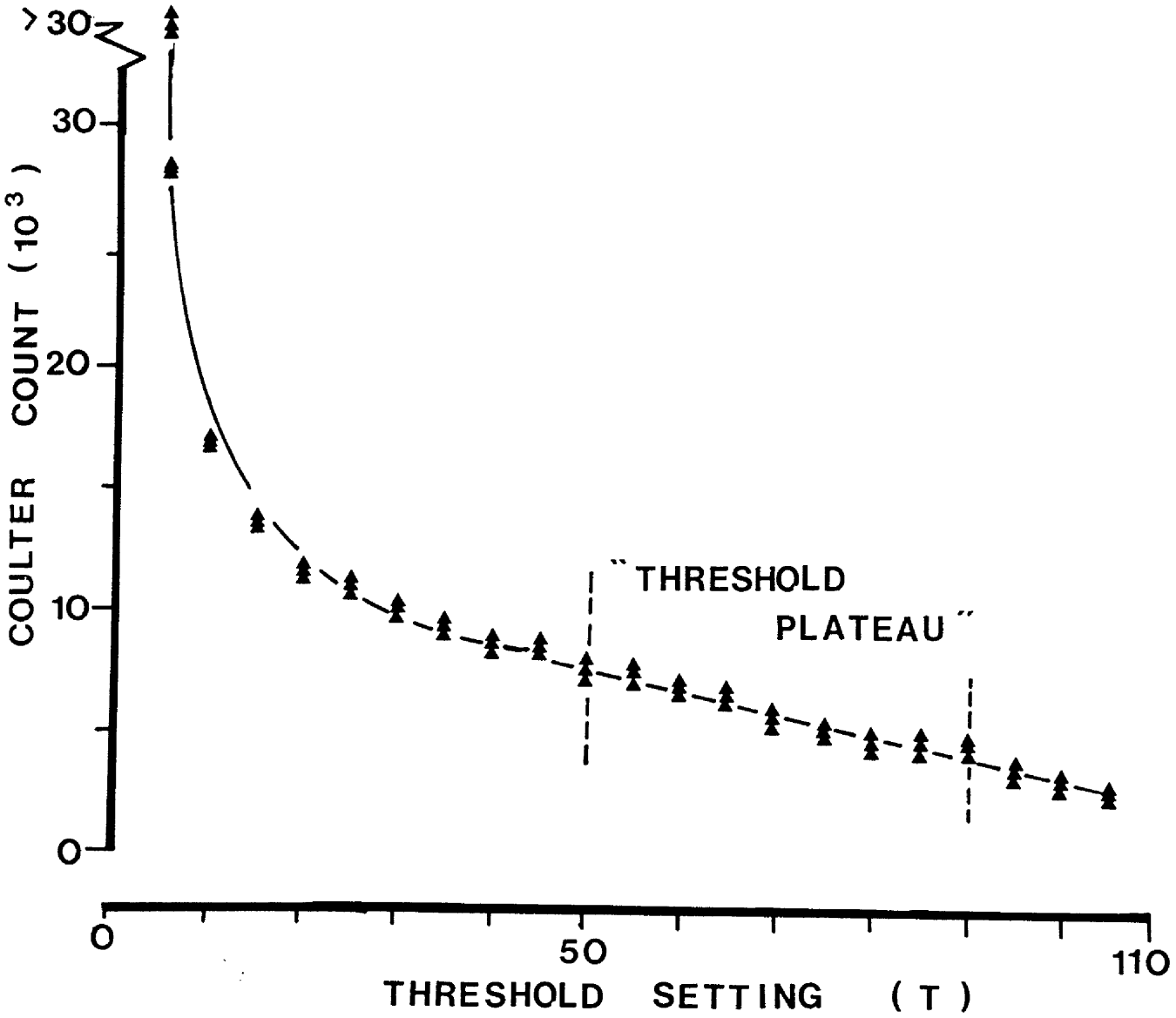
Post-mortem examination of some of the specimens infected with medium to high doses of marine yeasts revealed high concentrations of bacterial contamination from the inner and outer surfaces of the lesions, the gonads and inner coelomic lining, however, this was not quantitated. Additionally, yeasts of experimental infection were isolated in high numbers from the inner lesion area.

3.3. Enumeration of Coelomocytes

3.3.1. Calibration of the Coulter counter

To obtain the threshold setting suitable for the counting of *E. esculentus* coelomocytes a "threshold plateau" was obtained by graphically representing the relationship of the Coulter counter with change in the threshold setting as shown in Figure 73. The threshold setting (T) was selected from the mid-point on the "plateau" (which was about 70, and aperture current and attenuation settings at 8.0 and 0.707 respectively as recommended for counting mouse erythrocytes.

FIGURE 73. Relationship between Coulter count ($0.5ml^{-1}$) and threshold setting (T), (aperture current, B; 0.707 and attenuation constant, D; 8.0) for diluted whole coelomic fluid (1:200 ASW), to determine the "threshold plateau" and hence, the threshold setting suitable for enumeration of *E. esculentus* coelomocytes.


> 30
30
20
10
0
COULTER COUNT (10^3)
"THRESHOLD
PLATEAU"
0
50
110
THRESHOLD SETTING (T)

3.3.2. Total coelomocyte count

3.3.2.1. *Coulter counter*

In an attempt to compare the total coelomocyte counts (ml^{-1}) of healthy and "sick" specimens of *E. esculentus* the cells were enumerated using the Coulter counter.

Fresh samples of CF (and anticoagulant, 14mM EGTA) were withdrawn from healthy and "sick" (post-symptomatic animals previously injected with marine yeasts or *Ps. 111*) animals. The samples were diluted in filtered MBASW and 20ml volumes (held on ice) counted. The results are presented in Table 19. There was found to be a decline in the total coelomocyte count of the CF from sick animals as compared with the CF from healthy *E. esculentus*, the total coelomocyte count of the former was calculated to be 61% of the total coelomocyte count ml^{-1} of CF from healthy specimens.

3.3.2.2. *Haemacytometer*

The total coelomocyte counts (ml^{-1}) of both healthy and sick animals CF were also enumerated by the means of a Neubauar counting chamber (Table 20). The counts were found to be consistently lower than those obtained using the Coulter counter.

3.3.3. Density gradient coelomic fluid fractions

The CF's sampled from both healthy and infected (post-symptomatic or "sick" specimens) animals were separated by density gradient centrifugation. Diluted samples of each of the three fractions A (PL), B

TABLE 19. Total coelomocyte Coulter counts of both healthy and sick specimens of *E. esculentus*.

Sea urchin category	Number of *E. esculentus*	Coelomocyte count ($x10^6$ ml^{-1})		
		mean	SD	SEM
Healthy	28	5.34	3.40	0.64
Sick *	10	3.24	0.83	0.26

* Animals experimentally-infected with either yeasts or *Ps. III*.

Comparison of counts of sick versus healthy animals.

means: t = 2.09 p = > 5%
SD's: F = 0.06 p = > 5%

TABLE 20. Total coelomocyte haemacytometer counts of both healthy and sick specimens *E. esculentus*.

Sea urchin category	Number of *E. esculentus*	Coelomocyte count ($x10^6$ ml^{-1}) mean	SD	SEM
Healthy	13	2.38	2.49	0.69
Sick *	5	2.31	2.20	0.98

* Animals experimentally-infected with either yeasts or *Ps. III*.

Comparison of counts of sick versus healthy animals.

means: t = 0.032 p = > 10%
SD's: F = 0.78 p = > 5%

(CSC and VC) and C (RSC) were counted with the Coulter counter (Figure 74). It must be noted here that microscopic examination of each cell fraction revealed the incidence of "contaminating" coelomocytes from other layers e.g. vibratile cells (VC's) in fraction A, composed mainly of phagocytic leucocytes i.e. the fractions were not pure.

After experimental infection of *E. esculentus* with marine yeasts of *Ps 111* the differential coelomocyte counts (A, B and C) were observed to decline. This was particularly evident in the CSC and VC fraction where there was observed to be a 40% decline in the mean coelomocyte count of CF from infected animals. A 17% decline in the PL's and a 10% dcline in the RSC's were also observed in CF from this group of animals.

The majority of the coelomocytes of *E. esculentus* CF were phagocytic leucocytes, (PL's 65.55 $\pm$ SEM 2.46%), followed by the fraction containing vibratile and colourless spherule cells (VC's and CSC's 23.13 $\pm$ SEM 1.37%) and red spherule cells (RSC's 11.39 $\pm$ SEM 2.20%). The relative percentages of each fraction of CF of the total coelomocyte count are shown in Figure 75. The relative percentages of CF fractions from both healthy and "sick" animals remained fairly constant and there was evidence of only minor changes in the relative proportion of each coelomocyte type. A decline in the PL (A) and CSC and VC (B) fractions by 1% and a 2% increase in the RSC's (C) in CF from "sick" animals.

FIGURE 74. Differential coelomocyte Coulter counts ($log_{10} \pm$ SEM) of coelomic fluid from healthy and experimentally-infected specimens of *E. esculentus*.

(Healthy specimens, n = 22;
"sick" specimens, n = 7).

Where, A = PL's ; B = CSC's and VC's,
C = RSC's.

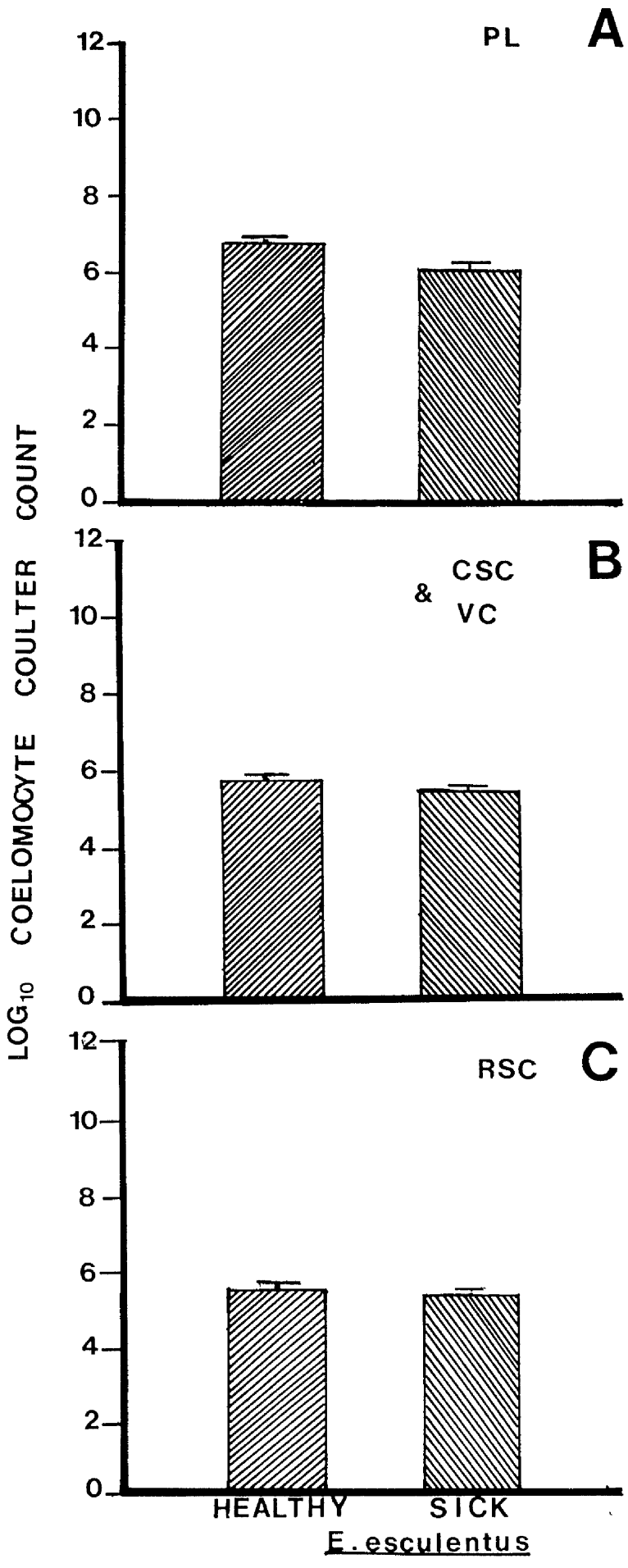
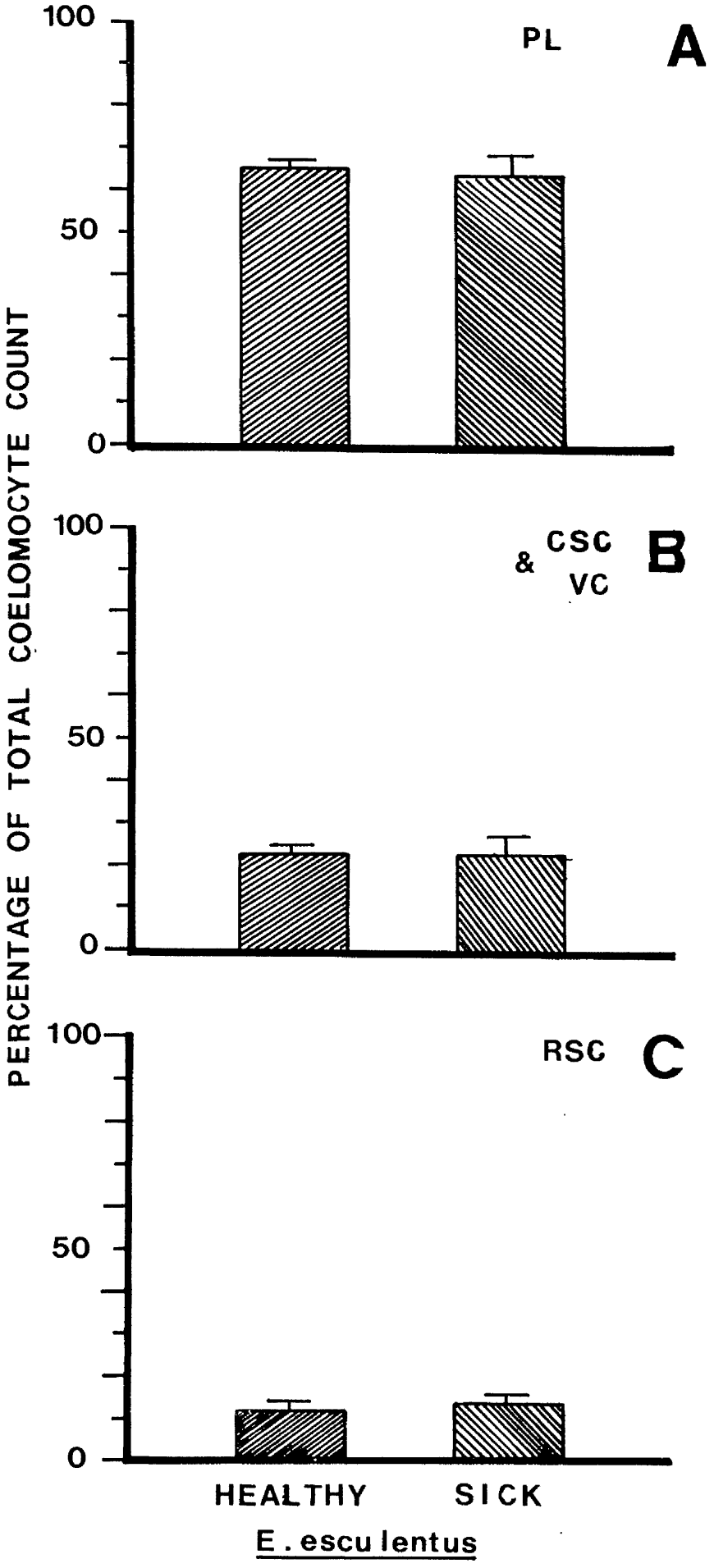

A
PL
B
CSC
&
VC
C
RSC
12
10
8
6
4
2
0
LOG10 COELOMOCYTE COULTER COUNT
HEALTHY
SICK
E. esculentus

FIGURE 75. Differential coelomocyte Coulter counts (± SEM) of coelomic fluid from healthy and experimentally-infected specimens of *E. esculentus* as percentages of the total coelomocyte counts.

(Healthy specimens, n = 22;
"sick" specimens, n = 7).

Where, A = PL's ; B = CSC's and VC's ;
C = RSC's.


100
50
0
PL
A
100
50
0
& CSC VC
B
100
50
0
RSC
C
PERCENTAGE OF TOTAL COELOMOCYTE COUNT
HEALTHY
SICK
E. esculentus

D I S C U S S I O N

1. MAINTENANCE OF *ECHINUS ESCULENTUS* IN RECIRCULATING ARTIFICIAL-SEAWATER AQUARIA

Although facilities for maintaining sea urchins in marine aquaria have existed for more than a hundred years (Hinegardener and Tuzzi, 1981), there are remarkably few accounts of keeping the adult animals in recirculating artificial-seawater aquaria (RASWA) in laboratories remote from the sea. Indeed, the only report of the maintenance of adult sea urchins in RASWA appears to be by Fridberger *et al.* (1979). So far it appears to have attracted few citations. These authors described experiences over more than five years of raising and keeping five different species of sea urchin, including *Echinus esculentus* under strict artificial conditions (closed-circuit aquaria with artificial seawater and light). However, the short publication lacked detailed methods specifically with respect to tank construction, a description of the biological filter bed and methods of management and desirable levels of water quality. Detailed general advice in keeping marine invertebrates however, was obtained from Spotte (1970), King and Spotte (1974), Kinne (1977), Committee on Marine Invertebrates (1981) and Mills (1985). One of the initial aims of this thesis was to describe a readily reproducible closed-circuit, artificial seawater system in which adult specimens of *E. esculentus* could be successfully maintained over extended periods of time and obtain comprehensive information for monitoring of the established RASWA. However, this work, despite its undeniable importance, did not form the main emphasis of the thesis and as such there has been no attempt to tackle this large area in great detail.

The preliminary aquarium systems used in the present investigation differed from the finally adopted design of RASWA in several aspects. The crucial change, on which the success of the aquaria were largely dependent, was the construction and maturation of the biological filter bed.

Firstly, the size and shape of the gravel is important in the construction of the biological filter. Small, angular gravel has a larger surface area for bacterial attachment (Spotte, 1970). Therefore, coarse, angular coral sand was found to be preferable to smooth, pebble varieties which were used here in the initial aquarium systems. Conversely, the circulation through the bed is impaired if the gravel grains are too small, resulting in anoxic areas where the growth of bacteria is impaired (Saeki, 1958). In the final aquarium system coral sand was used containing algal öolites, according to the manufacturer John Allen, Chelmsford, facilitating a suitable habitat for nitrifying and denitrifying filter bacteria due to its uniquely porous nature. The manufacturer also stated that algal öolites can accommodate hundreds of times more bacteria than beach sand or crushed shell. Gravel/sand measuring 2-5mm was shown to be best for most aquarium biological filtration systems by Saeki (1958). Kawai *et al.* in 1965 (cited by Spotte, 1970) determined that nitrifiers in the filter bed were 100 times more plentiful as those suspended in the water. As well as a suitable habitat for biological filtration bacteria, coral sand also serves as a buffer, since the practical pH for nitrification in marine systems is 7.5-8.3 (Spotte, 1970). Pure calcium carbonate is not readily soluble at the pH of seawater, but natural calcium carbonates containing a minimum of 4% magnesium, e.g. coral sand contains solubility sites which promote solution of the buffer in the presence of hydrogen ions (Goldizen, 1970). This was used in the final RASWA system. The replenishment of the alkaline reserve was also accomplished by the addition of chemical buffers "Sea Buff^{R}" to the RASWA based on carbonates and related to the equilibrium of the sea. A high rate of gas exchange, achieved by the air diffusing into the system (by surface agitation as filtered-water was sprayed over the aquarium surface), removes much of the CO_2, and aids in shifting the

equation to the left, while insuring that dissolved oxygen is at near saturation levels (Goldizen, 1970). Periodic sampling of the water in the final system gave an oxygen concentration of about 6 mg l^{-1} at 10°C. A biological filter bed may be compared to a huge respiring organism. When functioning properly it consumes a considerable amount of oxygen, the biological oxygen demand (BOD) (Hirayama, 1965, cited by Spotte, 1970).

In the natural environment toxic substances such as ammonia and its converted products (e.g. NO_3^- and NO_2^-) are normally diluted in the sea far below its toxic levels, but in a closed-circuit aquarium (RASWA), they can rapidly build up to toxic concentrations exceeding 30 mg NO_2 l^{-1} as measured by "Tetra Test ^{R}" colourimetric indicator kit. To counteract this it was necessary to establish in the aquarium a biological filter of nitrifying bacteria which would oxidize the ammonia to nitrite and then to nitrate. Nitrate ions are far less toxic to marine animals than ammonia by approximately three orders of magnitude (Kelley, 1965). Chemically, an abundance of nitrates causes a decrease in pH by replacing carbonate and bicarbonate ions and forming nitric acid (Honig, 1934). Thus, before a full load of animals can be introduced into the marine aquarium system it must first be "run-in" or "conditioned" (King, 1970, King and Spotte, 1974). The establishment of the nitrifying sequence in a new marine aquarium with the sand filter bed is an important process referred to as the "run-in" period by marine aquarists. A "conditioned" system was defined by Spotte (1970) as one in which the filter bacteria are in dynamic equilibrium with the routine formation of their energy sources. Biological filtration, defined as the mineralization of organic nitrogenous compounds, nitrification and denitrification, is accomplished by bacteria suspended in the water and attached to the coral sand of the filter bed. These bacteria entered the system in the form of an inoculation-concentrate "Sea Mature^R"

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + H^+$$

which was added to the aquarium water. The method of assessing whether the oxidation sequence of ammonia through to nitrite to nitrate was functioning was to monitor the nitrite. This was done by a semi-quantitative colourimetric method in which the measurable nitrite level was measured until it reached 0.5mg l^{-1} after daily additions of "Sea Mature^{R}". A decrease in the nitrite levels indicated a functioning oxidation sequence. This was confirmed by the addition of a source of ammonia (a sea urchin) and monitoring the fall in nitrite as it was converted to nitrate. A maturation period of about six weeks at 10°C was suggested by King (1970) and was confirmed in this investigation using the above method. It is widely accepted amongst marine aquarists that heterotrophic and autotrophic bacteria are the major groups present in the conditioned culture system (Spotte 1970; Committee on Marine Invertebrates, 1981). Heterotrophic species utilize organic nitrogenous compounds, excreted by marine invertebrates, as energy sources and convert them into simple compounds, such as ammonia. Most of the nitrification in a filter bed occurs on the upper gravel layers. Kawai *et al.* in 1965 (cited by Spotte, 1970) found that there were 10^5 ammonia oxidizers per gram of sand in the top layers of a mature marine biological system and 10^6 nitrite oxiders per gram. The incidence of both bacteria decreased with increasing depth. The mineralization of these organics is the first stage in biological filtration (Spotte, 1970). It is accomplished in two steps: ammonification, the breakdown of proteins and nucleic acids, producing amino acids and organic nitrogenous bases; and deamination, in which a portion of the organics and some of the products of ammonification are converted to inorganic compounds. Once organics have been mineralized by heterotrophs, biological filtration shifts to the second stage, which is nitrification. Nitrification is the biological oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) and of nitrate (NO_3^-) by autotrophic bacteria.

proteolysis

These organisms, unlike heterotrophs, require an inorganic substrate as an energy source and utilize CO_2. *Nitrosomonas* sp. and *Nitrobacter* sp. are the principal nitrifying bacteria in marine biological filtration systems.

In the initial stages of nitrification there is a time lag between the fall of ammonia and the oxidation of nitrite. This is because the growth of *Nitrobacter* is inhibited by the presence of ammonia (Spotte, 1970). Efficient oxidation of nitrite does not take place until most of the ammonia has been converted by *Nitrosomonas*. The third, and last stage in biological filtration is denitrification. This process is defined by Vaccaro in 1965 as a biological reduction of nitrate or nitrite to either nitrous oxide or free nitrogen and can be carried out by heterotrophic and autotrophic bacteria (cited by Spotte, 1970).

Mineralization, nitrification and denitrification are parts of the nitrogen cycle. The mechanisms in nature and captivity are the same, the effects are not. Captive animals are at the mercy of their limited environment and their lives depend on the rates of the vital conversions mentioned above.

The biological filter bed is a permanent installation. The gravel should not be taken out of the sytem and washed. This removes most of the detritus which supports a large population of the nitrifying bacteria and results in the necessity for re-establishment of the biological filter bed. However, heavy surface mats referred to as "Schmutzdecke" by Spotte (1970) are undesirable e.g. dropped spines, decaying seaweeds and faeces.

Mechanical and chemical filtration are also essential in the closed-circuit aquarium system (Spotte, 1970; King and Spotte, 1974). Mechanical

filtration is the physical separation and concentration of suspended particulate matter from the recirculated-water. This was accomplished by passing through suitable materials in the filter bed and external power filter pump. The functions of mechanical filtration in closed-system are three fold :

a. to reduce the turbidity of the water caused by suspended microorganisms and other particulate matter,
b. to lower the level of organic colloids, and
c. to remove accumulated detritus from biological filter bed.

Chemical filtration is the removal of substances (primarily dissolved organics, but also nitrogen and phosphorus compounds) from solution by adsorption on a porous substrate, or by direct chemical fractionation or oxidation. First, a reduction in the organic level decreases the number of available substrates for heterotrophic oxidation. This action lowers the oxidative potential of the system and helps keep it within its carrying capacity. Second, many of the organics present in solution have unfavourable effects on the aquatic animals. Activated carbon, or charcoal (in the external power filter-pump) is a porous substance with a high adsorptive capacity. The charcoal filter removes excess organic materials which discolour the water and also adsorbs hydrogen sulphide, methane and other toxic gases which may be produced by anaerobic bacteria (Roff, 1972).

The growth of algae on the sides of the RASWA was encouraged by adequate illumination and was useful in two ways. It was obsesrved to be used by *E. esculentus* as feeding and the photosynthetic algae have been reported to utilize inorganic nitrogen as ammonia, nitrite and nitrate (Roff, 1972, King and Spotte, 1974).

Another important change in the design of the final aquaria system was the replacement of laboratory tap water with distilled water. The former was found to be undesirable possibly due to the presence of chlorine and traces of copper (Spotte, 1970). Ideally, tap water must also be "aged" to expel chlorine, taking up to three days. The ASW was reconstituted according to SG and salinity, ensuring a suitable range for the well being of *E. esculentus* (1.0240 ± 0.002).

As well as the salinity directly affecting the animals, a report by Kawai *et al.* in 1965 (cited by Spotte, 1970) stated that nitrification was diminished as seawater of normal salinity was diluted or concentrated greater than ± 0.002 (SG = 1.025). Additionally, the bacteria are adversely affected by sudden fluctuations in the salinity, an effect which was also observed to be deleterious towards *E. esculentus*. For these reasons the SG was maintained at 1.0240 ± 0.002 with only gradual changes in the salinity during partial water changes and maintenance of water levels. These were tolerated by *E. esculentus* and allowed adjustment by the bacterial inhabitants of the biological filter bed to the change in SG (Spotte, 1970).

Finally, the routine monitoring of the aquarium water quality was necessary in the maintenance of RASWA suitable for maintaining *E. esculentus* in a healthy condition for prolonged periods. For example, a sudden change in temperature does not affect the bacterial population of the filter bed, but this factor is important with respect to *E. esculentus* which are stressed as a result of sudden temperature change. This was reflected by physical symptoms of "sickness", such as curled tube feet, flattening of spines, spine dropping etc. The bacterial viable count of the aquaria waters was also recorded routinely in an attempt to monitor changes in the population, as a result of mortality for example. However,

the bacterial count may not reflect the true total population. This may be due to the adhesion of the bacteria to the coral sand and tank walls and removal by the external power filter-pump. Additionally, many of the marine species may not be colony-forming on Zobell marine agar 2216E.

Regular partial water changes of at least 25% of the total volume per month as recommended by Goldizen (1970) were found to be necessary in maintaining the water quality of the RASWA. According to Spotte (1970) and King and Spotte (1974) this procedure dilutes accumulated nitrates, phosphates and organics, replenishes depleted trace elements and also replenishes the carbonate-bicarbonate buffering system, therefore assisting in pH control. A gradual decline in pH is a problematic feature of closed-system aquaria (Harvey, 1955).

Despite considerable gaps in the detail of the report by Fridberger *et al.* (1979) several specifications in their design were adopted here including mode of transport of sea urchins (although we took 2h compared with their 8h journey); similar aquaria volume (75-100l) although there was no information regarding the volume allowance per animal, or number/size animals per tank; ASW ("hv - Meeresalz") of a similar range of salinities 2.7-3.5% and pH (8.2); a similar method of illumination (60W tungsten lamp, 40cm above the water surface of "gro lux" fluorescent tube and conventional white tube of 20-40W, 20-40cm above water surface); and feeding with *Fucus* and *Laminaria* except Fridberger *et al.* also fed the animals with deep frozen spinach. It has been suggested that sea urchins may suffer from internal commensals that become parasitic when the former are fasted, though this has not been proven (Committee on Marine Invertebrates, 1981). Finally, it should be noted here that the RASWA apparatus was successfully transported and restablished in a different location (Wardlaw (1987) pers. comm.).

Thus, the basic differences between the waters of the open oceans and the water in a closed system aquarium such as the RASWA discussed above therefore have been summarized by Kelley (1965):

a. Nitrogen compounds appear in great abundance, initially as toxic ammonia, then as nitrite and finally as nitrate.
b. The alkaline reserve of the system decreases with an associated drop in pH.
c. The concentration of phosphate, sulphate, calcium and potassium ions increases.
d. The total organic content of the water increases.
e. Bacteria increase numerically, but with a sharp decline in the number of species.

By equipping a closed system with good aeration and an efficient filter, the two most serious problems - ammonia toxicity and loss of pH - can be greatly reduced confirming the observations of Saeki, 1958, Kelley, 1963 and Spotte, 1970.

Fridberger *et al.* (1979) do not define "healthy condition" of adult specimens in their system in terms of physical or bacterial contamination of the sea urchins coelomic fluid, which both may be used to assess trauma. The RASWA described in this thesis appear to have no adverse effect towards *E. esculentus* maintained either with regard to physical appearance or bacterial contamination of the coelomic fluid.

2. *IN VITRO* ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *E. ESCULENTUS* COELOMIC FLUID

Coelomic fluids (CF's) from hundreds of specimens of *E. esculentus* were shown to be highly bactericidal *in vitro* at 8°C towards the

marine *Pseudomonas* strain number *111* (Unkles, 1976, Wardlaw and Unkles, 1978; Service, 1982; Service and Wardlaw, 1985). Although there were quantitative differences in the antibacterial activity of individual urchins, none of the animals were completely lacking in this activity. Further work by Service (1982) and Service and Wardlaw (1985) showed that the bactericidal activity extended towards a panel of 23 strains of Gram-positive and Gram-negative bacteria. The sensitivities of the two groups were found to be similar. However, the average susceptibility of the Gram-negatives was somewhat higher than Gram-positives. Prior to the present study, all of the work on the bactericidal activity of *E. esculentus* coelomic fluid (CF) had been done with sea urchins maintained in natural running-seawater aquaria at the University Marine Biological Station, Millport. Therefore, having established RASWA suitable for maintaining sea urchins in a healthy condition for long periods of time in the Microbiology Research Laboratories, Glasgow, the second objective was to assess if the bactericidal activity of *E. esculentus* CF was reproducible with CF from animals maintained in a strict artificial environment (closed-circuit aquaria with artificial-seawater and light) for up to 3 months. The highly sensitive bacterial strain *Ps 111* was selected for this purpose. However, there was found to be no appreciable difference in the bactericidal activity of the coelomic fluid of specimens of *E. esculentus* maintained in RASWA at 10°C and sea urchins kept in running natural-seawater aquaria. The temperature of 10°C was chosen as the average typical temperature which the animals encounter in the natural marine environment.

There are many reports in the literature on the ubiquitous occurrence of yeasts in the marine environment (Fell and van Uden,1962; van Uden and Fell, 1968; Morris, 1968; Bahnweg and Sparrow, 1971; Morris, 1975;

Sieburth, 1979). However, there appears to be no information regarding the *in vitro* antifungal activity of sea urchin CF, animals which obviously encounter marine yeasts in their natural habitat. Thus, *E. esculentus* CF was tested *in vitro* towards eleven strains of yeasts representing four genera, *Candida*, *Debaryomyces*, *Metschnikowia* and *Rhodotorula* all isolated from a marine environment. Because there was no previous information on whether *E. esculentus* CF would exert antifungal activity it was essential to include a positive bacterial control strain. *Ps.111* was therefore selected as the "sentinel" control, due to its established sensitivity to *E. esculentus* CF, and was incorporated into the design of the experiments with the yeasts as mixed inocula. The inclusion of the bacterial control also allowed comparison of bactericidal activity of individual CF's and their corresponding antifungal activities. Yeast strains were selected from the National Collection of Yeast Cultures (NCYC) on the basis of their isolation from the marine environment and secondly, that they would survive or preferably grow at 10°C in artificial seawater (ASW) supplemented with a low level of nutrient (MBASW). Incubation periods were extended to 72 and in some cases 96h to allow elucidation of possible regrowth of the yeasts. The panel of marine yeasts displayed a range of sensitivities towards *E. esculentus* CF *in vitro* at 10°C. The sensitivity patterns fell into two broad groups; yeast strains which were reduced to SI's of less than 50 within 24h and remained static about this level during the proceeding 48-72h incubation period and the more resistant yeast strains which were reduced to SI's of about 50 with the incidence of regrowth in the following 48-72h incubatory period. However, *E. esculentus* CF in both groups was observed to exert an initial burst of fungicidal activity in the first 24h.

Candida marina (NCYC 784) appeared to be the most sensitive marine yeast to fungicidal activity of the CF with *C. torresii* (NCYC 786) and

Debaryomyces hansenii (NCYC 792) as the most resistant strains. Unfortunately, only three CF's were tested against each of these strains due to the limitation of time. It would be interesting, however, to pursue this further.

In an attempt to clarify whether the initial decrease in SI's was as a result of a genuine fungicidal effect or as a result of cellular agglutination of the yeasts, agglutination titration assays were done. However, the phenomenon of autoagglutination of the yeasts, *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) (10^6) in the presence of artificial seawater (ASW) diluent, coelomic fluid supernate (CFSN) and coelomic fluid lysate (CFL) masked the results. However, due to the dilute nature of the yeast suspensions in the antifungal/antibacterial test mixtures (about 500 cfu ml^{-1}) it is unlikely that agglutination played a major role in the decrease of the yeast or bacterial viable count in the microbiocidal tests. Agglutination of bacteria was shown by Service (1982) and Service and Wardlaw (1985) not to be responsible for bactericidal activity by *E. esculentus* CF. However, although agglutination may play a minor role in the antifungal activity of the CF, there was also a further marked decrease in the SI's of the yeasts in the CF's relative to the control fluids MBASW and CFSN indicating another mechanism or mechanisms involved in the antifungal activity together with the possible incidence of agglutination. There was also observed to be a range in the antifungal activities of CF's from individual animals. This was also true of the bactericidal activities of different CF's towards *Ps. 111*. The inclusion of the bacterial control strain in the *in vitro* assays allowed the comparison of antibacterial and antifungal activities of undiluted *E. esculentus* CF towards *Ps. 111* and the yeast strains *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783), respectively at

24 and 48h at 10°C. There was no correlation observed. However, further exploration work with a dilution assay would be useful here to elucidate any possible pattern of similarity between antibacterial and antifungal activities at lower dilutions. Because Service (1982) and Service and Wardlaw (1985) reported little difference between the sensitivities of Gram-positive and Gram-negative bacteria to *E. esculentus* CF *in vitro* (Gram-negative bacteria only slightly more sensitive), it is unlikely that the mechanism of bactericidal or fungicidal activity is cell-wall related. Yeasts possess a definite cell-wall structure which contains chitin among other compounds (Alexopoulos, 1962) and tests have not indicated any major matabolic or physiological differences in yeasts isolated from terrestrial or marine environments (van Uden and Fell, 1963).

Although Johnson (1969c) observed lysis of some bacteria in hanging drops of CF from *Strongylocentrotus*, it seems unlikely in *E. esculentus* that the antimicrobial activity of the CF was due to the release of a lytic enzyme because, in most cases, the SI's of *Ps. 111* did not reach zero until 24-48h and there was incomplete killing with the majority of the yeast strains. An enzyme would be expected to produce a more complete and more rapid effect. Also, on no occasion, when the cell suspensions were observed by phase-contrast microscopy, were there signs of cell lysis such as unusual morphological shapes or ghosts. Another possibility was considered; that the susceptibility of microorganisms to the CF may be related to their growth rate. For example, the more sensitive yeast *M. zobelli* (NCYC 783) has a shorter mean generation time (MGT) than *R. rubra* (NCYC 63) which suggests that more rapidly growing (and therefore, more rapidly metabolizing) yeasts are more susceptible to *E. esculentus* CF. The MGT's of the other marine yeast strains were not determined and therefore did not allow examination of these two variables. This would be

an interesting point for further study. Comparison of the antibacterial and antifungal activity of CF and CFSN, showed that the activity resided in the coelomocytes. In fact the supernate behaved as a growth medium for the *Ps. 111* and the yeasts.

The antimicrobial activity of *E. esculentus* CF was found to be dependent on the temperature of incubation of the test mixtures. CF incubated at lower temperatures of 5 and 10°C was more antifungal and antibacterial than at the higher incubation temperature of 23°C. However, the growth of the test organism in MBASW at these temperatures must be taken into account. Growth rate was shown to increase with a corresponding increase in the incubation temperature. It may be, therefore, that at 23°C a critical stage was reached at which the antimicrobial activity of the CF could no longer deal with the fast growth of the organisms at this temperature and so the activity appeared to be reduced. It is probable that 10°C, which is the normal ambient temperature of the animals, is best for studying antimicrobial activity.

Comparing the growth of the two marine yeasts, *R. rubra* (NCYC 63), *M. zobelli* (NCYC 783) and bacterial control strain *Ps. 111* in both natural (NSW) and artificial seawaters (ASW) at room temperature (approx. 22°C), superior growth in NSW from the Clyde Estuary was observed by all three organisms. The ASW "Tropic Marin^{R}" was also found to support better growth of all three organisms than the other ASW "Sea Salt^{R}". However, the exact constituents of both artificial sea salts were not specified by either of the manufacturers, except to say that the composition conformed closely to the natural material and contained the same vital trace elements found in NSW. Since better growth was evident in NSW it would be of advantage to use this as the control fluid (1% MB in NSW) in the *in vitro*

antimicrobial assays. However, NSW introduces biological variability and therefore requires ageing (gives the indigenous bacteria time to mineralize the organic matter and therefore minimize variability of organic content (Zobell , 1941)). There is also decreased availability of NSW due to distance to natural water sources. Conversely, ASW's are always available and batches are reproducible with minimal biological and chemical variation.

2.1 Echinochrome-A

So far it may be suggested from the data that the antifungal and antibacterial activity resided in the coelomocytes, particularly since the cell-free coelomic fluid supported growth of the marine yeasts and *Ps. 111.* To the contrary, Yui and Bayne (1983) reported that CFSN from *S. purpuratus* contained one or more humoral factors active against both Gram-positive and Gram-negative bacteria resulting in a decrease in their viable counts. It is also of interest to note that the majority of the yeasts grew better in the CFSN than in the MBASW. The reverse was true of *Ps.111.* CFSN would therefore be a better choice as a routine control fluid in *in vitro* antifungal assays in place of MBASW. However, *E. esculentus* CF which was sonicated using an ultrasonic disintegrator to form coelomic fluid lysate (CFL) was found to be highly bactericidal towards *Ps.111* and of the same order as the corresponding whole CF's. Conversely, CFL reduced the SI's of *C. haemulonii* (NCYC 787) to about 40 within 24h, and they remained static at this level over the 72h incubation. This result confirmed earlier reports that the locus of bactericidal activity was present in the coelomocytes but intact coelomocytes were not required for this activity (Messer and Wardlaw, 1979; Service, 1982; Service and Wardlaw, 1984). Conversely, antifungal activity was completely absent in CFL indicating the necessity for coelomocyte integrity. Because bactericidal activity was attributed chiefly to the napthaquinone pigment, echinochrome-A (Ech-A)

contained in the red spherule or morula cells (RSC) (Messer and Wardlaw, 1979; Service and Wardlaw, 1984) a purified crystalline extract of the pigment was tested against the marine yeast strains since there was no information on the antifungal activity of the napthaquinone pigment, specifically Ech-A towards marine yeasts. However, there are reports by Ciegler *et al.* (1981) who isolated two napthaquinone pigments; xanthomegin and viomellein from *Penicillium viridicatum* which possessed mycotoxic activity and Soderhall and Ajaxon (1982) who demonstrated inhibition of a crayfish fungal parasite *Aphanomyces astaci* by synthetic 2-methyl-1-, 4-napthaquinone. Additionally, Thomson (1957) in his book on "Naturally occurring quinones" reported potent fungicidal activity of 2-methoxy-1:4-napthaquinone, although no more details were given. Service and Wardlaw (1984) reported that Ech-A in CF from healthy specimens of the sea urchin *E. esculentus* ranged from 3 to 60 $\mu g\ ml^{-1}$ with a geometric mean of 14 ug ml^{-1}. Holland *et al.* (1967) and Johnson (1970) both working with *Strongylocentrotus* showed that Ech A migrated with particular coelomocyte proteins during electrophoresis. Service (1982) and Service and Wardlaw (1984) reported that Ech-A (up to 50 $\mu g\ ml^{-1}$) dissolved in seawater, with the aid of mammalian proteins as dispersants, was bactericidal or bacteriostatic towards six out of seven strains of marine Gram-negative and Gram-positive bacteria. Ech-A was found to be only sparingly soluble in seawater (Johnson, 1970) in the absence of mammalian proteins. Additional, but only circumstantial evidence that Ech-A may have antimicrobial activity in sea urchins was the report by Johnson and Chapman (1970) who observed an apparent barrier of Ech-A - bearing RSC's which separated diatom-infected spine tips in *S. franciscanus*. Pearse *et al.* (1977) also recorded that skeletal plates on apparently diseased specimens of *S. franciscanus* had a middle "red friable" layer, presumably due to Ech-A. Another report was that by Coffaro and Hinegardener (1977) who noted

that tissues of *Lytechinus pictus*, which had been damaged during grafting experiments, became reddened due to an influx of RSC's. Johnson (1969b) provided further circumstantial evidence for the possible antibacterial role of Ech-A when she observed the release of the pigment by the RSC's on contact with Gram-negative bacteria. Reports on the pigment as an algistat include that by Vevers (1966) who observed that pigments leached from an unspecified echinoid placed on a culture plate of cyanobacteria inhibited their growth and Whitton and McArthur (1967) demonstrated that 2,3-dichloro-1, 4-napthaquinone was toxic to the blue-green alga *Anacystis nidulans*.

No information existed on the antifungal activity of Ech-A from *E. esculentus*. In this study higher concentrations of Ech-A than were found in the CF of *E. esculentus* were tested against the marine yeast strains. Concentrations of up to three times of the maximum physiological concentrations found in *E. esculentus* did not appear to exert antifungal activity. However, a concentration of nearly four times that found in the CF was antifungal to some extent towards all seven yeast strains tested and the bacterial control *Ps. 111* was completely killed within 24h. Nevertheless, the SI's of the majority of the yeast strains fluctuated in the 2mg ml^{-1} BGG-MBASW control fluid, but in contrast the SI's of the yeasts were consistently lower in the Ech-A test fluid. Because of the behaviour of the yeasts in the control fluid containing the mammalian protein, an investigation of antifungal activity of Ech-A and its associated CF-protein *in vivo* is still required. It may be that such high concentrations of Ech-A are produced at localized points of infection *in vivo*, for example in the formation of pigmented lesions. This is discussed later.

Because Ech-A takes between 12-48h to achieve maximum bactericidal/ antifungal activity suggests that it acts on the bacterial or yeast metabolism rather than by disrupting the cell envelope/wall. This harmonizes with the suggestion made previously (Service, 1982) that the efficiency of antibacterial action of *E. esculentus* is influenced by the growth rate of the test bacteria. That is, rapidly metabolizing bacteria are more sensitive to bactericidal systems in CF. This aspect was not investigated in detail. Whitton and McArthur (1967) suggested the action of 2,3-dichloro-1, 4-napthaquinone on blue-green algae was due to its interference with specific enzymes or its taking part in photophosphorylation. But, this suggestion would not explain the effect on the heterotrophic bacteria or yeasts, although certain napthaquinones are known to form major components in the electron transport chain in bacteria (Dawes and Sutherland, 1976). The mechanism of Ech-A towards *Ps. 111*, *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) did not appear to be due to lysis since there was no decrease in optical density with time on exposure to high and low concentrations of Ech-A.

Certain chemically related substances to Ech-A (quinones) are precursors of melanin which is common in diverse species in the animal kingdom. Therefore, in this broader context, the production of Ech-A by sea urchins can be perhaps regarded as a specialized adaptation of a widely distributed biosynthetic mechanism. Melanin deposition may accompany host cellular defence responses (Smith and Ratcliffe, 1980; Soderhall, 1982; Ratcliffe *et al.*, 1982) in some invertebrates such as Crustacea and Jacobson and Millott (1953) reported the presence of melanin and its precursors in the sea urchin *Diadema antillarum*. One may speculate that the evolutionary branch to the sea urchins involved an ancestral creature which possessed biochemical pathways for both melanin and Ech-A. The

branch leading to the chordates adapted the former and the echinoid sub-branch, Ech-A (Figure 1).

The basis of antimicrobial activity of the napthaquinones, in general has not been extensively studied. Service (1982) and Service and Wardlaw (1984) reported the only investigations on the role of these pigments in echinoid antibacterial defence. Little is known on its biosynthesis, modes of storage and release from the RSC's including the mode of attraction of RSC's to point of infection/invading cells, mechanisms of bactericidal action and the role of its associated protein(s). These are all interesting points for further study.

2.2 Clotting

Clotting of the coelomocytes of echinoderms is well documented and it is a mechanism for damage repair. It possibly has a role in host defence such as encapsulation of invading objects (Bang and Lemma, 1962; Johnson, 1969c). It was observed that when exposed freely to the air *E. esculentus* CF formed a slimy clot within seconds, the clot at first pink rapidly turned red and finally to brown. The last process was slow and required between 12 and 24h at room temperature (approximately 22°C). This observation confirmed that made by Cannan (1927) with *E. esculentus* and Jacobson and Millott (1953) with *D. antillarum*. The latter author suggested this was an oxidative process and Cannan (1927) suggested that Ech-A exists in a partially reduced state in *E. esculentus*. Clotting of *E. esculentus* fluid, however, was not found to remove antibacterial activity (Unkles, 1976).

The clotting or aggregation of the coelomocytes in CF can be inhibited by two types of reagents. Boolootian and Giese, (1958) observed three types of cell clotting. The first is calcium-dependent; cells agglutinate

but retain their identity. In the second type of clot, cells agglutinate and fuse with one another to form a plasmodial clot, whereas in the third type cells agglutinate forming a mesh of fibres in which other cells are trapped. The latter two types of clots can be prevented by either using reducing agents or SH-binding chemicals. The clotting of the CF of *E. esculentus* CF was shown to be inhibited quite effectively by the Ca^{2+}-chelator, ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetracetic acid (EGTA) by Messer and Wardlaw (1979). Ca^{2+} has also been shown to be necessary in haemagglutinating activities in CF (Ryoyoma 1974a and b) and specifically *E. esculentus* (Parker, unpublished observation). The cells which appear to be responsible for the clots formed in *E. esculentus* CF are the phagocytic leucocytes (PL) which appear as rounded PL, which if left on ice for a short time, begin to form a retracted clot. The vibratile cells (VC's) may also have a role in the clotting of *E. esculentus* CF, since Bertheussen and Seljelid (1978) and Johnson (1969a) reported release of granula from these cells in *Stongylocentrotus* which were found to be responsible for rapid and extensive clotting of the CF. Because PL's enmesh other cells so readily it is essential to mix the aggregation inhibitor very thoroughly at the time of collection. Ca^{2+} is present at a concentration of 10mM, therefore a final concentration of 14mM EGTA is sufficient to sequester all the Ca^{2+} present in the MBASW and be present in excess. This concentration of EGTA did not inhibit the growth of either *C. haemulonii* (NCYC 787) and *D. hansenii* (NCYC 792) indicating that the yeasts do not require Ca^{2+} for growth or alternatively, have a more efficient calcium-chelator mechanism than EGTA. Another possibility is that the yeasts are able to ingest the EGTA-Ca^{2+} complex and remove the chelator. The EGTA at this concentration did not appear to affect the integrity of *E. esculentus* coelomocytes, as revealed by microscopic examination and trypan blue exclusion, reported by Messer and Wardlaw

(unpublished observations). However, 14mM EGTA is toxic to *Ps 111* indicating a requirement of Ca^{2+} by the bacteria for growth or cell integrity. Since the *Ps. 111* was to be included as a "sentinel" control in density gradient separated coelomocyte *in vitro* antimicrobial assays, the chelator was removed by washing with MBASW diluent before exposure of the mixed inoculum.

2.3 Density Gradient Separated Coelomocytes

The coelomocytes of the *E. esculentus* CF were separated using a stepwise, discontinuous "Ficoll^R"/"Triosil^R" density gradient containing the anticoagulant EGTA adapted from the method of Messer and Wardlaw (1979). Density-gradient centrifugation techniques have been used by previous investigators to separate coelomocytes of *Strongylocentrotus.* Lindsay *et al.* (1965) employed EDTA and a sucrose/seawater gradient, while Bertheussen and Seljelid (1978) used sodium metrizoate with a mixture of 50mM mercaptoethanol, 3mM caffiene and 2mM p-tosyl-L-arginine methyl ester (TAME) as anticoagulant.

Messer and Wardlaw (1979) adopted a method of separation of *E. esculentus* coelomocytes from that developed by Boyum (1968) for separation of leucocytes from human blood. The gradient was made up of a mixture of "Triosil^R", "Ficoll^R" and 14mM EGTA dissolved at different concentrations in seawater. The density gradient yielded four distinct bands of phagocytic leycocytes (PL's), vibratile cells (VC's), red spherule cells (RSC's) and colourless spherule cells (CSC's). However, the results obtained here in this investigation with a density gradient composed of "Ficoll^R" and "Isopaque^R" (the latter ingredient replaced "Triosil^R" used in the method of Messer and Wardlaw (1979)) dissolved in 14mM EGTA in ASW, produced three main bands after centrifugation composed of PL's, VC's and

CSC's and RSC's. This confirmed the result of Bertheussen and Seljelid (1979) who also found the band below the PL's now to be composed of a mixture of VC's and CSC's. After washing the EGTA from each coelomocyte band, because of its toxicity towards the control strain *Ps. 111*, the fractions were tested for antimicrobial and antifungal activity towards *Ps 111* and *C. haemulonii* (NCYC 787). This strain of marine yeast was chosen because it grew well in the control fluids and was sensitive to *E. esculentus* CF *in vitro*. The fraction containing a mixture of VC's and CSC's did not exert either antifungal or antibacterial activity *in vitro* at 10°C. In fact, it supported growth of both *C. haemulonii* (NCYC 787) and *Ps 111* of a similar magnitude to the control fluids MBASW and CFSN. However, functions for these cell types have been suggested. The potent haemagglutin (HA) for rabbit erythrocytes was reported to be localized mainly in the CSC's by Messer and Wardlaw (1979) and the release of granula from the VC's was found to be responsible for rapid and extensive clotting of CF immediately after removal from *S. droebachiensis* by Bertheussen and Seljelid (1978). This was prevented by the addition of a mixture of 50 mM mercaptoethanol, 3 mm caffiene and TAME.

The red pigmented RSC's band formed immediately above the "Ficoll^{R}/Isopaque^{R}" cushion had antibacterial activity but supported growth of the yeast. This observation upheld the earlier result in which Ech-A at physiological concentrations also supported growth of the marine yeasts as did CFL. However, the main and uppermost band on the gradient containing the PL's did exert antifungal activity towards *C. haemulonii* (NCYC 787) with SI's of 50 and less at 48 and 72h; a similar pattern of results to those obtained on exposure of the yeast to whole CF *in vitro*. Bertheussen (1981a) demonstrated avid phagocytosis of baker's yeast *in vitro* (*Saccharomyces cerevisiae*) by PL's of *S. droebachiensis* by scanning

electron microscopy (SEM). The same coelomocyte fraction was bactericidal towards *Ps. 111*, which was maintained in dynamic equilibrium over 48h. These observations confirm earlier conclusions, that the main locus of bactericidal activity appears to reside in the RSC's as Ech-A and intact cells are not wholly required although the involvment of the PL's in antibacterial activity must not be discounted. However, coelomocyte integrity is a pre-requisite for antifungal activity of *E. esculentus* CF, the cellular lysate CFL alone promoting growth of *C. haemulonii* (NCYC 787). It must also be taken into consideration that phagocytic activity of the PL fraction may have been partially inhibited towards the bacteria and yeasts and may be attributed to possible damage by the anticoagulant, centrifugation of the cells, or more importantly, the presence of other cells or humoral factors necessary for phagocytic activity to be efficiently expressed. For example, Bertheussen (1983) reported that complement-like activity in *S. daroebachiensis* was inhibited by Ca^{2+} concentration below 10mM, therefore removal of Ca^{2+} by EGTA to prevent clotting may also inhibit humoral factors which may be present in *E. esculentus* CF. However, Bertheussen (1981a) suggested direct binding by non-immunological surface receptors without the requirement of these substances. Johnson (1969c) reported that phagocytosis was of major importance in Pacific sea urchins against Gram-positive bacteria, and the same author (1970) showed by electron microscopy that a strain Gram-negative bacteria was also phagocytozed. No one prior to this investigation has demonstrated that phagocytosis occurs in CF of *E. esculentus*, although, evidence from other echinoid species suggested that it would almost certainly occur (Johnson, 1969b; Bertheussen and Seljelid, 1978). There appears to be only one report in the literature of phagocytosis of yeast cells *in vitro*, baker's yeast by Bertheussen (1981a). Bertheussen (1981a) described a method on how to obtain pure monolayers of

echinoid phagocytes *in vitro* and the ingestion of various particles (RBC, yeasts, *E. coli*, *V. anguillarum*, carbon, sephadex and latex) was monitored by SEM. Phagocytosis of baker's yeast cells and Vibrio were found to be less efficient than ingestion of RBC, *E. coli*, carbon, latex and sephadex.

Bertheussen (1981a) also reported that he found no opsonizing activity in cell-free CF of *S. droebachiensis* which indicated that humoral factors are not essential for the recognition of foreign substances by phagocytes. In mammals macrophages are mediated by humoral-recognition factors like immunoglobulins and complement, or it can take place by direct binding of a foreign surface to the membrane of phagocytic cells. The latter process is believed to be dependent on membrane receptors often termed non-immunological or foreign surface receptors. Conversely, a contradictory report by the same author (1983) documented complement-like activity in echinoid coelomic cell-free fluid. The substance was heat-labile, inhibited by Ca^{2+} concentration below 10mM and a low pH. Human-complement inhibitors also inhibited the lytic and opsonic activities in echinoid fluid. The presence of receptors for the C_3 component on echinoid phagocytes may prove important in understanding how echinoid coelomocytes recognize foreign particles. Bertheussen and Seljelid (1982) have suggested that echinoids may possess a system similar to the alternative pathway of complement in the vertebrates. There is no published information regarding the presence of humoral factors in the CF of *E. esculentus* and this would form a very interesting basis for further study.

The extent to which cell-cell cooperation may occur in antibacterial and antifungal activity also remains to be investigated. Improved separation techniques combined with the determination of the characteristic biological activities of the purified cell suspensions would also help resolve difficulties in coelomocyte nomenclature. More importantly

echinoid cells may give a better understanding of how the reticulo-endothelial or macrophage system evolved in the vertebrates, since the echinoderms belong to the same evolutionary line.

3. CLEARANCE AND PATHOGENICITY OF MARINE YEASTS FOR *E. ESCULENTUS*

Having shown that marine yeasts underwent a marked reduction in viable count on exposure to *E. esculentus* CF *in vitro*, the next stage of the investigation was to follow clearance of injected yeasts from the coelomic cavity of the intact animal.

Clearance from the coelomic cavities of sea urchins has been documented after injection into the coelomic cavity of several foreign particles including: bacteriophage T4 (Coffaro, 1978); erythrocytes, latex beads and carbon (Bertheussen, 1981a); bovine and human serum albumin (Hilgard and Phillips, 1968) and bacteria (Bertheussen, 1981a; Yui and Bayne, 1983). Specifically, clearance of bacteria from the coelom of *E. esculentus* has been reported by several workers at the University Marine Biological Station, Millport (Unkles, 1976; Wardlaw and Unkles, 1978; Service, 1982). They demonstrated high doses of marine *Pseudomonad, Ps 111* and mixtures of eight marine bacteria (10^9) were rapidly cleared to less than 10 ml^{-1} CF from the coelom of *E. esculentus* within 24-48 h post-injection, during which time the animals remained healthy. More detailed studies on the clearance of Gram-positive and Gram-negative bacteria from the coelom of *Strongylocentrotus purpuratus* were presented by Yui and Bayne (1983). The authors reported efficient clearance of three species of bacteria from the coelom although clearance rates differed for each strain. At 24h the overall reduction of Gram-positive bacteria was slightly lower than that of the Gram-negative strains. Viable counts were reduced, 90-99% in 3-6 h, after an estimated initial zero-time concentration 10^6-10^7 ml^{-1} CF. Clearance was observed to occur in at least two phases, the secondary

phase slower than the primary phase. The latter was observed to be exponential. Similarly, in this investigation injection doses of 10^9 *Ps. III* into the coelom of *E. esculentus* (notional zero-time concentration of 10^7 *Ps. III* ml^{-1} CF) were rapidly reduced by 99.9% within 4 h post-injection, thereafter, the bacteria were cleared more slowly to less than 10 ml^{-1} CF within 48 h and were undetectable at 3-6 days. This two-phase clearance was consistent with clearance of bacteria from *S. purpuratus* reported by Yui and Bayne (1983) although viable counts of *Ps. III* were not made within the 4 h period and therefore, primary clearance was not characterized further. In interpreting the results here two assumptions were made:

a. the bacteria were evenly distributed throughout the CF 40-90 min after injection, as suggested by Yui and Bayne (1983), and
b. that there was no leakage after injection of the suspension. This was later confirmed.

So far, all reports have been concerned with the clearance of bacteria from the coelom of sea urchins. There appears to be only one report in the literature on the injection of yeasts into the coelomic cavity. Bertheussen (1981a) injected baker's yeast, *Saccharomyces cerevisiae*, into the coelom of *S. droebachiensis* and a resultant 70% clearance of a dose of 5×10^8 yeasts was demonstrated within 2h post-injection. Complete clearance occurred within 24 h. The author quantitated the clearance by microscopic observation (SEM) in terms of the number of yeasts phagocytozed after 2h, 1-5 yeasts were internalized per phagocytic cell. However, compared to other types of injected particles, treated and untreated erythrocytes, latex and *Escherichia coli*, *S. cerevisiae* and *Vibrio* were found to be the least avidly phagocytozed (Bertheussen, 1981a). Because sea urchins are undoubtedly exposed to yeasts in the marine environment,

due to the ubiquitous occurrence of these fungi and because the CF *E. esculentus* has been shown to be antifungal towards selected strains of marine yeasts *in vitro* at 10°C, it was of interest to compare these studies with the clearance of injected marine yeasts from the coelomic cavity of *E. esculentus*. There appears to be no information on this topic. Another aspect important for study, on which there was no existing information, was the reaction of the whole animal to experimental infection in terms of the background bacterial contamination status of the CF, gross external physical appearance and the incidence of mortality. Two strains of yeast were chosen *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) on the basis of several criteria:

a. their distinguishable colony characteristics, and
b. growth in control fluids MBASW and CFSN *in vitro* at 10°C (the latter of which would indicate the necessary nutrients for the yeasts to grow *in vivo*).

Ps. 111 was incorporated into the clearance experiments as a "sentinel" control to allow comparison of clearance of bacteria and yeasts. Standardized suspensions of yeasts and bacteria in MBASW diluent were injected into the coelomic cavity via the peristomial membrane. Leakage after injection of the suspensions appeared not to occur, as detected by placing the injected sea urchins in a small volume of aerated ASW in a bucket and filtering 100 ml volumes of the water at time zero, 10, 20 and 30 min post-injection. Moreover, yeasts of experimental infection were very rarely detected from routine sampling of the water from RASWA. A dose of 10^7 yeasts was initially selected since in preliminary experiments sea urchins were injected with 1.0 ml suspension of *Ps.111* equivalent to 10 opacity units (OU), as determined with the International Standard Opacity Rod as reference. A 10 OU suspension contained approximately 10^9 bacteria

per ml. Similarly, in a preliminary investigation into whether clearance of yeasts actually took place, a suspension of 10 OU yeasts was prepared which corresponded to approximately 10^7 yeasts ml^{-1}. Preliminary experiments showed that such doses of *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) were rapidly cleared in the primary phase by up to 98% within 4h after injection. The remaining yeasts persisted at about this level in the longer, second phase of clearance until death of the animals at about 4 weeks. It appears that the remaining yeasts were persisting in the CF either at a static level, or in a state of dynamic equilibrium. This result of initial rapid clearance of the yeasts confirmed the report by Bertheussen (1981a). However, by contrast, the marine yeasts were incompletely cleared after a minimum of four weeks post-injection. Additionally, the incidence of elevated background bacterial contamination after one week of injection, to high levels in the proceeding weeks, was not recorded by other workers reporting bacterial clearance. To establish whether the phenomenon of clearance was dose-dependent, quantitative assays were done in which graded doses of the two yeast strains and the "sentinel" control strain *Ps. 111* were injected into the coelomic cavity of *E. esculentus*. Yui and Bayne (1983) in their studies determined primary and secondary-clearance kinetics for three species of bacteria from the coelom of *S. droebachiensis*. Viable counts were taken at 1, 1.5 and 6h post-injection. This allowed determination of the "Phagocytic Index" (K) equivalent to the slopes which were calculated using linear regression. The slope was found not to be strongly dose-dependent. However, calculation of K was not attempted in this investigation because viable counts were routinely determined on a weekly basis. Samples were taken less frequently than in preliminary qualitative studies, to minimize the incidence of background bacterial contamination in the CF, by sucessive puncturing of the peristomial membrane.

Ps. 111 was cleared completely at all doses, range, 6×10^4 - 10^{10}, although accompanied by an increase in the mortality rate of the animals with a corresponding increase in dose. Similarly, both *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 783) at only the lowest dose of 10^4 yeasts were completely cleared from the coelom within one week after injection A first-phase rapid clearance of the higher doses of yeasts, range 4×10^5 - 1×10^9, of up to 95% occurred within one week post-injection followed by a second slower phase of incomplete clearance. Moreover, the majority of the remaining yeasts after the initial stage of clearance persisted in the CF until the death of the animal. Similarly, as with *Ps. 111*-injected animals, increased mortality of the infected animals occurred with increase in the administered dose size. Death of all injected animals was accompanied by high background bacterial contamination of the CF, first appearing at low levels within one week after injection of the test organisms and reaching overwhelming proportions in some cases (10^7 ml^{-1} CF) just before death. This secondary bacterial contamination may have occurred:

a. via the point of inoculation in the peristomial membrane,
b. by transfer of the natural microbial flora of the peristomial membrane, although this is unlikely since surface sterilization with 95% (v/v) ethanol was done to minimize contamination by this route, or by
c. multiplication of existing bacterial flora present in the CF of *E. esculentus* as reported by Unkles (1976 and 1977) and also isolated here from the CF of artificially-maintained animals. Unkles (1976) isolated the main genera *Pseudomonas, Vibrio, Aeromonas, Flavobacterium, Acinetobacter and Moraxella* from the CF of *E. esculentus*.

Groups of control animals injected with sterile diluent (MBASW) only, were placed in both "infection" tanks containing experimentally-injected animals and alone in separate "storage" tanks. The latter category of control animals out-lived both experimentally-injected animals and accompanying control animals in the same tank, with minimal background contamination of less than 30 bacterial contaminants per ml CF. Conversely, the control animals present alongside the experimentally-infected sea urchins, particularly at the high doses (range: yeasts, 5 x 10^8-10^9; *Ps. 111*, 1-2 x 10^{10}) also died with overwhelming bacterial contamination, on a similar time scale. By contrast, at the lower doses (range: yeasts, 10^4 - 5 x 10^7; *Ps.111*, 6 x 10^4 - 5 x 10^9) the mortality rate was lower and typically one out of three control animals died. This high mortality rate of the control animals present in the same tank as the experimentally-injected animals may be attributed to several factors:

a. an increase in the bacterial viable count of the surrounding aquarium water due to the presence of moribund sea urchins,
b. transfer of bacterial contamination (perhaps potential pathogens) from experimentally-infected animals, possibly via injection sites or water-vascular system i.e. tube feet.

This would indicate a requirement for an open-system, running-seawater aquarium as opposed to the closed-circuit system used here, to minimize the spread of water-borne contamination between experimentally-infected and control animals.

Injection of suspensions of heat-killed yeasts (dose, 10^9) into the coelomic cavity of *E. esculentus* resulted in a background bacterial contamination of the CF. This first appeared at about one week after injection and fluctuated at levels between less than 10 and 5 x 10^4 ml^{-1}

CF. However, mortality was very low and 75% of the animals survived in excess of 18 weeks. This would indicate a requirement for live yeast suspensions to elicit responses recorded earlier.

Hence, conclusions made regarding the pathogenicity of the yeasts *R. rubra* (NCYC 63) and *M. zobelli* (NCYC 753) towards *E. esculentus* are limited due to the high mortality of the sterile diluent injected control animals deliberately placed alongside yeast-infected animals in the "infection" tank. Although the yeasts did not show net multiplication, the persistence of the fungi at a static level or in a state of dynamic equilibrium appears to suppress the host defence mechanisms of the animals allowing secondary bacterial contamination to supervene and kill the animals. By contrast, all doses of *Ps. 111* were cleared from the coelom in the majority of cases. High bacterial contamination and mortality also occurred which may again be due to overwhelming of the animals' defence by the injected material.

Physical changes in the external appearance of injected animals were also observed during the course of experimental infection with live inocula. Symptoms of "sickness" included loss of attachment of the animals from the side of the RASWA, lack of tube feet extension, flattening of spines and spine-tip breakage. Similar symptoms were recorded by Earll (pers. comm. 1985) in "bald sea urchin disease" of *E. esculentus* and by Jones and Schiebling (1985) when *Paramoeba* sp. were injected into the coelom of *S. droebachiensis*. Other symptoms were observed which took longer to develop, in particular were the appearance of red/black gelatinous lesions of a few millimetres in diameter, on the upper region of exterior surface of the test. These lesions were often seen to increase in size with time and often extended to as much as one third of the test

surface area sometimes accompanied by spine loss, green necrotic tissue and algal colonization. A typical lesion consisted of an area entirely deprived of appendages i.e. spines, pedicellariae, tube feet, and lined by swollen red tissue in the presence/absence of green-coloured necrotic tissue. In some cases animals exhibited several co-occurring lesions. Similar lesions were reported by several authors in other species of diseased sea urchin. Höbaus *et al.* (1981) documented victims of a mass mortality of *Paracentrotus lividus* in the Mediterranean in 1978 with one or more lesions of a few millimetres in diameter formed on the mouth side of the test and accompanying spine loss. Similarly, Maes and Jangoux (1984) also described lesion formation on the test surface of several species of sea urchin; *Sphaerechinus granularis, Psammechinus miliaris, P. lividus* and *Echinus esculentus.* As also shown by Johnson (1971) the red/black gelatinous belt was found to be composed of a loose-aggregate of cells presumed to be of coelomic origin e.g. phagocytic cells and red spherule cells (RSC's). The lesions produced on the test surface of *E. esculentus* infected with *Ps. 111* and yeasts were also found to be composed of a meshwork of entrapped cells RSC's, PL's, CSC's and VC's in that order of decreasing abundance as revealed on examination by light microscopy. The accumulation of red pigment was thought to be Ech-A. The lesion was also found to be infected with algae, bacteria and the organisms of experimental infection. This would suggest that mainly the RSC's and PL's seen to be involved in defence against invading foreign organisms. Cells of both types concentrate within the lesion where they form aggregations. The Ech-A released from the RSC's, probably by despherulation, acting as a disinfectant or algistat (Vevers, 1963; Johnson and Chapman, 1970). The green colouration often observed not due to algal colonization, were probably the first signs of necrotic tissue. The strong presence of the

PL's and synctia (plasmodia) formed by the phagocytes in the lesion area probably serve to phagocytose and to "wall off" foreign organisms. Hobaus (1979) suggested that synctia formed in wound regions of the body walls of *P. lividus* on disintegration, form a matrix rapidly sealing the wound in which ossicles are laid down for the test's repair. The increase of specialized mobile cells in pathologically altered body walls suggests that somewhere in the body of the sea urchin the proliferation, differentiation and maturation of these cells occur (Höbaus, 1979). Injured sea urchins are known to proliferate mobile cells in their axial organ, which have been suggested to be for emergency repairs of the test (Millott, 1967, 1969). The accumulation of Ech-A pigment may be likened to the deposition of black pigments in host response of arthropods to wounding or microbial attack. However, the main enzyme in melanin synthesis, phenoloxidase, is absent in sea urchins (Söderhäll and Smith, 1984). Melanization nearly always accompanies microbial or parasitic invasion. Therefore, it is reasonable to expect components released from such invaders to trigger the enzyme system in the host. For example, yeast cell walls were found to enhance phenoloxidase activity in *Galleria mellonella* insect larvae (Pye, 1974). Although this particular enzyme is not found in sea urchins, this enzyme has been isolated from other echinoderms (Jacobson and Millott, 1953; Millott, 1953). It was often observed that the tube feet outside the area of the lesion formation appeared healthy. This would imply that the disease agent involved in lesion formation is not efficaciously transported through the water-vascular system. Another change in physical appearance during the onset of infection was a characteristic darkening of the test colouration from pink/violet to a dark red/brown. This may be attributed to the influx of RSC's into the test layers and consequential degranulation of cells releasing Ech-A. It has been suggested that due to its bactericidal activity *in vitro* Ech-A may function as a general

disinfectant (Service, 1982; Service and Wardlaw, 1984) or as an algistat (Vevers, 1966). Interestingly, the incidence of algal infection of the spines and test of *E. esculentus* was observed to decrease with a corresponding increase in the darkening of the test colouration during an experimental infection extending over 8-9 weeks. Additionally, Johnson and Chapman (1970) speculated that the presence or absence of napthaquinone pigments in the spines of *S. franciscanus* influenced the degree of algal infection. In the later stages of experimental infection there were also changes in the colour and texture of the CF. Similar changes were reported in the CF of diseased *Strongylocentrotus* spp. by North *et al.* (1970-71). Healthy pink or colourless, viscous fluid became yellow, watery and "stringy" possibly due to coelomocyte lysis and release of Ech-A from the RSC's. A similar colour and texture change of the CF was observed during 24-96 h incubation of microbiocidal tests *in vitro*. This colour change of Ech-A from pink to pale/yellow, was suggested to be an oxidative process by Cannan (1927). Critically, further *in vivo* studies involving experimental infection of *E. esculentus* ideally requires a more accurate estimate of CF volume in individual specimens. Yui and Bayne (1983) derived the equation:

$$\text{CF (ml)} = 0.35 \times \text{weight (g)} - 4.2$$

and this was used in all clearance studies with *S. purpuratus*, the weight reported as the best indicator of CF volume. An investigation on the dispersal of injected microorganisms into the coelomic cavity of *E. esculentus* would also be advantageous. This would establish what proportion of inocula, if any, adhere to the inner side of the peristomial membrane, internal organs, or inner coelomic lining. Post-mortem analysis of several yeast-injected *E. esculentus* revealed high numbers of these

fungi concentrated in the inner lesion area and high numbers of bacterial contaminants in the gonads, inner coelomic lining and inner and outer lesion area. However, these observations were not quantitated. Additionally, timing of the distribution of injected material throughout the CF in *E. esculentus* has not been done. Yui and Bayne (1983) estimate 40-90 min for dispersal but they did not specify their methodology or specimen size.

Another aspect relevent to the study of microbial clearance from the coelom of *E. esculentus* is the incidence of CF clotting which undoubtedly occurs *in vivo* as well as *in vitro* in the presence of foreign material. These clots serve to close wounds (Bang, 1961; 1970). Coagulation may immobilize invading organisms and thereby prevent spread throughout the body cavity. This has been demonstrated *in vivo* by Bertheussen (1981a) in *S. droebachiensis*, who observed, after injection of a high dose of bacteria (2×10^9), almost immediately extensive coagulation of the CF in which the injected bacteria were trapped. The PL's have been implicated as playing a major role in the clotting process in sea urchins (Johnson, 1969c) facilitated by the release of acid mucopolysaccharides from the VC's (Johnson, 1969a; Bertheussen and Seljelid, 1978). This viscous, semi-gel quality disappeared after 12-24 h *in vitro*, but *in vivo* may be replenished by more VC's until the infected CF was cleared by phagocytosis (Bertheussen, 1981a).

There is little information regarding the quantitative nature of the coelomocytes in the CF of sea urchins and only one report on the coelomocyte profile of *E. esculentus* CF (Messer and Wardlaw, 1979). Bertheussen (1981a) found most cells in the CF of *S. droebachiensis* were phagocytes occupying 67% of the total coelomocyte population. Yui and

Bayne (1983) made differential coelomocyte counts of the CF of *S. purpuratus* with a haemacytometer. The average number of coelomocytes in the CF of healthy animals of this species was 10^7 ml^{-1} CF. The majority of cells were phagocytes (67.8%) which was consistent with that obtained by Bertheussen (1981a) for the same species of sea urchin, followed by VC's (16.8%), RSC's (10.5%) and CSC's (5%). Similar cell-profiles of the four coelomocyte types were obtained for the CF from healthy *E. esculentus* by enumeration with a Coulter counter with the differential cell populations as follows: PL's, 65.6%; CSC's and VC's, 23.1%; RSC's 11.4%. The mean total coelomocyte count was 5.3×10^6 ml^{-1} *E. esculentus* CF. However, parallel total coelomocyte counts enumerated with a haemacytometer were consistently lower. This was thought to be attributed to the presence of cellular debris in the CF not excluded by the threshold setting producing an artificially high count. Alternatively, the incidence of coelomocyte clumping was evident on occasions when counting undilute CF fractions in a haemacytometer. There was also evidence of unavoidable cell lysis attributed to disruption during density gradient centrifugation separation and pelleting during the washing procedure. Comparison of the total coelomocyte Coulter counts of CF from healthy and "sick" specimens of *E. esculentus* (latter category during late stages of experimental-injection with yeasts when symptoms of disease were visible) revealed consistently lower total counts of the latter fluid category in the region of a 39% decline. Differential coelomocyte counts (obtained by density gradient centrifugation of CF's from healthy and "sick" sea urchins) were found to be consistently lower, particularly the fraction containing the CSC and VC's (B) where a 40% decline in the mean coelomocyte count was observed. Additionally a decline in the PL's (A) of 17% and the RSC's (C) were also found.

A decline in each coelomocyte type in the CF from infected animals may be attributed to several factors including: coelomocyte migration to an infected area such as the test, clotting of the coelomocytes inaccessible by normal CF sampling methods, coelomocyte lysis or perhaps a combination of each of these factors. An increased incidence of cellular agglutination would possibly involve large numbers of mucoid-releasing VC's and PL's. Moreover, the characteristic darkening of *E. esculentus* test colouration observed during the course infection could be due to the influx of RSC's and their despherulation in the calcareous layers of the sea urchin test. The relative proportion of each cell type however, remained relatively constant in healthy and sick CF's, varying by only 1-2%.

A decline in the total coelomocytes and PL differential counts from "sick" *E. esculentus* CF may be attributed to cell lysis as it would appear in non-viscous, "stringy", yellow CF from this animal category. Alternatively, the decline may have been due to clotting or aggregation of the phagocytes (Johnson , 1969a). Yui and Bayne (1983) reported a decline in all coelomocyte types, with a change in the relative proportion of each cell type. The percentages of PL's and RSC's declined whilst the VC's increased although not appreciably.

The use of natural responses to injury or infection among invertebrates was a hallmark of Metschnikoff's work (1893) on invertebrates in his "Lectures on the Comparative Pathology of Inflammation", where he defined inflammation in terms of phagocytosis. This study strongly suggests that the mechanism of reduction in viable count of marine yeasts exposed to *E. esculentus* CF *in vitro* was primarily phagocytosis. Since there was a correlation between *in vitro* and *in vivo* reduction in viable count of the same strains of yeasts one may speculate that the mechanism of

clearance may also be phagocytosis. Phagocytosis has been demonstrated to occur both *in vivo* and *in vitro* with *S. droebachiensis* by SEM. Bertheussen (1981a) suggests that parallel phagocytic avidity *in vivo* and *in vitro* would indicate that humoral factors were absent or not essential for recognition of foreign substances by phagocytes. Demonstration and quantitation of *in vivo* and *in vitro* phagocytosis in *E. esculentus* CF would be a profitable area for further study.

Despite recent advances (reviewed by Cooper, 1976; Manning and Turner, 1976; Hildemann *et al.*, 1981) the phylogeny of immunity remains obscure. In particular, the mechanisms of invertebrate immunity are diverse, and many are poorly understood. The phylogenetic position of the echinoderms makes them pivotal to the understanding of the phylogeny of immunity and therefore the evolution of vertebrate immunity. The phenomenon of clearance may be used as one aspect of study of echinoderm immunology.

4. CONCLUSIONS AND PERSPECTIVES

4.1 Maintenance of *E. esculentus* in RASWA

This thesis describes detailed reproducible methods for the establishment and monitoring of RASWA in a laboratory remote from the sea. It describes experiences of over two years of successfully keeping adult specimens of *E. esculentus* in a healthy condition under artificial conditions. However, such a system was found to be unsuitable for whole animals infection studies in which *E. esculentus* were experimentally infected with live suspensions of marine yeasts and bacteria. An increased mortality rate, as a result of these infections, caused rapid and uncontrollable deterioration of the aquaria water-quality as meas ured by a rapid increase in nitrite and decline in pH. Therefore, further work on

the pathogenicity of marine yeasts ideally requires running-seawater aquaria which would prevent the accumulation of toxic substances and contaminating bacteria. Additionally, this system would require disinfection of waste seawater to avoid the release of potential pathogens into the sea. Alternatively, short-term infection experiments could be done in containers containing aerated ASW.

4.2 *In vitro* Antibacterial and Antifungal Activity of *E. esculentus* Coelomic Fluid

Although progress has been made in elucidating the mechanisms by which the sea urchin defends itself from invading microorganisms, it is still apparent that much remains to be learned about the mechanisms of the different coelomocyte types in the antimicrobial defence of *E. esculentus*.

A particularly rewarding line of research further to this work would be the mechanisms of involvment of the phagocytic leucocytes (PL's) in the antimicrobial defence of this animal , particularly since the PL's appear to play a major role in the fungicidal activity of this sea urchin. They have also been implicated in the killing of live bacteria and removal of dead bacteria after the action of other defence mechanisms in this study and in other echinoid species (Johnson, 1969b). Although Metschnikoffs (1884) fundamental studies were performed on the invertebrate *Daphnia*, recent work on phagocytosis has been focused almost exclusively on the higher invertebrates. The mechanisms governing attachment of particles, internalization and intracellular degradation in this group of animals are very complicated. Normal functioning of the cells requires the presence of many different substances, opsonins, some of which are not characterized. Phagocytic recognition is itself complex and involves at least three

different receptors, the Fc, C_3 and foreign surface receptor. Phagocytic recognition in some of the invertebrates appears to rely on non-immunological receptors on the surface of the cells. Therefore, the investigation of phagocytosis from such animals yields valuable information of "self"/"non-self" recognition. Bertheussen (1979) also suggests that the invertebrate phagocytic cell is probably also the effector cell during graft rejection and may represent the common ancestor of the vertebrate macrophage or lymphocyte. Interestingly, Kaplan and Bertheussen (1977) observed a striking similarity in the function of the foreign surface receptors of mouse peritoneal macrophages and echinoid phagocytes. Bertheussen and Seljelid (1978) have described a method for obtaining pure monolayers of phagocytes *in vitro* from *S. droebachiensis* which may usefully be applied to *E. esculentus*.

This investigation confirms evidence by Service and Wardlaw (1984) that the main locus of bactericidal activity lies in the Ech-A containing RSC's. However, although there is information regarding the structure and biosynthesis of the napthaquinone pigment, nothing is known of its mode of storage, the role of associated CF protein(s), release from the RSC's or mechanisms of antibacterial and antifungal action. All of these are interesting channels for further study.

There is also a need for clarification of the role of the vibratile cells in *E. esculentus* and whether as in *S. droebachiensis* (Bertheussen and Seljelid, 1978) it is involved in the clotting of the coelomocytes. The chemical nature and specificity of the haemagglutinin present in the CSC's of *E. esculentus* also merits further study. The role of the HA in the CSC's has been suggested to be as an opsonin, enhancing phagocytosis as in the oyster, *Crassostrea virginica* (Tripp, 1960) or the crayfish (McKay *et*

al., 1969). However, Fuke and Sugai (1972) found that ascidian HA did not activate phagocytosis since the coelomocyte types have been tested for antimicrobial activity in isolation. The possibility of coelomocyte cooperation in antimicrobial defence also merits investigation.

4.3 Clearance and Pathogenicity of Marine Yeasts For *E. esculentus*

The investigation demonstrates rapid primary clearance of high doses of injected marine yeasts from the coelomic cavity of *E. esculentus* within hours after injection. A second, slower phase in which the remaining yeasts persisted in the coelom over a period of weeks, or until death of the animals, was accompanied by secondary bacterial contamination of the CF. There are many aspects of this work which are still unclear and form an interesting basis for further study. A more detailed analysis of the viable count of bacteria and yeast during the first few hours after injection would allow the determination of particle clearance kinetics and therefore allow comparison of the rates of clearance. It would also be particularly interesting to investigate into whether this species of sea urchin exhibits memory or altered reactivity on secondary contact with biologically relative antigens, namely yeasts and bacteria.

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A P P E N D I X

APPENDIX 1A. Characteristics of NCYC yeast strains.

	Genera and species (NCYC number)					
	C. famata (789)	*C. famata* (799)	*C. guilliermondii* (145)	*C. haemulonii* (787)	*C. marina* (784)	*C. maris* (785)
Habitat	Seawater	Seawater	Seawater	Seawater	Seawater	Seawater
Colour on agar; W=white, C=cream, P=pink, B=brown.	C	C	C	C	C	C
Surface on agar; Sh=shiny; S=sly, D=dull.	Sh	S	Sh	D	D	D
Texture on agar; R=rough, S=smooth, W=wrinkled.	S	S	W	-	S	R
Ring in broth; - = absent, + = complete, +/- = incomplete.	-	-	+/-	-	+	-
Filamentous growth pseudomycelium ++ = well formed, + = illformed, - = absent.	-	++	++	+	+	-

APPENDIX 1A. (continued).

	Genera and species (NCYC number)					
	C. famata (798)	*C. famata* (799)	*C. guilliermondii* (145)	*C. haemulonii* (787)	*C. marina* (784)	*C. Maris* (785)
Habitat	Seawater	Seawater	Seawater	Seawater	Seawater	Seawater
Blastospores + = many, - = absent,	U	+	+	-	-	-
shape, U = unknown, NR = not relavent, O = oval.	NR	U	O	NR	NR	NR
Cells R = round, O = oval.	R/O	R/O	R/O	R	O	R/O
Arrangement S = single, P = pairs, C = chains, F = floccs.	P	S	C	F	P	P
Lipolytic	-	-	-	+	-	-

APPENDIX 1A. (continued).

	Genera and species (NCYC number)					
	C. famata (798)	*C. famata* (799)	*C. guilliermondii* (145)	*C. haemulonii* (787)	*C. marina* (784)	*C. maris* (785)
Habitat	Seawater	Seawater	Seawater	Seawater	Seawater	Seawater
Acid Production U = unknown, +/- = weak.	+/-	+	U	U	-	-
Fermentation						
Glucose	+	+	+	+	-	-
Galactose	-	-	+/-	-	-	-
Sucrose	-	+	+	+	-	-
Maltose	-	-	-	-	-	-
Cellobiose	U	U	U	U	U	U
Trehalose	U	U	U	U	U	U
Lactose	-	-	-	-	-	-
Melibiose	-	U	-	-	-	-
Raffinose	-	+	+	+	-	-
Metezitose	U	U	U	U	U	U
Inulin	U	U	U	U	U	U
Soluble Starch	-	-	U	-	-	-

APPENDIX 1A. (continued).

	Genera and species (NCYC number)					
	C. famata (798)	*C. famata* (799)	*C. guilliermondii* (145)	*C. haemulonii* (787)	*C. marina* (784)	*C. maris* (785)
Habitat	Seawater	Seawater	Seawater	Seawater	Seawater	Seawater
Assimilation (C)						
Glucose	+	+	+	+	+	+
Galactose	+	+	+	+/-	-	-
Sorbose	+	+	U	-	-	-
Sucrose	+	+	+	+	-	-
Maltose	+	+	+	+	-	-
Cellobiose	+	+	+	-	-	-
Trehalose	+	+	U	+	+	-
Lactose	+	-	-	-	-	-
Melibiose	+	+	-	-	-	-
Raffinose	+	+	U	+	-	-
Melezitose	+	+	U	+	-	-
Inulin	-	+	U	-	-	-
Soluble starch	-	-	+	-	-	-
Xylose	+	+	+/-	+	+	+/-
L-Arabinose	+/-	+	U	+/-	+	-
D-Arabinose	+	+	-	+	+	-
Ribose	-	+/-	U	-	+	-
Rhamnose	-	-	-	+	+	+
Ethanol	+/-	+	U	+/-	+	-

APPENDIX 1A. (continued).

	Genera and species (NCYC number)					
	C. famata (798)	*C. famata* (799)	*C. guilliermondii* (145)	*C. haemulonii* (787)	*C. marina* (784)	*C. maris* (785)
Habitat	Seawater	Seawater	Seawater	Seawater	Seawater	Seawater
Assimilation (N)						
NH_4 ($2SO_4$)	+	+	U	+	+	+
KNO_3	-	-	-	-	-	-
Ethylamine	+	+	U	+	+	+
Cadaverine	U	U	U	U	U	U

APPENDIX 1B. Characteristics of NCYC yeast strains.

	Genera and Species (NCYC number)				
	C. torresii (786)	*D. hansenii* (792)	*M. zobelli* (783)	*R. rubra* (63)	*R. Rubra* (797)
Habitat	Seawater	Seawater	Seawater	Vegetation	Seawater
Colour on agar: W = white, C = cream, P = pink, B = brown.	C	W	C	P	P
Surface on agar: Sh = shiny, S = sly, D = dull.	Sh	D	D	Sh	S
Texture on agar: R = rough, S = smooth, W = wrinkled.	S	S	R	S	S
Ring in broth: - = absent, + = complete, +/- = incomplete.	-	+	+	+	-
Filamentous growth Pseudomycelium ++ = well formed, + = ill formed, - = absent.	-	-	+	-	-

APPENDIX 1B. (continued).

	Genera and species (NCYC number)				
	C. torresii (786)	*D. hansenii* (792)	*M. zobelli* (783)	*R. rubra* (63)	*R. rubra* (797)
Habitat	Seawater	Seawater	Seawater	Vegetation	Seawater
Blastospores + = many, - = absent, shape, U = unknown, NR = not relevant O = oval.	-	-	-	-	U
	NR	NR	NR	NR	NR
Cells R = round, O = oval.	O	R/O	R/O	R/O	O
Arrangement S = single, P = pairs, C = chains, F = floccs.	P	P	P	S	S
Lipolytic	-	-	-	-	-

APPENDIX 1B. (continued).

	Genera and species (NCYC number)				
	C. torresii (786)	*D. hansenii* (792)	*M. zobelli* (783)	*R. rubra* (63)	*R. rubra* (797)
Habitat	Seawater	Seawater	Seawater	Vegetation	Seawater
Acid Production U = unknown +/- = weak	-	-	-	-	-
Fermentation					
Glucose	+	+	+	-	-
Galactose	-	-	-	-	-
Sucrose	-	+	-	-	-
Maltose	-	-	-	-	-
Cellobiose	U	U	U	-	U
Trehalose	U	U	U	-	U
Lactose	-	-	-	-	-
Melibiose	-	-	-	-	-
Raffinose	-	-	-	-	-
Melezitose	U	U	U	-	U
Inulin	U	U	U	-	U
Soluble Starch	-	-	-	-	-

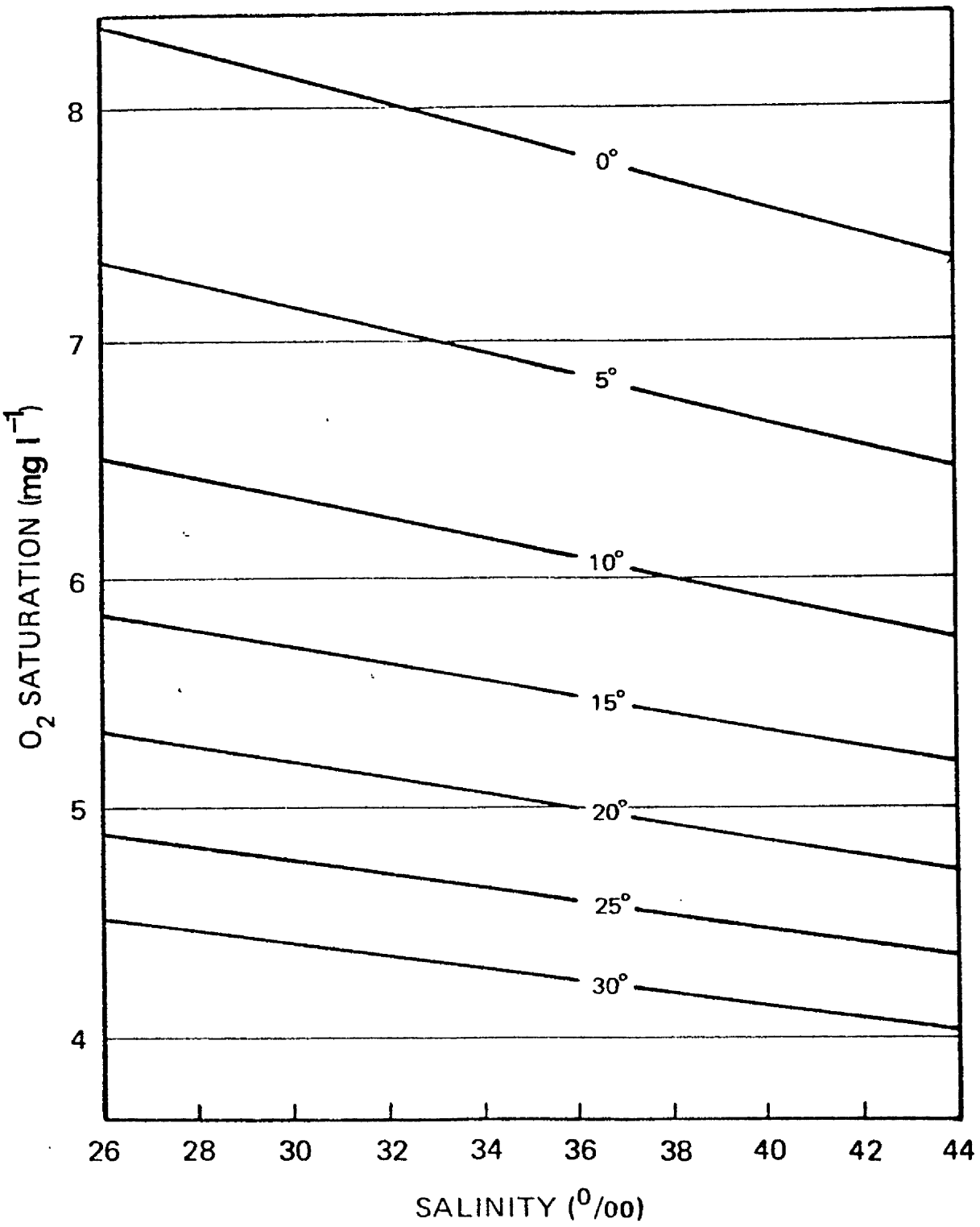
APPENDIX 1B. (continued).

	Genera and species (NCYC number)				
	C. torresii (786)	*D. hansenii* (792)	*M. zobelli* (783)	*R. Rubra* (63)	*R. rubra* (797)
Habitat	Seawater	Seawater	Seawater	Vegetation	Seawater
Assimilation (C)					
Glucose	+	+	+	+	+
Galactose	+/-	+	+/-	+	+
Sorbose	+	+	-	+/-	+
Sucrose	-	+	+	+	+
Maltose	-	+	+	+	+
Cellobiose	+	+	+	-	-
Trehalose	+	+	+	+	+
Lactose	-	-	-	-	-
Melibiose	-	+/-	-	-	-
Raffinose	-	+	-	+	+
Melezitose	-	+	+	+	+
Inulin	-	-	-	-	-
Soluble starch	-	-	-	-	-
Xylose	+	+/-	-	+	+
L-Arabinose	+/-	+	-	+	+
D-Arabinose	+	-	-	+	+
Ribose	+/-	+/-	+/-	+	+
Rhamnose	-	+/-	-	-	-
Ethanol	+/-	+	+/-	+	+

APPENDIX 1B. (continued).

	Genera and species (NCYC number)				
	C. torresii (786)	*D. hansenii* (792)	*M. zobelli* (783)	*R. rubra* (63)	*R. rubra* (797)
Habitat	Seawater	Seawater	Seawater	Vegetation	Seawater
Assimilation (N)					
NH_4 ($2SO_4$)	+	+	-	+	+
KNO_3	-	-	-	-	-
Ethylamine	+	+	-	+	+
Cadaverine	U	U	U	U	U

APPENDIX 2. Oxygen saturation of seawater of varying salinity (‰) at different temperatures (°C) (from Committee on Marine Invertebrates, 1981).


8
7
6
5
4
0°
5°
10°
15°
20°
25°
30°
O_2 SATURATION (mg l^{-1})
26
28
30
32
34
36
38
40
42
44
SALINITY ($^0/_{00}$)

APPENDIX 3. Preliminary recirculating artificial-seawater aquarium with external refrigeration system.

a. Artificial seawater "Sea Salt^{R}"

b. Power filter pump

c. Polypropylene tank casing

d. Plastic airlift tube

e. Water aeration spray

f. "Dorset Pea" smooth, quartz pebbles

g. Plastic filter base

h. External refrigeration system

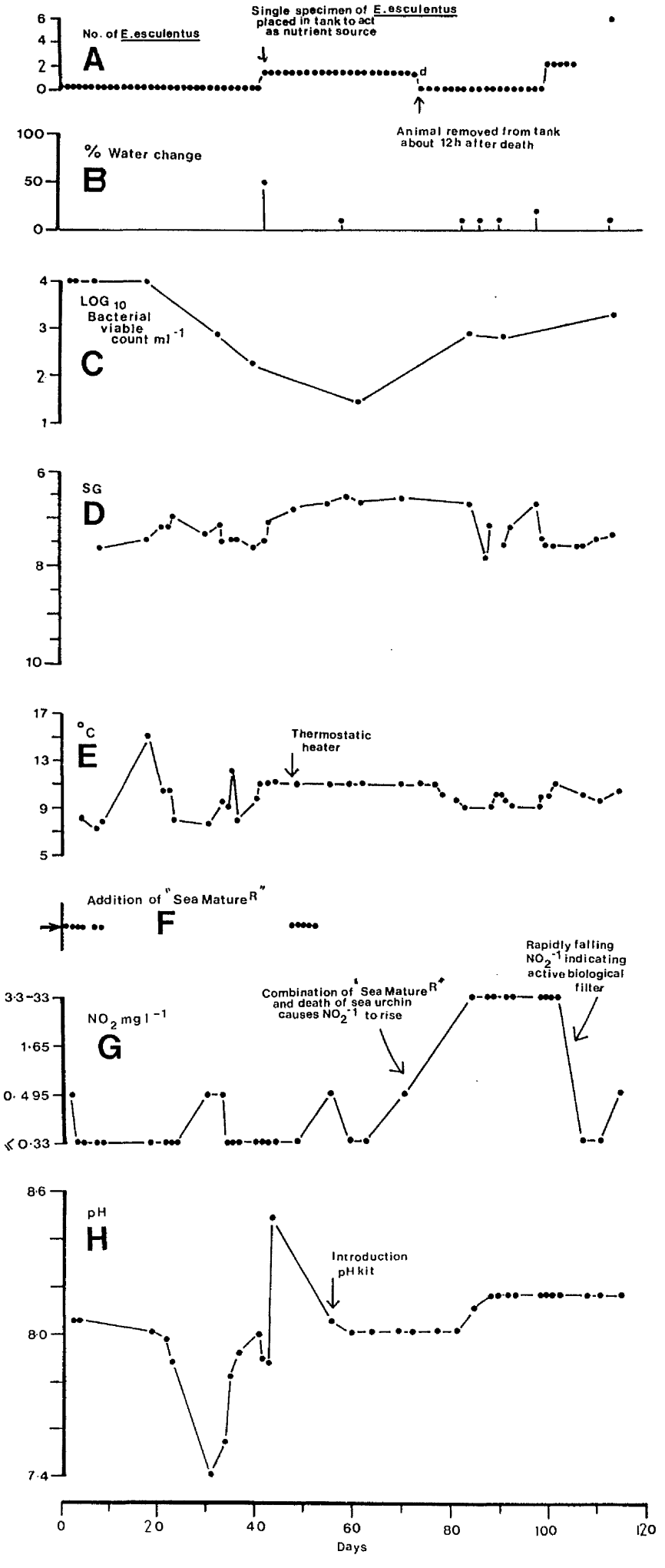
i. Recirculating coiled cooling tube (1 1/2 metres)

D. Direction of water flow

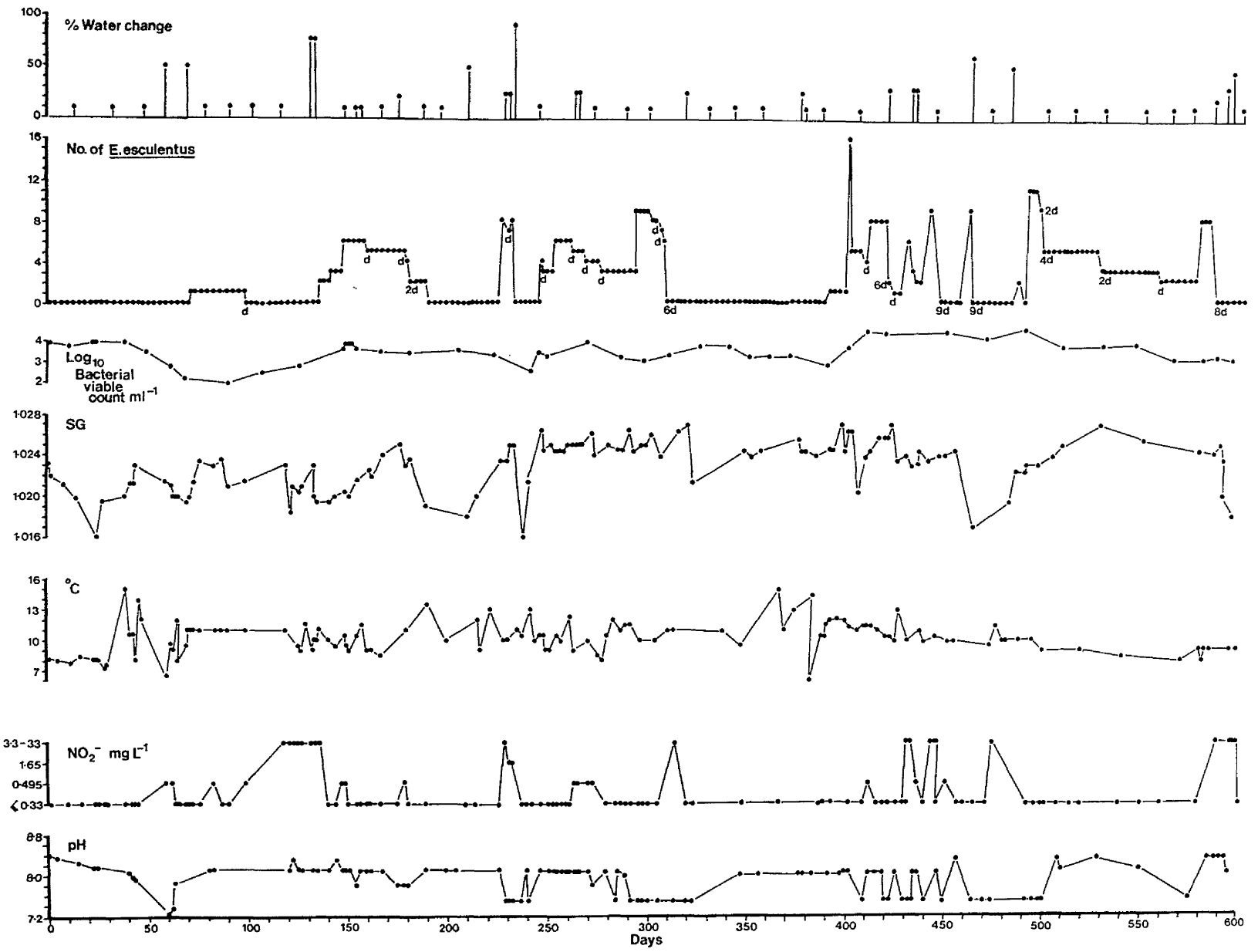
D
D
D
h
D
b
d
a
f
D
e
g
c

APPENDIX 4. Eight routinely measured parameters (A to H) of RASWA during the 'maturation' period of the biological filter bed.

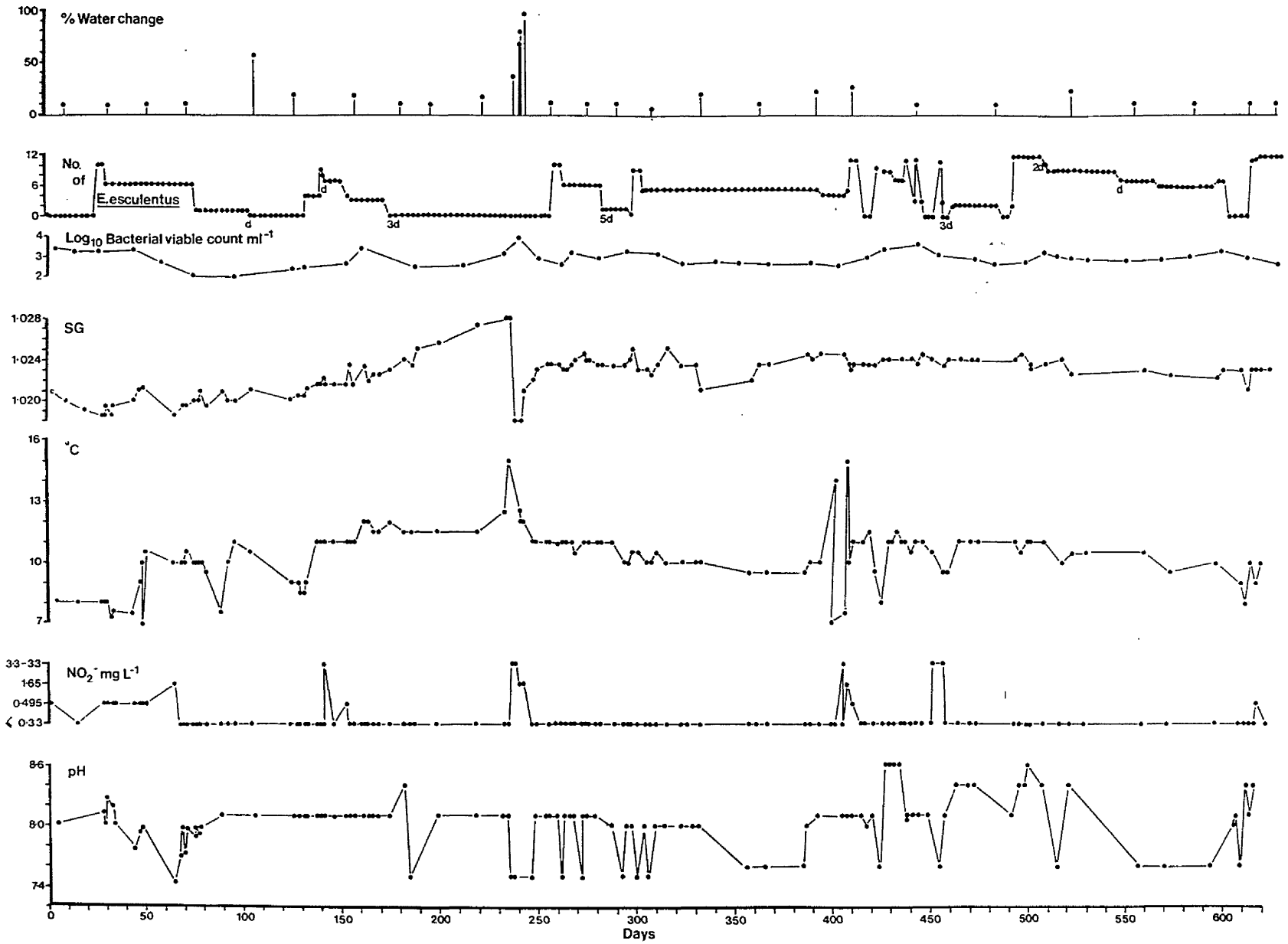
(d = Death of an animal)


Single specimen of E.esculentus placed in tank to act as nutrient source
No. of E.esculentus
A
Animal removed from tank about 12h after death
% Water change
B
LOG 10 Bacterial viable count ml -1
C
SG
D
°C
E
Thermostatic heater
Addition of "Sea Mature R"
F
Rapidly falling NO2 -1 indicating active biological filter
Combination of "Sea Mature R" and death of sea urchin causes NO2 -1 to rise
NO2 mg l -1
G
pH
H
Introduction pH kit
Days

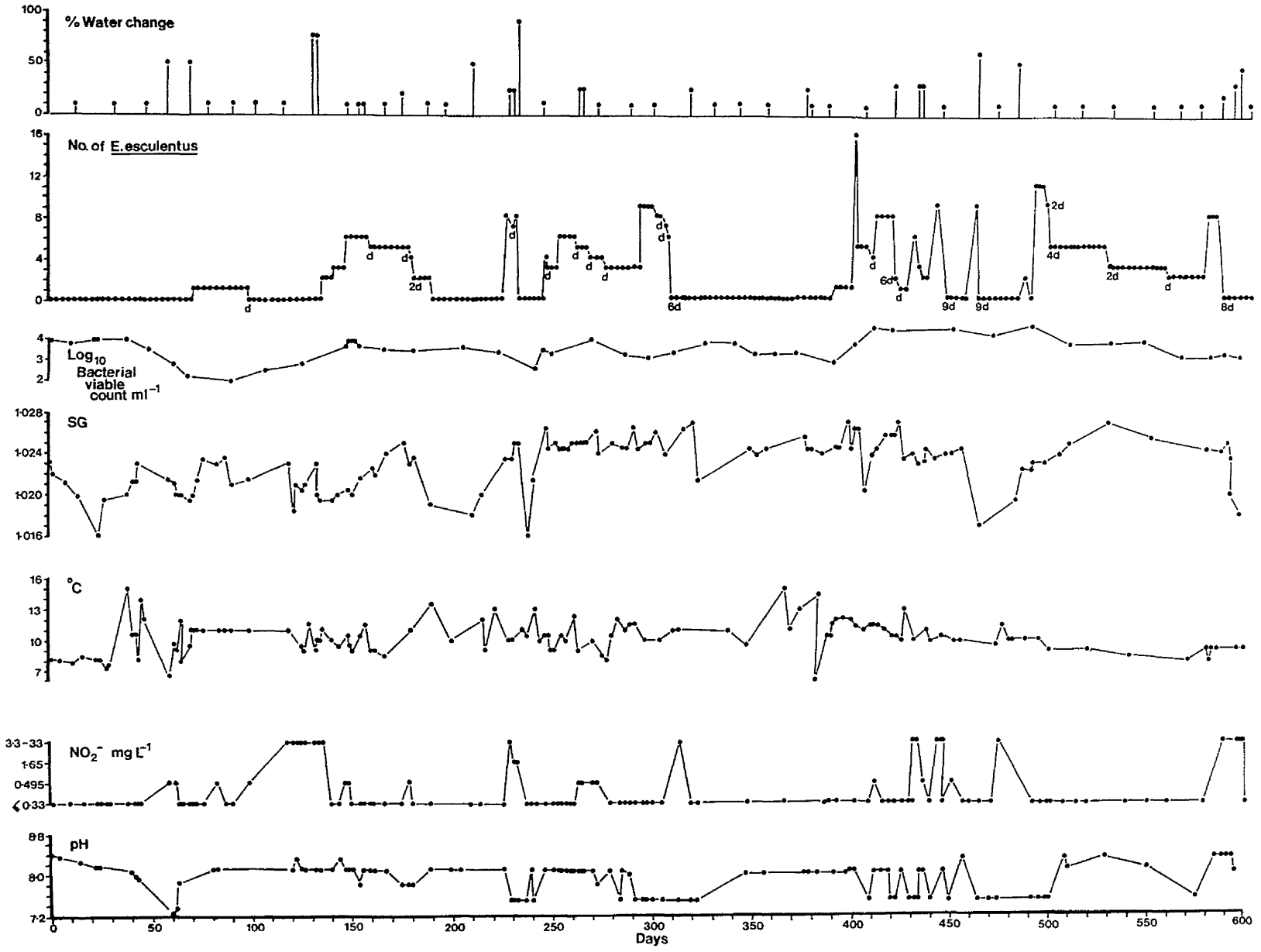
APPENDIX 5. Tank A (72 l), seven routinely measured parameters recorded over a 600-day period in the established aquarium system for maintenance *E. esculentus*.


% Water change
100
50
0
No. of E.esculentus
16
12
8
4
0
d
2d
6d
9d
4d
8d
Log10 Bacterial viable count ml-1
4
3
2
SG
1·028
1·024
1·020
1·016
°C
16
13
10
7
NO2- mg L-1
3·3-33
1·65
0·495
<0·33
pH
8·8
8·0
7·2
0
50
100
150
200
250
300
350
400
450
500
550
600
Days

APPENDIX 6. Tank B (76 l), seven routinely measured parameters recorded over a 600-day period in the established aquarium system for maintenance of *E. esculentus*.


% Water change
100
50
0
No. of E.esculentus
12
6
0
d
3d
d
5d
3d
2d
d
Log10 Bacterial viable count ml-1
4
3
2
SG
1·028
1·024
1·020
°C
16
13
10
7
NO2- mg L-1
3·3-33
1·65
0·495
< 0·33
pH
8·6
8·0
7·4
0
50
100
150
200
250
300
350
400
450
500
550
600
Days

APPENDIX 7. Tank C (76 l), seven routinely measured parameters recorded over a 630-day period in the established aquarium system for maintenance of *E. esculentus*.


% Water change
100
50
0
No. of E.esculentus
16
12
8
4
0
d
2d
6d
9d
8d
Log10 Bacterial viable count ml-1
4
3
2
SG
1·028
1·024
1·020
1·016
°C
16
13
10
7
NO2- mg l-1
3·3-33
1·65
0·495
<0·33
pH
8·8
8·0
7·2
0
50
100
150
200
250
300
350
400
450
500
550
600
Days

APPENDIX 8. Bactericidal activity of *E. esculentus* CF (n = 26) towards *Ps. III* and growth of the bacteria in MBASW control fluid at 10°C.

Experiment No.	Urchin No. or MBASW	Viable Count (and SI) at 0	24	48	72 h
		***			
1	1	nt	0 (0)	3 (5)	0 (0)
	2	nt	1 (2)	0 (0)	0 (0)
	3	nt	0 (0)	0 (0)	0 (0)
	4	nt	0 (0)	0 (0)	0 (0)
	MBASW	56 (100)	*C (⩾200)	C (⩾200)	C (⩾200)
2	5	nt	0 (0)	0 (0)	0 (0)
	6	nt	0 (0)	1 (13)	0 (0)
	7	nt	4 (50)	0 (0)	0 (0)
	MBASW	8 (100)	14 (175)	C (⩾200)	C (⩾200)
3	8	nt	6 (5)	2 (2)	0 (0)
	9	nt	82 (66)	**AC (⩾200)	C (⩾200)
	10	nt	30 (24)	250 (⩾200)	C (⩾200)
	MBASW	124 (100)	C (⩾200)	C (⩾200)	C (⩾200)
4	11	nt	14 (40)	0 (0)	0 (0)
	12	nt	6 (17)	300 (⩾200)	C (⩾200)
	13	nt	15 (43)	C (⩾200)	C (⩾200)
	MBASW	35 (100)	300 (⩾200)	C (⩾200)	C (⩾200)
5	14	nt	0 (0)	0 (0)	0 (0)
	15	nt	0 (0)	0 (0)	0 (0)
	16	nt	15(150)	50 (⩾200)	0 (0)
	MBASW	24 (100)	C (⩾200)	C (⩾200)	C (⩾200)
6	17	nt	6 (10)	5 (8)	184 (⩾200)
	18	nt	0 (0)	0 (0)	0 (0)
	19	nt	2 (3)	20 (34)	34 (58)
	MBASW	59 (100)	104 (⩾200)	C (⩾200)	C (⩾200)
7	20	nt	7 (12)	0 (0)	0 (0)
	21	nt	0 (0)	0 (0)	0 (0)
	22	nt	2 (3)	0 (0)	0 (0)
	MBASW	58 (100)	390 (⩾200)	C (⩾200)	C (⩾200)

* C - confluent growth
**AC - almost confluent growth
***nt - not tested

APPENDIX 8. (continued).

Experiment No.	Urchin No. or MBASW	Viable Count (and SI) at 0	24	48	72 h
8	23	nt	16 (50)	12 (38)	4 (13)
	24	nt	0 (0)	0 (0)	0 (0)
	MBASW	32 (100)	57 (178)	78 (⩾200)	C (⩾200)
9	25	nt	0 (0)	0 (0)	0 (0)
	26	nt	10 (22)	13 (28)	120 (⩾200)
	MBASW	46 (100)	52 (113)	AC (⩾200)	C (⩾200)

* C - confluent growth
**AC - almost confluent growth
***nt - not tested