PRODUCTION OF LOW-MOLECULAR WEIGHT TOXINS BY MARINE BACTERIA

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This work is dedicated to two very special people, Florence and Denis Gallacher. Also to the memory of Florence Boan.

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SUMMARY

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A tissue culture assay was developed which could detect sodium channel-blocking (SCB) toxins in as little as 0.1 ml of bacterial culture supernate. The assay was based on the principle that when ouabain (oub) and veratridine (ver) are added to a culture of mouse neuroblastoma (MNB) cells, an influx of Na^+ ions into the cell occurs which, if left unchecked, eventually leads to cell lysis. However, if SCB toxins are present, the effect of the above two neurotoxins is blocked and the cells survive.

Cell survival was measured with the vital stain, neutral red, and the absorbance of the pink colour, which developed after treatment with citrate-buffered alcohol, was measured with a microtitre plate reader. This allowed the cell protection, due to SCB toxins, to be calculated.

Standardisation of batches of chemicals, serum and culture conditions was vital to provide reproducible results.

The sensitivity of the assay was increased by stimulating differentiation of the MNB cells by growth in low concentrations of serum, or by addition of 10 mM HMBA (N, N'-Hexamethylene-bis-acetamide), or rat glioma medium to the culture medium.

SCB activity was found in both bacterial cell extracts and culture supernates. However, these samples also contained an inhibitor which, in the cell extracts, was heat sensitive, but in the culture supernates was heat-stable.

The inhibitor in the culture supernates was identified as NaCl, and arose from the high salt concentrations in the marine bacterial culture medium. This inhibitory effect could be reduced by several procedures such as; the use of growth media containing less NaCl, initial dilution of the sample in water, assaying the sample at dilutions of 1/16 or by charcoal extraction of the supernate.

Heating and ultrafiltration of the bacterial supernate and the TTX control yielded unexpected results, in that SCB activity decreased during the first 20 min of heating at 100°C, after which it recovered to its original value. Also, the SCB activity did not pass through an ultrafiltration membrane of 500 dal cut-off.

The tissue culture assay provided a means for large-scale screening of bacteria for SCB activity. Of isolates from Scottish coastal waters 36 % were SCB toxin producers, indicating that a wide range of bacterial isolates capable of this function exist. *Vibrio, Aeromonas* and *Pseudomonas* strains from type

culture collections also produced the toxin. Cultures of some of the SCB-producing strains were analysed by HPLC which indicated that neo-STX was present.

The assay therefore confirmed that SCB toxins are produced by bacteria and provides the first report that free-living bacteria capable of producing such toxins are widespread in the marine environment.

An investigation of culture conditions necessary for toxin production indicated that the toxins were produced during the stationary phase of the growth cycle and were probably products of secondary metabolism. Their production occurred after depletion of phosphate in the medium. SCB toxins could also be detected in seawater and *M. edulis* tissue extracts after exposure to SCB-producing bacteria.

The tissue culture assay was also used to detect SCB activity in mussel extracts, which had been contaminated by toxic dinoflagellates during an algal bloom. The results gave a high correlation when compared to the mouse bioassay and the method proved to be more sensitive than existing HPLC methods or the mouse bioassay.

The seasonal and geographical distribution of bacteria producing ciliostatic toxin (CT) implicated in the pathogenesis of oysters was also

investigated. The bacteria capable of this function varied seasonally, with the highest numbers occurring in the summer months, coinciding with high temperatures and vibrio numbers.

These isolates were also examined for SCB toxin production in which the highest toxin producers were found in the summer and early autumn.

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ABBREVIATIONS

asw: artificial seawater

CIF: Cell inhibitory factor

CMC: Carboxylmethyl cellulose

CT: Ciliostatic toxin

DMSO: Dimethyl sulphoxide

fcs foetal calf serum

GC-MS Gas chromatography-mass spectrometry

HMBA N,N' Hexa methylene-bis-acetamide

HPLC High performance liquid chromatography

ip: intraperitoneal

M1 medium 1

M2 medium 2

M3 medium 3

MAB: Monoclonal antibody

MB: Marine broth

ME: Mouse equivalents

MNB: Mouse neuroblastoma cells

MT: Microtitre plates

MU: Mouse units

NGF: Nerve growth factor

NMR: Nuclear magnetic resonance

oub: Ouabain

PLL: Poly-l-lysine

SIF: Supernate inhibitory factor

STX: Saxitoxin

tcu: Tissue culture units

TEM: Transmission electron microscopy

TLC: Thin layer chromatography

TLC-FAB: Thin layer chromatography-fast atom bombardment

TLC-FID: Thin layer chromatography-flame ionization detection

TTX: Tetrodotoxin

UV-Spec: Ultra-violet spectroscopy

VCT: Vibrio ciliostatic toxin

ver: Veratridine

INTRODUCTION.

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TOXIN PRODUCTION BY MARINE BACTERIA

Marine bacteria produce a diverse range of extracellular products, some of which are toxins. The latter have been defined as: "any single (or multiple) chemical entity produced by one species, capable of producing pathological changes in a second species." (Walker and Masuda, 1990).

This thesis deals with marine bacterial toxins which cause economical losses in the shellfish industry. Two aspects are considered:

1. Toxins accumulated in bivalve molluscs which have adverse effects on human health, thereby causing public scares and decreased sales.
2. Pathogenic or potentially pathogenic bacteria which can wipe out stocks of bivalve larvae.

In consideration of this, two groups of low molecular weight, non-protein, toxins produced by marine bacteria were investigated. Most emphasis has been given to the sodium channel-blocking (SCB) toxins, namely tetrodotoxin and the paralytic shellfish poisons (PSP); the second group studied were the ciliostatic toxins implicated in vibriosis of shellfish.

SODIUM CHANNEL-BLOCKING TOXINS

Tetrodotoxin

Japanese fugu is, according to Vietmeyer (1984) "one of the most mysterious creatures of the sea " (Fig 1); it is perhaps the world's most poisonous fish, yet in Japan it is the epitome of gourmet dining (Fig. 2). "Delicious, ugly, deadly poisonous and ultimately edible" (Vietmeyer, 1984), it contains tetrodotoxin (TTX) which, with a mouse LD50 of 10 µg/kg i.p. (Fuhrman, 1986), is calculated to be equivalent to a lethal dose of approximately 0.5 mg for a 70 kg man (Mosher, 1986) and is one of the most poisonous non-protein substances known to man.

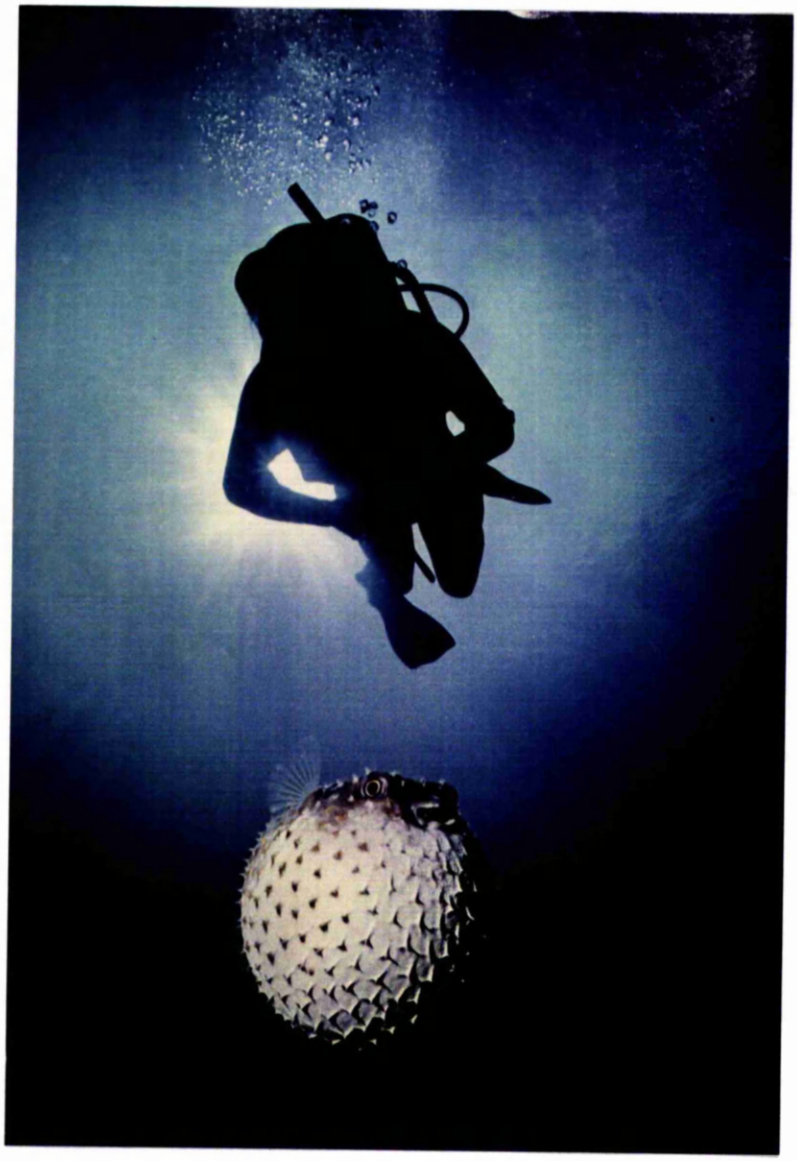
All fugu cooks in Japan must be licenced, but even so it is the gastronomic version of Russian Roulette. Sometimes a diner still loses the gamble as 60% of puffer poisonings are fatal (Vietmeyer, 1984).

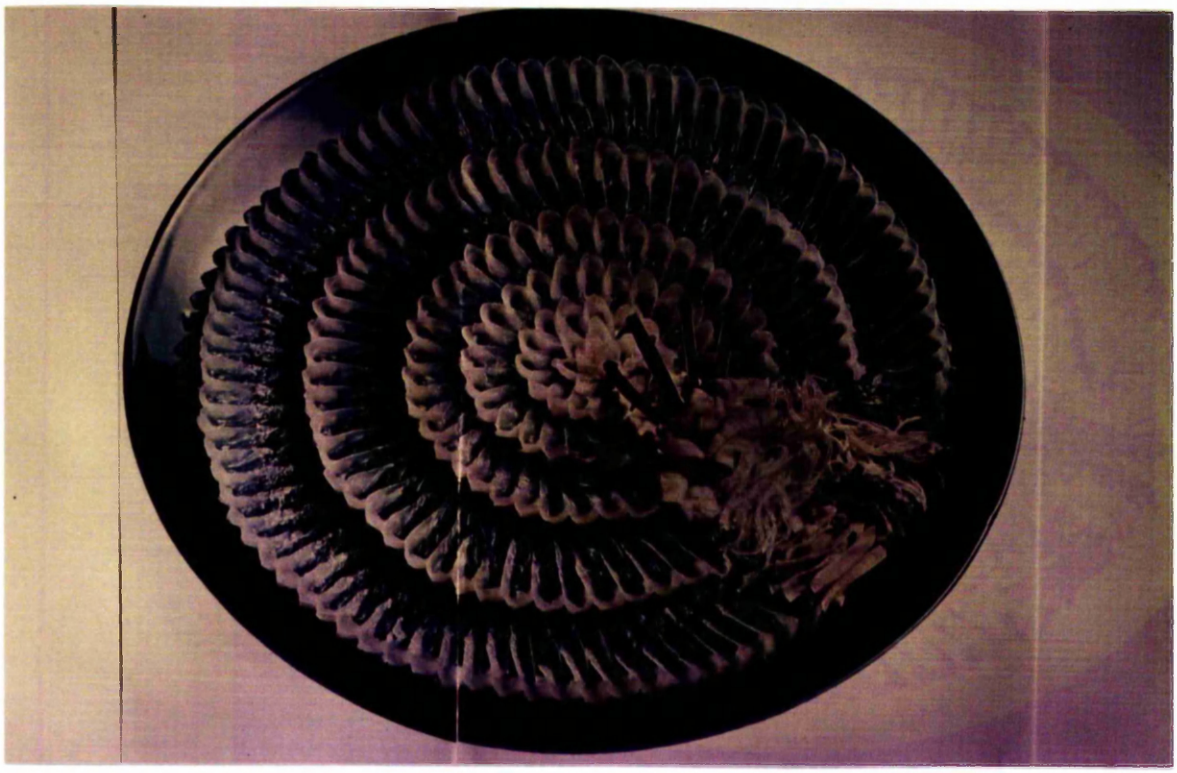
"Last night he and I ate fugu.
Today I help carry his coffin".

(Senryu verse).

The attraction of fugu is the presence of traces of TTX which apparently leads to a delicious tingling sensation in the extremities accompanied by a feeling of warmth and euphoria; the first sign

FIGURE 1 Puffer fish. (Reproduced by kind permission of the National Geographic Magazine).





that something is going very wrong is the appearance of the same sensation in areas not extreme and the disappearance of the euphoria (Prince, 1988).

A restaurant owner in Japan described the symptoms: "It is a terrible death, even though you can think very clearly; your arms and legs become numb, it becomes impossible to sit up, you can't speak, can't move and soon can't breathe" (Vietmeyer, 1984).

Numbness of the lips and tip of the tongue is the first warning, followed by a headache, abdominal pain, armache, wobbling gait, vigorous vomiting, ataxia and stupor. Aphasia becomes severe, dyspnea develops, blood pressure drops, complete ataxia over the entire body occurs followed by paralysis of sensation. The patient is fully aware of what is happening but becomes comatose shortly after and develops respiratory arrest. The symptoms can appear between twenty minutes and three hours after the intake of poison; death can occur anytime during this period. (Konosu *et al.*,1979).

History of TTX

The name tetrodotoxin originates from the order *Tetradontiformes* to which puffer fish belong. The bones of the fish Tetraodontidea have been excavated from Stone Age relics (Konosu *et al.*, 1979), and a

puffer was identified on the tomb of the Egyptian Pharoah Ti, of the Vth dynasty, 2500 BC. Its poisonous properties are believed to have been known to the Egyptians (Mosher *et al.*, 1964).

The earliest Chinese materia medica, the Pen T'so Chin (1-2 B.C.) lists the eggs of Tetraodon fish among its drugs, and an accurate account of the toxicity of liver, eggs and ovaries in a fish which seems to be fugu was described by Chaun Yaufung during the Sui Dynasty (AD 581-617); (Mosher *et al.*, 1964).

Puffer fish poisoning is not limited to Japan and the Middle East, being also reported by the early Spanish in Mexico, by a Mexican historian in Califonia and by Captain Cook, in the journals of the voyage of HMS Resolution in 1774 (Fuhrman, 1986), as well by the fictional character James Bond (Fleming, 1957; 1958)!

TTX has also been implicated as the causal agent in the process of zombification by which zombies, "the living dead" of Voodoo culture in Haitian folklore, occur (Yasumoto and Kao, 1986).

In 1883, Charles Reny reported the results of the first experimental work on fugu and listed five of the most poisonous species, in which the gonads were the most toxic (Fuhrman 1986).

In the first comprehensive study of the pharmacology of TTX in humans, hypertension and respiratory depression were described by Takahashi and Inoko in 1889, who also noted that the heart continued to beat after respiration stopped (Fuhrman, 1986).

Purification of the active principle of fugu poisoning began in the late 19th century and by 1911, Tahara had obtained a preparation which was about 4% pure; this was used in many pharmacological studies and was awarded a U.S. patent in 1913 (Fuhrman 1986).

Isolation of crystalline TTX was achieved in 1950 by Yokoo, who called the product spheroidine. This was later shown to be identical to the toxin isolated in 1952 by Tsuda and Kawamura, named tetrodotoxin, and the latter name has prevailed.

Once crystalline TTX was available, a race ensued to solve its structure. The results were presented at a Symposium of the International Union of Pure and Applied Chemistry in April 1964 by four different groups (Fuhrman, 1986).

Structure of TTX

Tetrodotoxin has the empirical formulae $C_{11}\ H_{17}\ N_3\ O_8$ (Mosher *et al.*, 1964); it has one guanidine group, six hydroxyl groups and one hemilactal functional

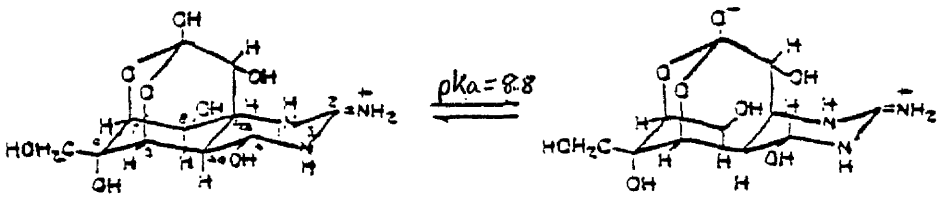
group (Maruyama and Noguchi, 1984). The structure which is shown in Figure 3 (Mosher, 1986) is based on extensive degradation studies and five separate x-ray crystallographic investigations on TTX derivatives (Mosher, 1986).

TTX, is stable in the crystalline state and in dilute acids, e.g. acetic acid; where it exists in the protonated hemilactal form, however, it is degraded by stronger acids, e.g. 0.1 M HCl. Strong bases form the carboxylate anion, which has only a transistory existence and soon decomposes to the fluorescent quinazoline derivative which is used in the detection and analysis of TTX.

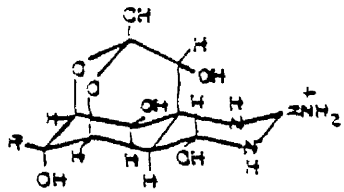
Crystalline TTX is not soluble in water, but is very soluble in dilute acid, from which it crystallizes slowly when the solution is brought back to neutrality. It does not dissolve to any appreciable extent in neutral organic solvents, such as methanol.

Derivatives of TTX

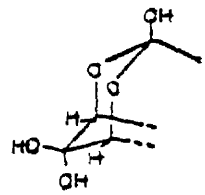
Several derivatives of TTX occur (Fig. 3) naturally and are often found alongside TTX. For instance Goto *et al.* (1985) reported that during isolation, TTX is in equilibrium with anhydro-TTX, which is readily converted to TTX in solution, particularly at low



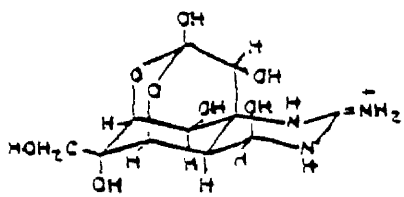
Tetrodotoxin*



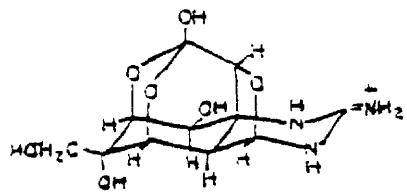
Chiriquitoxin**



Nortetrodotoxin**



4-epitetrodotoxin***



Anhydrotetrodotoxin***

* protonated deprotonation relation of C-10-OH group

** analogues modified in the C6 position.
Chiriquitoxin has an unidentified group (R) of 121 mass units. nor-TTX is shown as the ketone hydrate on C6 which exists in complex equilibrium with the ketone and lactone form.

*** Positions H and OH on C4 in 4-epi-TTX are reversed from those in TTX. Anhdro-TTX is formed by dehydration between C4 and C9.

(adapted from Kao 1966, and reproduced by kind permission of Pergamon Press).

pH. Conversely, TTX changes into a hydrated form in solution, equilibrium being 4 parts of TTX to 1 part of anhydro-TTX (Simidu *et al.*, 1987).

The derivatives of TTX share the specificity of the parent toxin in blocking only the Na^+ channel and occupying the same receptor (Kao, 1986). However, modification of the TTX structure at the guanidine group leads to products that are inactive, or at least have greatly reduced toxicity and sodium channel-blocking (SCB) activity. On the other hand, the TTX structure can be modified at the C6 and C11 regions of the molecule and still retain substantial activity. This was demonstrated by the isolation of chiriquitoxin (Fig. 3) along with TTX from the eggs of a Costa Rican frog (Pavelka *et al.*, 1977).

Chiriquitoxin has a toxicity similiar to that of TTX, with the same basic structure, but with a substituent of 121 mass units in place of the C11 CHOH group, and is less stable (Fuhrman, 1986). Also, chiriquitoxin blocks not only the Na^+ channel but also interferes with the movement of potassium ions (Kao, 1986).

In Table 1, the toxicity of TTX, STX and their derivatives are compared to some other well-known toxins.

TABLE 1. Relative potencies of TTX, STX, their derivatives and other well-known toxins

Toxin	Toxicity (μg/kg)*
TTX(pH 7.8)	10.0
TTX(pH8.8)	3.7
Chiriquitoxin	10.0
Nor-TTX	0.8-2.5
4,epi-TTX	4.0
Anhydro-TTX	0.2
STX(pH 7.25)	10.0
STX(pH 8.25)	6.0
Neo-STX	10.0
dc-STX	2.0
GTX II	2.0
GTX III	4.0
Botulinum toxin	0.00003
Diphtheria toxin	0.3
Sodium cyanide	10,000.0

(Adapted from Mosher et al., 1964 and Kao, 1986)

* Refers to minimum lethal dose by intraperitoneal administration to mice.

Origin of TTX

Puffer fish are distributed throughout most warm seas and some temperate ones and were originally thought to be the only species from which TTX could be isolated (Fuhrman, 1986). Tani (1945) examined 19 species of puffer; all were toxic to various degrees, but with intra- and inter-species variation.

Initially, TTX was considered to be a metabolic product of these toxic fish perhaps evolved for protection against predators (Furham, 1986). In 1964, however, a toxic substance originally discovered in the 1930's in the eggs of the Californian newt or salamander, *Taricha torosa* and named tarichatoxin, was also discovered in the adults, embryos and eggs of various species of Western American newts of the Genus *Taricha* and was unequivocally identified as TTX (Mosher *et al.*, 1964).

Subsequently, TTX has been isolated from animals of eight different classes in six different phyla (Table 2); such diversity of toxic species made the theory of metabolic production of TTX unlikely for the following reasons.

1) Diversity of species

There are no common factors or relationships in the

TABLE 2 Animals from which SCB toxins have been isolated

Phylum	class	common name	TTX	PSP	Reference
Chordata	Pisces	puffer fish	+	+	Tani, 1945; Lalone, 1963; Konosu, 1979; Kodama et al., 1984;Onue, 1984; Nakamura et al., 1984, 1985; Endo et al., 1988; Laobripatr et al., 1990;
"	"	parrot fish	+	-	Yasumoto et al., 1986a
"	"	goby fish	+	-	Hashimoto et al., 1971; Noguchi et al.,1971,1973
"	Amphibia	newts	+	-	Yasumoto et al., 1988; Fuhrman, 1986; Yotsu et al., 1990
"	"	atelopid frogs	+	-	Kim et al., 1975; Pavelka et al., 1977
Echinodermata	Asteroidea	starfish	+	-	Noguchi et al., 1982; Maruyama et al., 1984,1985
Arthropoda	Crustacea	crabs	+	+	Yasumoto et al., 1981, 1983, 1986a; Noguchi et al., 1983, 1986; Llewellyn 1989
"	Merostomata	horseshoe crab	+	+	Fusetani et al., 1983
Mollusca	Cephalopoida	blue ringed octopus	+	-	Scheumack, 1978; Hwang et al., 1989
"	Gastropoda	ivory shell	+	-	Yasumoto et al., 1981; Noguchi et al., 1981
"	"	trumpet shell	+	+	Narita et al., 1981; Sakai et al., 1987
"	"	frog shell	+	-	Noguchi et al., 1984
"	"	lined moon shell	+	-	Narita et al., 1984; Jeon et al., 1984 Hwang et al., 1990
"	"	green turban shell	-	+	Kotaki et al., 1981
Platy-helmithes	Turbellaria	flat worm	+	-	Miyazawa et al.,1986; Jeon et al., 1986
		poison worm	+	-	Thuesen et al., 1988
Nemertini	"	ribbon worm	+	-	Ali et al., 1990

groups of animals in which TTX has been found and there are only limited numbers of toxic species within these groups.

2) Cultured puffer fish are not toxic.

However, cultured fish became toxic when they were fed toxic livers, but not pure TTX, or when fish were in close contact with toxic fish (Matsui *et al.*, 1985).

3) Inter-species, intra-species, geographical and seasonal variations.

Inter- and intra-species variation in toxicity occurs in most of the species examined (Table 2), including the freshwater puffer (Laobhripatr *et al.*, 1990). Toxin concentrations in the same species also varied between locations (Hashimoto *et al.*, 1979) and with the season, e.g. the toxicity of the ribbon worm was highest in May, dropped sharply in September and increased by the following May (Ali et al., 1990). Other authors confirmed this finding in a variety of species. The principal toxin in the same geographically-close species also varied, e.g. in the xanthid crab, *Zanthid aureus*, either TTX or PSP was overwhelmingly dominant (Yasumura *et al.*, 1986). This was also true for the trumpet shell (Sakai *et al.*, 1987).

4) Tetrodotoxin Precursor

Palvelka (1977) suggested that since TTX was not extracted from eggs of an atelopid frog by water but was released with 3 % acetic acid, the acid was required to generate the toxin from a bound precursor. The toxin, once released, was water-soluble. TTX and small amounts of anhydro-TTX were also detected in the RNase T2 hydrolyslate of a non-toxic high molecular weight fraction separated from a highly toxic puffer liver. Kodama proposed that this was an inactive form of TTX bound in some unknown way to RNA present in the preparation (Kodama *et al.*, 1983a). A high molecular weight precursor of PSP was previously isolated from the scallop digestive gland by the same technique (Kodama *et al.*, 1982).

5) Chemical structure.

The unique chemical structure of TTX seemed unlikely to originate from any common biogenic pathway and experiments with radiolabelled precursors demonstrated a lack of metabolic incorporation (Mosher and Fuhrman, 1984).

These observations cast doubt on an endogenous source of TTX within the diverse group of animals. However, an exogenous source via the food chain seemed plausible. TTX-containing flatworms were proposed as the source of the toxification of

pufferfish (Jeon *et al.*, 1986) and TTX-containing starfish were found in the midgut of the toxic trumpet shell (Narita *et al.*, 1981; Noguchi *et al.*, 1982). However, the diverse eating habits and food supplies of such widely different creatures, as those listed on Table 2, made the theory of a food source less attractive and it also failed to explain the lack of toxicity in cultured fish.

Bacterial production of TTX

The proposal that TTX was produced by microorganisms living symbiotically within the animals seemed a reasonable explanation for some of the above observations. This possibility was first examined by Matsui, who postulated that symbiotic bacteria were involved in the toxification of puffer fish (Matsui *et al.*, 1985). This was confirmed by the isolation of a TTX-producing bacterium from the xanthid crab, *Atergatis floridus* by Noguchi *et al.* (1986) and the subsequent isolation of other TTX-producing bacteria from a variety of animals (Table 3).

However, Yasumoto *et al.* (1986) demonstrated that TTX was produced by bacteria and transmitted to other animals through the food chain in a coral reef environment.

Therefore, although bacteria may be the primary

TABLE 3 Bacteria producing SCB toxins

Bacteria	origin	medium	Volume of medium (ml)	Incubation time (days)	temp. (°C)	MU	Toxicity ng/ml	Cells or supernate	Toxin	Method	Author
Aphanizomenon flos-aquae	Cyanobacterium	-	-	-	-	-	-	cells	neo-stx, STX	-	Ikawa et *al.*, 1982
V. fischeri	xanthid crab	M.2	500	10	25	-	-	both	TTX, anhydro-ttx	HPLC, GC-MS UV spec;	Noguchi et *al.*, 1986
Shewanella alga	red calcareous alga	3%NaCl 1% polypeptone	12,000	12	25	-	16.6	both	"	HPLC, TLC mouse	Yasumoto et *al.*, 1986
V. alginolyticus *V. damsela* *Staphlococcus* *V. alginolyticus*	starfish	M.2	1000	3 10	25 25	213 -	42.6 -	supernate "	TTX, 4-epi, anhydro	HPLC, GC-MS UV spec	Narita et *al.*, 1987
V. alginolyticus	Fugu	M.2	500	7	25	2	0.3	cells	TTX	HPLC, GC-MS mouse	Noguchi et *al.*, 1987
V. cholerae *V. hydrophila*	-	-	-	-	-	-	0.1	-	SCB	brian membrane	Tamplin et *al.*, 1987
V. alginolyticus *V. anguillarum* *V. costicola* *V. harveyi* *V.parahaemolyticus* *Photobacterium phosphoreum* *A. hydrophila* *A. salmoncida* *Plesiomonas shiselloides*	culture collections	M.3	400	1	20	5	2.5	cells	anhydro-ttx	HPLC, GC-MS mouse	Simidu et *al.*, 1987
Alteromonas tetrodonis *V. pelagia*	Fugu	0.5 % polypeptone 0.25 % yeast extract 0.01 % FePo 0.5 % Tris-HCl	12,000	10	28	-	-	supernate	TTX, 4-epi anhydro	HPLC, GC-MS	Yotsu et *al.*, 1987
V. damsela *Vibrio sp.* *Pseudomanas sp* *Citrobacter freundi* *Acinetobactar sp.*	trumpet shell	?	1000	7-10	25	-	-	-	-	HPLC, GC-MS UV spec;	*Narita et *al.*, 1989
V. alginolyticus *Vibrio sp.* *Alteromonas*	horseshoe crab	-	-	-	-	-	-	-	-	-	*Kungsuwan et *al.*, 1989
Bacillus *Pseudomonas* *Alteromonas* *Vibrio*	blue-ringed octopus	M.1	400	1-2	20	5 3	2.5 1.5	cells	TTX	HPLC, GC-MS, TLC, elecrophoresis	Hwang et *al.*, 1989
V. alginolyticus	arrowworms		250	1	30	-	280-790	both	TTX	HPLC, tissue culture	Thuessen & Kogura 1989
Bacillus *Micrococcus* *Acinetobacter* *Aeromonas* *Alteromonas* *Moraxella* *Vibrio*	sediment		400	3	25	-	-	cells	TTX	"	Do et *al.*, 1990
Moraxella sp	dinoflagellate	M.2	1,000	3	25/4	20	2	cells	STX GTX, STX	HPLC, TLC HPLC, mouse tissue culture	Kodama et *al.*, 1988, 1989 Kodama et *al.*, 1990
Bacillus sp.	dinoflagellate	MB/SW	4,000	10/14	15	-	-	cells	GTX's, STX	HPLC, tissue	Ogata et *al.*,1990
Streptomyces	sediment		200	5/7	27	-	-	cells	TTX	HPLC, GC-MS, tissue culture culture	Do et *al.*, 1991

*In Japanese refer to Tamplin, 1990.

producers of TTX, the food chain may also be involved.

Bacterial species from the genera *Vibrio*, *Alteromonas*, *Staphylococcus*, *Aeromonas*, *Photobacterium*, *Pleisiomonas*, *Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Bacillus*, *Micrococcus*, *Moraxella* and *Streptomyces* have all been implicated as TTX producers (Table 3). Members of the genus *Vibrio* seem to be the most common isolates, particularly the species *V. alginolyticus*. This is hardly surprising since the internal flora of several of the toxic marine animals are predominantly vibrios, followed by *Pseudomonas*. This is the case with puffer fish (Noguchi *et al.*, 1987; Sugita *et al.*, 1988), xanthid crab, *Atergatis floridus* (Noguchi *et al.*,1986) and the starfish *Astropecten polyacathus* (Narita *et al.*, 1987). However, it is important to note that other animals such as oysters (Lovelace *et al.*, 1968; Grischkowsky and Liston, 1974; Austin *et al.*, 1988 and Utisalo 1988), lemon sole and skate (Liston, 1957) have microflora consisting predominately of vibrios but are not toxic. Also, Sugita *et al.* (1988), found that 100 cultured specimens of the normally toxic species *F. rubripe rubripe* were all non-toxic despite the presence in their intestinal flora of high numbers of vibrios. Therefore, although TTX-

producing ability has been reported over a wide spectrum of genera, only a limited number of species seem to be involved (Table 3).

Tamplin (1990), a co-author in the report that *V. cholerae* was found to produce SCB activity (Table 3) criticises the current findings by stating that "production by some bacteria has not been validated since TTX and anhydro-TTX are described as difficult to detect using HPLC and GC-MS methods and do not show activity in the mouse bioassay."

Paralytic Shellfish Poisons

For several centuries, medical records have described cases of food poisoning from eating mussels and clams at certain times of the year (Schantz, 1986) and with symptoms identical to those described previously for TTX.

The first Canadian record of this type of poisoning was from the voyages of Captain George Vancouver (1793), in his ship the Discovery, when one of his crew died and several became ill after eating toxic mussels (Waldichuk, 1990).

One of the most puzzling phenomena with this type of poisoning was its sporadic occurrence, the only clue to its presence was when human cases of poisoning began to appear, generally quite suddenly

and only lasting a week or two. The actual cause was not known until 1927 when outbreaks of mussel poison occurred along the central Californian coast. Sommer observed blooms of the dinoflagellate *Gonyaulax catanella* near the mussel beds and suspected that the dinoflagellates, upon which the mussels were feeding, might be poisonous.

Acidic extracts of the dinoflagellate and of the mussels, killed mice in the same manner (Sommer and Meyer, 1937; Sommer *et al.*, 1937). The mussels bound 90% of the poison in the dark gland or hepatopancreas and showed no physical signs of harm that would distinguish toxic from non-toxic animals.

This led Needler (1949), Prakash (1963) and others to observe that the dinoflagellate *Gonyaulax tamarensis* (*Alexandrium tamarensis*) caused clams and scallops along the north-east coast of North America and England to become poisonous.

Saxitoxin, which is chemically quite distinct from TTX, was first isolated in large quantities from the Alaska butter clam, *Saxidomus giganteus*, in 1957 (Shimizu, 1989). In 1975, four new toxins named gonyautoxins (GTX) I,II,III and IV were isolated from the soft shell clam, *Mya arenaria*, exposed to a massive red tide caused by *A. tamarensis* in Massachusetts (Shimizu, 1989). Since then, 18 derivatives of the tetrahydropurine structure of STX

(Mr. 299 dal) have been identified (Fig. 4) the potency of which vary considerably (Table 1). These toxins are collectively known as Paralytic Shellfish Poisons (PSP) (Schantz, 1986) and act on the Na^+ channel in the same manner as TTX.

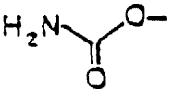
Origin of paralytic shellfish poisons

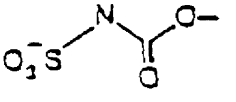
Red tides (Fig. 5), both toxic and non-toxic, have occurred throughout recorded history (Anderson, 1989), however, they now constitute a problem of global dimensions for fisheries, mariculture and public health (Fig. 6), since over the past 20 years the magnitude and geographic locations have increased (White, 1990). (For a full summary of toxic algal blooms and their effect on shellfish, the table of Shumway (1990), should be consulted). Therefore, the elucidation of mechanisms of toxification are of vital importance.

The pioneering work of Sommer and colleagues (1937) and the large body of data accumulated since then show that PSP are associated with the growth of dinoflagellates which then accumulate in filter-feeders and may be further distributed through trophic transfer.

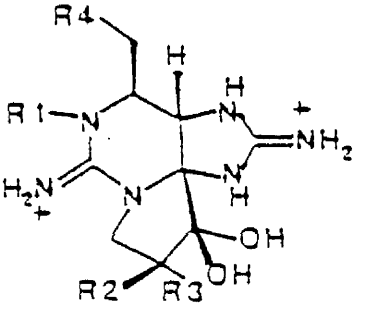
Three morphologically distinct genera of dinoflagellates are associated with PSP (Hall,

R1	R2	R3	Carbamate Toxins	Sulfamate Toxins	Decarbamoyl * Toxins
H	H	H	STX	B1	dc-STX
OH	H	H	NEO	B2	dc-NEO
OH	H	OSO_3^-	GTX I	C3	dc-GTX I
H	H	OSO_3^-	GTX II	C1	dc-GTX II
H	OSO_3^-	H	GTX III	C2	dc-GTX III
OH	OSO_3^-	H	GTX IV	C4	dc-GTX IV

R4: 

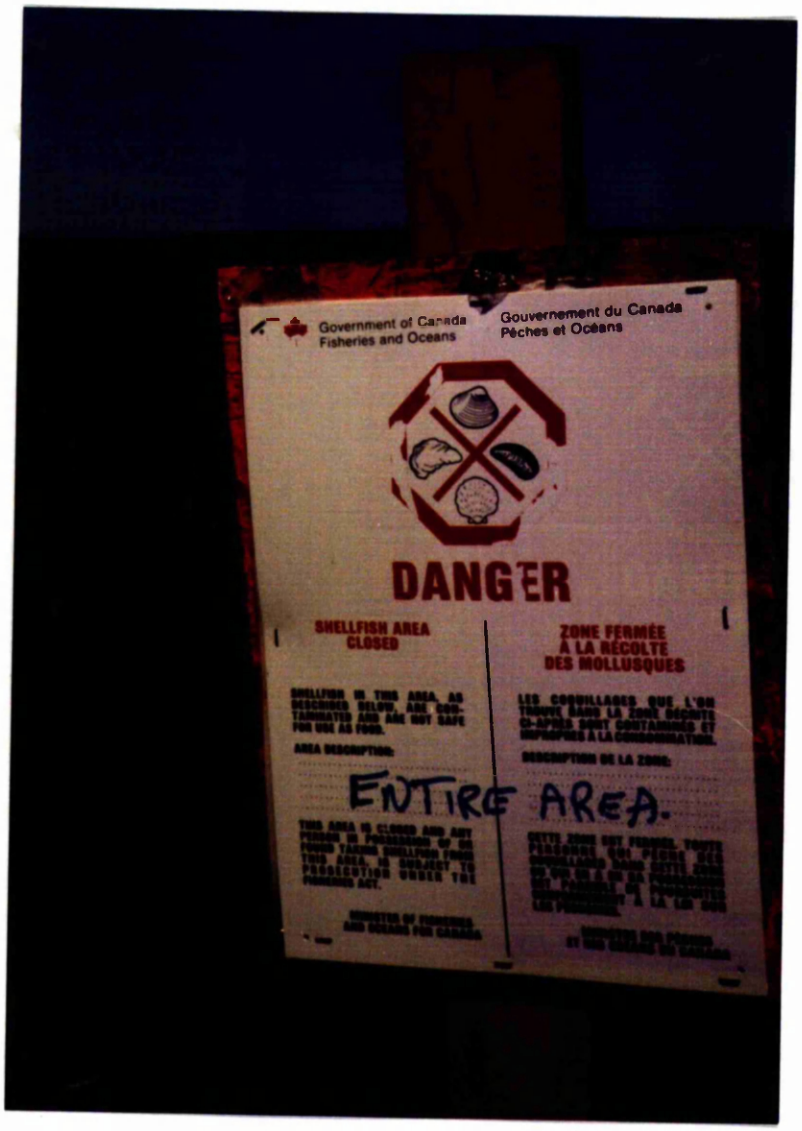
R4: H 

R4: HO—



* Several of the decarbamoyl toxins have not been reported but are postulated to occur in nature based on the presence of the others. (Adapted from Sullivan, 1990).

FIGURE 5 Red tides along the north-east coast of England, in the summer of 1990. (Taken from 29,000 feet and reproduced by kind permission of Prof. A.C. Wardlaw, Glasgow University, Microbiology Department).


Government of Canada
Fisheries and Oceans
Gouvernement du Canada
Pêches et Océans
DANGER
SHELLFISH AREA CLOSED
ZONE FERMÉE À LA RÉCOLTE DES MOLLUSQUES
ENTIRE AREA.

1990). However, PSP have also been isolated from several animals, e.g puffer fish, marine snails and xanthid crabs (Table 2) and also from a strain of macroalgae (Yasumoto *et al.*, 1983b). Several other points should be noted:

1) A dietary food source, particularly in the xanthid crab, is not evident and the sporadic distribution among individual speciments was difficult to reconcile with diet (Hall, 1990).

2) Many dinoflagellates involved in red tides do not produce confirmed biotoxins, even among known toxic organisms e.g. *Alexandrium* and *Gymnodinium* (Shimizu *et al.*, 1990), in the same locality (WHO, 1984).

3) As with TTX, PSP-containing animals also exhibit inter- and intra-species, geographic and seasonal variations as does the macroalga *Jania spp.*.

4) The toxicity of the dinoflagellate *Alexandrium* can be highly variable and intra- and inter-species variations occurs. Toxin profiles vary with various growth conditions, e.g. growth stages, salinity, cell cycle, temperature and light (Anderson *et al.*, 1990; 1991; Ogata *et al.*, 1989); also, it has been proposed that the toxin content of all species increased with decreasing growth rate.

5) The toxicity of scallops increased to a maximum when the population of *A. tamarenisis* was decreasing; the scallops showed toxicity for several months with a rise and fall of levels during the period when apparent supply of the toxin was suppressed (Kodama and Ogata, 1988).

6) Differences in toxicity were observed amongst various dinoflagellate sub-clones which were derived from a single cloned strain (Kodama and Ogata, 1988), Kodama concluded that toxin production of *A. tamarensis* was not a hereditary characteristic.

7) Kodama identified PSP from particles of bacterial size separated from seawater during a period when bivalve toxicity increased in the absence of toxic dinoflagellates and also from particles of a similiar size separated from cell-free cultured medium of *A. tamarensis* (Kodama *et al.*, 1990a).

8) As with TTX, PSP have been identified in RNase T_2 hydrolysates of a non-toxic, high molecular weight fraction obtained from the digestive gland of the scallop *Patinopecten yessoensis* and from RNA of the dinoflagellate *Protogonyaulax* (Kodama *et al.*, 1983b). An RNase hydrolysate of the hepatopancreas of a horseshoe crab also contained

PSP. However, these results were not always reproducible (Fusetani *et al.*, 1983).

As with TTX, these factors have led to the investigation of bacterial involvement in the toxification of dinoflagellates.

Bacterial involvement in PSP production.

Bacteria are universally associated with algae in the ocean. The fact that many algae grow more slowly, if at all, in axenic conditions suggests that the associations constitute a form of symbiosis (Tostesen *et al.*, 1989) and as such, the role of bacteria in the toxicity of toxic algae has been considered.

Bacterial transformation.

Differences in toxin profile between the intoxicated animal and the apparent dinoflagellate source often occurred by selective retention and metabolic transformation by the accumulating organs. (Hall, 1985). This is of great importance as the sulphamates (Fig. 4), which have very low toxicity and are easily transformed to the carbamates (Fig. 4) of high toxicity, are generally a major component of the toxin mixture of the dinoflagellate and therefore constitute a substantial reservoir of cryptic or latent potency (Hall, 1985). This

conversion of toxins between dinoflagellates and contaminated shellfish has been reported in scallops, mussels and oyster (Oshima *et al.*, 1990) and can be carried out by bacteria such as *Pseudomonas* and *Vibrio* (Kotaki *et al.*, 1985).

Extracellular bacteria

The association of external bacteria in toxin production of *A. tamarensis* was considered by Shilo and Aschner (1953) who reported that bacteria may alter the toxicity of *P. brevis*. This organism produced organic compounds which stimulated bacterial growth, which in turn led to synthesis of growth factors required by dinoflagellates (Wilson, 1955).

Riquelme *et al.* (1989), found that the extracellular organic carbon produced by the toxic diatom *Asterionella glacialis* stimulated the growth of the dominant strain of *Pseudomonas* but did not have any effect on the *Vibrio* strains present.

Romalde *et al.*(1990a) proposed that a "Phycosphere" existed during a dinoflagellate bloom, under the influence of which the bacterial activity could be altered, with the possible selective stimultion of certain bacteria. This occurred with strains of *A. tamarensis* and *P. catenella* which

selectively influenced the growth of their associated bacteria. However, from different isolates of the same dinoflagellate, different predominant bacteria were obtained in culture (Nelinda *et al.*, 1985). This has also been reported for *Ostreopsis lenticularis* and *Gambierdiscus toxicus* (Tostesen *et al.*, 1989). The diversity of bacterial strains in the early stages of all the blooms was very low, with only one or two species in abundance (Romalde *et al.*, 1990 a,b). Vibrio numbers during a bloom have been reported to increase (Romalde *et al.*, 1990a,b; Buck and Pierce, 1989), however, others have reported that they decrease (Riquelme *et al.*, 1987; Romalde *et al.*, 1990a).

Several workers have investigated the role of dinoflagellate-associated bacteria in toxin production. Kodama and Ogata, (1988) found toxin in *A. tamarensis* and *Ptychodiscus brevis* cultured under axenic conditions, whereas Tostesen *et al.*, (1989) reported that *Gymnodinium veneficum* lost toxicity in axenic culture.

Nelinda *et al.*, (1985) did not detect any toxicity from a pure culture of dominant bacteria isolated from *A. tamarensis*, nor was toxicity detected in extracts of bacteria isolated from *Ostreopsis lenticularis* and *Gambierdiscus toxicus* (Tostensen *et*

al., 1989). However, peak *Ostreopis* cell toxicities, in the stationary phase of growth, were correlated with a significant increase in the bacteria directly associated with these cells (Tostesen *et al.*, 1989). Paradoxically, Tostensen stated that "changes in the quantity of bacteria directly associated with microalgal surfaces and extracellular matrices during culture growth may be related to variability and degree of toxicity in these laboratory cultured benthic dinoflagellates."

Intracellular bacteria

Kodama and co-workers have been the main protagonists for the view that toxic bacteria are present in dinoflagellates, since bacteria are believed to occur within the cell in some species of dinoflagellates (Leedale, 1969; Silva, 1979, 1982). Silva hypothesized that intracellular bacteria associated with *A. tamarensis* were involved in toxicity. Kodama and Ogata (1988), using transmission electron microscopy, (TEM) demonstrated rod-shaped bacteria in 10 - 15% of strongly toxic dinoflagellate clones. However, Taylor (1990) stated that Kodama's TEM did not show bacteria clearly and intensive examinations by several other groups have been unable to demonstrate bacteria within cells of

the most toxic species of *Alexandrium* even though they appeared to be quite common in the nucleus of other non-toxic dinoflagellates (Nelinda *et al.*, 1985).

Nevertheless, Kodama isolated an intracellular bacterium from *A. tamarensis* identified as *Moraxella spp.*, which produced STX (Kodama *et al.*, 1988). Due to the small quantities of toxin produced and the differing PSP profiles between the bacterium and the dinoflagellate, he proposed that *A. tamerensis* enhanced toxin production by the bacteria and converted STX to its various derivatives.

Later work indicated a positive relationship between the toxicity of *A. tamarensis* and the associated bacterial numbers. In addition, bacteria were not obtained from non-toxic dinoflagellates (Kodama and Ogata, 1988; Kodama *et al.*, 1989). Toxin was produced by *Moraxella spp.* only after 64 h in nutrient-rich medium. However, starved conditions induced toxicity after 24 h which increased considerably up to the 10th day (Kodama *et al.*, 1990b). Later, Kodama's group reported that the amount of toxin produced by the *Moraxella* varied even when grown under the same conditions, indicating that unknown factors regulated toxin production. They also isolated a toxic *Bacillus sp.* from *Gymnodinium catenatum* (Ogata *et al.*, 1990).

The occurrence of PSP in bacteria has a precedent in that both STX and neo-STX have been identified as the neurotoxins produced by the freshwater Cyanobacterium Aphanizomenon *flos aquae* (Sasner et al.,1984) and the biosynthetic pathway for their production has been elucidated (Shimizu et al., 1984).

Mode of Action of SCB Toxins

Sodium channel-blocking (SCB) toxins prevent nerve conduction by a highly specific and reversible blockage of the inward movement of Na^+ through the cell membrane of the activated neuron (Mosher and Fuhrman, 1984). In order to understand this mechanism and the subsequent use of tissue culture as an assay, the generation of nerve conduction will be discussed.

Generation of nerve conduction

The resting cell

The following account of the generation of nerve conduction is edited from Darnell *et al.*, (1990). With the exception of water, the major chemical substances in animal extracellular fluid are sodium and chloride ions, whereas the intracellular fluid contains high concentrations of potassium and organic molecules.

All cells under resting conditions have an electrical potential across the plasma membrane orientated so that the inside of the cell is negatively-charged with respect to the outside. The resting membrane potential is determined by the differences in ion concentrations of the intracellular and extracellular fluid and the permeability of the plasma membrane to different ion species i.e. the membrane is 50-75 times more permeable to K^+ ions than to Na^+ ions. Since Na^+, K^+ and Cl^- ions are present in the highest concentration they generally play the most important roles in the generation of resting membrane potential.

The value of the equilibrium potential for any ion depends on the concentration gradient for that ion across the membrane; the larger it is the larger the equilibrium potential, due to a higher electrical flux required to balance the concentration difference.

The permeability of the membrane to a given ion species depends upon the number of open channels through which an ion can cross the membrane. For a given concentration gradient, the greater the membrane permeability to an ion species the greater the contribution that ion species will make to the membrane potential.

At -70 mV, neither Na^+, or K^+ are at equilibrium potential, therefore there is a net movement of Na^+ into the cell and K^+ out. However, Na-K ATPase pumps Na^+ back out and K^+ back in to the cell at a ratio of $3Na^+$: $2K^+$. This unequal transport directly increases the separation of charge across the membrane.

Electrical signals

Transient changes in the membrane potential from its resting level constitute electric signals which can alter cell activities. One such signal is the action potential, which is the rapid alteration in membrane potential, that may last only 1 ms, during which the membrane potential may change from -70 mV to +30 mV and then repolarise to the original value. This is the method by which the nervous system communicates over long distances. Only a few types of cells, e.g. nerve, muscle, cardiac and some gland cells have plasma membranes capable of this function (excitable membranes).

In the resting state, the open channels are predominently permeable to K^+ and Cl^-. Almost all the Na^+ ion channels are closed and the resting potential is much closer to the K^+ equilibrium potential. However, during an action potential, this is markedly altered and a transient change in

membrane ion permeability occurs. When the membrane potential is less negative (i.e. depolarized), Na^+ channels open, increasing the membrane's permeability to Na^+ ions several-hundred fold, allowing Na+ ions to rush into the cell. Therefore, more +ve charge is entering in the form of Na+ ions than is leaving in the form of K^+ ions and the polarity reverses, becoming +ve inside and -ve outside the membrane.

Depolarization opens the channels so that the membrane permeability to Na^+ ions increases and Na^+ ions diffuse into the cell. This addition of net positive charges further depolarises the membrane which, in turn produces a still greater increase in Na^+ ion permeability lasting for around 1 ms, allowing approximately 6000 Na^+ ions to pass through. The Na^+ channels then close spontaneously and enter an inactive state from which they cannot be opened, thereby limiting, for several milliseconds, the number of action potentials that a neuron can conduct. This restores the membrane potential to its resting level and can be accelerated by the opening of a special set of K^+ channels, thereby increasing K^+ ion permeability and leading to a rate of K^+ diffusion out of the cell that is greater than the rate of Na^+ ion diffusion into the cell. Thus the membrane potential is

rapidly returned to its resting level and the channels return to the closed, activable state where they will reopen in response to depolarization.

Not all depolarizations lead to an action potential. These only occur after enough Na^+ channels are open so that Na^+ entry exceeds the flux of K^+ out of the cell. Once generated, each action potential triggers, by local current flow, a new one at an adjacent area of the membrane hence allowing nerve conduction over long distances.

The Na^+ and K^+ channels that determine the resting potential of the cell, "the resting or leakage channels", are not voltage-dependent; they do not open or close during the action potential and, as judged by the effects of inhibitors, are different proteins from the voltage-gated channels. (Darnell *et al.*, 1990).

The sodium channel

In the mammalian brain, sodium channels consist of a heterotrimeric glycoprotein complex of α (260 Kdal), ß1 (36 Kdal) and ß2 (33 Kdal) subunits, whereas the Na^+ channel from mammalian skeletal muscle contains only subunits of 260 Kdal and 38 Kdal which are analogous to the α and B1 subunits of the brain Na^+ channel (Catterall, 1985).

The amino acid sequence of the channel proteins has been deduced from cDNA sequences from both the electric eel and the rat and consists of four homologous internal repeat sequences of around 100 amino acids, each containing five hydrophobic segments at equivalent positions, together with a characteristic segment with several positive charges (Prince, 1988). These sequences are connected and flanked by shorter stretches of non-homologous residues and do not have a hydrophobic leader sequence at the N-terminus (Catterall, 1985).

For details of the proposed topological model of the folding of the channel polypeptide across the membrane and the general location of the trans-membrane pore and gating charge, the reader is referred to Noda *et al.* (1986).

The physiological function of the channel is to induce transient fluctuations in the membrane potential by opening a conducting pathway which is selective for Na^+ ions (Agnew *et al.*, 1986).
The channels open, or "gate," in response to a range of different stimuli that permutate the population distribution among a set of possible channel conformations, i.e. kinetic and thermodynamically stable changes in formation which depend on voltage (Agnew *et al.*, 1986). Upon depolarization, for example, Na^+ channels pass through multiple closed

states before activation; this requirement for each domain to undergo conformational change in order to achieve activation provides a structural basis for the sigmoid time curve of channel activation (Catterall, 1985).

The Na^+ channel can therefore be described as a catalyst for the reaction whereby Na^+ ions pass through an otherwise insulating high-resistance membrane.

However, distinct, although small, differences exist among channels from different sources (Agnew *et al.*, 1986) and certain quantitative physiological and pharmacological properties of Na^+ channels differ among neuronal, skeletal, muscular and myocardial cells (Strichartz, 1988). Three distinct but structurally similar, Na^+ channel genes are expressed in the mammalian brain (Agnew *et al.*, 1986).

Action of SCB toxins

TTX and STX bind competitively (Strichartz *et al.*, 1986) to the principal functional component of the Na+ channel, the transmembrane protein known as the α subunit (Catterall, 1985), thus causing highly specific and reversible blockage of the inward movement of Na^+ ions through the cell membrane of an

activated neuron (Mosher and Fuhrman, 1984). As little as 1-10 nM TTX in nerve and muscle can cause this blockage (Hurst, 1985) without any effect on K^+ ions (Mosher and Fuhrman, 1984); (note that chiriquitoxin does affect K^+ ion transport, Kao, 1986).

Some excitable tissues, e.g. neuronal cells and innervated mammalian skeletal muscle, express Na+ channels that are highly sensitive to TTX. Conversely, denervated skeletal muscle and cardiac muscle cells express Na^+ channels which are 100 to 1000 times less sensitive to TTX (Frelin *et al.*, 1990).

The TTX/STX binding site is also recognized by conotoxins from the venom of *Conus georgraphus* which specifically inhibit Na^+ channels from innervated skeletal muscle and tissue from the eel *Electropax* in a similiar way to TTX. However they do not act upon Na^+ channels from neuronal tissue or TTX-insensitive Na^+ channels of denervated muscle and heart (Frelin *et al.*, 1990).

Active groups of both TTX and STX which are essential for blocking the channel are listed on table 4. There are remarkable stereospecific similarities between these active groups although the molecules of STX and TTX are chemically quite different (Kao, 1986).

TABLE 4. Active groups of tetrodotoxin and saxitoxin

TTX	STX
(1,2,3 Guanidinium)	7,8,9 guanidinium
C-9 - OH	C-12-OH
C-10 -OH	C-12-OH
C-4 - OH	-
(C-8-OH)	carbonyl

(groups in parentheses cannot be verified by direct experimental data. Adapted from Kao, 1986 and reproduced by kind permission of Perqamon Press)

Although, TTX and STX act upon Na^+ channels with the same physiological consequences, the chemistry of binding differs between these two toxins. Binding and blocking of both are inhibited at low pH (5.5) and high pH (8.5) and by high concentrations of polyvalent cations in the bathing solution (Strichartz *et al.*, 1986). However, binding of STX is more sensitive than that of TTX to elevated cation concentration and is less sensitive to increases in temperature.

Also, excitable tissues from certain animals that are less sensitive to the effects of one of these toxins sometimes show near normal sensitivity to the other (Strichartz et al., 1986). Therefore, the molecular interactions of TTX and STX with the Na^+ channel are not identical, e.g. in the rainbow trout and the Atlantic puffer (*Spheroides*), STX exhibits a faster rate of action and recovery than TTX. This has been interpreted to mean that the receptors are different but overlap (Yamamori and Nakamura, 1988).

In shore crabs, a seasonal fluctuation in sensitivity to STX has been observed and attributed to the presence of red tide blooms; the resistant shore crab remained sensitive to TTX, to which it was not normally exposed, even through sensitivity to STX was reduced (Barber, 1988a).

It was later found that STX-resistant crabs contained a high molecular weight protein which was not observed in STX-sensitive crabs and demonstrated that crabs sensitive to STX can be rendered insensitive in a dose dependent manner (Barber, 1988b).

Several neurotoxins other than TTX and STX exert their influence on the sodium channel (Table 5) by binding to different receptor sites and these have been used extensively in Na^+ channel research. (Catterall, 1985).

One such substance, veratridine, is used in the tissue culture assay which was employed extensively in this thesis.

Detection of SCB Toxins

Several methods have been used to identify SCB toxins (Table 6), but the principal ones, for both TTX and PSP's, are HPLC (high pressure liquid chromatography) and the mouse bioassay.

Instrumental analysis

Tetrodotoxin

Instrumental methods for detection of TTX include HPLC and although variations of the technique exist (Nakumura *et al.*, 1985, Nagashima *et al.*, 1987), the method of choice is that of Yasumoto which has been

TABLE 5 Neurotoxin receptor sites associated with the sodium channel.

Receptor	Ligand site	Physiological effect
1.	Tetrodotoxin Saxitoxin	Inhibit transport
2.	Veratridine Batrachotoxin Aconitine Grayanotoxin	Cause persistent activation
3.	α-Scorpion toxins Sea anemone toxins	Inhibit activation Enhance persistent activation
4.	β-Scorpion toxins	Repetitive firing Shift activation
5.	Brevetoxin	Repetitive firing Enhance persistent activation

(adapted from Catterall, 1985; and reproduced by kind permission of Elsevier Science Publishing Company).

TABLE 6 Methods for detecting SCB toxins

Method	Reference	Sensitivity (ng/ml)
Pig ileum	Hamad, 1957	5.0
Frog nerves	Kao, 1963	9.6
	Strong, 1973	2.5
Crab nerves	Keynes, 1967	4.5
Lobster nerves	Moore, 1967	95.7
Mouse assay	Evan, 1972	200.0
	Onue, 1984	-
	WHO, 1984	40 µg*
Fluorimetric	Nunez et al., 1976	340.0
Spectroscopy	Svenaga, 1978	30.0
Crude brain membranes	Davio and Fontelo, 1984	0.8-0.15
	Huot et al., 1989	<1
HPLC	Yasumoto and Michishita, 1985	4.4-3700
	Nakamura et al., 1985	20.0
	Nagashima et al., 1987	-
	Yotsu et al., 1989	9.6-9450
	Sullivan & Wekell, 1986	10-30 µg*
TLC-FAB	Nagashima et al., 1988	100.0
TLC-FID	Ikebuchi et al., 1988	40.0
Monoclonal antibodies	Huot et al., 1989	100.0
Tissue culture	Kogure et al., 1989	0.2

* STX as expressed as µg STX equiv/100 g mussel extract

progressively modified through the years (Yasumoto *et al.*, 1982, 1985, Yotsu *et al.*, 1989).

Other common analytical techniques include gas chromatography linked to a mass spectrophotometer (GC-MS), ultra-violet spectroscopy (UV-spec), nuclear magnetic resonance (NMR), electrophoresis and fluorimetric spectroscopy (Nunez *et al.*, 1976, Suenaga, 1978). All of these methods involve laborious extractions of toxins from large volumes, using charcoal and Biogel columns, followed by treatment with sodium hydroxide to produce the C9-base fluorescent derivative; GC-MS and NMR also involve further derivatisation.

Less commonly used methods include thin-layer chromatography linked to fast atom bombardment (FAB) mass spectrometry (Nagashima *et al.*, 1988) and thin-layer chromatography with flame ionization detection (TLC-FID), (Ikebuchi *et al.*, 1988).

Most of the methods can be criticised for their lack of sensitivity, e.g. ^{3}H and ^{13}C NMR show poor resolution due to the hemilactal-lactone tautomerism (Yasumoto *et al.*, 1988). Also, many of the published chromatographs for HPLC analysis are not clear, and the technique is cumbersome and prone to regular failure.

A non-instrumental chromatographic method is thin-layer chromatography (TLC), but again sensitivity is

very low.

Paralytic shellfish poisons

Unlike TTX, instrumental methods for detection of PSP toxins involve almost solely HPLC. Liquid chromatography was first used, in conjunction with the fluorescent technique of Bates & Rapoport (1975), by Buckley *et al.* (1978) and later improved by using HPLC (Sullivan and Iwaoka 1983). Subsequent improvements published by Sullivan and colleagues (Sullivan *et al.*, 1985, Sullivan and Wekell, 1986) represent advances over both his 1983 method and the original fluorescence method and have achieved detection limits of 10-30 µg STX equiv/100g (Sullivan and Wekell, 1986).

The use of HPLC in place of the mouse bioassay for determining toxicity levels in mussel extracts has been examined by 3 groups and high correlations were found between the methods (Table 7). However, Waldock *et al.* (1991) reported that with the large bloom which occurred off the north-east coast of England, in the summer of 1990, the HPLC method was not sufficiently rapid or robust to support the large spatial and temporal survey required for several marine species. Co-elution of compounds with different toxicities could also be a problem.

TABLE 7. Correlation coefficients between HPLC and the mouse bioassay.

Correlation coefficient	Sample size	Reference
0.90	68	Sullivan *et al.*, 1985
0.82	19	Fileman, 1988
0.79	14	Waldock *et al.*, 1991

Immunochemical methods

As immunochemical methods of detection are, in general, sensitive and relatively inexpensive, efforts have been made to produce antibodies to the SCB toxins.

A monoclonal antibody (MAB) to tetrodonic acid was raised by Watabe *et al.* (1989), but the reactivity to TTX was rather low. Two monoclonal antibodies of high sensitivity to TTX and no cross-reactivity with STX were produced by Huot *et al.* (1989a) and a detection limit of 100 nq/ml was achieved for TTX .

Several workers have also produced anti-STX antibodies (Johnson *et al.*, 1964; Carlson *et al.*, 1984; Chu and Fan, 1985) and monoclonal antibodies (Davio *et al.*, 1985; Huot *et al.*, 1989 a,b), two of which neutralized the activity of STX. A polyclonal antibody with a high affinity for STX, but which cross reacted to various degrees with other derivatives, has also been tested (Cembella *et al.*, 1990).

This has led to the commercial availability of two separate ELISA kits for STX. However, these often give false positive results (Taylor, 1990). The large numbers of toxins in the STX toxin family poses another problem for ELISA kits because, although they may be of lower toxicity than the

parent compound, they may be converted within the animals to more toxic forms and therefore, must still be detected.

There is also a radioimmunoassay for STX (Hall, 1985) which is reportedly 1000 times more sensitive than the mouse assay. However, the shelf life of the assay vials was short and the method was not very sensitive for STX derivatives (Hurst, 1985).

Bioassays

Bioassays for detection of SCB toxins include the use of animal nerves, crude brain membranes and flies (Table 6). However, such techniques were generally too slow and cumbersome to allow the analysis of large numbers of samples and in some instances they require expensive equipment.

The mouse bioassay

The major bioassay and official method for both TTX and PSP toxins (Association of Official Analytical Chemists, 1984) is the mouse bioassay. In this assay, male mice of a specific strain, weighing between 19-22 g are injected i.p. with serial dilutions of the test preparations. The time of death for the various dilutions is recorded and compared to tables or a standard dose-response curve

for TTX or STX (which give responses with different slopes).

One mouse unit (MU) for STX is defined as the "amount of toxin which will kill a 20 g mouse in 15 min" when 1 ml of acidified extract of mussel tissue is injected intraperitoneally. This has been reported to be equivalent to 200 ng STX (Fileman, 1988) or 180 ng STX (Waldock *et al.*, 1991).

For TTX, one mouse unit is defined as "the dose of toxin which kills a 20g mouse in 30 min" (Kodama *et al.*, 1985), however, some variations in this definition exist (Mosher *et al.*, 1964; Palvelka *et al.*,1977; Hashimoto *et al.*, 1971). The actual amount of TTX attributed to a mouse unit has also varied, e.g. 1 MU = 143 ng TTX (Mosher *et al.*, 1964) or 220 ng TTX (Yasumoto *et al.*, 1985). However, the 30 min death time is most commonly used and 1 MU for TTX is generally quoted as 200 ng TTX (Evans, 1972; Thuesen and Koqure, 1989). By extrapolation, the lethal dose of TTX in humans, has been calculated to be 10,000 MU (Maruyama and Noguchi, 1984; Onüe, 1984)

Detection limits for STX, using the mouse bioassay, are quoted as 32 - 58 µg STX equiv/100g shellfish (Hurst, 1985) or 40 µg STX equiv/100g (WHO, 1984). The accuracy is given as ± 20 %. The established action level at which shellfish are

considered unfit for human consumption is 80 µg STX equiv/100g (Fileman, 1988) which is equivalent to 400 MU/100g (AOAC, 1984). The comparative potencies of the various PSP toxins by this assay are listed by Sullivan and Wekell (1986).

The major disadvantages of the assay are that variations in sensitivity can be caused by differences in sex, weight and strain of the animal. Also, the pH and concentration of NaCl in the sample can cause variations (Evans, 1972), e.g. even 0.1 % NaCl will reduce the TTX assay to 65 % of the true value. Other interfering substances may be present in crude preparations (Onue, 1984) and close to the detection limit, the toxin concentrations may be underestimated by as much as 60 % (WHO, 1984).

As each analogue of the STX has a different specific toxicity, the mouse assay may dramatically misrepresent the true toxin concentration on a molar basis. This is especially important if a large percentage of the less toxic analogues are present (Boyer *et al.*, 1985). It has also been claimed that the mouse assay is not sufficiently sensitive to detect concentrations of TTX commonly found in natural samples (Kogure *et al.*, 1988a).

Tissue culture assay

Nerve cells are characterised by their unique morphological appearance, the possession of exciteable membranes and specialized biochemical machinery (Kimhi 1976).

The use of tissue culture to study the properties of such cells was first investigated by Augusti-Tocco and Sato (1969). They found that 79 % of a mouse neuroblastoma (MNB) cloned cell line, obtained from a spontaneous tumour maintained since 1940 by serial transplantation in strain A/J mice and adapted to *in vitro* culture in 1967 (Klebe and Ruddle, 1969), was capable of generating action potentials. Subsequent work (Nelson *et al.*, 1969), showed that while normal mature neurons usually do not divide, at least some neuroblastoma tumour cells retained the ability to divide *in vitro* and also exhibited properties expected of neurons. Most of the cells had active membranes in which action potentials could be blocked by TTX, suggesting that a Na^+ ionophore identical to nerve axons was present in those cells (Nelson *et al.*, 1971; Spector, 1973).

It was then established that the transition of a culture of neuroblastoma cells from the actively-dividing state to confluence of the monolayer was characterized by the synthesis of various enzymes involved in neurotransmitter metabolism, enhancement

of electrical excitability and some degree of process formation (Kimhi, 1976).

The use of cultured MNB cells provided an excellent model system with which Catterall and Nirenberg (1975) studied the action potential Na+ ionophore by ion flux methods. Catterall used various clones of the mouse neuroblastoma cell line C1300 at the stationary phase of growth, in a series of elegant experiments which established the mode of action of several neurotoxins and their interactions with the Na^+ channel (Catterall and Nirenberg, 1973; Catterall, 1975 a,b; Catterall and Ray, 1976; Catterall, 1977).

Initial experiments, involving veratridine (ver) resulted in a marked increase in passive Na^+ permeability detectable by measurements of $^{22}Na^+$ uptake (Catterall and Nirenberg, 1973). Since the relatively low intracellular Na^+ ion concentration was maintained by the action of Na^+/K^+ activated ATPase, which exchanges internal Na^+ ions for external K^+ ions against the concentration gradient of these ions, the Na^+ ion uptake was initiated by inhibiting the action of this pump with ouabain (oub).

The stimulation of uptake was half-maximal at 0.5 mM oub and complete at 2.0 mM oub. The half-maximal

uptake of $^{22}Na^+$ for ver was 0.04 mM and approached completion at 0.1 to 0.2 mM. In the presence of 5 mM oub, external Na^+ ions entered the cells, following a logarithmic time course with T 1/2 = 30-50 min; ver at 100 µM reduced T 1/2 to 5-10 min, without changing the equilibrium intracellular Na^+ concentration (Catterall, 1975a). This effect was completely inhibited by 1 µM TTX. Using 5.0 mM oub and 0.1 mM ver the half-maximal inhibition of action potential was 3 to 80 nM TTX, depending on the clone used. Complete inhibition occurred at 300 nM TTX (Catterall and Nirenberg, 1973), later reported to be 11 nM (Catterall, 1975a); a diagrammatic representation of the action of oub/ver/TTX on a MNB cell is shown in Fig 7.

Lowering Na^+ ion concentration in the medium lowered the dissociation constant for TTX, suggesting that competitive interaction between TTX and Na^+ ions may occur (Catterall, 1975a). It has subsequently been reported that blocking of the channel by TTX is antagonized by Na^+ ions, Ca^{2+} ions and H^+ ions (Strichartz and Castle, 1990).

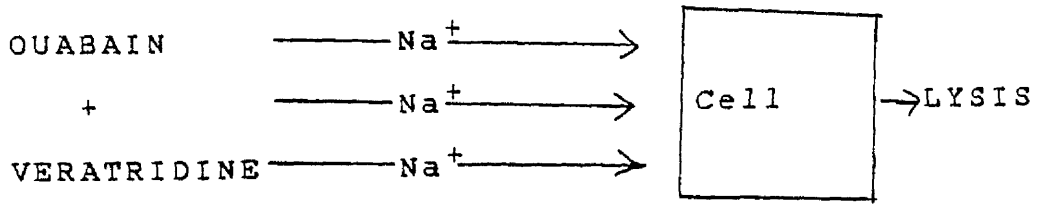
It was later shown that ver is only a partial agonist which activates about 8 % of Na^+ channels at saturation (West and Catterall, 1979) and is inhibited competitively by divalent cations such as Ca^{2+} and non-competitively by TTX (Catterall, 1975a).

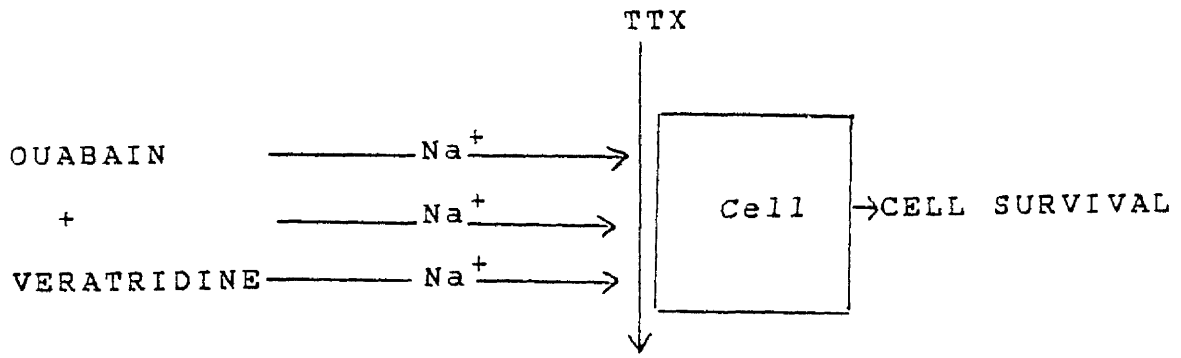
Further experiments using these cell lines involved the use of other neurotoxins e.g. scorpion toxin, and led to the present theory on the action of the Na^+ channel.

The tissue culture method of Catterall was modified by Kogure *et al.* (1988a) to form a quantitative assay for the detection of TTX and STX. Using the same cell line, oub/ver and principles previously established, the influx of Na^+ ions, if left unchecked, caused swelling of cells, weakening of intracellular organelle structure, loss of typical neuronal shape, and eventual cell death (Kogure *et al.*, 1989). Addition of TTX prevented this (Fig. 7). Live and dead cells were observed microscopically within 8 h of toxin treatment and cell rounding was assessed quantitatively.

An important feature of this assay was its direct utility for sodium channel-blocking activity, in that the presence of SCB toxins resulted in normal cell viability. At the optimum concentrations of ouabain (1 mM) and veratridine (0.05 mM), 1 nM TTX prevented the cells from dying (20 %), whereas with 100 nM TTX, 90 % of the cells survived. The authors stated that the minimum detectable level of TTX was 3 nM (Kogure *et al.*, 1988a) or 1 ng

FIGURE 7 Diagrammatic representation of the interaction of neurotoxins with a MNB cell.


OUABAIN
+
VERATRIDINE
Na+
Na+
Na+
Cell
LYSIS


TTX
OUABAIN
+
VERATRIDINE
Na+
Na+
Na+
Cell
CELL SURVIVAL

(Kogure *et al.*, 1989) which is equivalent to 10^{-3} MU and has a sensitivity of at least 2-10 times greater than HPLC (Kogure *et al.*, 1988a).

This method has since been used to detect TTX in marine sediments (Kogure *et al.*, 1988b) in eubacteria and in actinomycetes isolated from these sediments (Do *et al.*, 1990, 1991), in bacteria isolated from arrowworms (Thuesen and Kogure, 1989) and in investigations of STX-producing bacteria from dinoflagellates (Kodama *et al.*, 1990a; Ogata *et al.*, 1990). However, its use has only been reported by Kogure and his colleagues and thus, awaits independent confirmation. This is probably due to several problems associated with the assay, the modification of which forms a large section of this thesis.

CILIOSTATIC TOXINS

Aquaculture of Shellfish

The ever-increasing world population and the decline in natural populations of both fish and shellfish has encouraged interest in aquaculture (Trust, 1986). Shellfish such as mussels, oysters, clams, crabs, lobsters and shrimps have been subjected to varying degrees of cultivation in different parts of the world by high-density commercial production,

made possible by the development of intensive husbandry systems for the early life stage of the molluscs.

In Scotland, six species of shellfish are cultured, with 457 tonnes of mussels, 1.4 million oysters, 105,000 scallops and 1.4 million queen scallops farmed in 1990 (Munro, 1991).

Diseases of shellfish in aquaculture

Unfortunately, the development of aquaculture has been accompanied by the appearance of a variety of debilitating and serious diseases. Such infections typically occur in the development of high-density, highly-stressed husbandry of many species (Elston, 1984).

Epizootics of disease can completely destroy productivity for extended periods and may be caused by bacteria, fungi, protozoa or viruses. However, bacteria are the most common agents (Elston, 1984), causing recurrent problems which result in sudden mass mortalities of cultured bivalve larvae and spat.

Initial work into the association of bacteria with disease of bivalve larvae was done by Walne (1958). Subsequently, there have been numerous worldwide reports implicating bacteria as agents of disease in

bivalve molluscs (Elston, 1984).

Vibriosis (bacillary necrosis), caused by members of the genus *Vibrio*, remains the most common cause of massive losses within hatcheries and nurseries for bivalves (Elston, 1984). The pathogenicity of this disease is not understood and the virulence factors are not defined. Although direct infection of larvae tissues by bacterial cells have been reported (Elston and Leibovitz, 1980; Elston et al., 1982), mechanisms for invasion have not been established.

For bivalves, Elston (1984) states that "while bacterial disease clearly mediated by an essential infection of bacteria cells occurs, toxin-mediated disease also occurs, the distinction between these two modes of pathogenesis may not always be so clear cut, it is likely that complex forms of disease occur where infection of bacterial cells essential to cause disease is facilitated by toxic metabolites."

The role of toxins in vibriosis

The involvement of toxins in bivalve disease was first suggested from two laboratories, those of Brown and Losee (1978) and DiSalvo (1978). Brown and Losee provided evidence which suggested that vibrios produced toxins and that massive bacterial

invasion was not necessary for necrosis to occur in bivalves. DiSalvo reported the production of a heat-stable, water-soluble exotoxin from the supernate of *V. anguillarum* isolated from moribund larvae, which inhibited swimming and contributed to death of oyster larvae. Subsequently other toxins have been reported by several authors (Table 8) and this led Nottage and Birkbeck (1986; 1987 a,b; 1989), to investigate the role of toxin(s) in vibrio infections of oysters.

Preliminary work on oyster spat and *M. edulis* gill tissue demonstrated that the 72 h culture supernates of 12 *Vibrio* strains were toxic to spat. Subsequently, a spat lethal factor of 39 Kd, proteinase activities of 40 Kd and 80 Kd, haemolytic activity of 20 Kd and ciliostatic activity (to mussel gill tissue) of <5 Kd were identified from the culture supernate of *Vibrio sp.* NCMIB 1338. Of these, only the ciliostatic activity was heat-stable (Nottage and Birkbeck, 1986).

Since a major pathological feature in bivalve vibriosis is extensive necrosis, subsequent work focused on the effect of the proteinase factor of *V. alginolyticus* NCIMB 1339 on 2 day-old larvae. Crude supernate and the <5 Kd fraction were, however, more toxic than the purified protease (Nottage and

TABLE 8 Toxins implicated in the vibriosis of shellfish

Toxin	Origin	Source of bacteria	Reference
heat stable exotoxin	*V. anguillarum*	moribund larvae	Di Salvo, 1978
heat labile exotoxin	-	-	Jeffries, 1982
heat labile exotoxin	*Vibrio sp.*	oyster	Brown & Roland, 1984
proteinase	*Vibrio sp.*	moribund larvae	Brown, 1984
lethal toxin proteinase haemolysin ciliostatic toxin	*Vibrio sp.* NCIMB 1338 *V. alginolyticus* NCIMB 1339	bivalve	Nottage & Birkbeck, 1986; 1987 a, b and Nottage et al., 1989
exotoxin	*V. anguillarum* *V. tubiashi*	moribund larvae	Lodeiros et al., 1987

Birkbeck, 1987b). This led to further work on the low molecular weight fraction, with the finding that it was both lethal to oyster larvae and ciliostatic to *Mytilus* gill sections. This was of interest, as ciliostatic toxin could be an important virulence factor in bivalve larvae, since cilia are of crucial importance in swimming and in the generation of feeding and respiratory currents. Thus, any interruption of ciliary function would be disadvantageous. Early signs of vibriosis are a drop in food consumption, erratic swimming which eventually stops, and abnormal ciliary movement (Helm and Smith, 1971; Jeffries, 1982; Elston, 1984). *Vibrio* cells also inhibited the feeding of adult bivalves (McHenery and Birkbeck, 1986).

Nottage *et al.*, (1989) partially purified the low molecular weight factor, named vibrio ciliostatic toxin (VCT) and discovered a second, weaker ciliostatic factor which was haemolytic.

The distribution of ciliostatic toxins (CT) amongst a range of bacterial strains was also investigated, with CT produced by 77 % of the bivalve vibrio strains, 100 % of the fish vibrio strains, 24 % of the environmental isolates identified as *Vibrios* and 36 % of other species of environmental isolates, mainly *Aeromonas*. Therefore, most of the pathogens produced CT, whereas fewer of

the environmental isolates, which were not examined for virulence, produced the toxin(s). Since organisms other than vibrios produced CT, it was suggested that their production was not limited to *Vibrio* species in the aquatic environment. It was also found that VCT could be detected in seawater inoculated with *V.alginolyticus* NCIMB 1339, to a titre of 10^3/ml which increased to 10^6 viable cell count per ml in a 48 h period.

These results led to the investigation, in this work, of the geographical and seasonal distribution of CT—producing bacteria in the environment and their relationship, if any, to reported seasonal increases in *Vibrio* numbers.

OBJECTS OF RESEARCH

The objects of this work were three-fold; the first was to confirm reports, originating recently from Japan, that marine bacteria produce tetrodotoxin, (TTX) and to determine how common such bacteria are in the marine environment. A tissue culture assay was developed to this effect. However, since the assay was applicable to sodium channel-blocking (SCB) toxins in general, the work was extended to include paralytic shellfish poisons (PSP).

The second objective was that, if bacteria were found to produce SCB toxins, the cultural conditions that promoted toxin production were to be investigated.

The third objective was to determine, at two Scottish fishfarms, the seasonal distribution of bacteria capable of producing ciliostatic toxin (CT), a substance which is implicated in the pathogenesis of vibriosis of shellfish.

MATERIALS AND METHODS

MATERIALS AND METHODS

BACTERIAL STRAINS

This study investigated the ability of a variety of bacteria to produce low-molecular weight toxins. This included 19 marine bacteria and 3 non-marine bacteria of known designation , a full list of which can be found in Appendix 1. Unidentified marine bacteria (956 isolates) were also investigated. These isolates were obtained from the inlet source of seawater at 2 fish farms; at the S.F.I.A. Marine Fish Cultivation Unit, Ardtoe and at Golden Sea Produce Ltd., Hunterston and were cultured and stored as described later.

The known marine isolates were taken from freeze dried ampoules, reconstituted in marine broth and plated on to marine agar plates. Cultures were maintained in marine agar covered with paraffin (Buell and Weston, 1947). The non-marine strains were treated in the same manner with the exception that they were cultured in nutrient broth or agar.

Various bacteriological media were used and full particulars are given in Appendix 2.

DEVELOPMENT OF THE TISSUE CULTURE ASSAY

MNB C1300 N-2a Cell Line

Mouse neuroblastoma (MNB) cell line C1300 originated from a spontaneous tumour maintained since 1940 by serial transplantation in strain A/J mice (August-Tocco and Solo, 1969).

The clone Neuro-2A was established from a spontaneous tumour of a strain Albino mouse (Klebe and Ruddle, 1969). A culture in the 150th serial passage was submitted to the American Type Culture collection in May 1969, and was supplied by Flow Laboratories in its 167th passage as a cell monolayer in a 80 cm^2 flask.

Maintenance of MNB cells

The reagents required and the media formulations for maintaining these cells and for the tissue culture assay are listed in Appendices 3 and 4. All media components and culture flasks were supplied by Gibco, with the exception of foetal calf serum (fcs), which was supplied by Flow Laboratories.

Upon arrival, the flask containing the MNB cells was incubated overnight at 37°C in air, afterwhichthe cells were removed with trypsin-EDTA (2 ml), washed twice in RPMI 1640 and split into four 80 cm^2 tissue culture flasks, containing

maintenance medium (20 ml) with 10 % fcs and incubated at 37°C.

The MNB cells were then split every 4 days, with excess cells stored in liquid nitrogen. In order to do this, the excess cells were added to plastic universal bottles, centrifuged at 1000 x *g* for 5 min (MSE Minor S centrifuge) and resuspended in tissue culture grade plastic freezing vials containing 1 ml of fcs with added dimethyl sulphoxide (10 % v/v DMSO). The bottles were then sealed using red-hot tweezers and stored in liquid nitrogen.

In later experiments the MNB cells were cultured in 175 cm^2 flasks containing maintenance medium (40 ml) in which the fcs concentration had been reduced to 5 %.

Tissue Culture Assay

The tissue culture assay required ouabain (oub), veratridine (ver) and on occasions tetrodotoxin (TTX) all of which were supplied by Sigma. The preparation of these stock solutions is described under "solutions for tissue culture" in Appendix 4. Also required were neutral red (0.05 % w/v), citrate-buffered alcohol and Hank's balanced salt solution (HBSS), the chemicals for which were all

supplied by BDH; the formulations are also described in Appendix 4.

Only flasks containing a confluent layer of MNB cells were used in experiments, and 24 h before use, fresh maintenance medium was added to each flask. The number of flasks of MNB cells required could be calculated on the basis that one 175 cm^2 flask contained a sufficient number of MNB cells to seed four microtitre (MT) plates (96 well, flat-bottomed microtitre plates, with lids; Gibco Nunc).

Trypsin-EDTA (2 ml for a 80 cm^2 flask and 5 ml for a 175 cm^2 flask) was added to each flask and incubated at 37°C for 5 min; the flasks were shaken to loosen the cells and the resultant supernates were decanted and mixed. The cell suspension was centrifuged (1000 x *g* for 5 min), the supernate discarded and the cell pellet resuspended in an appropriate quantity of RPMI (4 ml of RPMI per 80 cm^2 flask and 8 ml of RPMI per 175 cm^2 flask). This solution was then split into the appropriate number of universal bottles (each MT plate required 1 universal bottle containing 2 ml of MNB cell suspension) and centrifuged (1000 x *g* for 5 min), after which the MNB cells from each universal, were resuspended in 22 ml of suspension medium to give approximately 1 X 10^7 MNB cells/ml.

Each cell suspension (200 µl per well) was then seeded into MT plates excluding the last four wells, which were the dye blank controls. The plates were incubated for 24 h at 37°C after which the medium was tipped off.

Veratridine and oub were added at the required concentration to the appropriate wells in 50 µl quantities followed by 100 µl of the sample. Controls included ver alone, oub alone and cells alone with the total volume in each well adjusted to 200 µl using RPMI; each sample was done in quadruplicate. Since oub/ver combinations kill the MNB cells, cell death controls were incorporated and these consisted of 4 wells containing oub and ver (50 µl of each), made up to 200 µl total volume with a solution which was an exact replica of that in the sample; for example, if bacteria in MB culture supernate was to be tested at a 1/16 dilution, i.e 1 ml of sample in 15 ml of RPMI, the medium added to the oub/ver controls consisted of 1 ml of MB in 15 ml RPMI.

After a further 24 h incubation the solutions were again decanted. Neutral red (0.05 % w/v), 200 µl, was added to each well and the plates were incubated for 30 min at 37°C. Subsequently, the dye was removed and the cells washed once with HBSS before

the addition of 200 µl/well of citrate-buffered alcohol. After at least 1 h incubation at room temperature, the absorbance of the resultant pink colour was measured using a Titertex Multiscan MC Microplate Reader with a 540 nm filter. This method is summarised in Fig. 8.

The % cell survival was calculated by taking the average absorbance reading for each sample and applying the formula:

$$\frac{\bar{x}\ \text{absorbance of sample}}{\bar{x}\ \text{absorbance of control}} \times 100 = \%\ \text{survival}$$

$\bar{x}$ is the average of 4 absorbance readings per sample.

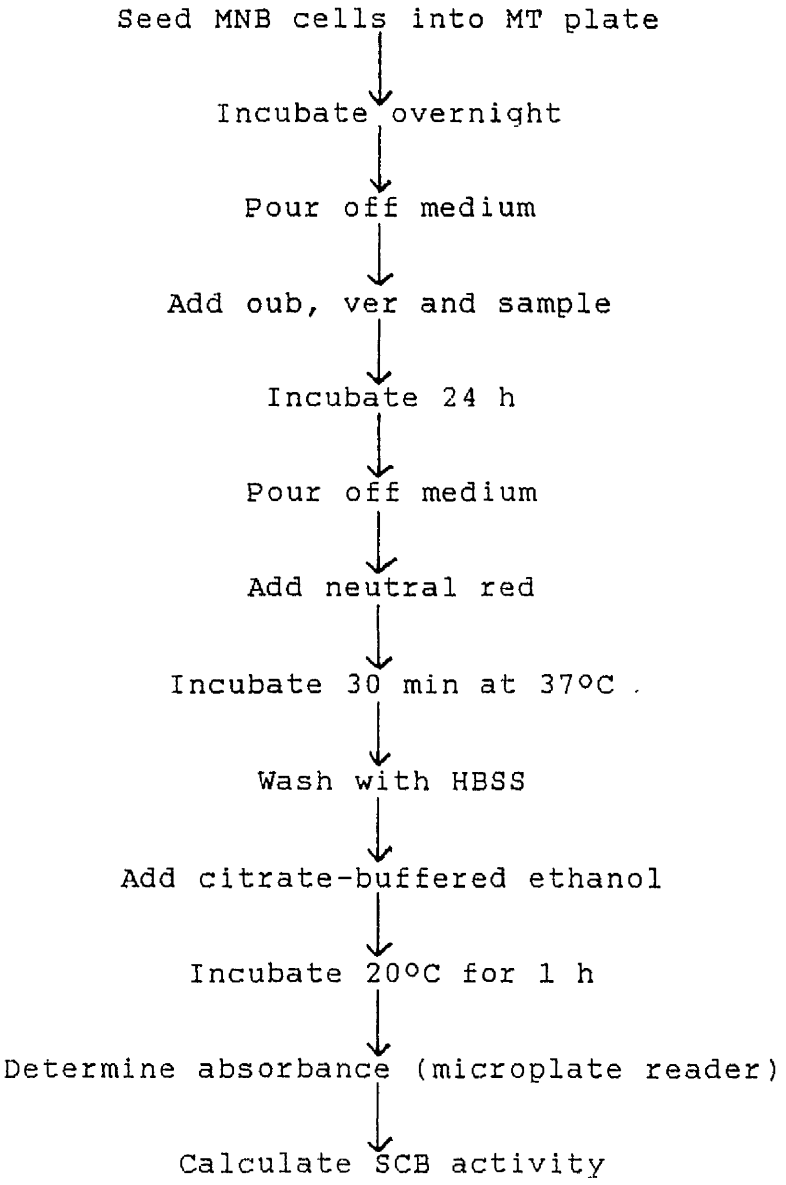
The control consisted of 4 wells which only contained MNB cells and RPMI.

The % protection, which is equivalent to the SCB activity (%), was calculated by:

$$\frac{A-B}{100-B} \times 100 = \%\ \text{protection} = \%\ \text{SCB activity}$$

Where A = % survival of MNB cells in the presence of oub/ver and the sample, and B = % survival of the MNB cells in the presence of oub and ver only.

Tissue Culture Assay



Use of MTT

MTT (3-(4,5-dimethylthiazol-2-y-1)-2, 5-diphenyltetrazolium bromide) was investigated as an alternative dye to neutral red. MNB cells, in suspension medium, were seeded into wells of MT plates at 10^4, 10^5, 10^6 and 10^7 MNB cells/ml in 100 µl aliquots. MTT stock solution was prepared, as described in Appendix 4, and 10 µl added directly to each well, which still contained the suspension medium. The plates were incubated at 37°C for 4 h after which acidic isopropanol (100 µl of 0.04 M HCl in isopropanol), as per the method of Mossman (1983), was added to the wells and mixed.

In later experiments the suspension medium was removed before the addition of the acidic isopropanol and the resultant blue colour measured on a microplate reader at 570 nm.

Preparation of precoated microtitire plates

A stock solution of poly-l-lysine P2636 (PLL; Sigma) was prepared as described under "solutions for tissue culture" (Appendix 4). PLL solution (200 µl) was dispensed into each well of a 96-well MT plate and left for 5 min, after which the solution was removed by aspiration, and the plates thoroughly

rinsed with sterile deionised water and allowed to dry.

Determination of optimum ouabain and veratridine concentrations

Stock solutions of oub and ver were prepared as described in Appendix 4, and were further diluted with RPMI to give final concentrations (in each well) of 0.1 to 1.0 mM oub, and 0.05, 0.1 and 0.15 mM ver. Ver and oub (50 µl of each), at each concentration, were added to the wells of a MT plate in quadruplicate and the total volume of the well adjusted to 200 µl with RPMI. Controls included oub alone at each concentration, ver alone at each concentration and MNB cells alone. The optimum oub/ver concentrations were those for which the combined toxins caused maximal cell death but individually caused <10 % cell death.

Dose-response curve of TTX

Tetrodotoxin (TTX; Sigma) was prepared as a stock solution as described in Appendix 4. This stock solution (313 µM), was diluted 1/100 with RPMI to give a solution of 3130 nM which was further diluted to give a range of concentrations of 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 250, 500, 750 and 1000 nM, calculated as the final concentration of

TTX in the well after the addition of each solution (100 µl/well). Each sample was added in quadruplicate to wells of the MT plates containing MNB cells and oub/ver (50 µl each of oub/ver; as described for the tissue culture assay).

Controls included TTX/oub, TTX/ver, oub, vet, TTX and cells alone, each made to the 200 µl volume with RPMI. The % cell protection at each concentration of TTX was calculated as described previously.

Time course for the assay

MNB cells seeded onto MT plates were incubated with either oub/ver, oub/ver/TTX, oub alone, ver alone, or TTX alone, following the procedure described under the tissue culture assay, with the exception that the MNB cells were exposed to the above combinations for various periods from 1 h to 28 h.

Use of differentiating agents

C6 medium

The first agent examined was C6 medium, which consisted of maintenance medium, in which the formulation was adjusted to include a 1:1 ratio of the supernate, in which rat glioma cells had been grown, to RPMI. The full formulation of this is described in Appendix 3.

The C6 rat glioma cells were obtained from Dr. Ailsa Somerville, Southern General Hospital, Glasgow and were maintained in the same manner as the MNB cells, with maintenance media containing 10 % fcs and split every 4 days. The supernate from each spilt was pooled and stored frozen (-20°C) until required for incorporation in the C6 medium.

Two tissue culture flasks (80 cm^2) were inoculated with 1 ml of RPMI containing 3 x 10^7 MNB cells. The first flask contained the C6 medium, whereas, the second flask contained maintenance medium. Both flasks were maintained at 37°C for 4 days, after which the media were removed and the MNB cells incubated for a further 5 min in 2 ml of trypsin-EDTA solution. Loose cells were then removed, washed in RPMI, resuspended in suspension medium and applied to the tissue culture assay. The oub/ver concentrations used were 0, 0.05, 0.1 and 1.0 mM and 0, 0.01, 0.025, 0.05 mM respectively, with or without added TTX to a final concentration of 30 ng/ml.

Other differentiating agents

The effects of serum limitation, DMSO, HMBA, valinomycin and aminopterin on the MNB cells was investigated. The preparation of stock solutions of

the above and subsequently, the tissue culture media incorporating these solutions, are listed in Appendix 3.

Twelve tissue culture flasks (80 cm^2) containing maintenance medium, were inoculated with 1 ml of RPMI containing 3 X 10^7 MNB cells and incubated at 37°C with a medium change at the 3rd day. By the fourth day the cells had reached confluence, after which, the maintenance medium from each flask was replaced with medium containing one of the differentiating agents (2 flasks per agent), except for the two flasks which were the controls and remained in maintenance medium. The fcs concentration in the media was 5 % except in the case of serum limitation, where it was reduced to 2%. These flasks were maintained at 37°C, for a further 7 days, with media changes every 24 h, after which, the MNB cells from duplicate flasks were combined and applied to the tissue culture assay.

DETECTION OF SODIUM CHANNEL BLOCKING ACTIVITY IN BACTERIA

Examination of Bacterial Cell Extracts

Strains OK1, OK2, GFB, GFC, NCIMB 829 and NCIMB 1903 were analysed for sodium channel-blocking (SCB)

toxin production (Appendix 1). Each of the above strains were inoculated into flasks, each containing 30 ml of four different media, M1, M2, M3 and MB (Appendix 2) and incubated at 25°C on a rotary shaking incubator (100 oscillations/min) for 24 h. The cultures were centrifuged at 7800 x *g* for 30 min (Sorvall RC-5B centrifuge with an SS34 angled rotor at 10,000 rpm at 5°C), the supernate poured off and the cell extracts resuspended in 30 ml of 0.1 % (v/v) acetic acid and sonicated for 10 min. The cells were then split into 2 X 15 ml aliquots, one of which was heated for 20 min at 100°C and the other was untreated. Both sets of extracts were filtered and the filtrates freeze dried. The resultant solids were each resuspended in 15 ml of RPMI and further diluted by 1/2, 1/5 and 1/10, again in RPMI, and applied to the tissue culture assay.

This process was repeated with strain GFC in MB, with the difference that the bacterial cells obtained were examined over a two-fold dilution series and 7-day culture supernate was also tested.

Examination of Culture Supernates

In the preliminary investigation into SCB production by bacterial culture supernates, strains OK1, OK2, GFB, GFC, NCIMB 829 and NCIMB 1903, were inoculated

into flasks containing 30 ml of either M1, M2, M3 or MB. These were incubated at 25°C for 7 days, after which, the culture was centrifuged (7800 x g for 30 min) and the supernate removed and stored frozen until tested in the tissue culture assay at dilutions, in RPMI, of 1/2 and 1/4.

In later work, the effect of strains GFC and OK1 cultured in either M2 or MB, on the tissue culture assay, was investigated by the method described above, but over a two-fold dilution series.

Prior to this, the effects of the individual media on the MNB cells was investigated by applying two-fold dilutions, in RPMI, of M1, M2, M3 and MB to the tissue culture assay.

Investigating the inhibitory factor

MB containing added TTX was prepared by adding 0.3 ml of the TTX stock solution (0.2 mg/ml; Appendix 4) to 100 ml of MB to give a solution of 600 ng/ml TTX (1880 nM). This was used both in the heating and the ultrafiltration experiments where, 0.1 ml of the solution, was added to 0.1 ml of oub/vet therefore, the final concentration in the well was 30 ng/ml (94 nM).

Effect of heat on the supernates

Culture supernate (10 ml of 7 day culture) of strain GFC, or MB containing added TTX, was heated at 100°C for 5, 10, 20 and 30 min, allowed to cool and applied, along with unheated supernate, to the tissue culture assay, in a series of two-fold dilutions with RPMI. The experiment was repeated, with further samples heated for 40, 50, and 60 min.

Ultrafiltration of the supernates

The preliminary experiment in the ultrafiltration of 7 day culture supernates, of strain GFC, involved centrifugation at 7000 x *g* for 20 min in microfuge tubes, which contained 10,000 and 30,000 molecular weight cut-off filters (Millipore, Herts., 10,000 and 30,000 NMWL filter units). The resultant filtrate was tested in a two-fold series of dilutions in the tissue culture assay.

Further experiments involved passing 50 ml of supernates, or MB containing added TTX, through Amicon filters YC05, YM2, YM5 and YM10 and YM30, which have molecular weight cut-off's at 500 d, 1 Kd, 5 Kd, 10 Kd and 30 Kd respectively (Amicon, Surrey), until only a 10 ml volume remained. However, the MB containing added TTX was concentrated only to 40 ml upon ultrafiltration with the 500 d molecular weight cut-off. The filtrate,

concentrate and untreated supernate were applied, in series of two-fold dilutions in RPMI, to the tissue culture assay.

SCB production by strain GFC cultured in different strengths of marine broth

Strain GFC was inoculated into full-strength, 1/2-strength, 1/3-strength and 1/4-strength MB (Appendix 2) and cultured for 7 days at 25°C, after which, an indication of growth was obtained by measuring the absorbance at 600 nm.

The cultures were centrifuged (7800 x *g*, 30 min) and the supernate applied to the tissue culture assay in a series of two-fold dilutions in RPMI.

Increasing the total salt content of the diluent

The effect of increasing the total salt concentration was investigated by adding culture supernates of strain GFC in RPMI, containing added MB or NaCl, to the tissue culture assay, over a two-fold dilution series, and comparing the results to those obtained with RPMI only, as diluent.

Diluents containing added MB were prepared by mixing MB with RPMI, initially at ratios of MB:RPMI of 1:1, 1:2 and 1:3, and later at ratios of 1:5, 1:19 and 1:29.

RPMI containing added salt was prepared by adding NaCl (0.07 g) to 10 ml of RPMI to give a diluent containing 120 mM of added NaCl. The 2.4 mM and 12 mM NaCl solutions used in subsequent experiments were prepared by diluting the 120 mM NaCl solution by 1/50 or 1/10. Since RPMI contained 94 mM NaCl, addition of the NaCl solution increased the NaCl concentration in the diluent to 96.4, 106 and 214 mM NaCl respectively.

Dilutions in water

A 1/4 dilution of 7-day culture supernate of strain GFC in M B, was made in deionised H_2O, followed by subequent two-fold dilutions in either full-strength RPMI or half-strength RPMI, and tested in the tissue culture assay. This was repeated for strain GFC in M.2, with the initial deionised water dilution being at 1/2, followed by subsequent dilutions in either full-strength or 1/2-strength RPMI (1/2 strength RPMI was prepared by dilution with deionised water).

Charcoal extraction of the supernate

In this work, samples were extracted with charcoal obtained from Sigma and the procedure followed was adapted from Noguchi *et al.*, 1986.

Culture supernate (7 day) of strain GFC in MB, and MB containing added TTX, were charcoal extracted by adding 10 ml of the supernate to 0.6 g of charcoal in a universal container and shaking (rotary incubator, 100 osc/min) for 20 min. At this point, any SCB activity from the supernate should be bound to the charcoal. The samples were centrifuged (7800 x *g* 30 min) and the initial supernate discarded. RPMI (10 ml) was added and the samples were agitated for a further 10 min, centrifuged again (7800 x *g*, 30 min), and the resultant supernate again discarded.

Freshly prepared acidified alcohol (5 ml of 1 % acetic acid containing 20 % ethanol) was added and the sample shaken for a further 10 min. Any SCB activity present was extracted from the charcoal by this procedure and was, therefore, contained in the supernate. The charcoal was removed by centrifugation and the acid extract filtered through Whatman no. 1 filter paper, evaporated to dryness (approx 1 h) and reconstituted in 10 ml of RPMI. The resuspended extract was then subjected to two-fold dilutions in RPMI and applied to the tissue culture assay and the results compared to the untreated supernate.

SCB Activity of Various Bacterial Isolates

Culture supernates of bacteria pathogenic to fish and shellfish, a marine *Pseudomomas* strain and 3 non-marine bacteria were tested for SCB activity (information on these bacterial strains is given in Appendix 1). The culture supernates were obtained by inoculating the marine strains into 30 ml of MB which were incubated at 25°C (rotary incubator 100 osc/min) for 24 h. The non-marine strains were incubated into 30 ml of nutrient broth and incubated, with shaking, at 37°C for 24 h.

Each sample was centrifuged (7800 x *g* 30 min) and the culture supernate applied to the tissue culture assay at a 1/16 dilution in RPMI.

Preparation of Bacterial Samples for Analysis by High Pressure Liquid Chromatography (HPLC)

Overnight bacterial cultures of strains GFC, NCIMB 829, A862 and A1096 (the latter 2 strains were obtained from the Ardtoe water samples) were inoculated (1 %) into 2 x 1 L of MB for each strain. The flasks were incubated on a rotary shaker (100 osc/min) for 24 h at 25°C, after which the 2 x 1 L for each strain were pooled and centrifuged (3800 x *g*, 30 min). The supernate was retained, freeze-dried

and the dry weight of the powder measured. The powder was split into two equal aliquots and extracted for either PSP or TTX as described below.

PSP extraction

This extraction procedure was adapted from that used by the Ministry of Agriculture, Fisheries and Food (MAFF), Burnham-on-Crouch, England, for the analysis of mussel extracts.

The powder from each sample was reconstituted in 20 ml 0.1 M HCl and left overnight. The mixture was centrifuged (7800 x *g*, 30 min) and the resultant supernate filtered through Whatman no.1 filter paper and stored frozen until used.

TTX Extraction

This procedure was adapted from that of Noguchi *et al.* (1986). The powder from each sample was reconstituted in 20 ml of 1 % acetic acid/ 20 % ethanol in distilled water and left overnight. It was then heated (100 °C) for 1 h to remove the acid after which 2 ml of 2 M NaOH (prepared by dissolving 8 g NaOH in 100 ml water) was added and the sample was boiled for a further 45 min. This was adjusted to pH 4-5 with 1 M HCl and centrifuged (7800 x *g*, 30 min); the resultant supernate was

stored frozen (-20 °C) until required.

HPLC analysis

Both PSP and TTX extracts were sent to MAFF, Burnham-On-Crouch for HPLC analysis. However, due to constraints on resources, only the PSP extracts were analysed by the procedure described by Waldock *et al.* (1991). The chromatograms obtained were analysed by the calculation described in Appendix 6.

CULTURE CONDITIONS FOR SCB PRODUCTION

Growth of Bacteria in MB

Strain GFC in a shaking culture

Strain GFC was inoculated into 30 ml of MB and cultured at 25°C in a rotary incubator (100 osc/min). After 20 h, 20 ml of the culture was removed and centrifuged (7800 x *g*, 30 min). The cells obtained were washed twice in MB, after which they were resuspended to 20 ml with M B.

The washed cell suspension (10 ml) was added to 500 ml of MB and 9 ml samples were removed every hour, for 12 h, followed by samples at 24 h, 72 h, 168 h, and 336 h. This was later repeated with samples taken every 2 h for 24 h.

The viable cell counts were obtained for each

sample by plating 0.1 ml of ten-fold dilutions, in MB, of the culture supernate, onto marine agar plates and incubating the plates for 48 h at 20°C.

SCB activity was measured by centrifuging (7800 x *g*, 30 min) the samples and testing the resultant culture supernates, at 1/8 and 1/16 dilutions in RPMI, in the tissue culture assay.

Strain GFC in static culture

The above procedure was repeated with the difference that strain GFC was incubated as a static culture at 25°C for 7 days.

Other strains

Strains OK1, NCIMB 829, NCIMB 1903 and A1096 were treated as described above for 3 days with samples taken every hour up to the 12th hour after inoculation and thereafter at 24 h and 72 h.

Growth of Strain GFC In Seawater

An overnight culture of strain GFC in MB (30 ml) was centrifuged (7800 x *g*, 30 min) and the supernate discarded. The remaining cells were washed 3 times in artificial seawater (asw; Tropic Martin, Tropical Marine Centre, Herts. Appendix 2) before resuspension in 30 ml of asw. This suspension, which

contained approximately 10^9 cfu/ml was diluted by 10^5-fold, in asw, and 1 ml added to 100 ml of asw to give a final concentration of 10^2 cfu/ml. The culture was shaken on a rotary incubator (100 osc/min; 25°C) for 7 days. Samples (5 ml) were removed at intervals of 24 h for the first 3 days and then at day 7.

The viable count was obtained by inoculating marine agar plates with 0.1 ml of each sample, over a series of ten-fold dilutions in asw, and incubating the plates at 20°C for 48 h.

To investigate the SCB activity the samples were centrifuged (7800 x *g*, 30 min) and the resultant supernate was applied to the tissue culture assay in a series of two-fold dilutions in RPMI.

Growth of strain GFC in seawater with or without added carbon sources

An overnight culture of strain GFC in M B (10 ml) was centrifuged (7800 x *g*, 30 min) the supernate discarded, the cells washed 3 times in asw and resuspended in 10 ml of asw. This 1x suspension of cells was diluted 1/10 and 1/100 in asw and 0.3 ml of each solution was added to flasks containing 30 ml of asw, or asw with added glucose (20 mM) or sucrose (20 mM; Appendix 2). A 10x suspension of

the cells was prepared by resuspending washed cells obtained from 10 ml of culture in 1 ml of seawater and adding 0.3 ml to each flask as above.

All of the flasks were incubated at 25°C (rotary incubator 100 osc/min), for 24 h, after which viable counts were obtained by plating out ten-fold dilutions of each sample (0.1 ml) on to marine agar and incubating the plates for 48 h at 20°C.

To investigate the SCB activity the samples were centrifuged (7800 x g, 30 min) and the resultant supernates applied to the tissue culture assay at a 1/8 and 1/16 dilution in RPMI.

The SCB activity was expressed as ng TTX equiv/cell, which was calculated from comparing the % SCB activity to the TTX standard curve to obtain the concentration of TTX in nM. This was multiplied by the dilution factor, converted into ng/ml and divided by the viable count.

Experiments involving Phosphate

The reagents and stock solutions required for the phosphate experiments are listed in the Appendix 5.

Peptone medium

Growth of strain GFC in seawater with added peptone. Peptone (Oxoid L37) was added to asw at

concentrations of 0.1, 0.5 and 1 % (Appendix 2) and strain GFC was cultured in each for 24 h, at 25°C (shaking culture 100 osc/min), after which the absorbance reading (600 nm) of each sample was obtained, as a measure of cell growth. Since the medium containing 0.5 % peptone allowed substantial growth of strain GFC, this medium was subsequently used in the experiments in which additional phosphate was added.

Peptone medium with added phosphate

Stock phosphate solution (Appendix 5) was added to flasks containing 30 ml of peptone medium (0.5 % peptone in asw) such that the final concentration of added phosphate ranged between 0 to 100 µg/ml P, as described in Appendix 2.

Growth of strain GFC in peptone medium with added phosphate

Cells from an overnight culture of strain GFC were obtained by centrifugation (7800 x *g*, 30 min) of 30 ml of culture supernate. The cells were then washed 3 times in peptone medium in which they were finally resuspended to a volume of 30 ml. Each of the above flasks containing peptone medium (30 ml) with added PO_4 and the control containing only peptone medium,

were inoculated with 0.3 ml of the washed cell suspension and incubated for 24 h at 25°C. Samples (0.1 ml) of each were then inoculated over a series of ten-fold dilutions on to marine agar plates (Appendix 2) which were incubated at 20°C for 48 h to obtain the viable count.

To determine the SCB activity of the supernates the samples were centrifuged (7800 x g, 30 min) and the resultant supernates tested in a series of two-fold dilutions in RPMI by the tissue culture assay.

An amendment to the procedure was subsequently made, whereby the supernates were charcoal-treated prior to addition to the tissue culture system.

Addition of phosphate during the culture of strain GFC

Strain GFC was inoculated into 5 flasks containing peptone medium (30 ml). Each flask was cultured for 24 h, however, at 16 h, 18 h, 20 h and 22 h in the growth cycle, stock phosphate solution (50 µg/ml P; Appendix 5) was added to the relevant flask, except for the control flask which did not contain additional phosphate. At 24 h the cultures were centrifuged (7800 x *g*, 30 min) and the resultant supernates applied to the tissue culture assay in a series of two-fold dilutions.

In a later experiment, the culture supernates were charcoal-extracted before addition to the tissue culture assay.

Colorimetric determination of phosphate

The method used was that of Chen *et al.* (1956) whereby 0.1, 0.2, 0.3 ml etc., of stock PO_4 solution (Appendix 5) was adjusted to a total volume of 4 ml with deionised water. Colour reagent (4 ml; Appendix 5) was added and the samples were mixed well before incubation at 37°C for 2 h. The absorbance due to the resultant blue/green colour was read at 820 nm against a reagent blank.

A standard dose response curve was obtained by plotting the absorbance values for each dilution of the standard against the PO_4 concentration.

The samples were tested by taking 1 ml and adjusting the volume to 4 ml with deionised water to which 4 ml of colour reagent was added followed by incubation as above. The resultant absorbance values were compared to the standard graph to obtain the concentration of phosphate (µg/ml P) present in each sample.

ANALYSIS OF MUSSEL EXTRACTS BY THE TISSUE CULTURE ASSAY

Uptake of Strain GFC by *Mytilus edulis*

The protocol for the maintainance of bacteria with mussels was adapted from Birkbeck and McHenry (1982). Washed cells, from an overnight culture of strain GFC in MB, were added to a final concentration of 2.1 X 10^7 cfu/ml in a jar containing 5 mussels (*M. edulis*) in 100 ml of aerated asw. A similiar jar in which bacteria were not added served as a control. Samples of asw were taken at 1 h, 3 h and 24 h and viable counts obtained, by plating the sample (0.1) in ten-fold dilutions in asw, on marine agar followed by 48 h incubation at 20°C.

The soft parts of the mussels were combined and homogenized as described by Hwang *et al.* (1989), with 3 volumes of 1 % acetic acid in methanol for 5 min and centrifuged (7800 x *g* for 30 min). This was repeated 3 times after which the supernates were combined and the solvent evaporated at 100°C for about 1 h. The residual solid was redissolved in 10 ml RPMI and applied in a series of two-fold dilutions to the tissue culture assay.

Analysis of Mussels Intoxicated With PSP

Mussel (*M. edulis*) samples labelled T64, T87, T125, T55, T538, T59, collected and extracted by MAFF (Burnham-on-Crouch), during the phytoplankton bloom on the north-east coast of England in 1990, were supplied for tissue culture analysis, as 2 ml aliquots in 0.1 M HCl.

Initial analysis was on extract T64, which was diluted 1/10 in RPMI and split into 2 x 5 ml aliquots. One of the aliquots was heated at 100°C for periods of 10 min, 20 min, 30 min, 40 min and 60 min, whereas the other aliquot was untreated. These samples were then diluted in a two-fold series in RPMI, up to a 1/1024 dilution, and applied to the tissue culture assay. The other mussel extracts were analysed as described above, but the heated extract was treated for 60 min at 100°C.

The titre obtained for each extract, by the tissue culture assay, was defined as the dilution at which the extract demonstrated 50 % SCB activity.

LOW MOLECULAR WEIGHT TOXINS FROM BACTERIA ISOLATED FROM SCOTTISH COASTAL WATERS

Isolation and Testing of Marine Bacteria for VCT and SCB

Isolation and culture of bacteria

Water samples were obtained monthly from 2 locations, Hunterston and Ardtoe, over a 2 year period. The water temperature was recorded at the time of sampling.

In the first year (Oct. 1987 - Sept. 1988) each sample (0.1 ml) was plated undiluted, and at 1/10 and 1/100 dilutions in sterile asw on marine agar and Thiosulphate citrate bile salt sucrose agar (TCBS), before incubation for 7 days at 20°C. The number of colonies formed were counted and all of the isolates, of the colony type which was dominant on the plate, were inoculated into bijou bottles containing marine agar and incubated for 48 h at 20°C before being stored at 4°C under paraffin (Buell and Weston, 1947).

In the second year of sampling (Sept. 1989 to Sept. 1990) the procedure described above was followed, with the exception that half-logarithmic dilutions were made and all of the bacterial isolates on the marine agar plate, at whichever dilution contained approximately 50 colonies , were isolated and replated to obtain pure cultures of each isolate, directly onto marine agar plates which were incubated for 48 h at 20°C. Subsequently, each sample was cultured in MB for 72 h at 25°C and,

thereafter, centrifuged (7800 x *g*, 30 min) to obtain the culture supernate which was stored frozen (-20°C) until use.

The samples in survey 1 were later replated onto marine agar plates and treated as above to obtain the culture supernates.

All of the culture supernates were tested for vibrio ciliostatic toxin (VCT) by the mussel gill assay (see below), but only the culture supernates, obtained in the second survey at Ardtoe, were tested for SCB activity, by applying a 1/16 dilution of each supernate, to the tissue culture assay.

The mussel gill assay

With a few minor amendments the assay used was that of Nottage and Birkbeck (1986). Mussels (*Mytilus edulis*) of 50-70 mm shell length, which had been maintained in aerated asw at 10°C until use, were forced opened and sections of *ca* 5 mm^2 excised from the gill tissue. These gill sections were placed in individual wells of 100mm-square vented, tissue culture petri plates (Flow Laboratories), containing 1 ml of 25 % a.s.w. (with penicillin 100 iu/ml and streptomycin 100µg/ml). Bacterial culture supernates (1 ml) were then added to each well in triplicate, except for the controls, where 1 ml of

the media used to culture the bacterial strain was added. The plates were incubated at 20°C for 24 h, afterwhich the integrity of the section and activity of the ciliated epithelial cells was recorded by visual inspection, using a Beck inverted microscope.

RESULTS

RESULTS

1. DEVELOPMENT OF THE TISSUE CULTURE ASSAY

In initial experiments, mouse neuroblastoma (MNB) cells at 1.4 x 10^5/ml were seeded into each well of the microtitre (MT) plates and incubated for 24 h before addition of 1 mM ouabain (oub) and 0.05 mM veratridine (ver), individually and in combination. Cells with added saline served as controls. The plates were incubated for a further 3, 6 or 24 h and observed microscopically as described by Kogure *et al.* (1988).

With plates incubated for 3 or 6 h, it was extremely difficult to distinguish between dead and live cells, although, at 24 h in both the sample and control wells, some cells looked unhealthy and debris was visible in the medium; but, the viable cells could not be confidently counted.

Thus, it was decided to incorporate a vital stain in the assay to determine the proportion of live cells and to facilitate quantification with a microtitre plate reader. Diluents other than saline were also investigated.

Use Of a Microplate Reader

Neutral red solution (200µl, 0.05 % w/v in saline) was added to each well of the MT plate containing MNB cells which had been incubated for 24 h and the plate was incubated for a further 30 min at 37°C, to allow the viable cells to absorb the stain. This was followed by washing three times with saline to remove free dye. Addition of 200 µl of citrate-buffered ethanol to each well produced a pink colour, the absorbance of which could be measured at 540 nm by the microplate reader and which provided a measure of the number of viable cells.

An absorbance reading of between 0.8 and 1.3 (540 nm) was obtained in control wells containing 3×10^7 MNB cells/ml after neutral red staining, and with RPMI as the diluent. However, cells were readily dislodged on washing. To solve this problem several approaches were considered.

Adhesion Of Cells

Use of MTT

The dye MTT (Appendix 4), was substituted for neutral red as a vital stain, since its conversion into a coloured crystalline product by cellular dehydrogenases made the washing of the cell monolayer unnecessary. Unfortunately, the coloured

crystalline product was not readily dissolved in the acidic isopropanol solvent described by Mossman (1983). However, the crystals dissolved satisfactorily if the medium was removed prior to addition of the solvent.

Time was not spent on examining procedures to dissolve the crystals and the assay as it existed was considered impractical.

Decreasing the number of washes

A second approach to reducing cell loss on washing was to decrease the number of washes, i.e. one after the treatment with toxins and 3 after incubation with neutral red.

To explore this, MNB cells were dispensed into MT plates at concentrations of 3.6×10^6/ml and 1.2×10^7/ml and subjected to various wash procedures, the results of which were observed microscopically and by neutral red staining.

By microscopy, an increase in cell loss was clearly observed as the number of washes increased; this was confirmed by staining the cells (Table 9). Even a single wash caused unacceptable cell loss. However, at least one wash after the neutral red stain was required to remove excess dye. Therefore,

TABLE 9: Effect of washing procedures on the retention of MNB cell monolayers in MT plates. The plates were inoculated at two MNB cell concentrations and the A_{540} taken as an empirical measure of the retained cells after incubation, followed by either no washing or up to 4 washes.

Number of washes		Absorbance (540 nm ± SEM) of the retained cells from the initial inoculum(/ml)	
before staining	after staining	3×10^6	1.2×10^7
0	0	0.200 ± 0.040	0.750 ± 0.070
0	1	0.072 ± 0.013	0.072 ± 0.005
2	2	0.043 ± 0.003	0.043 ± 0.005
1	1	0.074 ± 0.008	0.074 ± 0.006

methods of improving the adhesion of the cells were considered.

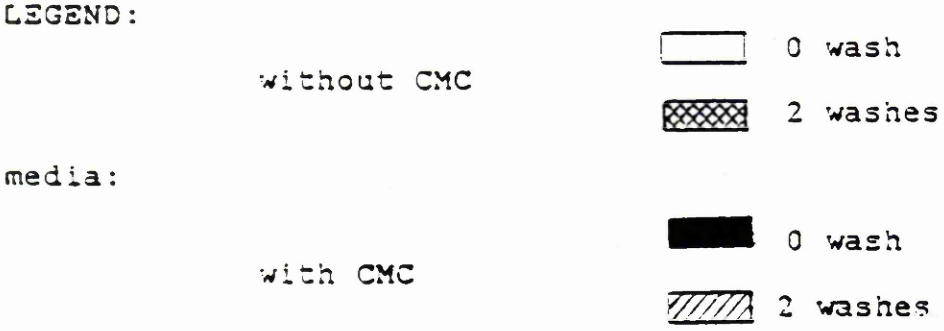
Carboxylmethyl cellulose and poly-l-lysine

Addition of carboxylmethyl cellulose (CMC) to culture medium improves the monolayer formed (Ross *et al.*, 1972) and poly-l-lysine (PLL) increases cell adhesion (McKeehan and Ham, 1976). In order to investigate their possible use in the assay, modified RPMI medium, with or without added CMC, was used to suspend two concentrations of MNB cells cultured in MT plates, precoated with solutions of either 0, 0.1, or 1.0 mg/ml PLL. A comparison was made of the cell retention before and after washing, by measuring the resultant absorbance (540nm).

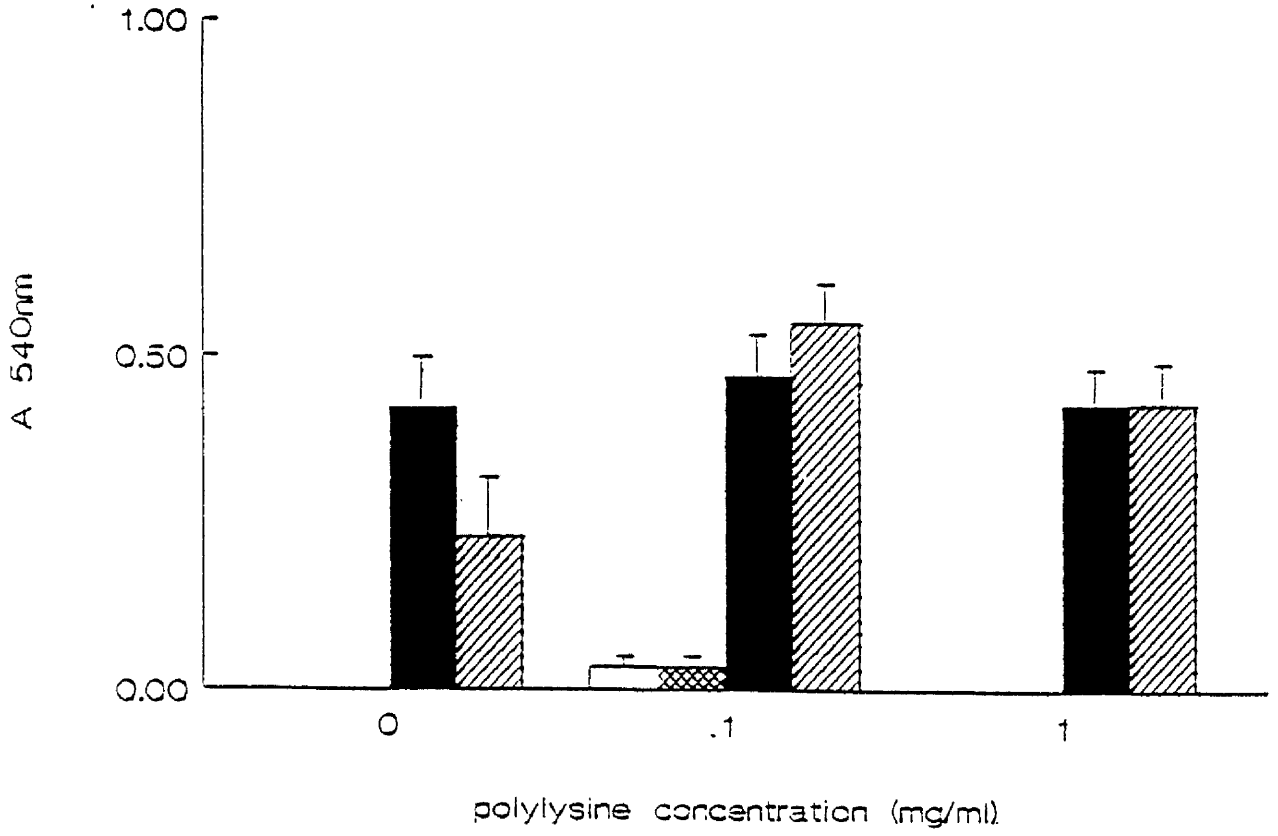
As shown in Fig. 9, cells which had been suspended in modified RPMI medium containing CMC yielded significantly higher absorbance values than those suspended in the medium without CMC, although a decrease in cell retention still occurred upon washing.

However, by using MT plates pretreated with PLL, the loss of cells upon washing was reduced. PLL also increased the retention of cells cultured without CMC, but increased retention occurred when CMC was present.

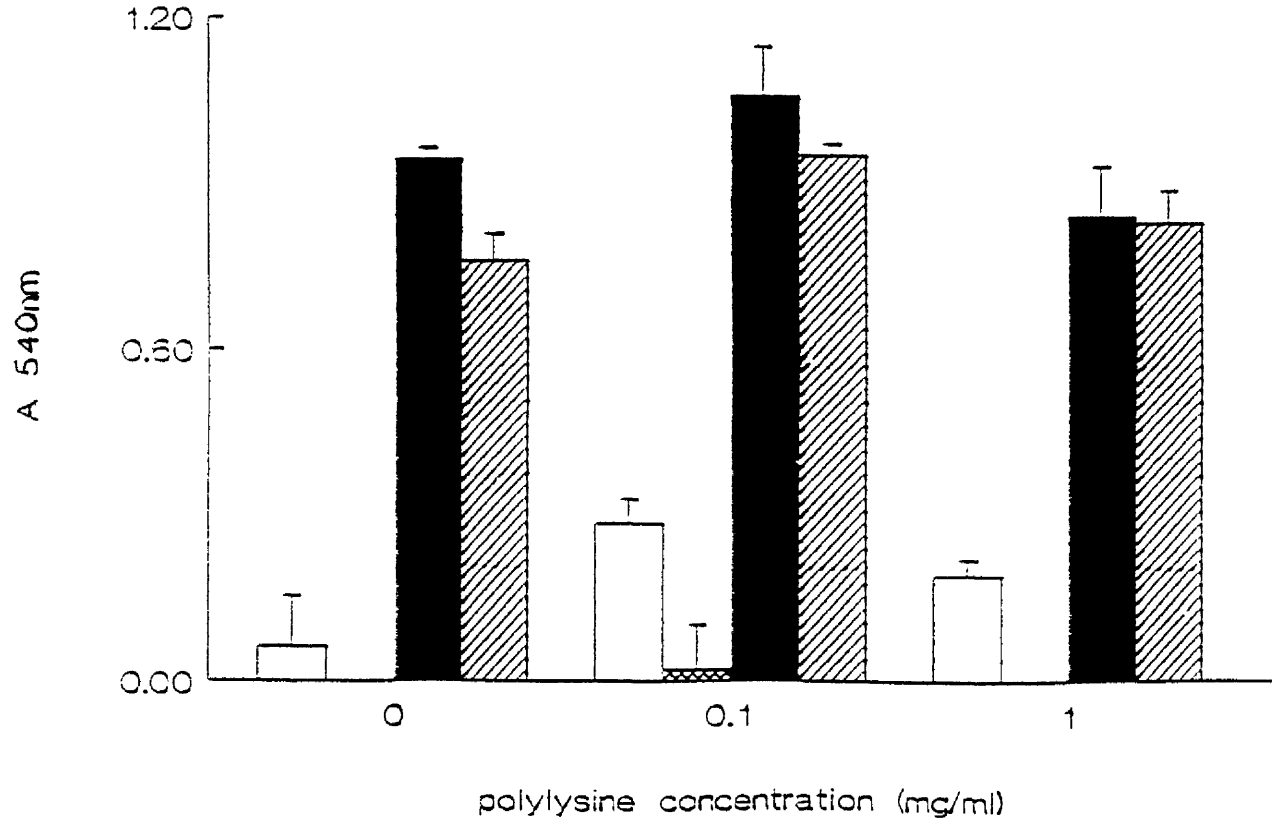
FIGURE 9: Effect of inoculum size, number of washes and presence or absence of CMC and PLL, on the retention of MNB cells in MT plates (as measured by A_{540} ± SEM).



Inoculum of 5 X 10^6 cells/ml



inoculum of 1 x 10^7 cells/ml



Unheated serum

In all of the above experiments, modified RPMI medium containing heated serum (56°C for 30 min.) was used for MNB cell growth. Therefore, a comparison was made of the cell retention obtained, from the absorbance values, upon neutral red staining of cells, cultured in medium containing unheated serum.

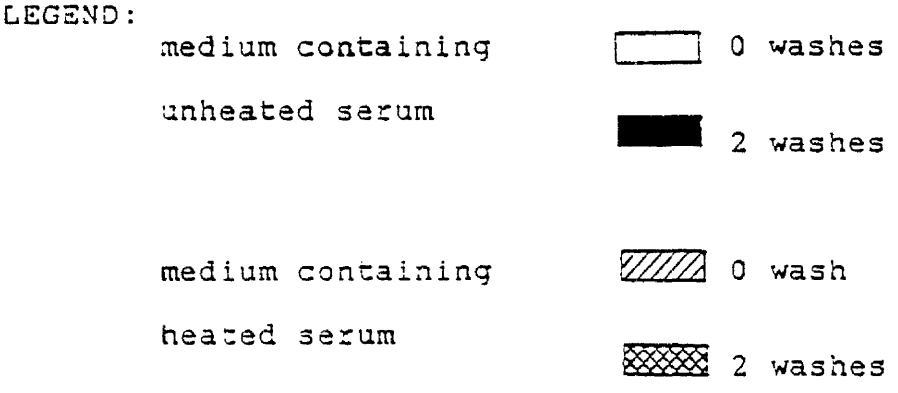
Figure 10 clearly indicates that the MNB cells cultured in modified RPMI medium containing heated serum demonstrated a large decrease in retention, both before and after washing; however, this was vastly improved when the serum was unheated. Therefore, in subsequent work, the serum was untreated.

Diluent

Initially, saline was used as the diluent for all materials in the assay and for controls. In the controls, however, dead cells were observed, therefore, alternative diluents were tested.

After overnight incubation of the MNB cells, the medium was decanted, replaced by various solutions (Table 10) and incubated for a further 24 h, after which the cells were stained with neutral red and the absorbance at 540 nm measured as an indicator of

FIGURE 10 Effect of culturing MNB cells in growth media containing heated (56°C for 30 min) or unheated serum, on the retention of the cells in MT plates before and after washing (as measured by A_{540} ± SEM).



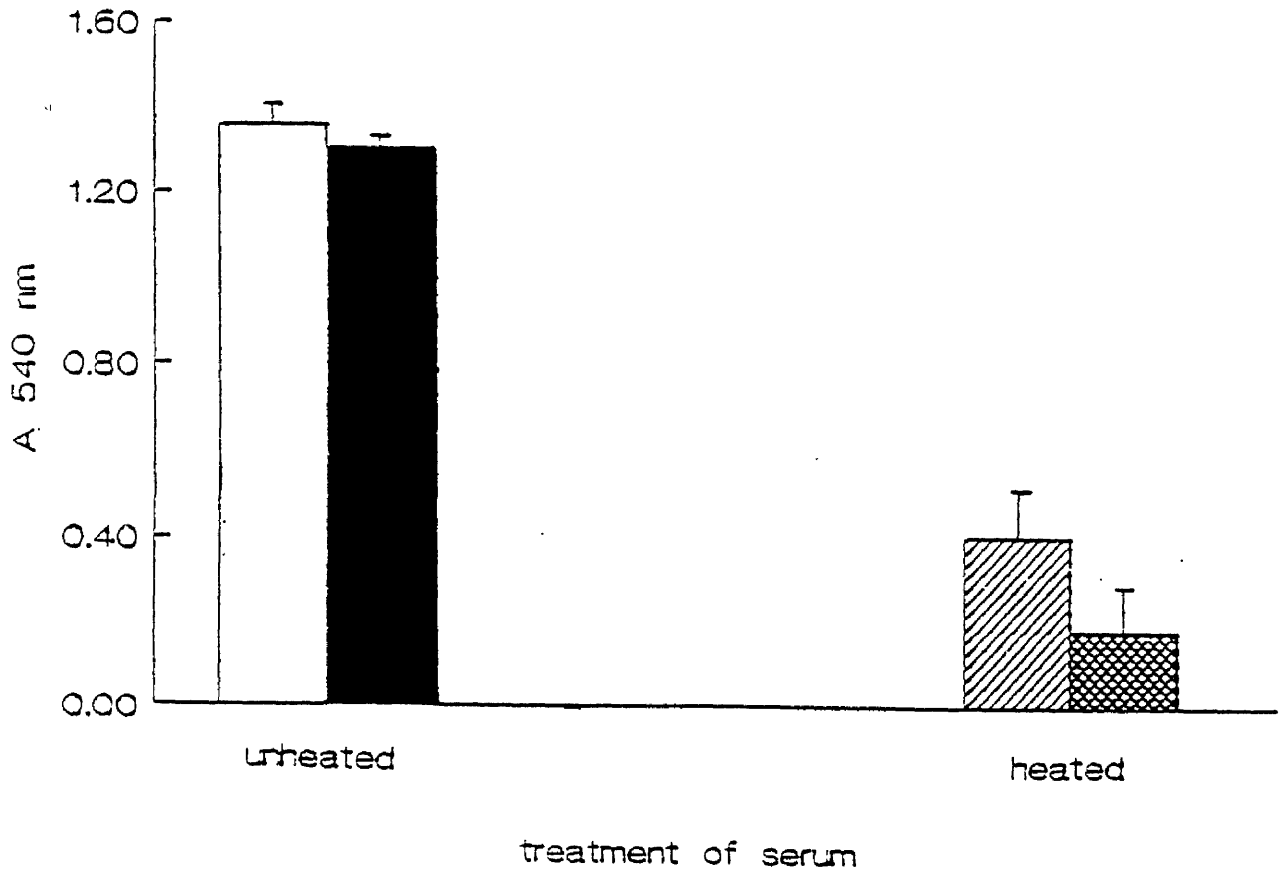

1.60
1.20
0.80
0.40
0.00
A 540 nm
unheated
heated
treatment of serum

TABLE 10 Effect of various diluents on the viability of MNB cells in MT plates (as measured by the A_{540} ± SEM)

Diluent	Absorbance of viable cells (A_{540} ± SEM)
RPMI 1640	1.370 ± 0.028
RPMI:HBSS(1:1)	1.360 ± 0.021
HBSS	0.752 ± 0.023
Saline	0.492 ± 0.016

the number of viable cells (Table 10).

The decrease in absorbance found with saline as diluent indicated that it had an adverse effect on MNB cell viability, as did HBSS. However, a dramatic improvement in the number of viable MNB cells was found when RPMI alone or mixed with HBSS were used as diluents. Therefore, RPMI 1640 was incorporated as the diluent for oub/vet/TTX and the cell controls.

In summary, best results were obtained when MNB cells were grown in culture medium containing unheated serum. The subsequent tissue culture assays were done with suspensions of approximately 1.0 x 10^7 cells/ml prepared in culture medium containing CMC (suspension medium). RPMI 1640 was used routinely as diluent and the MNB cells were treated with 1 wash, with HBSS, after neutral red staining.

Optimum Concentration Of Ouabain And Veratridine

Several combinations of different concentrations of oub and ver were assayed, in a MT plate, to find the optimum concentrations at which the two compounds individually had little effect on cell viability (<10 % cell death) but in combination killed the MNB cells. In the event, the optimum concentration varied widely in different experiments, as did the percentage cells killed (Table 11). This was thought

TABLE 11 Use of different batches of serum, in growth media for MNB cells, and the subsequent effect on the optimum concentrations of oub and ver required to cause cell death.

Serum Batch	Date	Concentration (mM) ouabain	Concentration (mM) veratridine	Cell death (%) (± SEM)
A	30.11.89	1.00	0.050	70 ± 2.5
B	13.12.89	0.05	0.025	20 ± 1.0
C	16.01.90	0.02	0.020	40 ± 3.6
D	05.02.90	0.10	0.025	40 ± 2.6
E	15.03.90	0.80	0.025	30 ± 2.2
F	11.05.90	0.10	0.050	55 ± 1.1
G	18.06.90	0.40	0.100	60 ± 3.0
G	12.07.90	0.40	0.100	55 ± 1.8
G	14.08.90	0.50	0.100	80 ± 3.4
G	20.10.90	0.50	0.100	35 ± 3.1
G	11.03.91	0.40	0.100	40 ± 1.0
G	26.03.91	0.50	0.100	50 ± 0.9

to be due to batch variations in the oub/vet or in the serum. Indeed, a more consistent reaction of MNB cells to the toxins was achieved when the same batch of serum was used consistently from June 1990 (Table 11) onwards. However, fresh batches of oub or ver were still titrated before use.

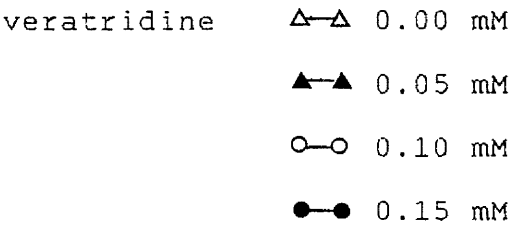
In the example shown in Fig. 11, oub was essentially non-toxic at concentrations up to 0.4 mM, after which cell death increased. On the other hand, ver was not toxic at concentrations up to 0.10 mM. The combination which fulfilled the stated conditions and which gave maximum cell death (60 %) was 0.4 mM oub and 0.1 mM ver. However, in later experiments, using the same concentrations of oub/ver, some variations in the quantity of cell death still occurred between titrations; this was probably due to slight variations in culture conditions (i.e. incubation periods).

Dose-Response Curve for Tetrodotoxin

The response of oub/ver treated cells to TTX was investigated over the range of 0-3000 nM and a standard dose-response curve obtained (Fig. 12). There was a linear response of log dose against probit with a regression coefficient of 0.99. The 50 % end point was at 50 nM TTX (15.9 ng/ml).

FIGURE 11 Effect of various concentrations of oub and ver on % death of MNB cells.

LEGEND:



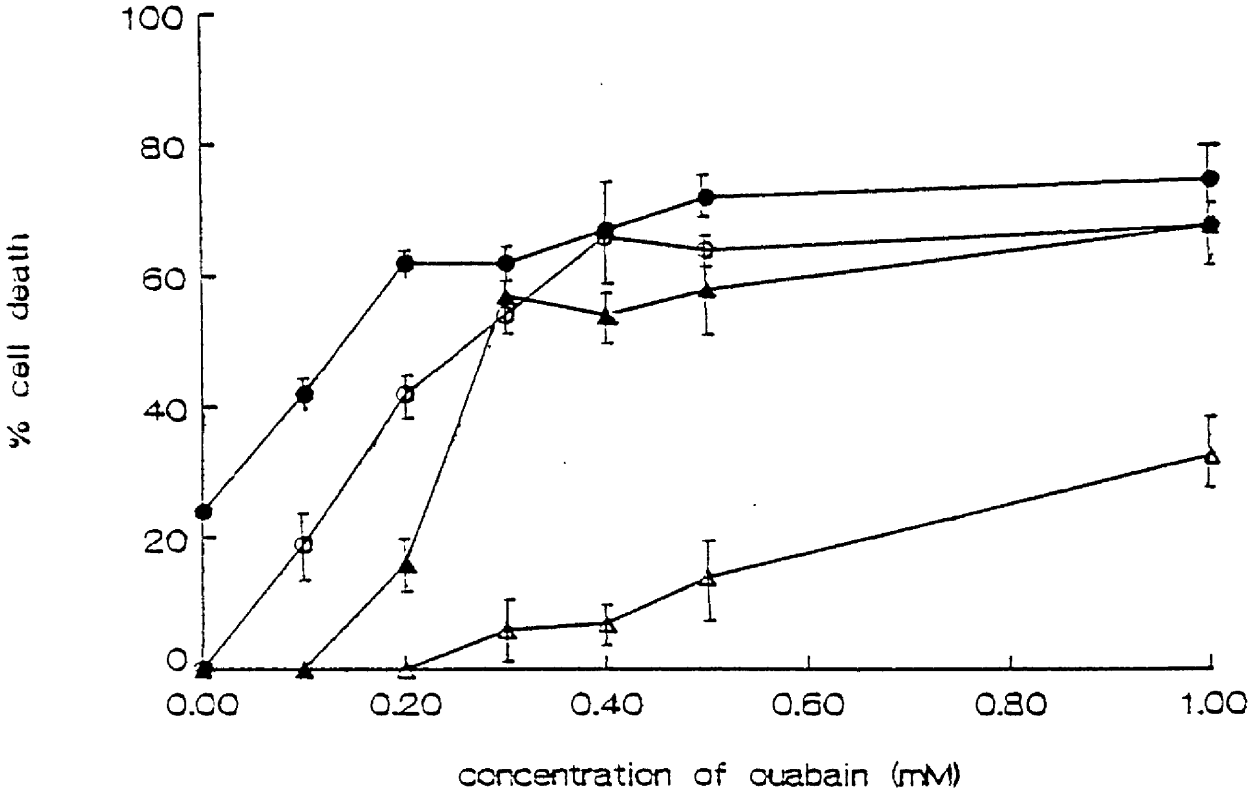
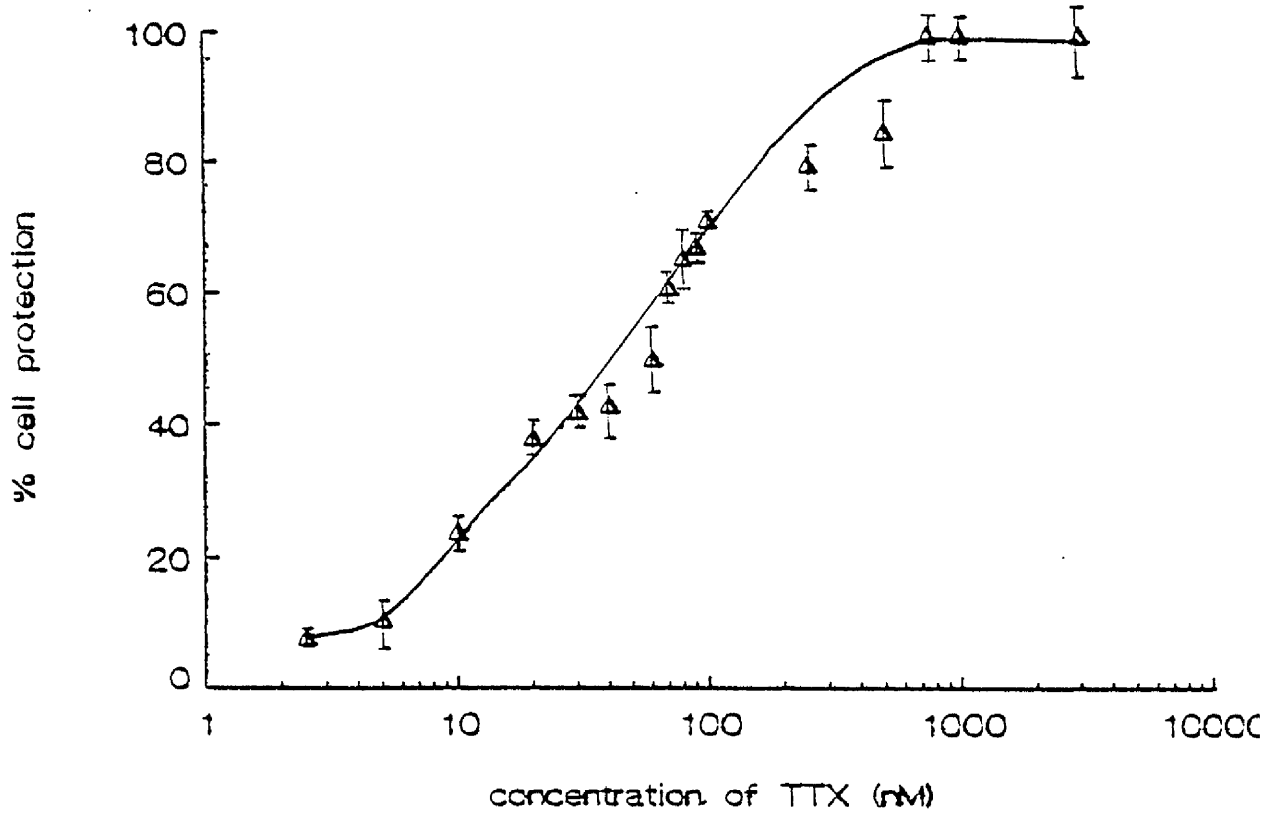
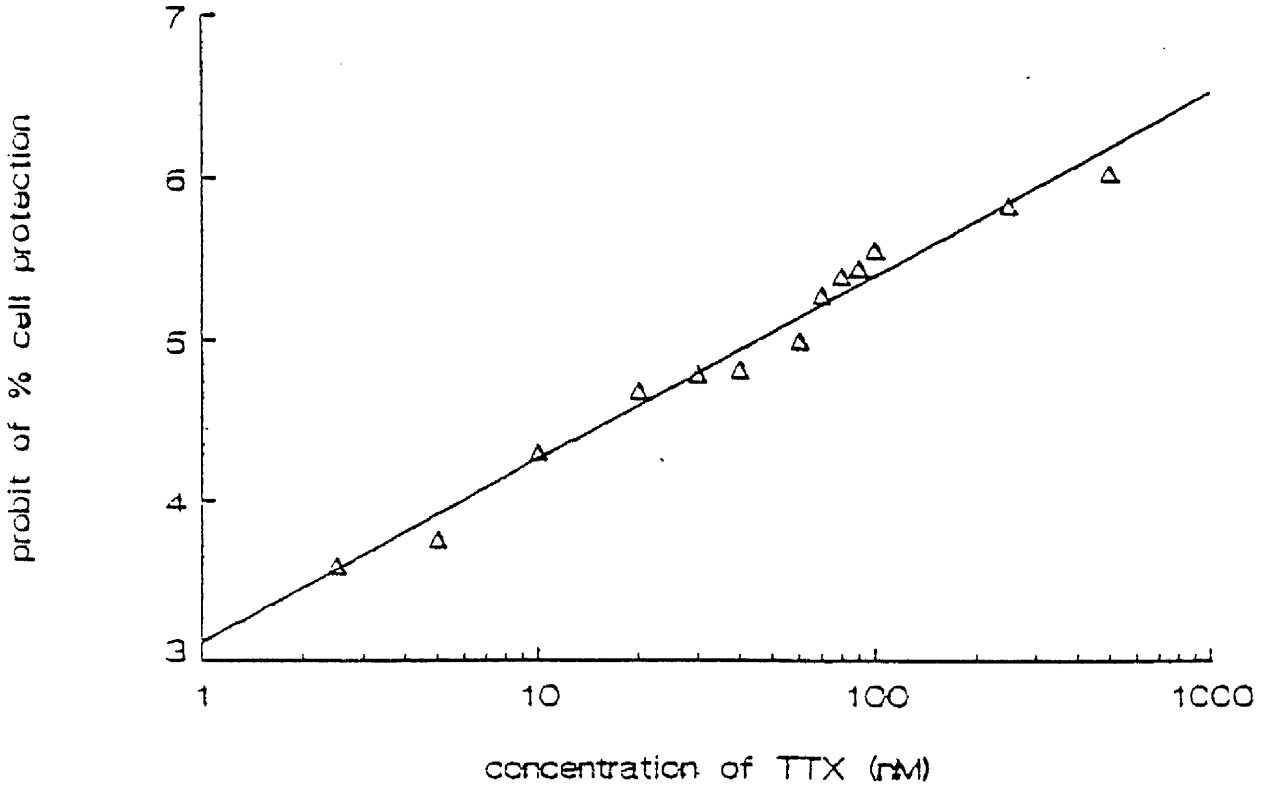

100
80
60
40
20
0
% cell death
0.00
0.20
0.40
0.60
0.80
1.00
concentration of ouabain (mM)

FIGURE 12 Dose response curve for TTX acting on MNB cells in the presence of oub/ver (upper figure) and its probit conversion (lower figure).


100
80
60
40
20
0
% cell protection
1
10
100
1000
10000
concentration of TTX (nM)


7
6
5
4
3
probit of % cell protection
1
10
100
1000
concentration of TTX (nM)

Time Course For The Assay

The time required for combinations of oub and ver to cause cell death and for TTX to protect cells was investigated by incubation of toxin-treated cells for periods from 1 to 28 h. The controls of RPMI, oub alone, ver alone, TTX alone and in combination with oub or ver, did not have any adverse effect on cell viability. The oub and ver treated cells were still viable up to 6 h. However, cell death was clearly observed after overnight incubation; TTX offered full protection throughout this period (Fig. 13).

The Use Of Differentiating Agents To Enhance The Susceptibility Of MNB Cells To Oub/Ver

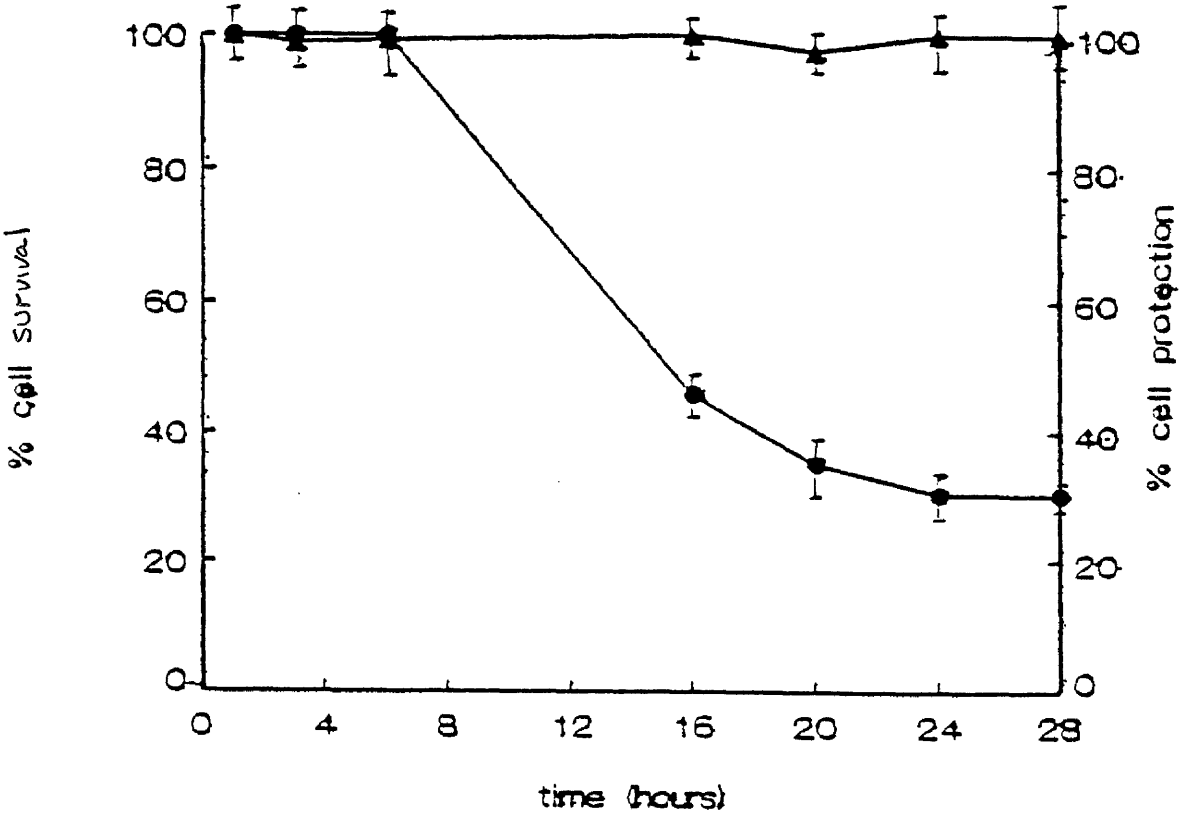
As the tissue culture assay for SCB toxins is based on the control of excitable sodium channels in the cell membranes of MNB cells, an increase in the concentration of such channels enhances the sensitivity of the assay. Many agents are known to induce differentiation of neuroblastoma cells and a number were therefore tested for their effect on the sensitivity of cells to oub and ver.

FIGURE 13 Effect of incubation with oub/ver on MNB cell death, or cell protection on the addition of TTX over a 24 h period.

LEGEND:

●—● oub/ver

▲—▲ oub/ver/TTX


100
80
60
40
20
0
% cell survival
% cell protection
0
4
8
12
16
20
24
28
time (hours)

C6 culture medium

Culture medium in which rat glioma cells had previously been grown was mixed with an equal volume of RPMI, which after supplementation with fcs and antibiotics (C6 medium) was used in the culture of MNB cells. This allowed comparison of the excitability of these treated cells with control cells grown in normal maintainance medium.

The MNB cell monolayer, grown in maintainance medium reached confluency after 3 days, whereas the cells cultured in C6 medium took 4 days. The MNB cells from both cultures were used to seed MT plates which were incubated with either oub/ver or oub/ver/TTX.

Cells grown in C6 medium were more sensitive to the lethal effect of oub and ver; cell death increased by 30-40 % with a corresponding 20 % increase in TTX protection at a ver concentration of 0.05 mM (Fig. 14)

C6 medium, although beneficial, was not convenient for routine use as large quantities of the glioma supernate would have been required.

Other differentiating agents

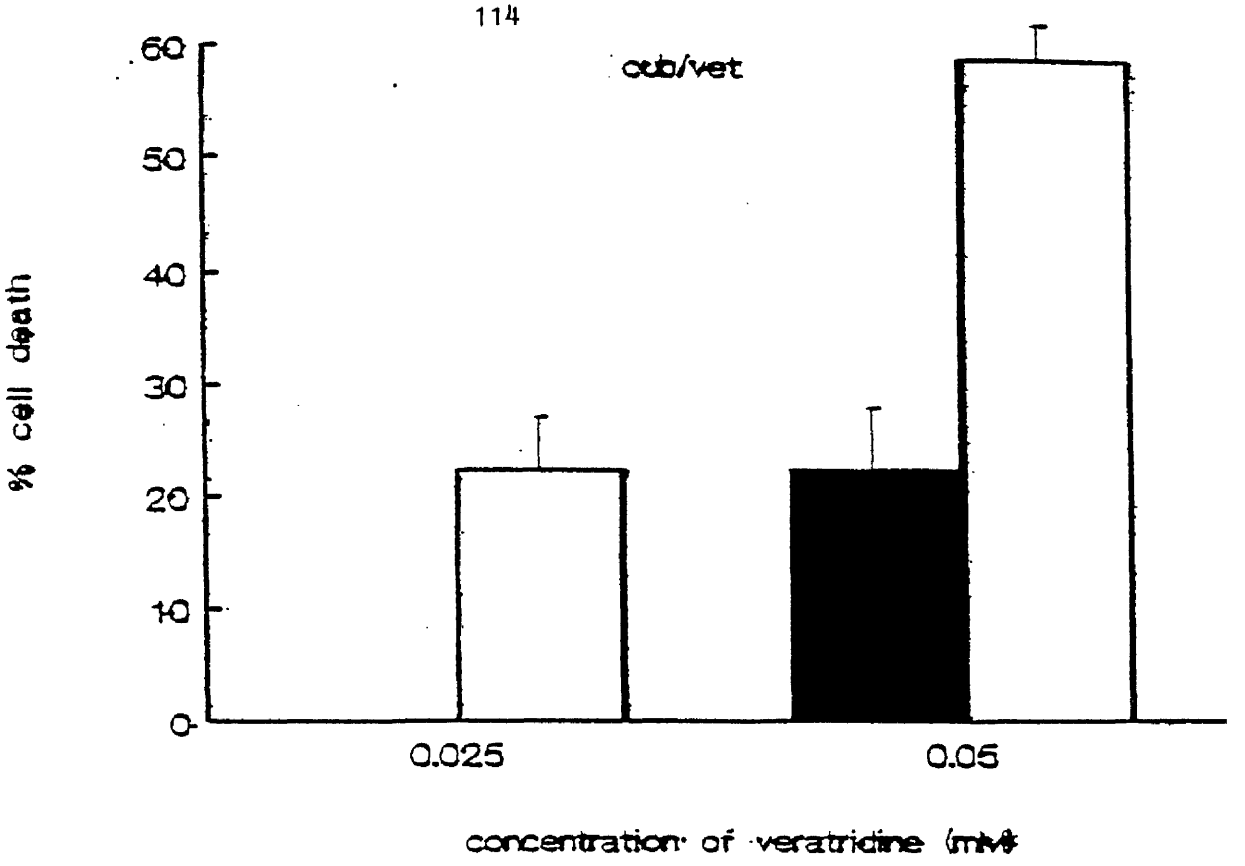
Several other differentiating agents viz; 2 % Dimethyl sulphoxide (DMSO), N,N'-Hexamethylene-bis-

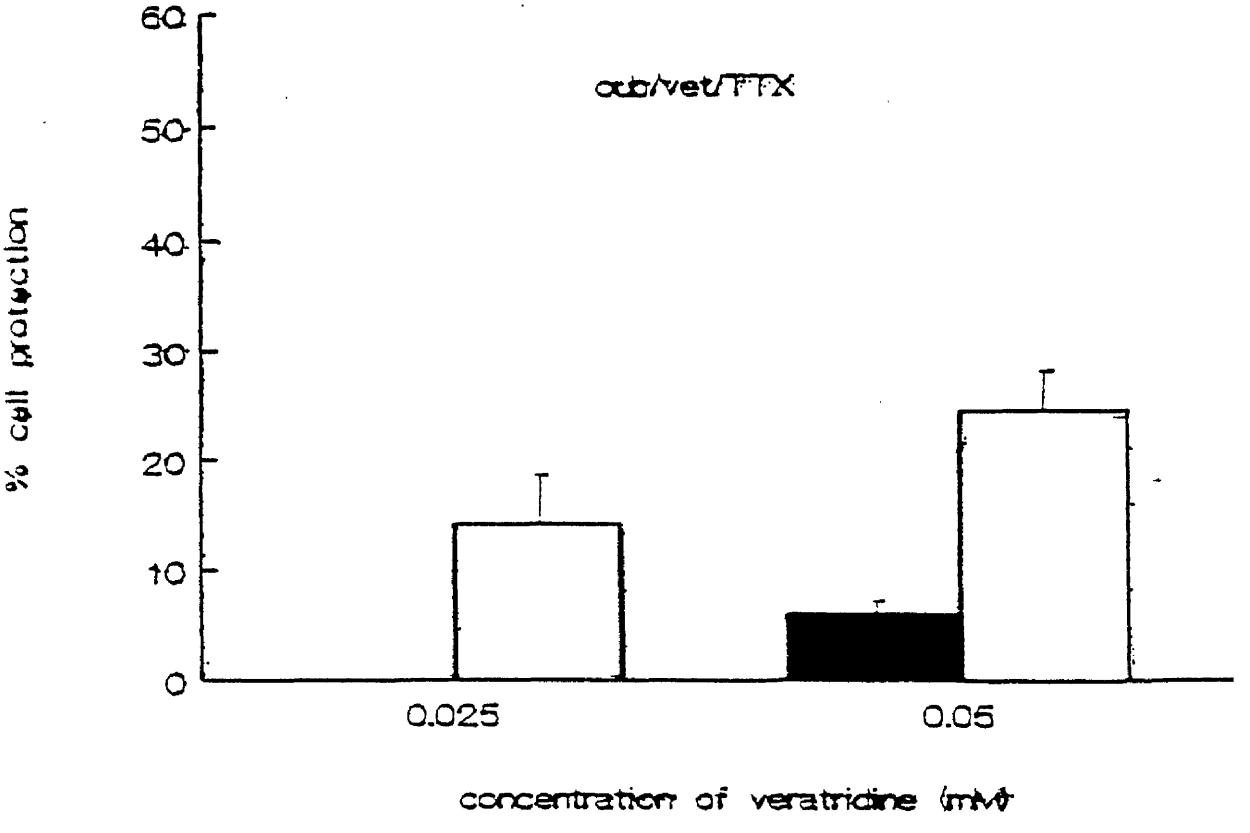
FIGURE 14 Effect of C6 medium compared to maintainance medium on the cell death of MNB cells on incubation with oub/ver or cell protection on the addition of TTX.

LEGEND:


maintainance medium


C6 medium


oub/vet
% cell death
60
50
40
30
20
10
0
0.025
0.05
concentration of veratridine (mM)


oub/vet/TTX
% cell protection
60
50
40
30
20
10
0
0.025
0.05
concentration of veratridine (mM)

acetamide (HMBA; 2.5 and 10 mM), valinomycin (1 µM and 0.01 µM), aminopterin (10 µM and 1 µM) nerve growth factor (NGF; 50 and 100 ng/ml) and serum limitation (2 % v/v) were investigated.

Each of the above, was incorporated into maintenance medium containing 5 % serum, which was reduced to 2 % in the serum limited sample. Cells were grown to confluence, maintained for a further 7 days in media containing the differentiating agent, seeded onto MT plates and the sensitivity to oub and ver measured.

Maintainance medium containing aminopterin at both concentrations killed the MNB cells within 3 days. Also, a large percentage of the cells maintained in medium containing valinomycin at 1 µM were dead, however, the remaining viable cells were elongated.

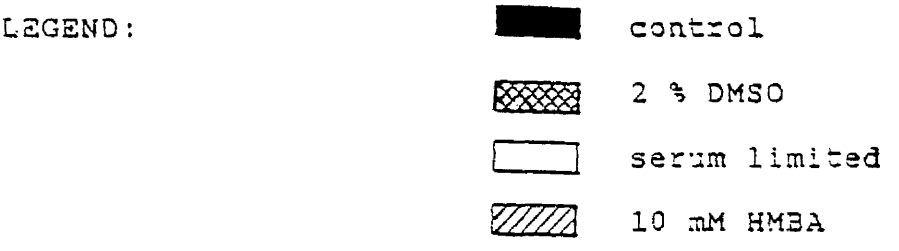
MNB cells grown in medium containing both concentrations of NGF, 2.5 mM HMBA or 0.01 µM valinomycin, exhibited the same morphology and response to oub/ver as the control, at all concentrations of oub/ver tested.

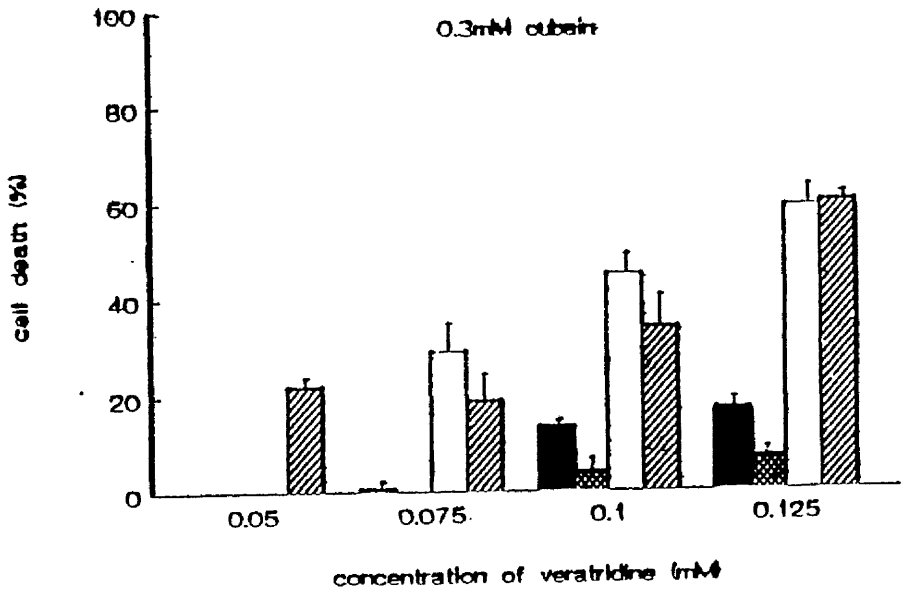
The morphology of MNB cells, cultured in maintainance medium supplemented with 10 mM HMBA or under serum limited conditions, seemed elongated with processes extending from the cell body. These cells also exhibited similiar increases in cell

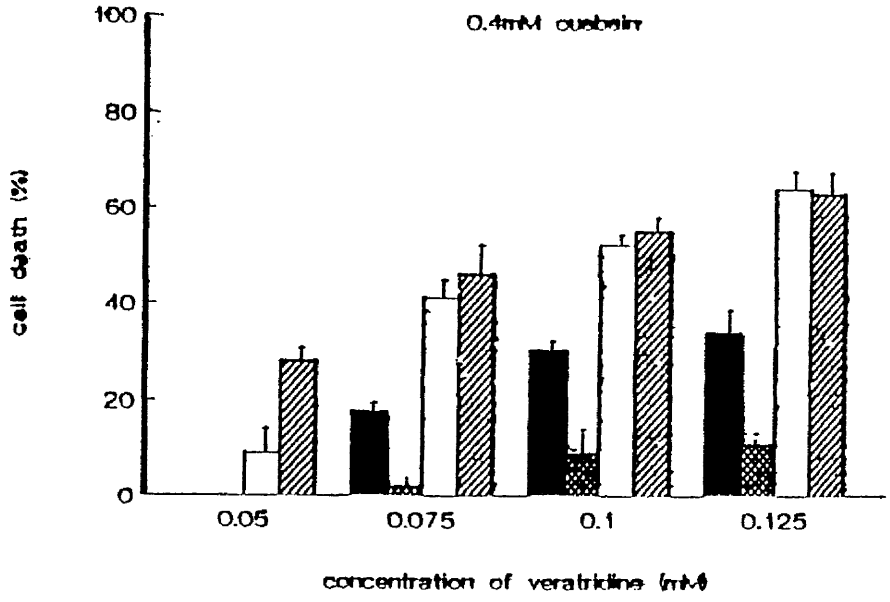
death of between 20 and 35 %, when compared to the control, at various concentrations of oub/ver (Fig. 15). However, medium containing 10 mM HMBA increased the sensitivity of the cells to oub/ver at lower concentrations of the two toxins than occurred under serum limited conditions.

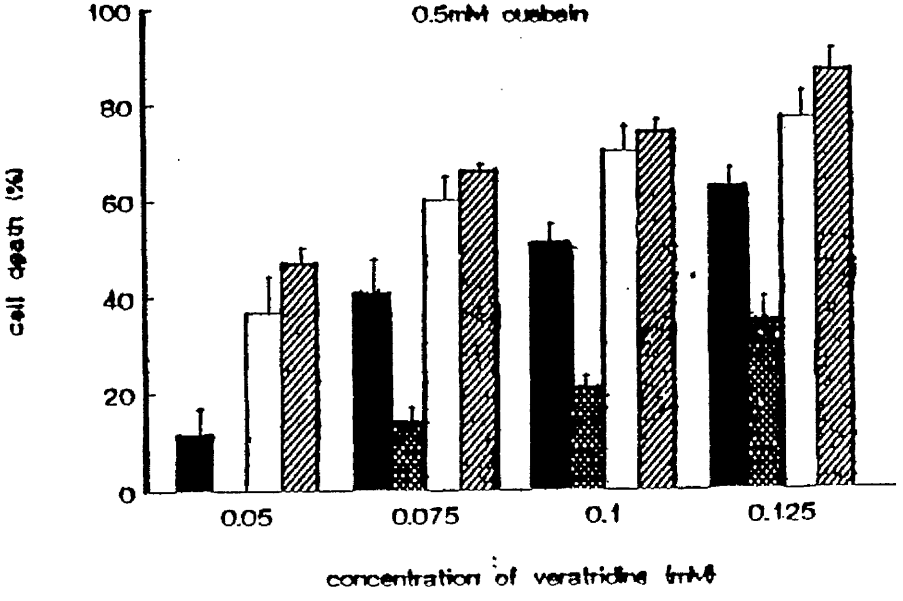
In DMSO-supplemented medium, MNB cells larger than the controls were observed. The nucleus could be clearly seen and the cells formed a matrix with short thick processes. However, a marked decrease in the sensitivity of MNB cells to oub/ver was found; MNB cell death decreased by up to 30 % compared to the control (Fig. 15).

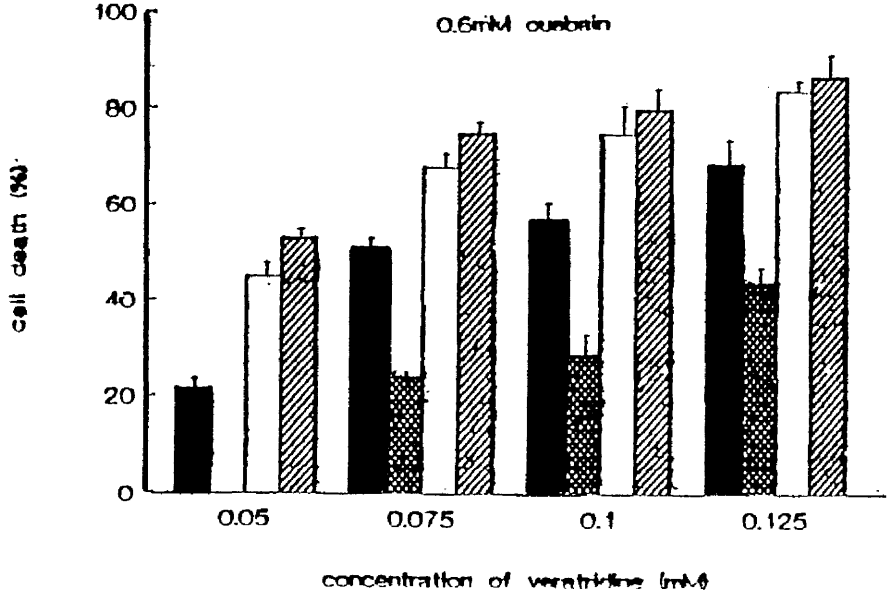
FIGURE 15 Effect of culturing MNB cells in serum limited medium or medium containing either Hexamethylene-bis-acetamide (HMBA) or Dimethyl sulphoxide (DMSO), on cell death, induced by varying concentrations of oub/ver.




0.3mM ouabain
cell death (%)
100
80
60
40
20
0
0.05
0.075
0.1
0.125
concentration of veratridine (mM)


0.4mM ouabain
cell death (%)
100
80
60
40
20
0
0.05
0.075
0.1
0.125
concentration of veratridine (mM)


0.5mM ouabain
cell death (%)
100
80
60
40
20
0
0.05
0.075
0.1
0.125
concentration of veratridine (mM)


0.6mM ouabain
cell death (%)
100
80
60
40
20
0
0.05
0.075
0.1
0.125
concentration of veratridine (mM)

DETECTION OF SODIUM-CHANNEL BLOCKING ACTIVITY IN BACTERIA

Bacterial Cell Extracts

In initial experiments, cell extracts from 24 h cultures of 6 strains of marine bacteria were either heated at 100°C for 10 min, or left untreated and analysed for SCB activity by the tissue culture assay.

With the exception of OK1, the unheated cell extracts were not toxic to the MNB cells at dilutions of 1/10, however, SCB activity was only detected in strain GFC cultured in M2 and MB. Upon heating, SCB activity was detected in all of the cell extracts examined and varied with both the media and the strain (Table 12).

In subsequent experiments, SCB activity of the supernate and cell extract, either heated or unheated, of strain GFC cultured in MB, were compared. In the unheated cell extract, SCB activity was not detected until the supernate was diluted to 1/8, whereas when heated, the activity was detected at a 1/1 dilution (Fig. 16), although in smaller quantities. These results indicated that the cell extract contained a heat-labile inhibitor (CIF) of SCB activity.

In the culture supernate, as with the unheated

TABLE 12 Effect of a) culture medium and b) heating at 100°C for 10 min on the SCB activity of cell extracts, from 6 strains of marine bacteria.

Strain	Heating at 100°C for 20 min	SCB activity* of cells derived from medium			
		M1	M2	M3	MB
OKI	heated	+	++	++	++
	unheated	c/t	c/t	c/t	c/t
OK2	heated	+	+	±	nd
	unheated	-	-	-	nd
GFB	heated	+	+	+	+
	unheated	-	-	-	-
GFC	heated	++	++	+	+
	unheated	-	+	-	+
829	heated	++	+	+	++
	unheated	-	-	-	-
1903	heated	+	+	++	nd
	unheated	-	-	-	nd

*SCB activity was determined with TTX as a standard and the results expressed as TTX equivalent (ng/ml). -,0; ±,<10; +,10-40; ++, 40-100; c/t,cytotoxic;nd,not done

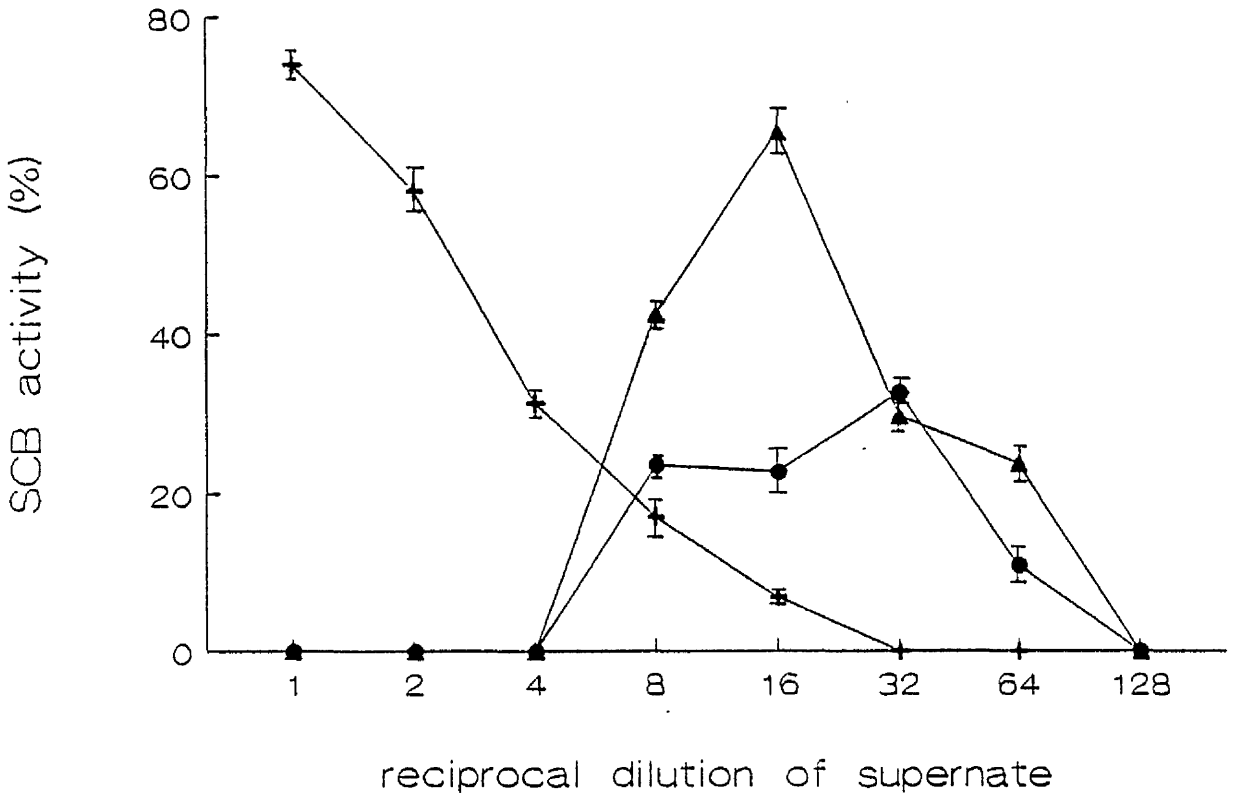
FIGURE 16 Distribution of the SCB activity (as measured by % protection of oub/ver treated MNB cells) between the supernate and cell extract of bacterial strain GFC. The effect of heating the cell extract at 100°C for 10 min was also examined.

LEGEND:

▲—▲ culture supernate

●—● cell extract

+—+ cell extract (heated)


80
60
40
20
0
SCB activity (%)
1
2
4
8
16
32
64
128
reciprocal dilution of supernate

cell extract, SCB activity was not detected until the supernate had been diluted to 1/8 with maximum activity occurring at a dilution of 1/16 (Figs. 16 & 17). Therefore, the culture supernate also contained an inhibitor (SIF) of SCB activity.

As the SCB activity in the culture supernate was higher than that of the cell extracts, supernates were subsequently used to investigate the SCB activity of bacteria.

Culture Supernates

Prior to examining culture supernate for SCB activity, the cytotoxicity of the 4 different media to the MNB cells was investigated. The media were toxic to the cells only when undiluted, with the exception of M1 which was slightly toxic at 1/2.

Preliminary experiments involved the culture supernates of each of the 6 bacterial strains, grown for seven days, in four different media. Trace amounts of SCB activity at a dilution of 1/4 in only M2 was found for all of the 6 strains.

Further investigations concentrated primarily on the strains GFC and OK1 cultured in either M2 or MB and tested over a two-fold dilution series. These gave inverted v-shaped dose response curves similiar to that already described for strain GFC, with

inhibition of the protective activity of SCB toxins at high concentrations of supernate (Figs. 17 & 18).

When the SCB activity of strain GFC was converted into probits (Fig. 17) the quantity of toxin which protected 50 % of the MNB cells was calculated as 1350 nM TTX equiv. (430 ng TTX equiv/ml).

Further experiments with strains GFC and OK1 in M1 and M3, GFB and 1903 in M3, 829 in MB and OK2 in M1 all showed similiar inverted v-shaped dose response curves. Therefore, irrespective of the strain or media, an inhibitory factor (SIF) was present in the culture supernate which masked the detection of SCB activity in all 6 strains tested. To identify the factor a number of experiments were undertaken.

Investigating the nature of the inhibitory factor

Effect of heating on the IF and SCB activity

The effect of heat on the stability of the SIF was investigated. Preliminary work on strain OK1 culture supernate indicated that, although the cytotoxic factor (shown in Fig. 18) was destroyed upon heating (100°C for 30 min), the SIF was not.

When GFC supernate was heated at 100°C for up to 1 h, the SIF factor was not destroyed. However, the SCB activity decreased by approximately 50 % within the first 5 min of heating and full activity was not regained until the sample had been heated for at

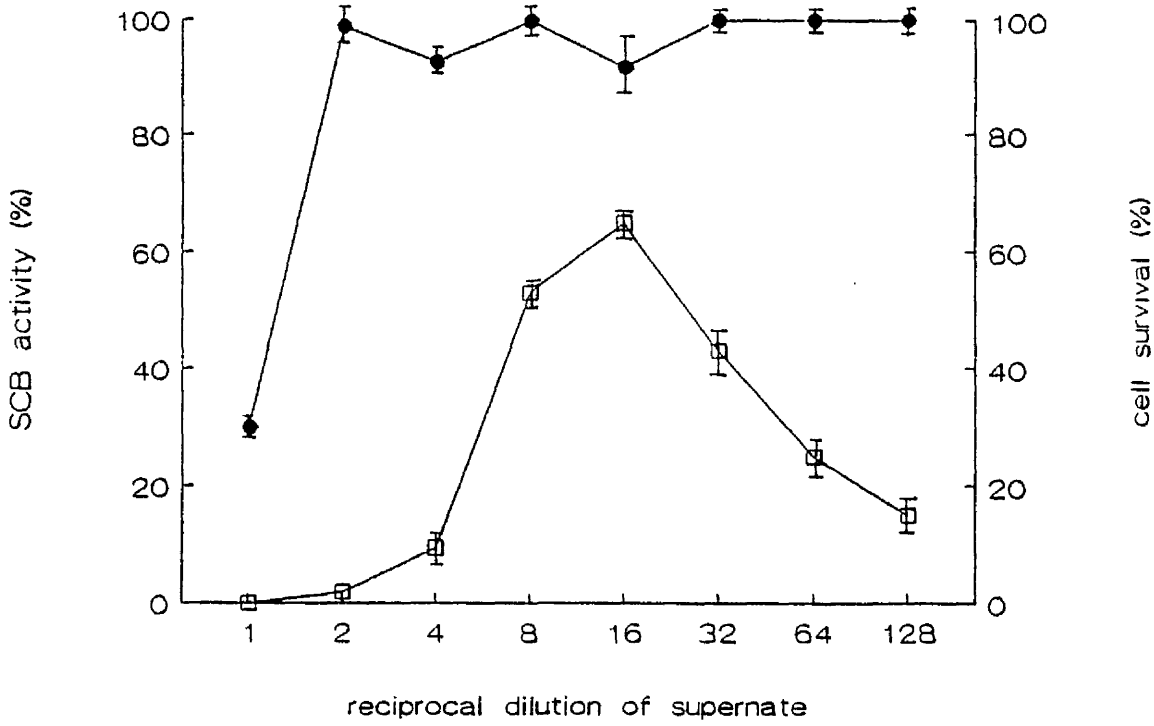
FIGURE 17 SCB activity, in the presence of oub/ver, of strain GFC supernate cultured in MB and cell survival in the absence of oub/ver (upper figure). The lower figure shows the SCB activity of GFC culture supernate expressed as probits.

LEGEND:

□–□ % SCB activity

●–● % cell survival

GFC in MB



GFC in MB

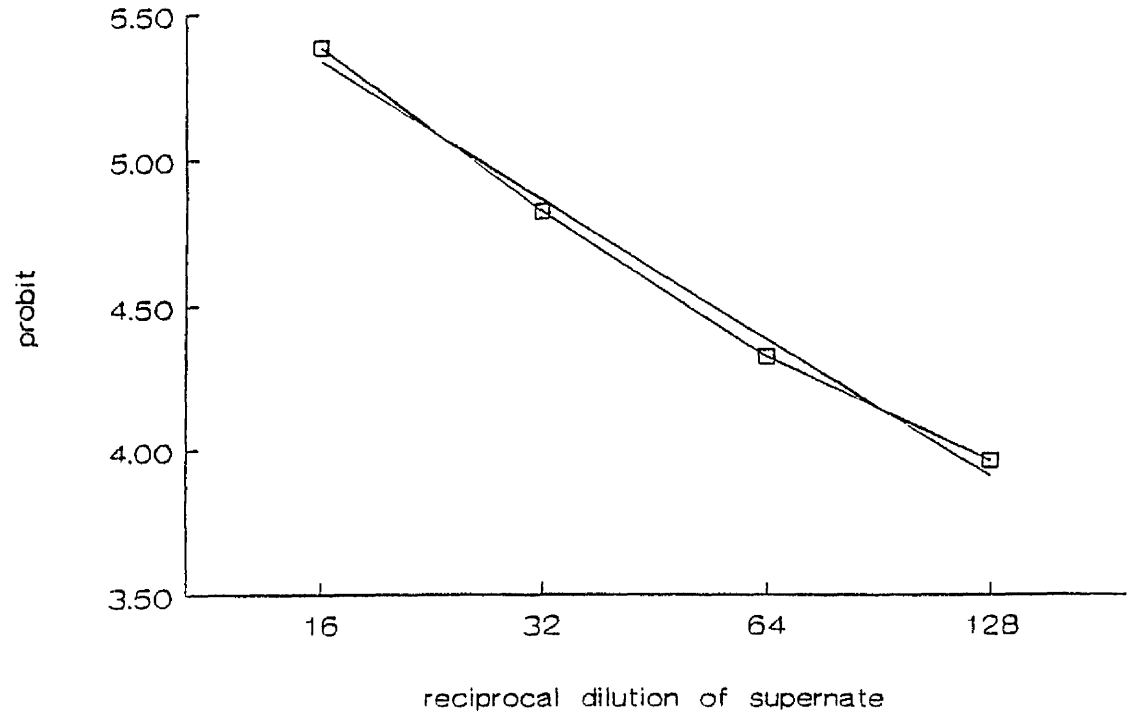


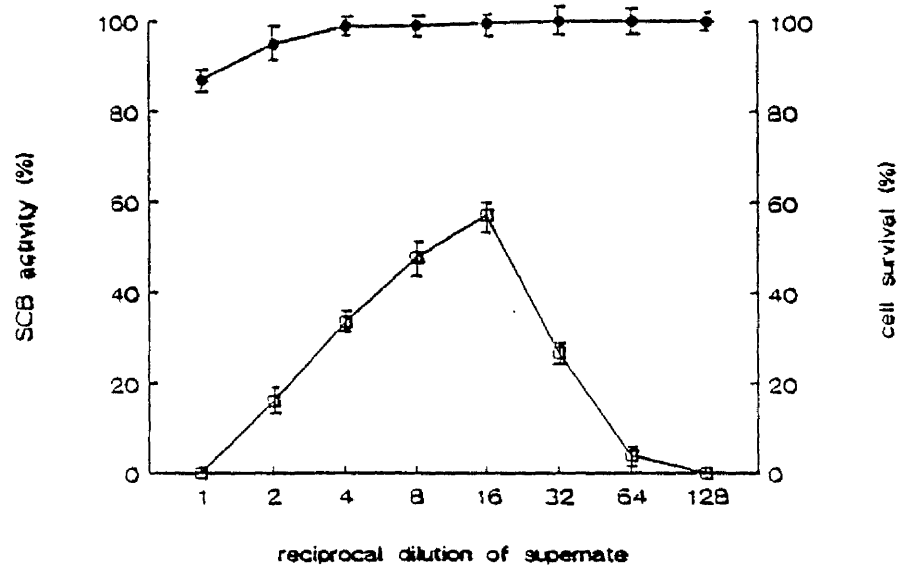
FIGURE 18 SCB activity, in the presence of oub/ver, of culture supernates of strain GFC in M2 and OK1 in either M2 or MB and cell survival in the absence of oub/ver.

LEGEND:

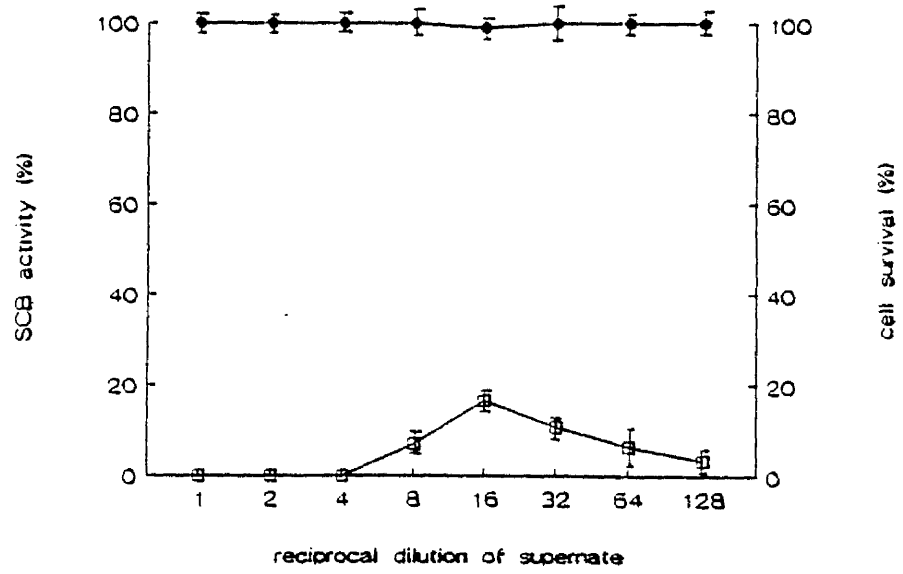
□–□ % SCB activity

●–● % cell survival

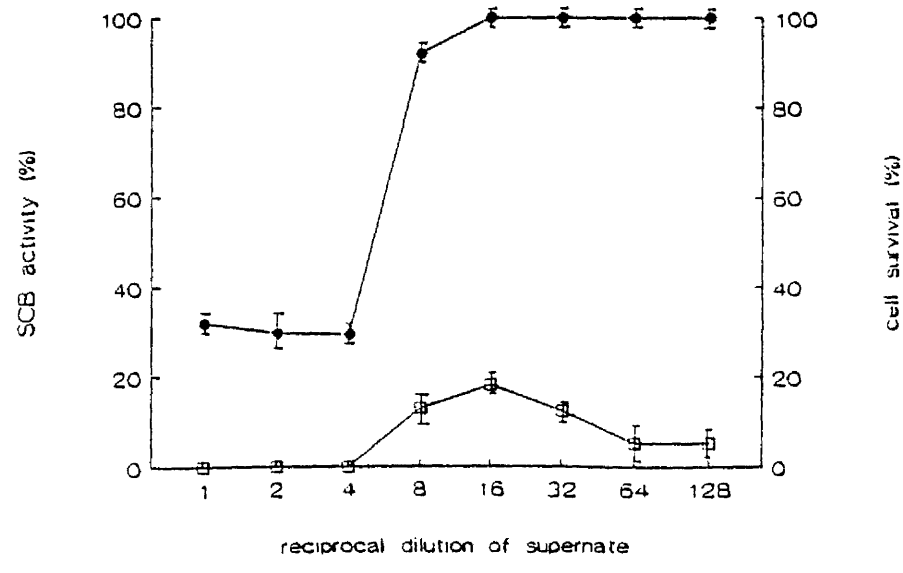
strain GFC in M2



strain OK1 in M2



strain OK1 in MB



least 30 min (Fig. 19a), after which it remained stable for up to 1 h.

When MB containing added TTX was heated at 100°C for up to 30 min and examined for SCB activity, the SIF was found in only the unheated sample and to a lesser extent than with the bacteria. However, the decrease in SCB activity upon heating for up to 20 min still occurred (Fig. 19b).

Effect of Ultrafiltration on the IF and SCB activity

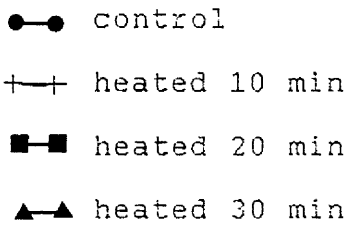
Ultrafiltration was used to investigate the molecular size of the SIF. In initial experiments centrifuge tubes containing either a 10 Kd or 30 Kd cut-off membrane were used to ultrafilter, by centrifugation, culture supernates of strains GFC and OK1. The filtrates of these two strains, when assayed by tissue culture, still contained the SIF.

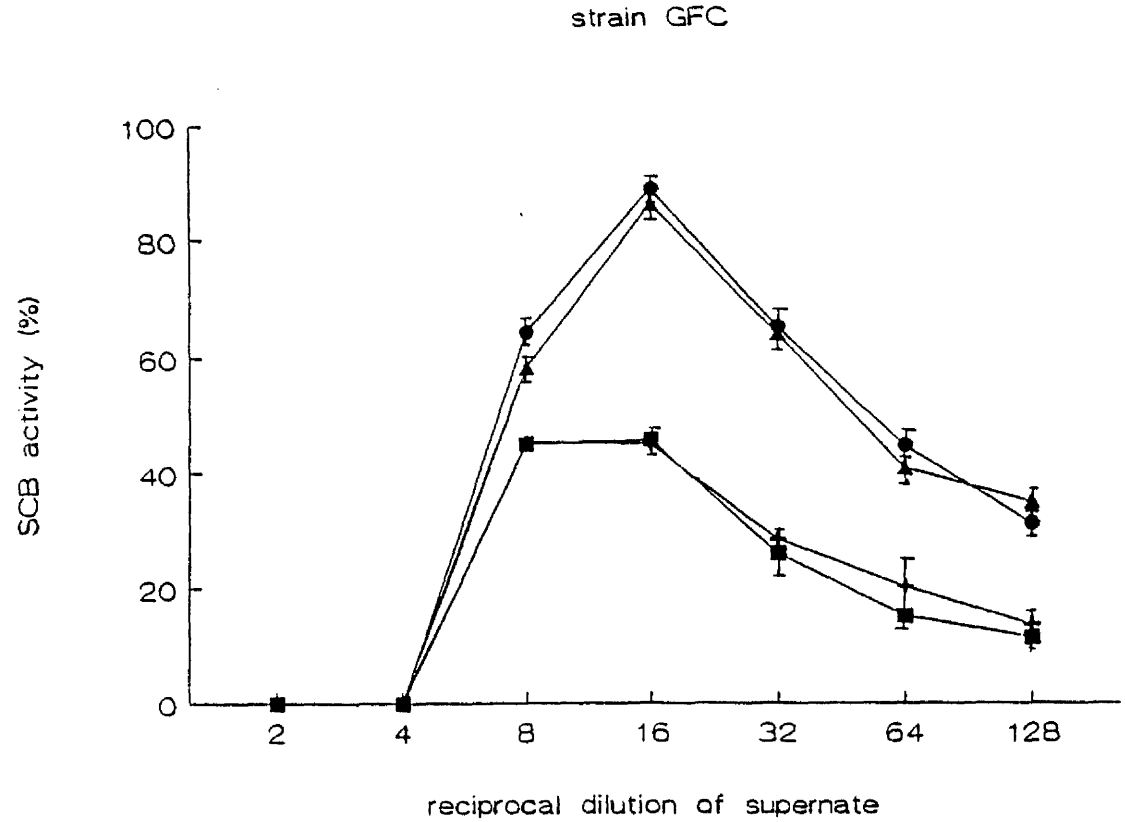
In further experiments the SIF and SCB activity in strain GFC crude culture supernate was compared to the filtrate and concentrate after ultrafiltration by Amicon cut-off filters over the range 500 d, 1 Kd, 5 Kd, 10 Kd, and 30 Kd molecular size.

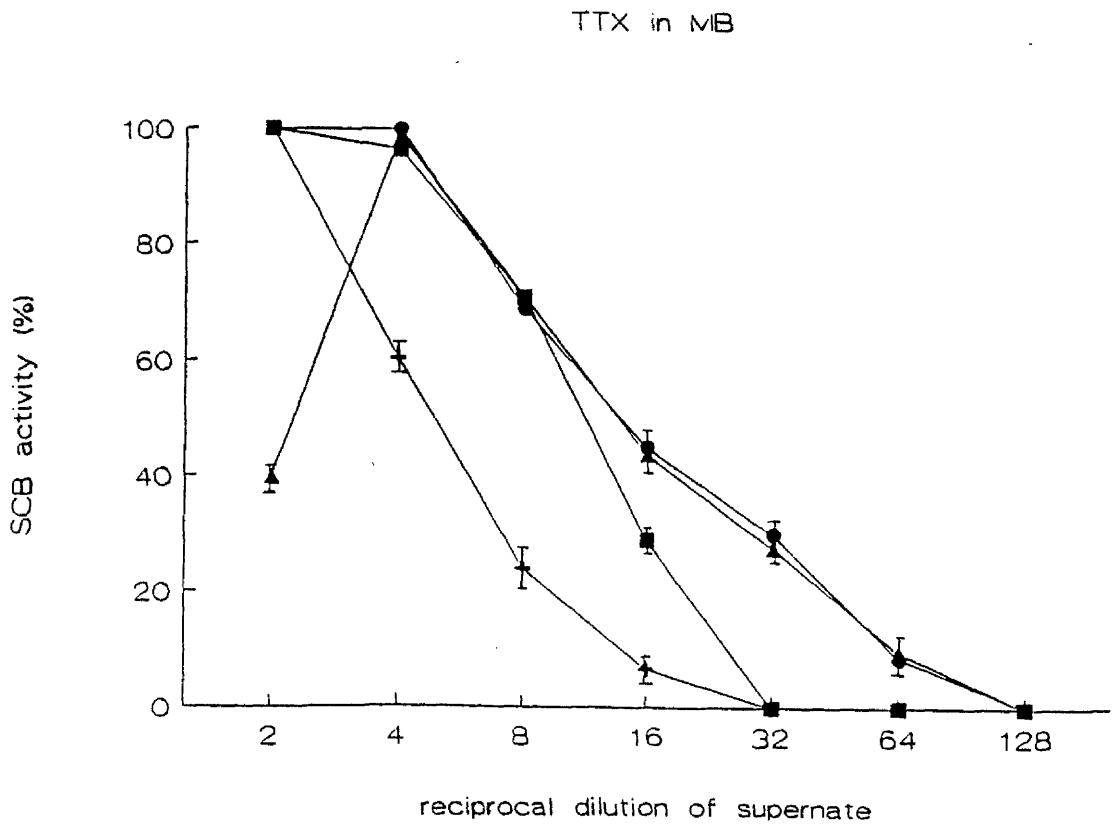
The SIF was not removed in the filtrate or concentrate of the supernates upon ultrafiltration with membranes of 1K or above. However, it was partially removed in the 500 d concentrate,

FIGURE 19 Effect of heat, at 100°C for up to 30 min, on the SIF and SCB activity of a) strain GFC culture supernate and b) TTX in MB.

LEGEND:




strain GFC
SCB activity (%)
100
80
60
40
20
0
2
4
8
16
32
64
128
reciprocal dilution of supernate


TTX in MB
SCB activity (%)
100
80
60
40
20
0
2
4
8
16
32
64
128
reciprocal dilution of supernate

indicating that it consisted of a substance with a molecular weight of <500 d. However the SCB activity did not pass through the 500 d filter but remained in the 5x concentrate and was present at higher levels than in the crude supernate (Fig. 20a).

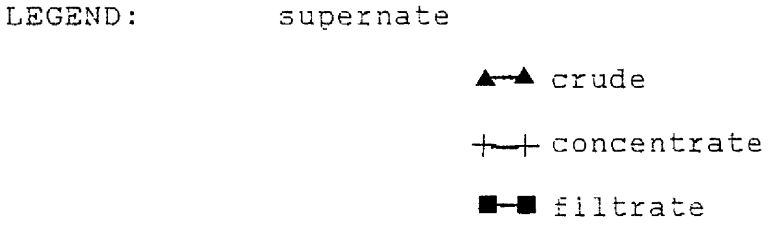
This effect was reproducible and was also found when MB containing added TTX was ultrafiltered at 500 d, although a corresponding increase in the SCB activity of the 1.25x concentrate was not observed (Fig. 20b).

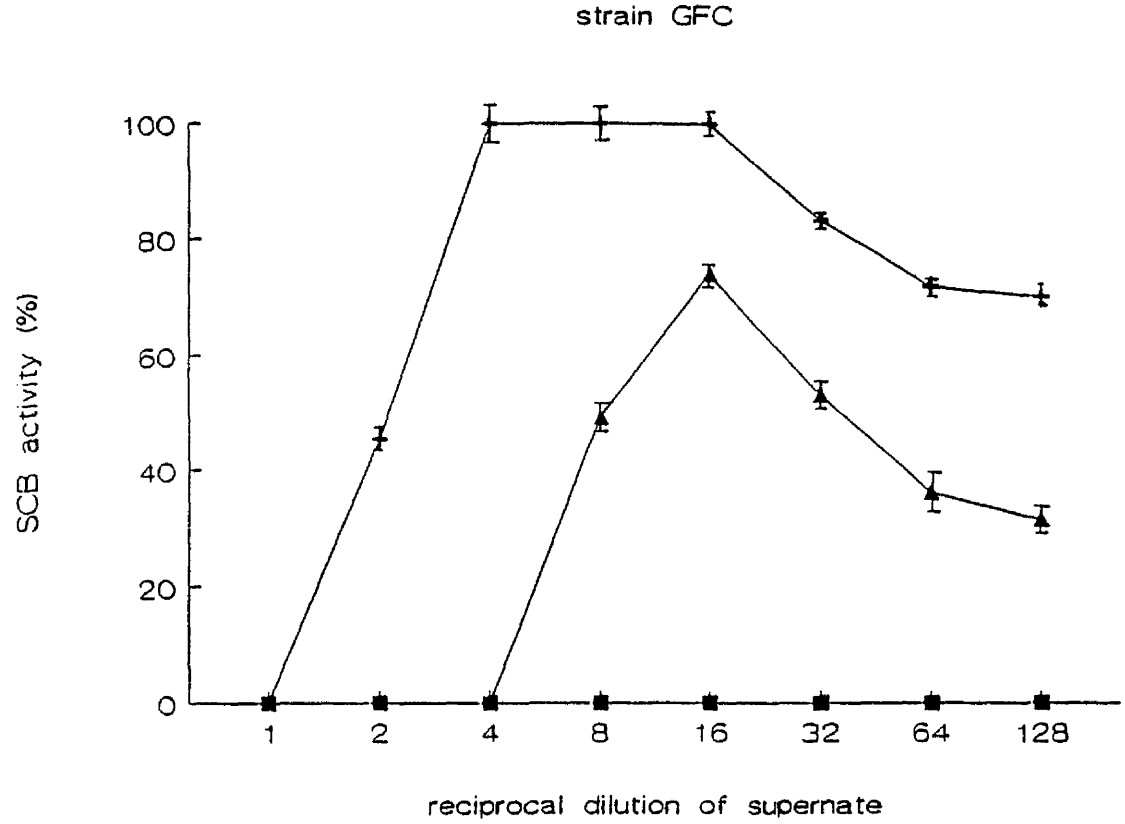
Growth in different strengths of marine broth

Strain GFC was cultured in various strengths of MB and the resultant SCB activity compared to that found on culturing the strain in full-strength MB.

In the supernate of strain GFC cultured in full-strength MB, the SCB activity exhibited the previously observed inverted v-shaped dose-response curve, with maximum activity at a dilution of 1/16. Whereas, in supernate obtained from 1/4-strength MB, the effect of the SIF decreased and maximum SCB activity occurred at 1/2 dilution. This was also found, but to a lesser extent, in the supernates from 1/2 and 1/3-strength MB which both showed maximum SCB activity at 1/4 dilution (Fig. 21). However, growth of strain GFC in these samples also

FIGURE 20 Effect of ultrafiltration (500 daltons) on the SIF and SCB activity of the filtrate and concentrate of a) strain GFC supernate and b) TTX in MB.




strain GFC
SCB activity (%)
100
80
60
40
20
0
1
2
4
8
16
32
64
128
reciprocal dilution of supernate

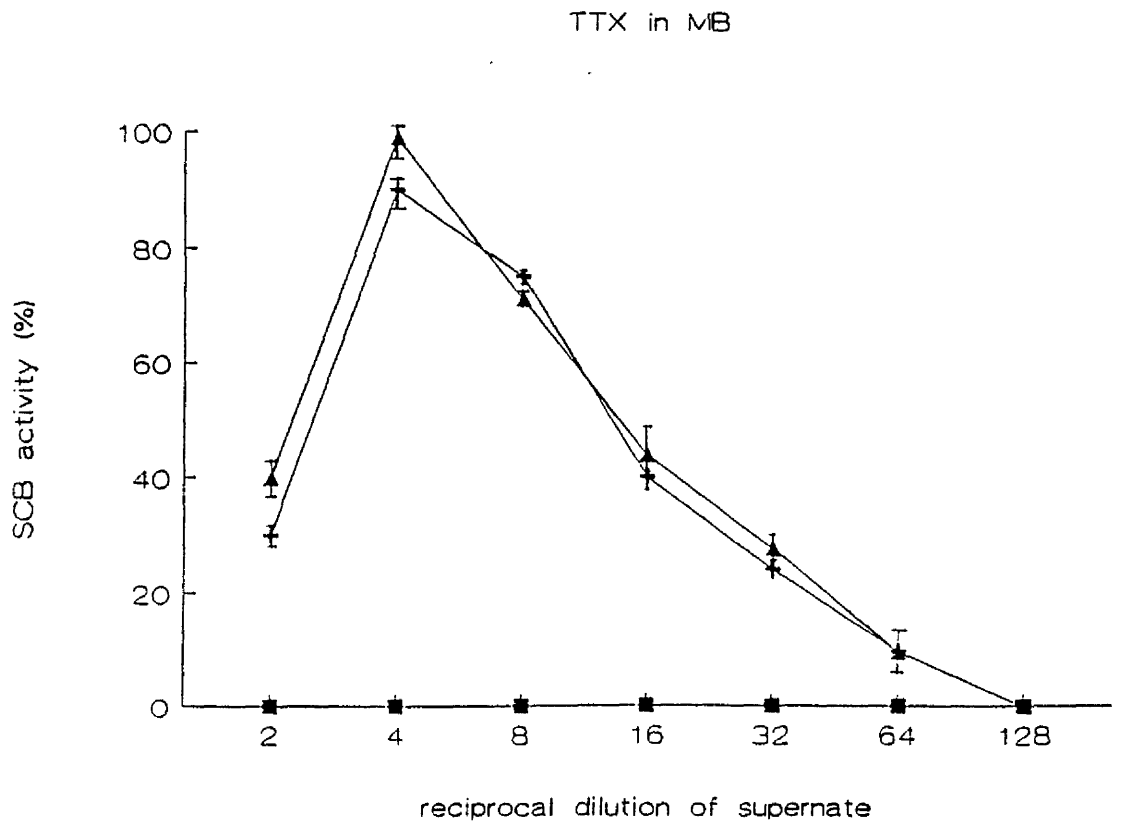
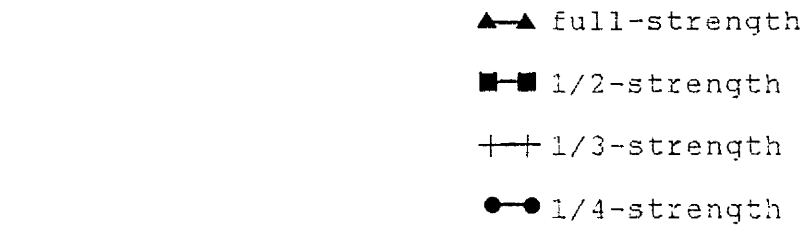
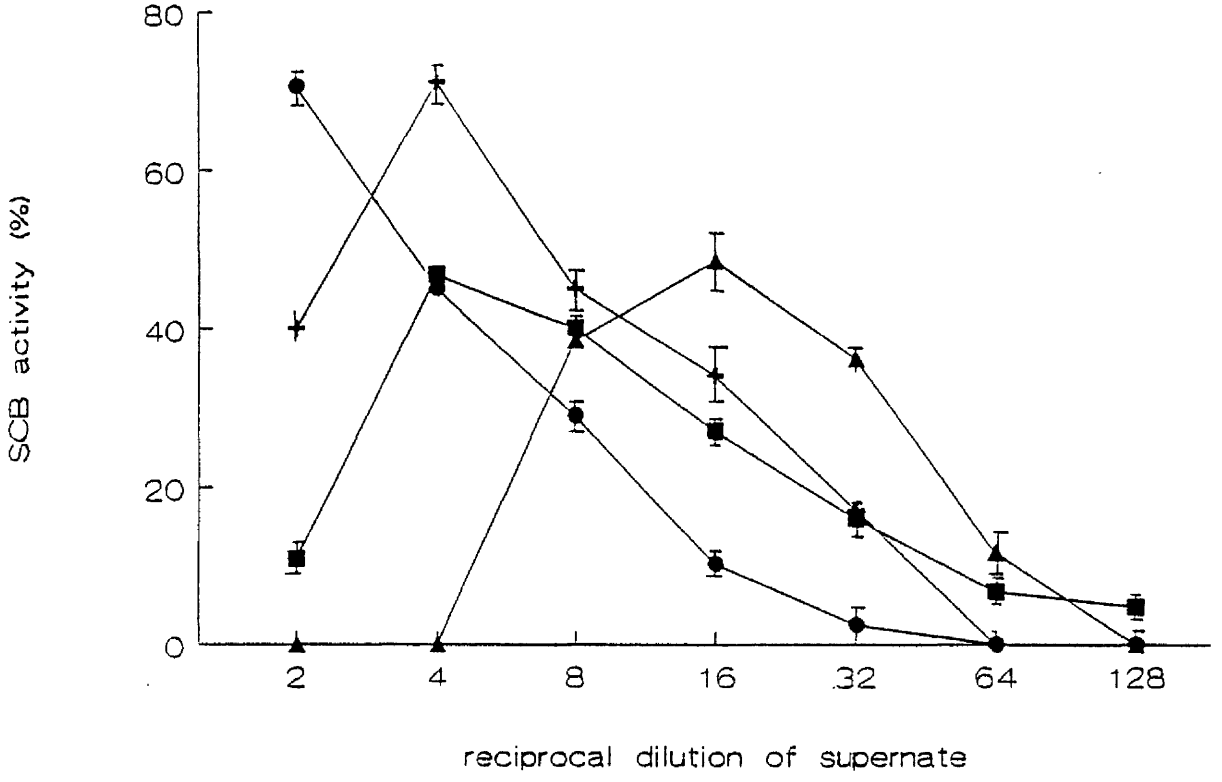

TTX in MB
SCB activity (%)
100
80
60
40
20
0
2
4
8
16
32
64
128
reciprocal dilution of supernate

FIGURE 21 Effect of diluting the MB, in which strain GFC was grown, on the SIF and SCB activity in culture supernates.




80
60
40
20
0
SCB activity (%)
2
4
8
16
32
64
128
reciprocal dilution of supernate

decreased as the strength of MB used for growth decreased (Table 13).

The effect of MB on the SCB activity of bacterial culture supernates was then studied by the tissue culture assay.

Effect of MB on SCB activity

The effect of MB on the detection of SCB activity in supernates was examined by diluting strain GFC supernate, cultured in MB, with a diluent which was a mixture of MB and RPMI at amounts of 1 + 1, 1 + 2, and 1 + 3. When these samples were applied to the tissue culture assay, SCB activity was not detected at any dilution, although culture supernate diluted in RPMI alone showed the inverted v-shaped dose-response curve.

This experiment was repeated with diluents of MB:RPMI at higher dilutions of 1 + 4, 1 + 18, 1 + 28 and RPMI alone. As the levels of MB in the diluent decreased an increase in SCB activity could be observed (Fig. 22).

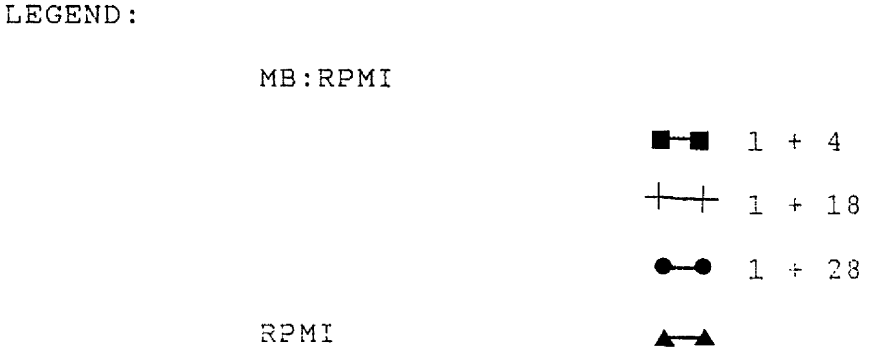
Effect of NaCl on SCB activity.

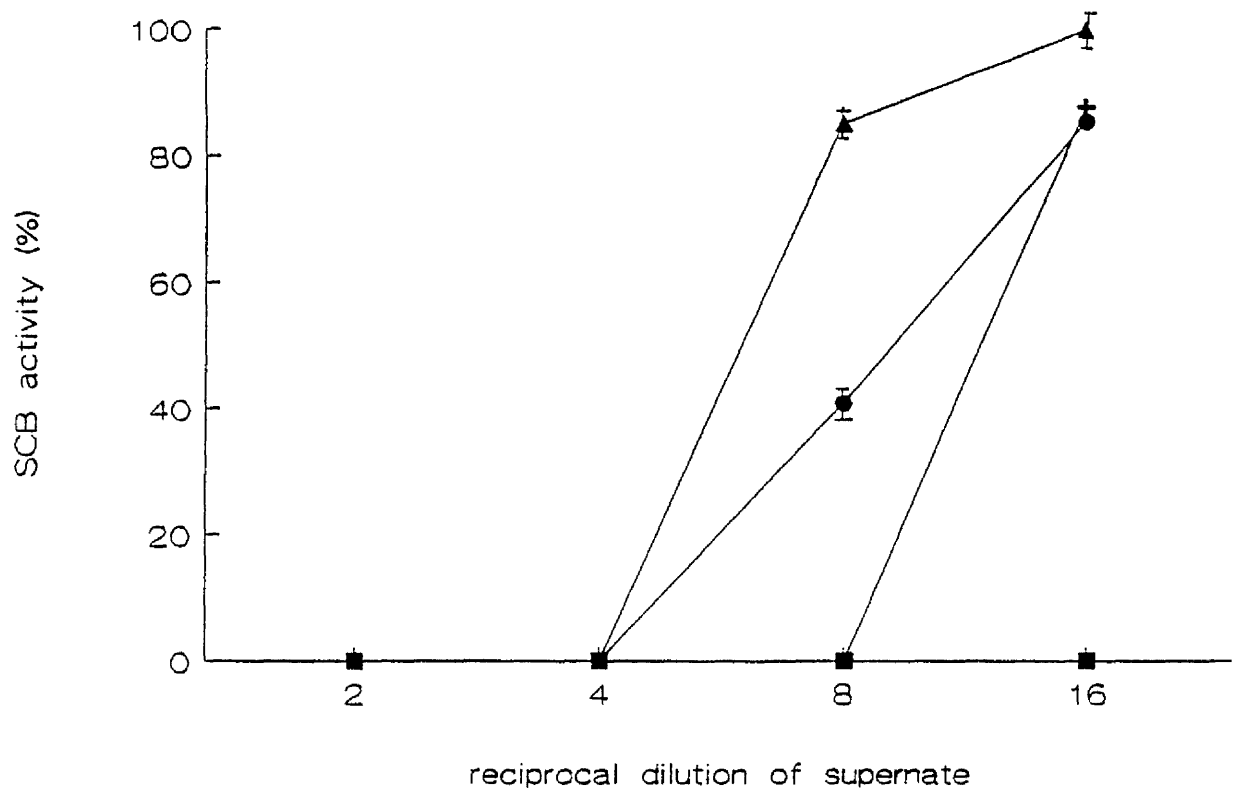
Since NaCl is the major constituent of MB, its effect on the SCB activity was determined by diluting strain GFC culture supernate with RPMI containing added known quantities of NaCl and

TABLE 13 Growth of strain GFC in different strengths of MB for 24 h.

Strength of MB	Absorbance (540nm) at 24 h
full	1.590
1/2	1.465
1/3	0.945
1/4	0.427

FIGURE 22 Effect of different mixtures of MB and RPMI (as diluent) when assaying strain GFC culture supernates for SCB activity.




100
80
60
40
20
0
SCB activity (%)
2
4
8
16
reciprocal dilution of supernate

assaying the samples by the tissue culture method. This clearly indicated that the effect of the SIF factor on the SCB activity decreased as the total NaCl concentration in the diluent decreased (Fig. 23). When the NaCl concentration in each dilution of strain GFC culture supernate (MB) was calculated, it was observed that the SCB activity was not detected until the NaCl levels had decreased (Table 14).

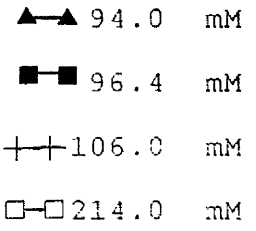
Use of a diluent containing less salt

The effect of a dilution series containing less total NaCl than is found by dilution with RPMI, on the SIF and SCB activity, was examined by initially diluting strain GFC supernate, obtained from either M2 or MB, with water followed by full-strength or 1/2-strength RPMI, and the results compared to those found with RPMI only as diluent (Fig. 24).

In strain GFC/MB culture supernate, a decrease in the SIF was observed with both the diluents used although, with water followed by full-strength RPMI, the difference was very small. However, a large decrease in the SIF was found with both diluents for GFC cultured in M2, although, as above, the samples diluted in 1/2-strength RPMI caused the greatest reduction in the SIF.

FIGURE 23 Effect of RPMI containing known quantities of NaCl as diluent when assaying strain GFC culture supernate for SCB activity.

LEGEND: Total NaCl concentration in RPMI



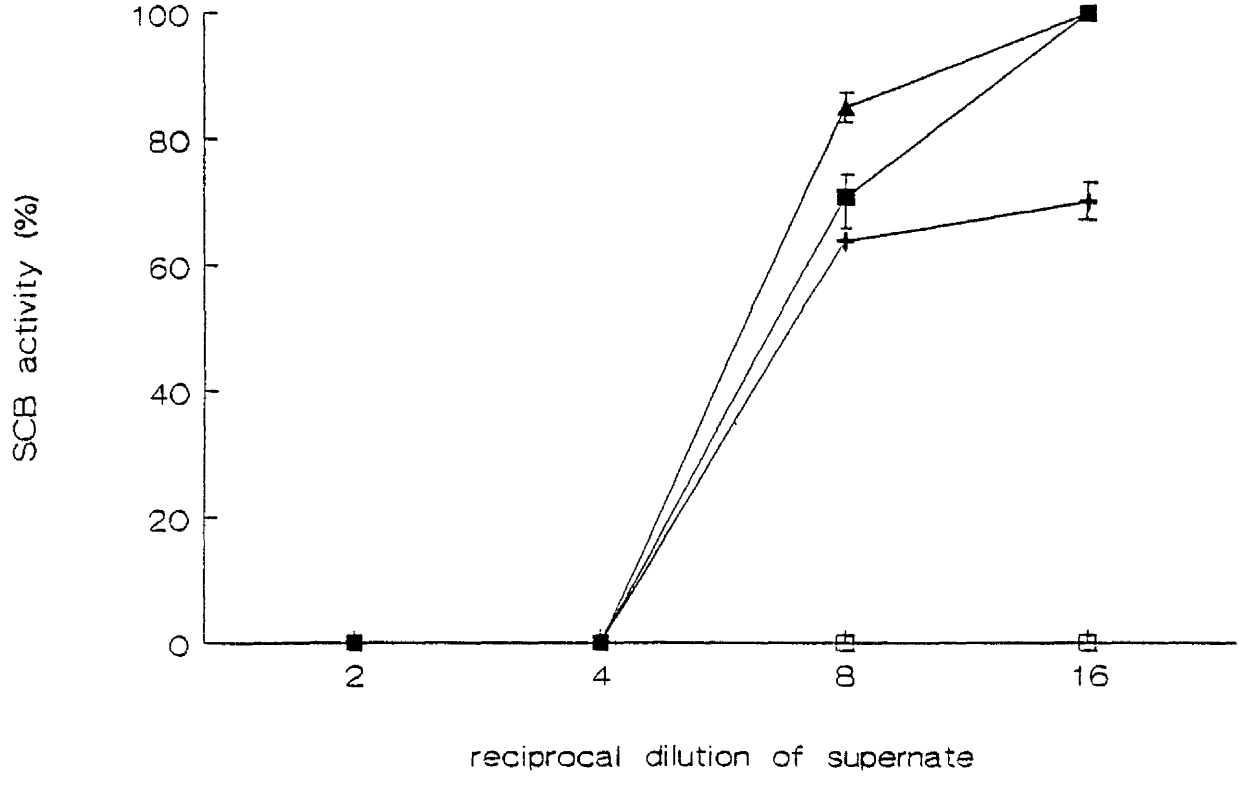
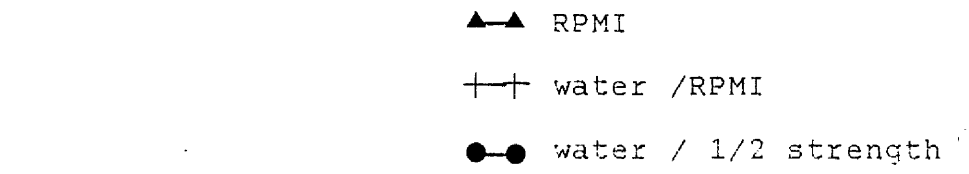

100
80
60
40
20
0
SCB activity (%)
2
4
8
16
reciprocal dilution of supernate

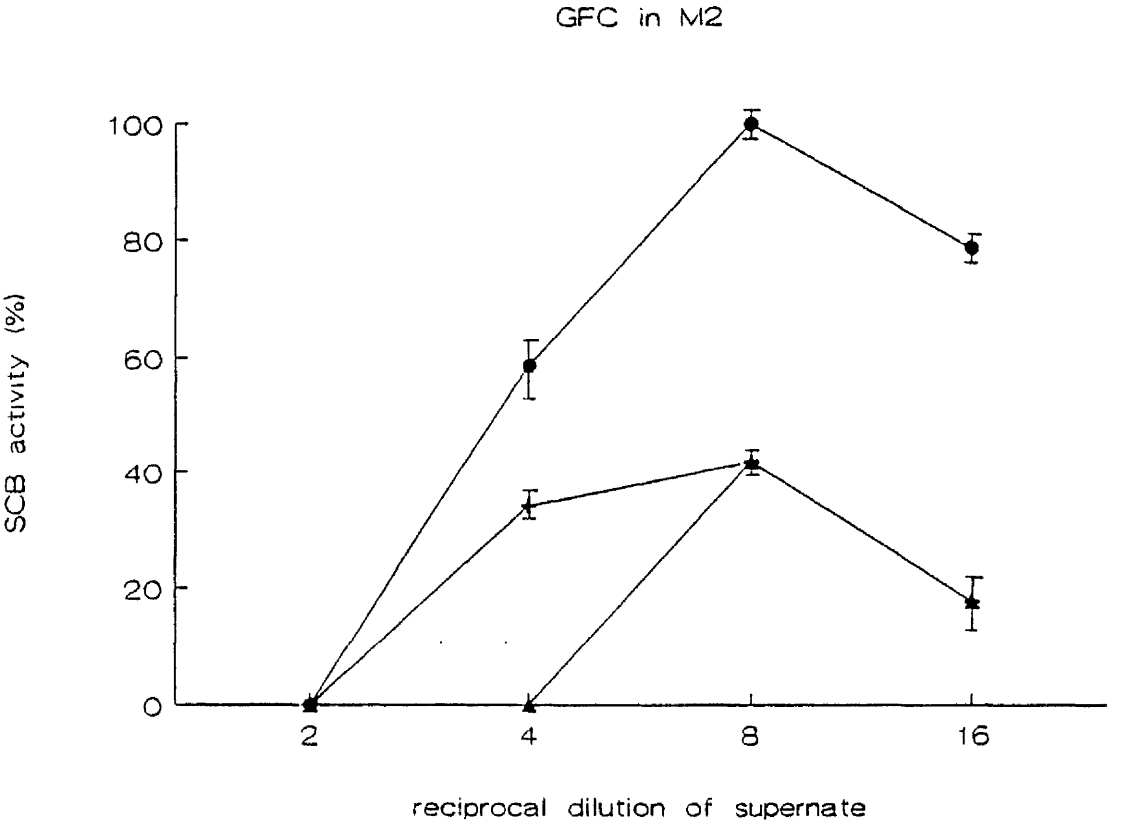
TABLE 14 Influence of supernate dilution and the calculated final concentration of NaCl on the SCB activity of strain GFC culture supernate.

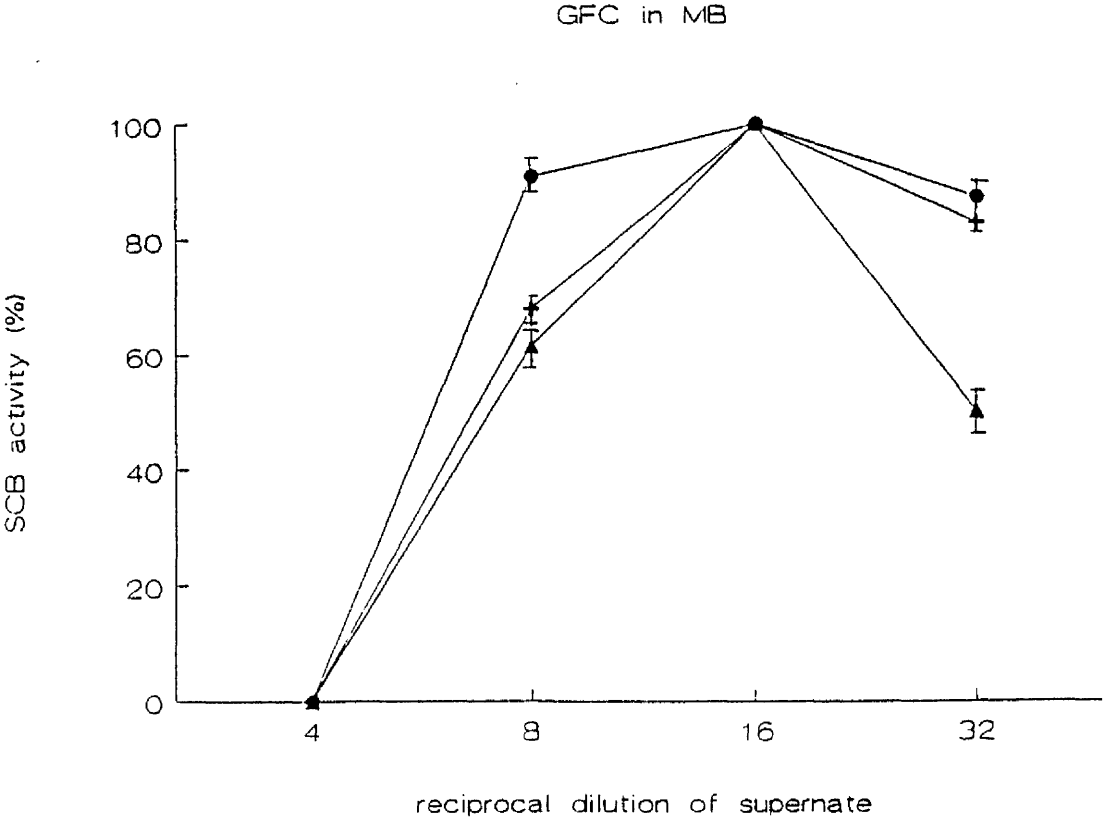
Supernate Dilution	*calculated NaCl concentration(mM)	SCB activity (%) (± SEM)
1/2	154	0
1/4	124	0
1/8	109	42.5 ± 1.7
1/16	101	65.5 ± 1.1

* The final NaCl concentration in the well is based on MB containing 332 mM NaCl and RPMI containing 94 mM RPMI.

FIGURE 24 Effect of an initial dilution in deionised water of strain GFC supernate, cultured in either M2 or MB, followed by further dilutions in RPMI or 1/2-strength RPMI on the SCB activity of the supernate.




GFC in M2
100
80
60
40
20
0
SCB activity (%)
2
4
8
16
reciprocal dilution of supernate


GFC in MB
100
80
60
40
20
0
SCB activity (%)
4
8
16
32
reciprocal dilution of supernate

Charcoal treatment of supernates

Supernate of strain GFC cultured in MB and MB containing added TTX were charcoal extracted and the resultant SCB activity compared to that of untreated samples. The SIF was effectively removed by the charcoal treatment, however, the amount of SCB activity decreased by up to 50 % (Fig. 25).

Screening of Various Bacteria for SCB Activity

Preheated (100°C for 30 min) culture supernates of various marine bacterial strains were analysed for SCB activity. Of the 11 *Vibrio* strains examined (excluding the Japanese strains), 8 produced SCB activity. Only 1 of the 2 *Aeromonas* strains tested produced SCB activity as did the only *Pseudomonas* strain tested. Three species of non-marine bacteria were also examined, but SCB activity was not found. In the SCB toxin producers, the level of activity varied widely between strains (Table 15).

Preliminary Identification Of Toxins Produced By Marine Bacteria

Supernates of bacterial strains A1096, A862, GFC and NCMIB 829 cultured in MB were analysed for PSP (paralytic shellfish poisons) by HPLC at MAFF, Burnham on Crouch. A compound which co-eluted with

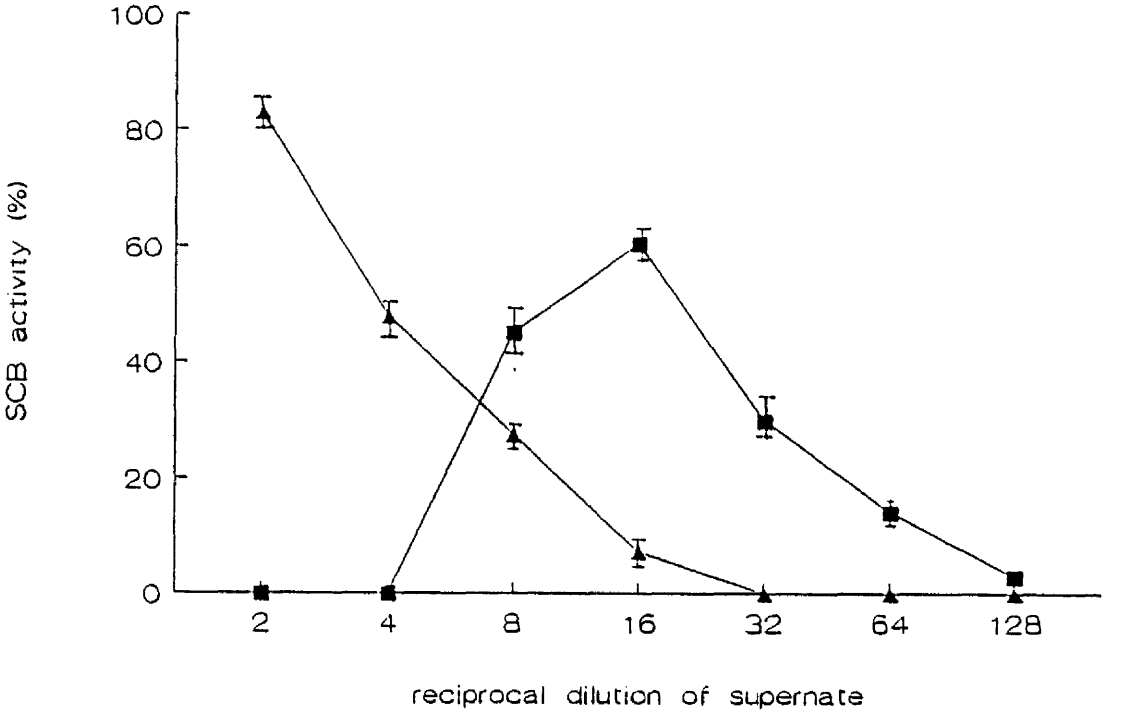
FIGURE 25 Use of charcoal to extract SCB activity from a) strain GFC culture supernate and b) TTX in MB.

LEGEND:

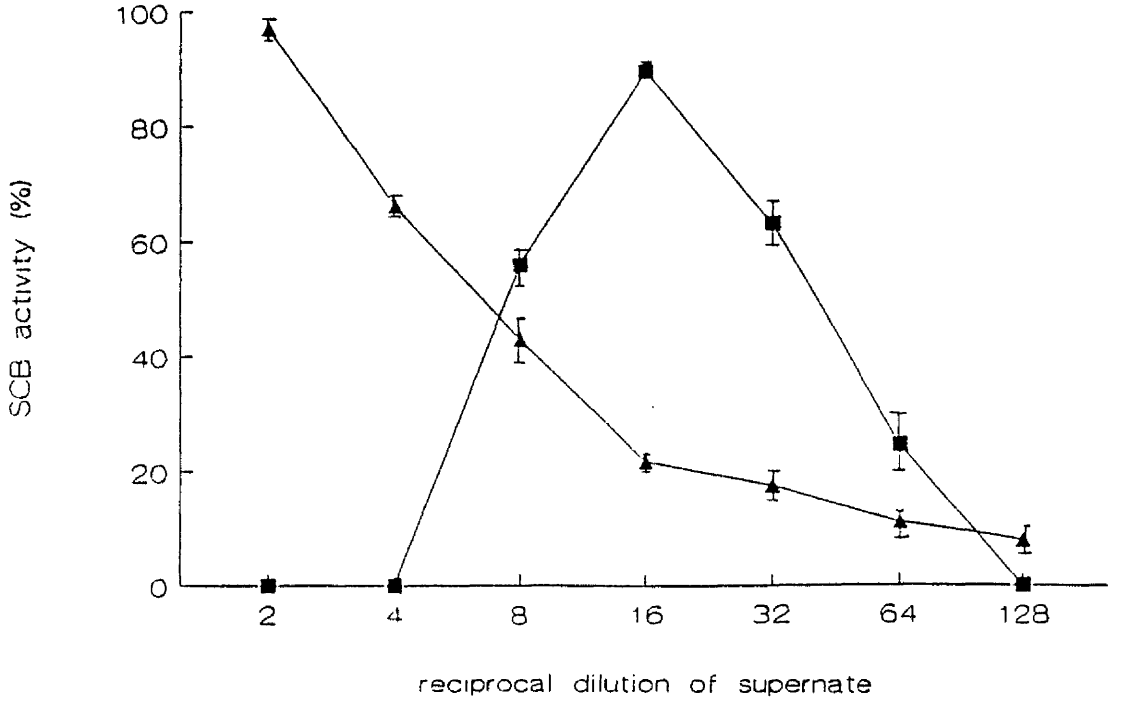
■—■ untreated supernate

▲—▲ charcoal extracted supernate

GFC in MB



TTX in MB



bacteria

Bacterium		Source	SCB** activity
Alteromonas tetraodonis	GFC	puffer fish	+++
Vibrio (Listonella) pelagia	GFB	puffer fish	++
Shewanella alga	OK-1	alga (*Jania* sp)	+++
Vibrio (Listonella) pelagia biovar II	OK-2	alga (*Jania* sp.)	+
V. alginolyticus	NCIMB 1903nt	horse mackerel	+
V. alginolyticus	NCIMB 1339*	hard clam	-
V. anguillarum	NCIMB 329nt	sea trout	+++
V. anguillarum	" 91079*	turbot	++
V. anguillarum	" 2981*	oyster larvae	+
V. anguillarum	" 4979*	oyster larvae	-
V. anguillarum	" 5679*	oyster larvae	±
V. tubiashii	B1*	oyster larvae	+++
V. tubiashii	B2	oyster larvae	+
V. tubiashii	B55nt	oyster larvae	-
V. tubiashii	T61nt	abalone larvae	+++
Aeromonas salmonicida	3.101	brook trout	±
Aeromonas salmonicida	80628	Atlantic salmon	-
Pseudomonas	1-1-1	sea water	+
Escherichia coli B	NCTC 10537		-
Bacillus subtilis	NCC 3610		-
Staphylococcus aureus	NCTC 7121		-

*strains reported by Nottage et al., (1989) to produce CT

nt: not tested for CT

**SCB activity was determined with TTX as a standard and the results expressed as TTX equivalent (ng/ml):

- 0; ±<20; + 20-100; ++100-200; +++ > 200

neo-STX (neo-saxitoxin) was found in every sample, each of which also contained a compound which may be a degradation product of STX. A second batch of supernates, on HPLC analysis, gave similiar results. However, due to the number of other peaks in the chromatograms, thought to be due to impurities, the identification of neo-STX was considered to be only tentative. Nevertheless, assuming the compound was neo-STX, the equivalent concentrations of STX and mouse units were calculated by MAFF (Table 16).

TABLE 16 HPLC analysis for PSP of the culture supernates of four bacterial strains, with the results expressed as the equivalent concentration of STX and in mouse units.

Strain no.	Toxin concentration in supernate					
	neo-STX (µmol/L)		STX (µgSTX equiv/L)		SCB activity (MU/g)	
	exp.1	exp.2	exp.1	exp.2	exp.1	exp.2
A1096	0.68	n/d	248	n/d	2.4	n/d
A862	7.62	6.38	2773	2322	24.0	20.6
NCMB829	4.74	4.45	1727	1620	8.6	16.2
GFC	12.19	8.17	4439	2974	26.0	14.4

n/d not done; for calculations see Appendix 6

CULTURE CONDITIONS FOR SCB PRODUCTION

SCB Activity In Relation To Growth

Strain GFC

To determine at which stage of the growth cycle SCB toxins were produced, strain GFC was cultured in marine broth and was sampled at different points, viz. mid-logarithmic phase, end of logarithmic phase, early stationary phase, late stationary phase and decline phase, for growth and SCB activity.

The strain commenced exponential growth after 3½ h and entered stationary phase at approximately 8 h; thereafter, a slight decline in cell numbers occurred between 24 and 72 h followed by a sharper decline over subsequent days (Figure 26a). SCB activity was not detected during the period of exponential growth but appeared from 24 h onwards and remained stable for 7 days, after which a slow decline in activity was observed (Fig. 26a). This was found in both untreated supernates and the charcoal-treated samples.

In further experiments, viable count, phosphate concentration (µg/ml P) and the SCB activity of strain GFC was examined in greater detail. As before the strain entered exponential phase at 3½ h which

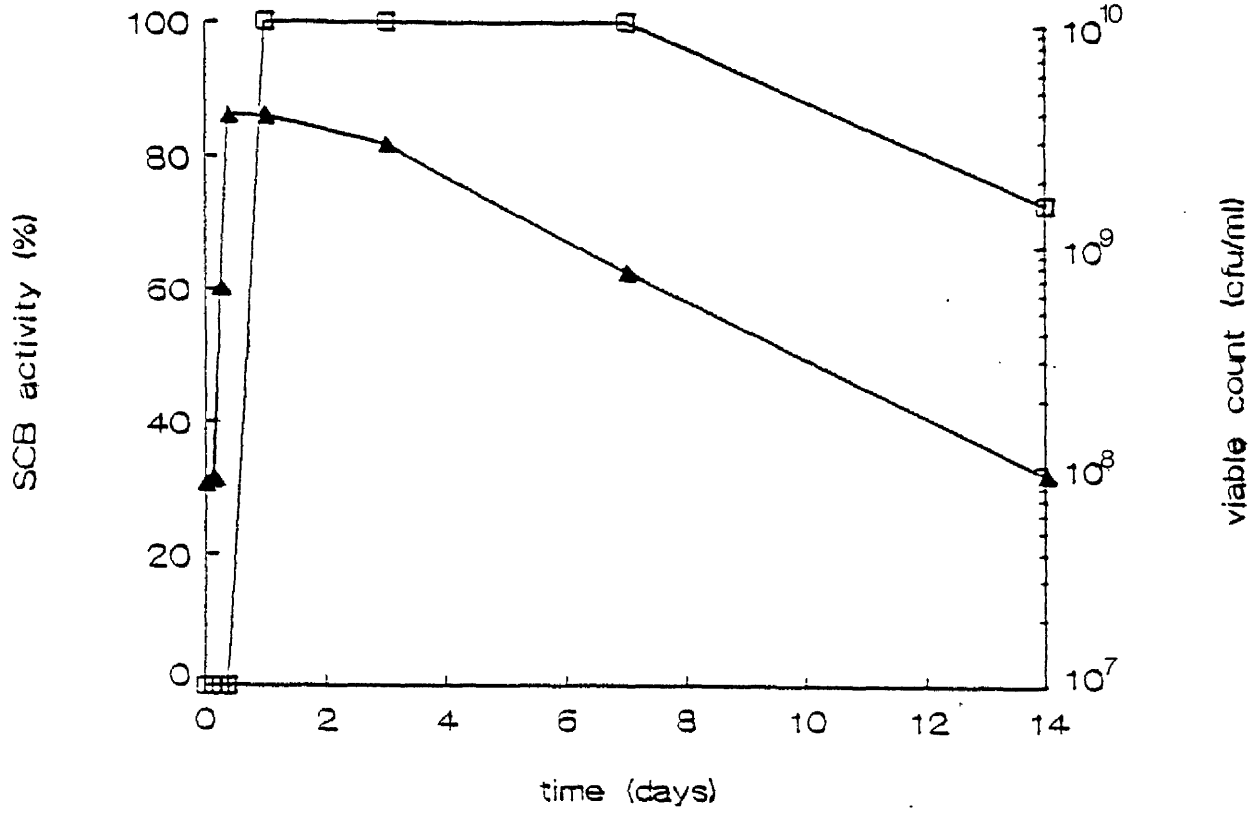
FIGURE 26 Effect of the growth cycle of strain GFC on the SCB activity in the culture supernate in a) a shaking culture over a 14 day period (upper figure) and b) a static culture over a 7 day period (lower figure).

LEGEND:

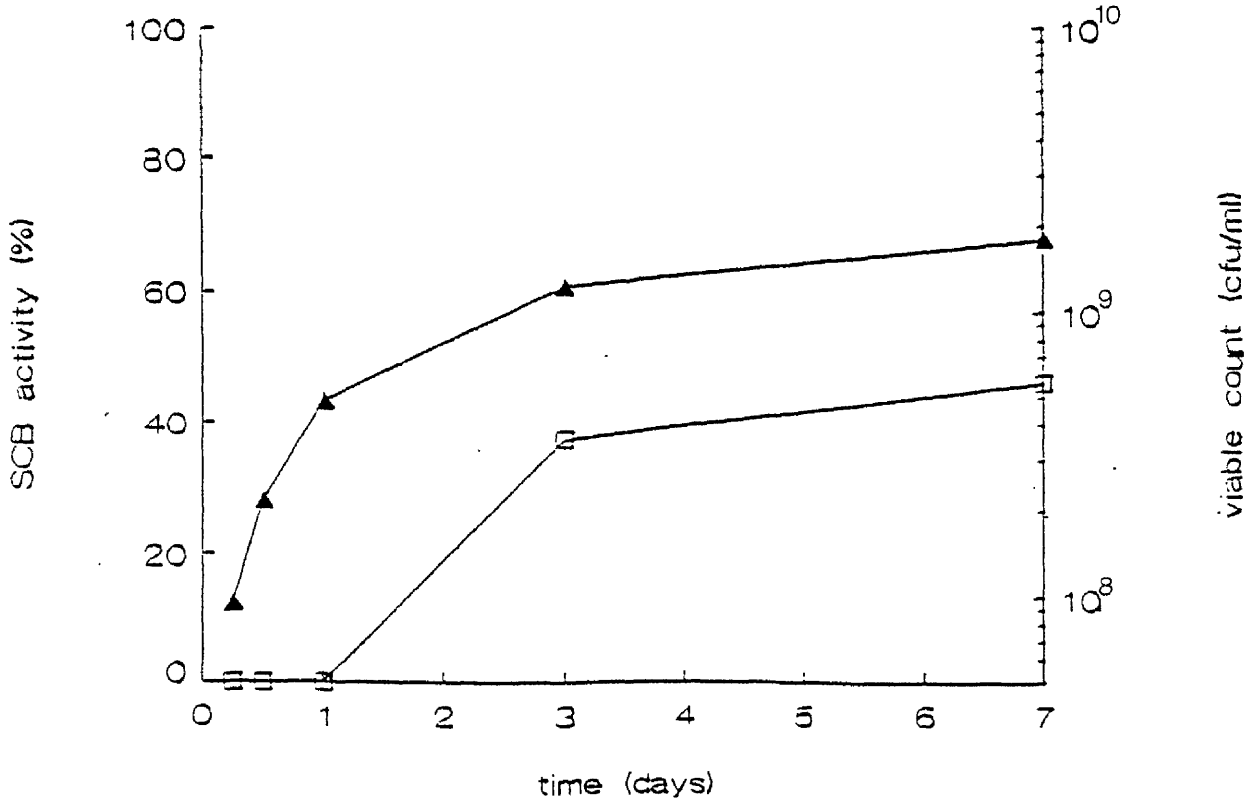
▲—▲ viable count

□—□ SCB activity

strain GFC in shaking culture



strain GFC in static culture



continued up to 8 h. This coincided with a sharp drop in the phosphate concentration in the medium which was depleted by the beginning of the stationary phase. SCB activity was first detected in the culture supernate obtained during the stationary phase at 18 h (Fig. 27).

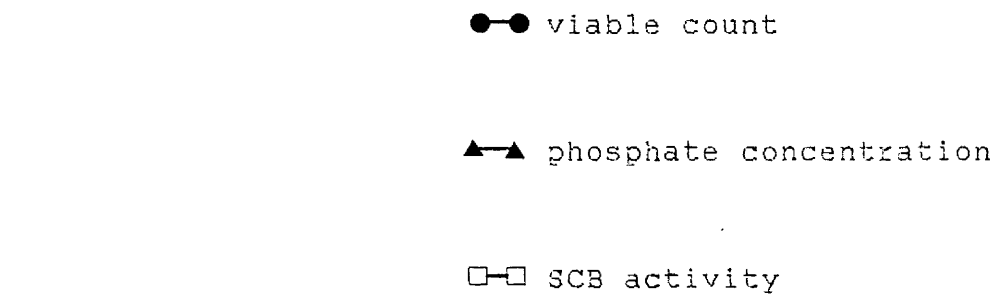
The same strain cultured under static conditions entered stationary phase at 72 h at which point SCB activity was detected. However, under these conditions, the cell numbers were lower by approximately 60 % as was the SCB activity (Fig. 26b).

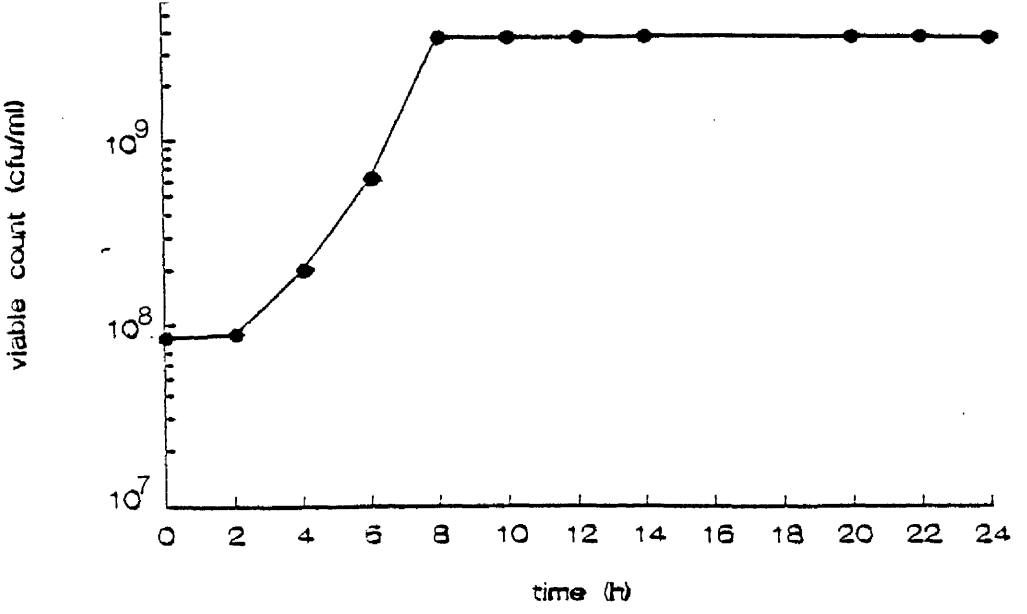
Other strains

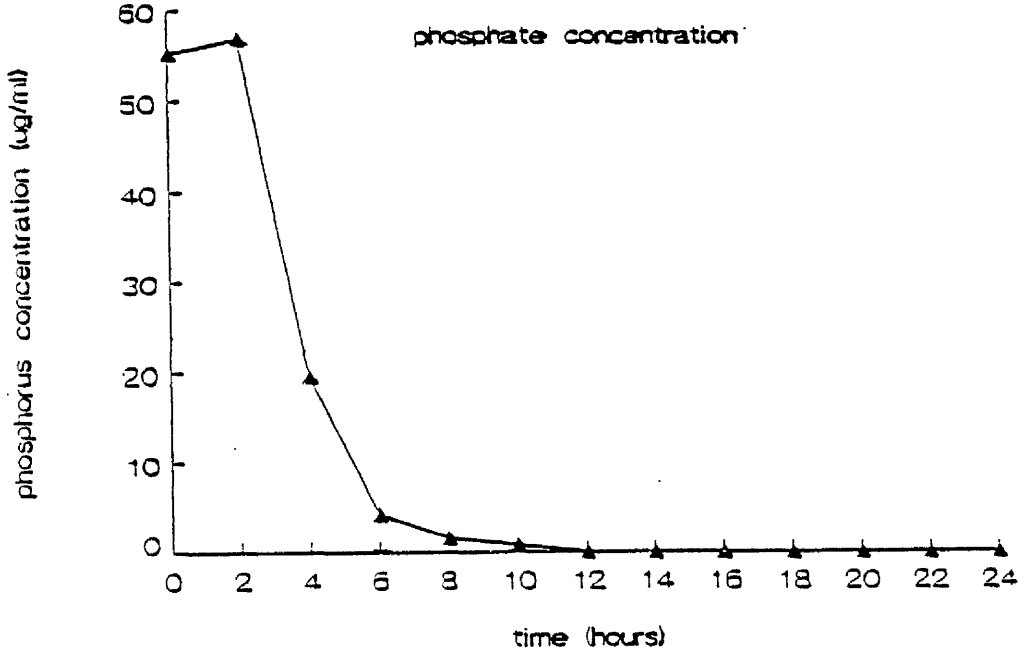
Strains 1903 (Ardtoe isolate), NCIMB 1096, NCIMB 829, and OK1 were also cultured in marine broth and examined for SCB activity over a 3-day period. In all 4 cases, SCB activity was detected in the culture supernate as the cells entered stationary phase at approximately 12 h (Fig. 28). This differed from strain GFC, in which, SCB activity could not be detected until the cells had progressed well into stationary phase which had commenced at 8 h. However, similiarly to GFC, all strains exhibited maximum SCB activity at 24 h.

After 3 days, the 4 strains showed slight

FIGURE 27 Comparison of the viable count of strain GFC, the phosphate concentration and SCB activity in the culture supernate, over a 24 h period.




viable count (cfu/ml)
10^9
10^8
10^7
0 2 4 6 8 10 12 14 16 18 20 22 24
time (h)


phosphate concentration
phosphorus concentration (ug/ml)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24
time (hours)

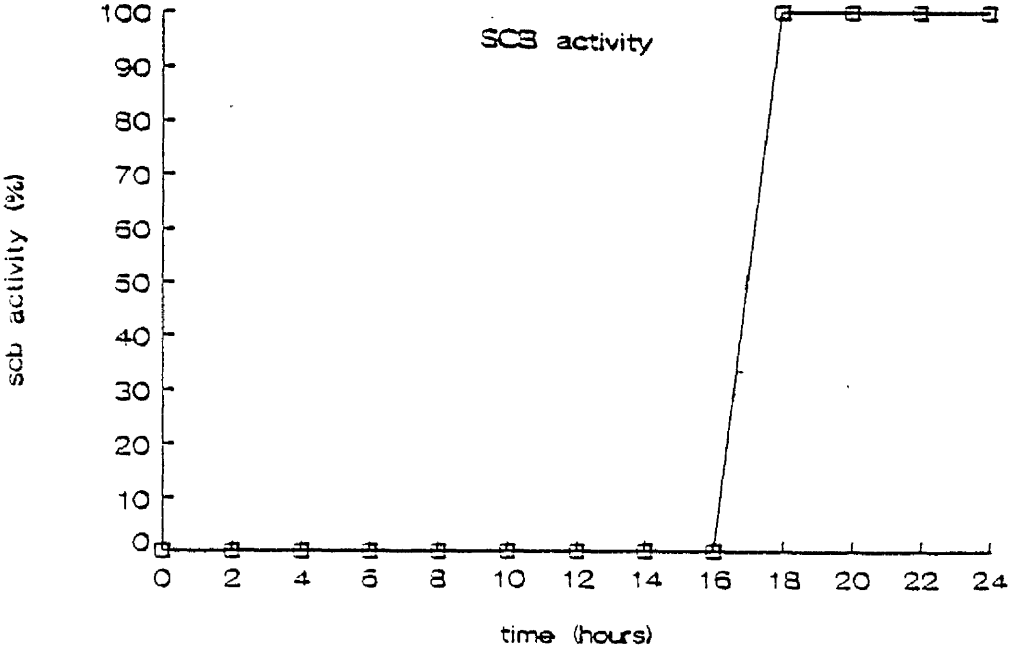
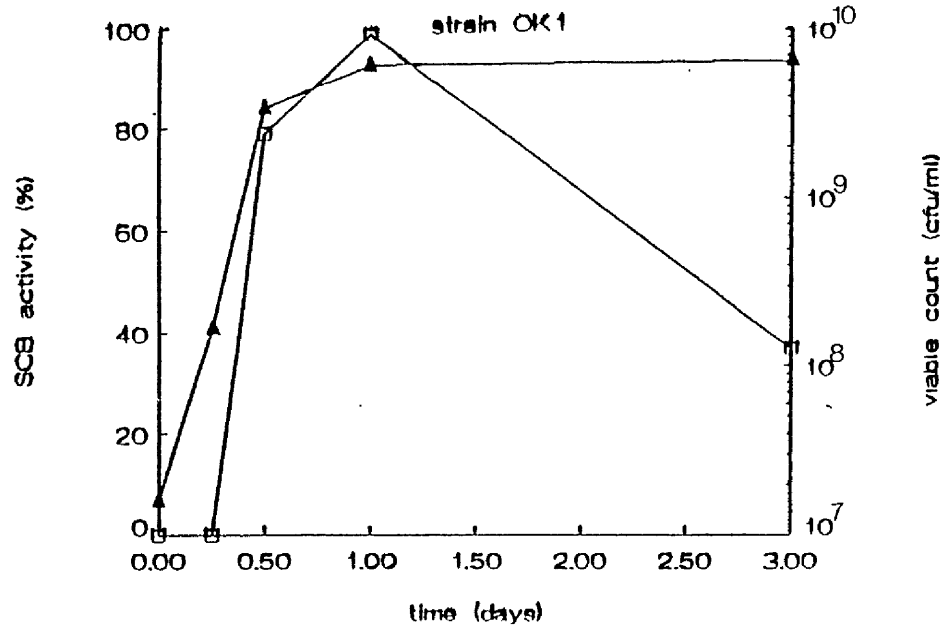

SCB activity
scb activity (%)
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24
time (hours)

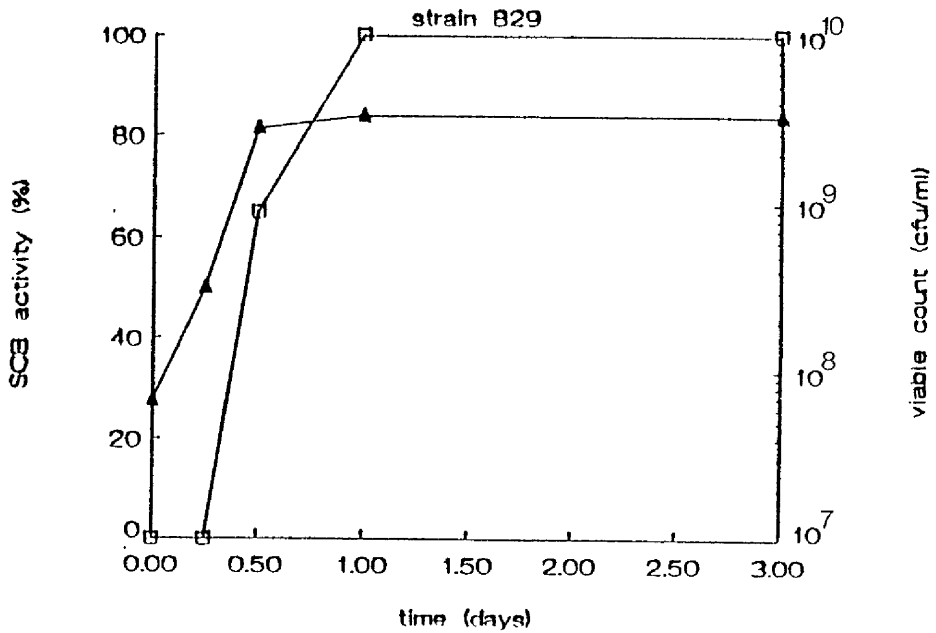
FIGURE 28 SCB activity in culture supernates of 4 marine bacteria at different stages of the growth cycle.

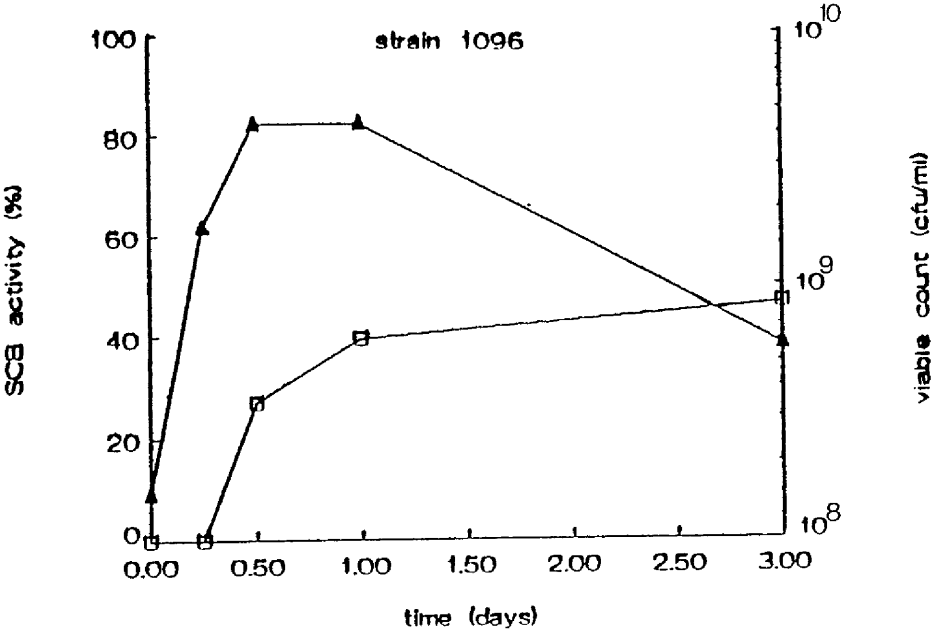
LEGEND:

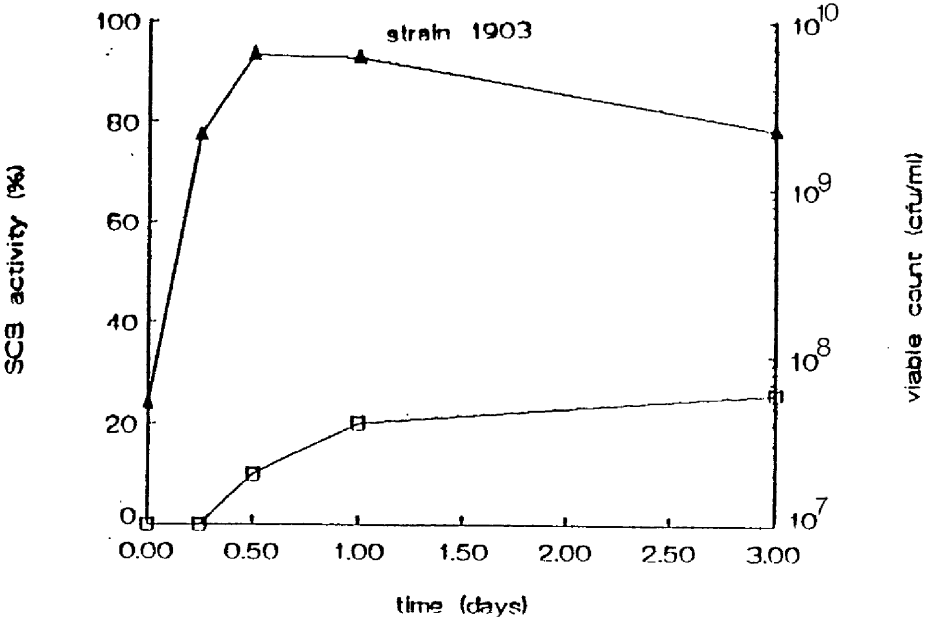
▲—▲ viable count

□—□ SCB activity


strain OK1
SCB activity (%)
viable count (cfu/ml)
time (days)
0.00
0.50
1.00
1.50
2.00
2.50
3.00


strain 829
SCB activity (%)
viable count (cfu/ml)
time (days)
0.00
0.50
1.00
1.50
2.00
2.50
3.00


strain 1096
SCB activity (%)
viable count (cfu/ml)
time (days)
0.00
0.50
1.00
1.50
2.00
2.50
3.00


strain 1903
SCB activity (%)
viable count (cfu/ml)
time (days)
0.00
0.50
1.00
1.50
2.00
2.50
3.00

variations, in that, strain OK1 exhibited a sharp decrease in SCB activity while the cell numbers remained stable. However, with strain 829, both SCB activity and cell numbers remained the same, whereas, in strains 1903 and 1096 the cells lysed but the SCB activity was stable.

Overall, in each of the 5 strains examined, SCB activity was produced in stationary phase and was stable for up to 3 days, with the exception of OK1.

SCB Activity Under Nutrient Limiting Conditions

Growth in seawater

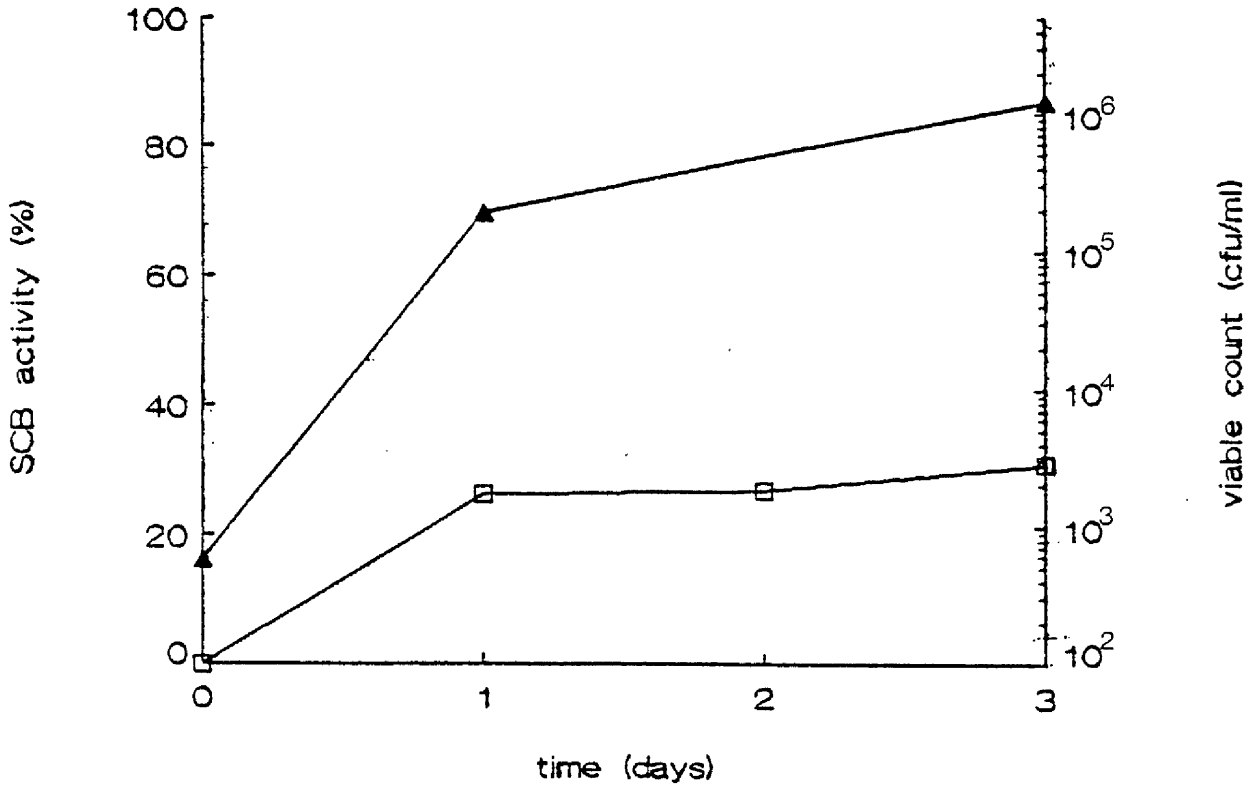
Marine broth and the culture media tested in the previous experiments provided rich growth conditions which would not be found in normal marine environments. Therefore, in order to investigate SCB toxin production by bacteria under nutrient-limited conditions, strain GFC was inoculated at low concentration into artificial seawater and samples were tested for up to 3 days for viable cell concentrations and SCB activity. Cell numbers increased 2000-fold within 3 days and SCB activity was readily detected at a dilution of 1/16 in cell free supernates within 24 h (Fig. 29). This pattern was duplicated when the supernates were tested at a dilution of 1/8 or, after charcoal treatment, at 1/2

FIGURE 29 Viable count and SCB activity in supernates of strain GFC cultured in seawater.

LEGEND:

▲—▲ viable count

□—□ SCB activity


100
80
60
40
20
0
SCB activity (%)
0
1
2
3
time (days)
10^6
10^5
10^4
10^3
10^2
viable count (cfu/ml)

and 1/4 dilutions. Therefore, GFC was capable of some multiplication in seawater and produced a significant quantity of SCB toxin(s).

Growth in seawater with or without added carbon sources

This experiment examined the effect on the SCB activity of inoculating strain GFC at ten-fold differences in cell numbers, into seawater with, or without, either glucose or sucrose being present as a carbon source. After incubation for 24 h the viable count of each sample and the SCB activity was determined. These results,in general, indicate that as the bacterial cell numbers increase over a 10^3-fold range, the concentration of toxin produced decreased by 10^2-fold, even in the presence of an added carbon source. Therefore, optimum SCB toxin production occurred at low bacterial numbers (Table 17).

Another unexpected observation was the low bacterial numbers found in the asw with added glucose, indicating that this carbon source may have an adverse effect on cell viability (Table 17).

TABLE 17 Effect of culturing, for 24 h, ten-fold variations in strain GFC cell numbers in seawater, with or without added carbon sources , on the SCB activity of the supernate.

Dilution	Inoculum	SW			SW/glucose			SW/sucrose		
		viable count (cfu/ml)	SCB activity (%)	SCB activity (ng TTX equiv/cell)	viable count (cfu/ml)	SCB activity (%)	SCB activity (ng TTX equiv/cell)	viable count (cfu/ml)	SCB activity (%)	SCB activity (ng TTX equiv/cell)
1/100	5.3×10^5	2.63×10^5	16.6	7.76×10^{-5}	$<10^3$	10.5	-	7.64×10^5	6.0	1.33×10^{-5}
1/10	5.3×10^6	2.53×10^6	21.9	1.41×10^{-5}	8×10^3	23.6	4.46×10^{-3}	1.4×10^6	21.0	2.19×10^{-5}
1x	5.3×10^7	2.39×10^7	25.0	1.60×10^{-6}	$<10^5$	23.9	-	2.25×10^7	26.0	1.82×10^{-6}
10x	5.3×10^8	3.54×10^8	29.3	1.44×10^{-7}	9.3×10^6	28.0	5.21×10^{-6}	2.02×10^8	27.0	2.14×10^{-7}

Effect Of Phosphate on SCB Production

Addition of phosphate

The effect of phosphate concentration on SCB activity was examined when GFC was cultured in artificial seawater containing 0.5 % peptone, with phosphate added at 10-100 µg/ml P. Seawater was chosen as the base medium to keep the initial phosphate concentration to a minimum; peptone was also added at the minimum concentration which allowed abundant cell growth (Table 18).

A problem which emerged in this experiment was the decrease in MNB cell death obtained in the oub and ver controls as the phosphate levels increased, thus reducing the margin by which the SCB activity could be detected and as a result, decreasing the sensitivity of the assay, e.g. at 1/16 dilution of the control containing 0 µg/ml P of supernate 40 % cell death occurred with oub/ver but this decreased to 10% cell death on the addition of the control containing 100 µg/ml P. This was thought to be due to the increase in total salt concentration in the medium due to the addition of phosphate buffer which consisted of the monobasic and dibasic potassium phosphates. To overcome this problem the supernates were subsequently treated with charcoal.

All of the cultures contained similiar

TABLE 18 Growth of strain GFC (as measured by A_{600}) in seawater with peptone added at different concentrations.

Concentration of peptone (%)	Absorbance (600 nm)
0.0	0.000
0.1	0.510
0.5	2.020
1.0	2.445

concentrations of bacterial cells after 24 h, i.e. 2×10^9 cfu/ml although in the cultures with added 60 or 100 µg/ml P the cells formed large clumps and were difficult to count. Charcoal-treated undiluted supernates were examined by tissue culture and demonstrated a clear decrease in SCB activity as the phosphate concentration increased (Fig. 30).

Addition of phosphate at different points in the growth curve

In these experiments 50 µg/ml P was added to the culture of strain GFC at varying times in the stationary phase of its growth cycle in order to examine the effect on SCB production.

Addition of phosphate up to 20 h in the growth cycle blocked the expression of SCB activity in the untreated culture supernate after 24 h incubation whereas, in a separate experiment using a charcoal-treated sample, the SCB activity was detected at these times; however, on the addition of phosphate at 16 h the quantity of SCB toxin produced was greatly reduced (Table 19).

When the phosphate levels in the media of each sample after 24 h incubation were measured it was found that only a very small quantity of the

FIGURE 30 Effect of incubating strain GFC for 24 h, in media containing different concentrations of added phosphate, on the SCB activity of the culture supernates.

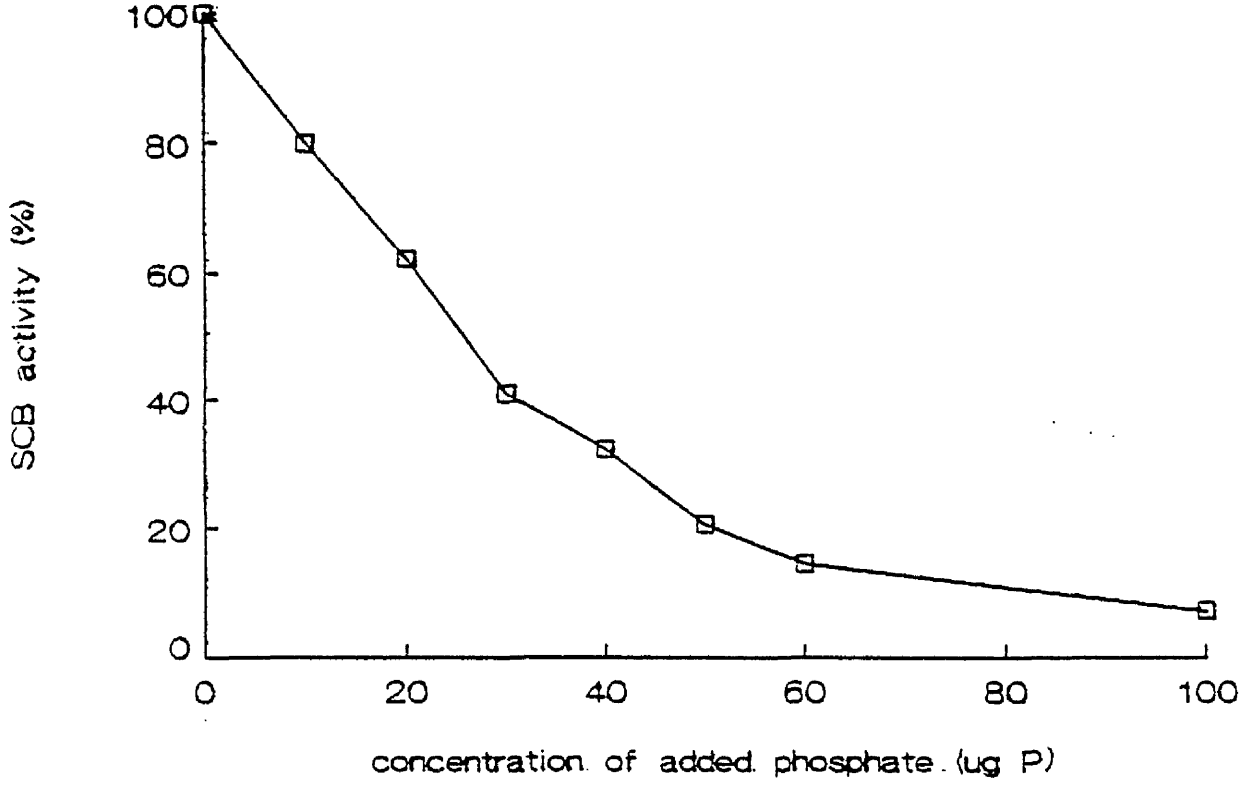

100
80
60
40
20
0
SCB activity (%)
0
20
40
60
80
100
concentration of added phosphate (ug P)

TABLE 19 Residual phosphate concentrations in culture supernates of strain GFC after the addition of phosphate to the media at different points in the growth cycle. The associated effect on SCB activity is also shown.

Time phosphate added (h)	Toxin production at 24 h (% cell protection)		Phosphorus concentration at 24 h (µg/ml P)****
	EXP.1**	EXP.2***	
16	0	36	45.0
18	0	100	47.5
20	0	100	47.5
22	25.7	100	48.0
24*	49.8	100	1.5

* no phosphate added

** untreated supernate at 1/8 dilution

*** undiluted charcoal extracted supernate

**** measured from experiment 1

phosphate added was utilised by the bacteria (Table 19).

ANALYSIS OF MUSSEL EXTRACTS BY THE TISSUE CULTURE ASSAY

Uptake Of Strain GFC By *M. edulis* And Extraction Of SCB Activity From The Tissue

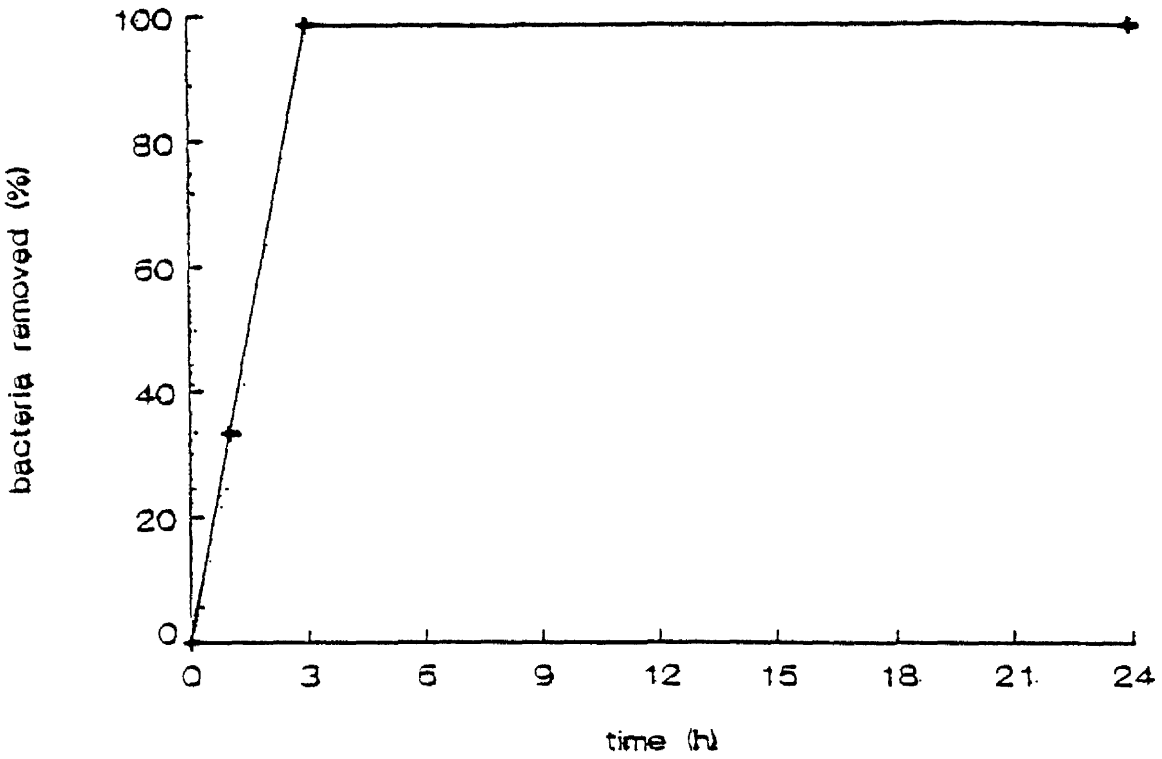
To determine whether mussels were able to accumulate sodium channel-blocking (SCB) toxins by filter-feeding, 5 mussels (*Mytilus edulis*) were exposed to a suspension of strain GFC cells (2.1 x 10^7cfu/ml) in a volume of 100 ml for 24 h at 10°C, with aeration. The bacteria were rapidly removed from suspension (Fig. 31), with <2 % remaining after 3 h.

At 24 h the mussel flesh was homogenized, subjected to the extraction procedure of Hwang et al. (1989) and the tissue extracts examined for SCB activity. Undiluted extracts produced 100 % SCB activity, which decreased in relation to dilution (Fig. 31).

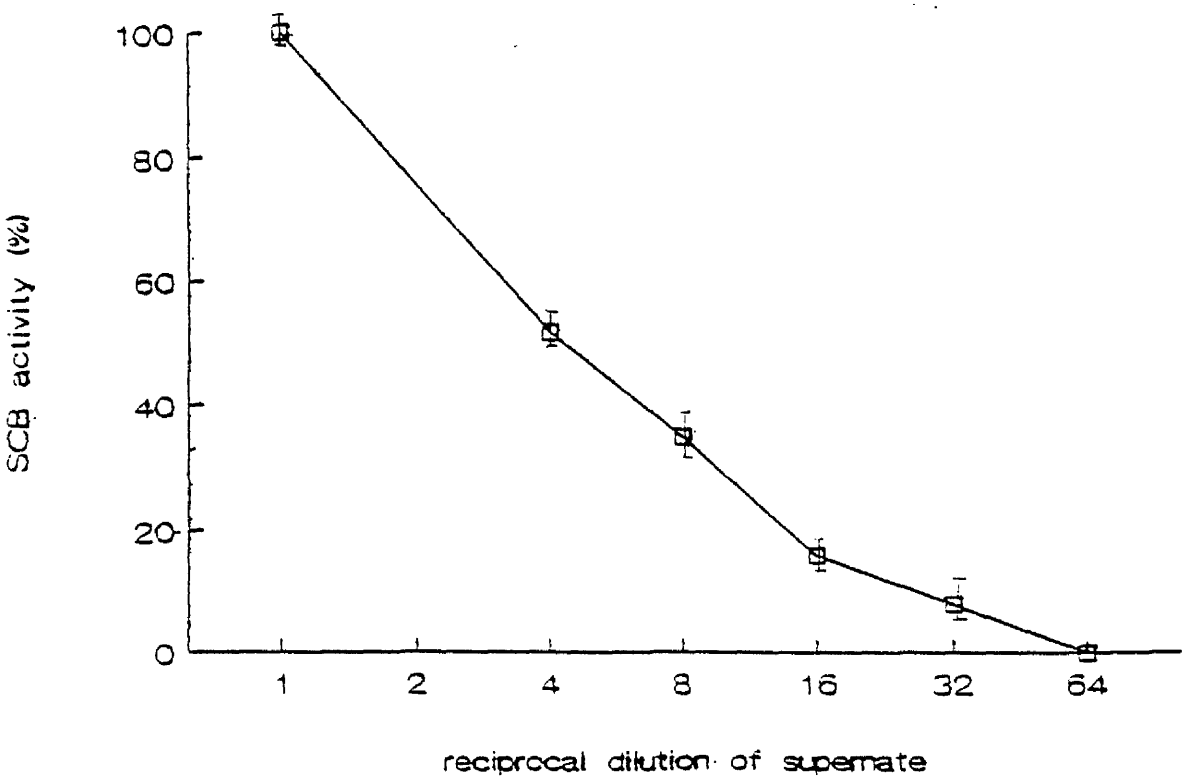
This experiment showed that *M. edulis* removed strain GFC from seawater within 3 h and SCB activity could be extracted from the flesh 24 h later.

FIGURE 31 Removal of bacteria by *M. edulis* (upper figure) and the SCB activity extracted from their tissues after 24 h (lower figure).

removal of bacteria



SCB activity of mussel extract



Analysis Of Mussels Contaminated With PSP

To examine the sensitivity of the tissue culture assay for detecting PSP (paralytic shellfish poisons), 6 different extracts from mussels which had been exposed to a toxic phytoplankton bloom, which occurred off the north-east coast of England in the summer of 1990, each containing various quantities of PSP toxins (as determined by HPLC and the mouse bioassay), were obtained from MAFF, Burnham-on-Crouch.

On analysis by the tissue culture assay, SCB activity was detected in all 6 samples. However, the most toxic extract (T64) produced a substance which masked the SCB activity. This inhibition was progressively destroyed by heating at 100°C, and the SCB activity detected increased with increasing time of heating, reaching a maximum at 60 min, with an 8-fold increase in titre (Fig. 32a).

Subsequently, the other extracts, were analysed both before and after heating for 60 min at 100°C. These varied in their response to heating, some showing up to a three-fold increase in titre, while others were unchanged (Table 20).

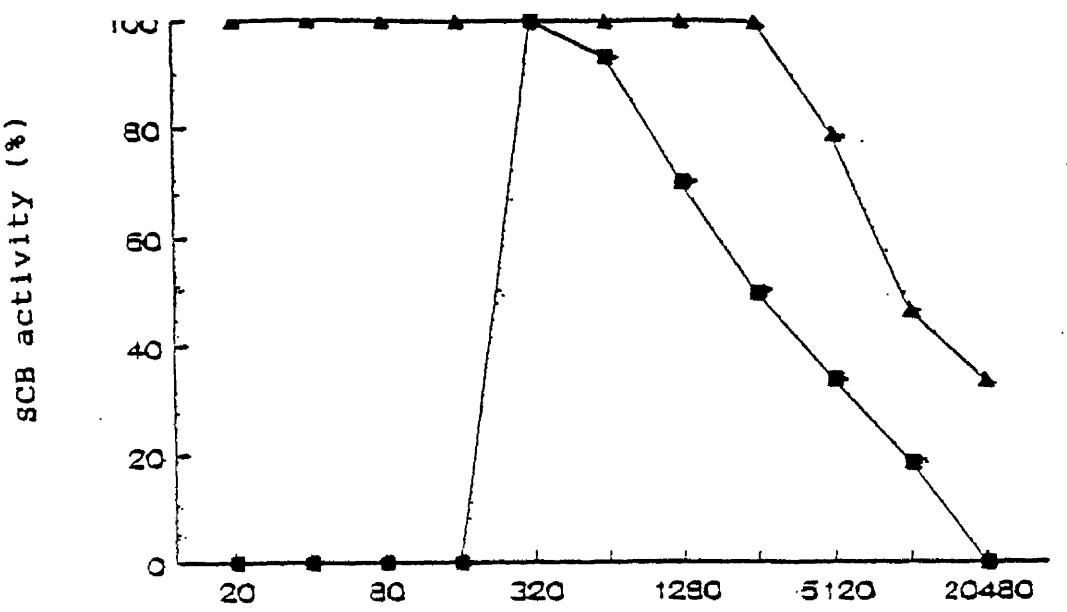
The values obtained for the SCB activity by the tissue culture assay were converted into probits (Figs. 32b,c & 33) in order to linearise the dose

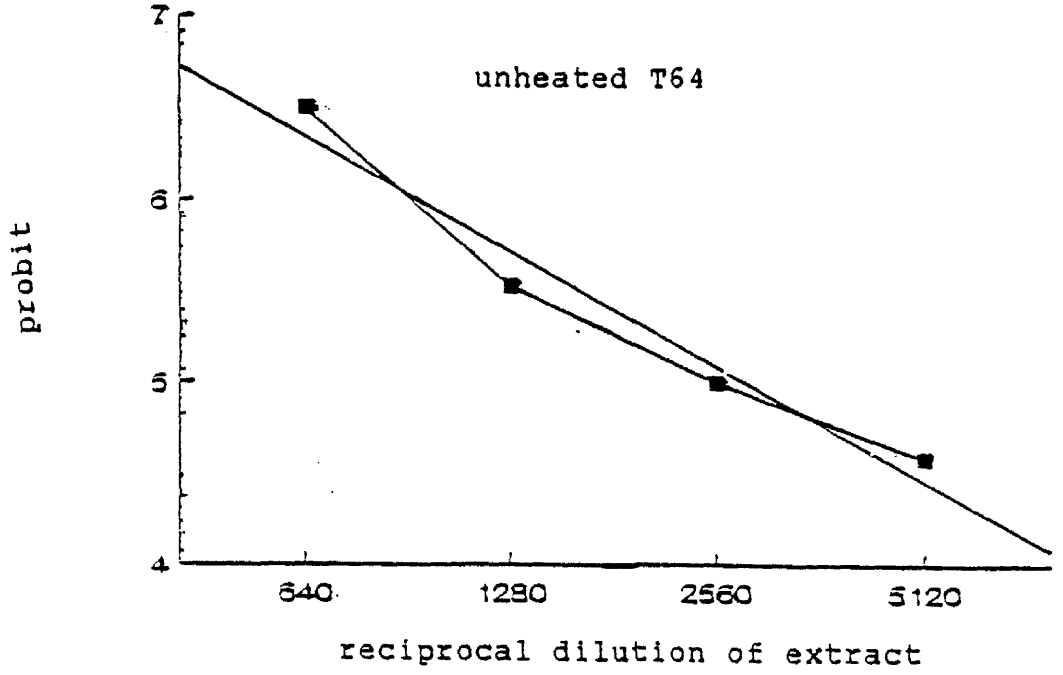
FIGURE 32 The SCB activity of heated (100°C for 60 min) or unheated T64 mussel extract as analysed by a) the tissue culture assay and b & c) their probit conversions.

LEGEND:

▲—▲ heated

■—■ unheated


SCB activity (%)
100
80
60
40
20
0
20
80
320
1280
5120
20480
reciprocal dilution of extract


unheated T64
probit
7
6
5
4
640
1280
2560
5120
reciprocal dilution of extract

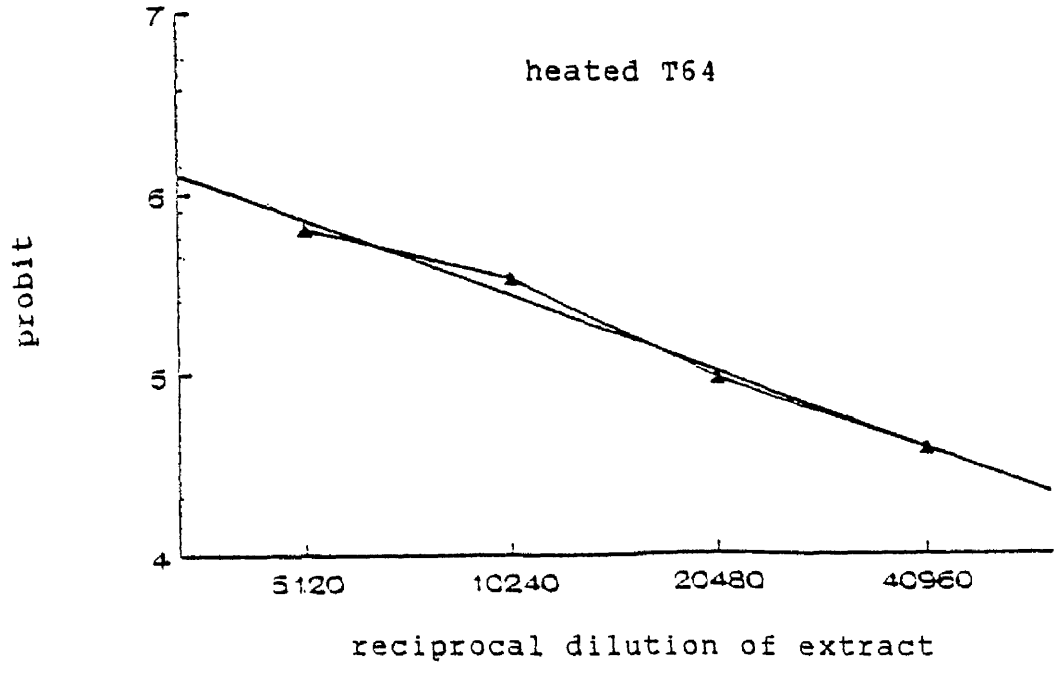

heated T64
probit
7
6
5
4
5120
10240
20480
40960
reciprocal dilution of extract

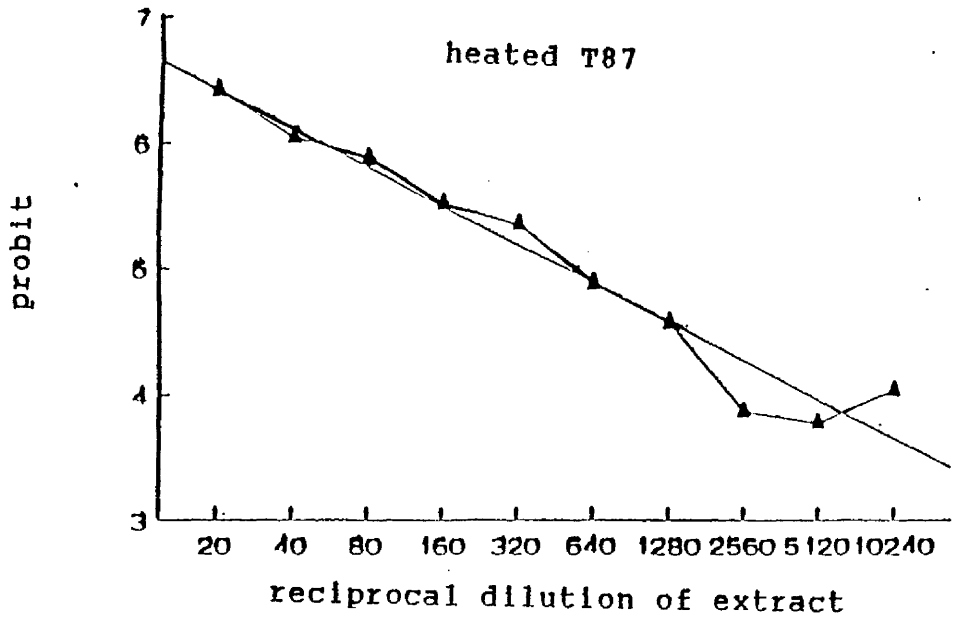
TABLE 20 Comparison of the toxicity levels obtained when mussel extracts were analysed by HPLC, the mouse bioassay and by the tissue culture assay.

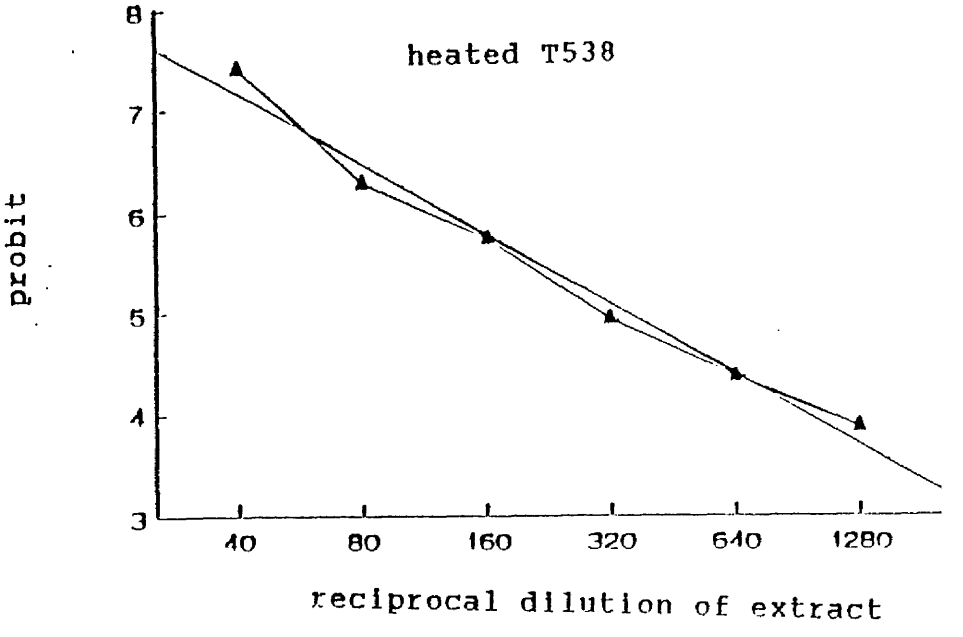
Sample no.	HPLC* Total toxins* (μM)	HPLC* Total STX (μg STX equiv/L)	HPLC* Mouse equivalents (ME/100 g)	Mouse bioassay* Mouse units (MU)	Tissue culture assay heated (tcu)	Tissue culture assay unheated (tcu)
T64	61.89	15,183.0	16870	19881	16630	2820
T87	15.59	713.4	5081	3199	534	657
T538	4.33	47.3	1023	1020	350	156
T125	9.90	404.0	2684	328	516	280
T55	1.39	0	235	245	149	122
T59	1.26	0	102	209	288	85

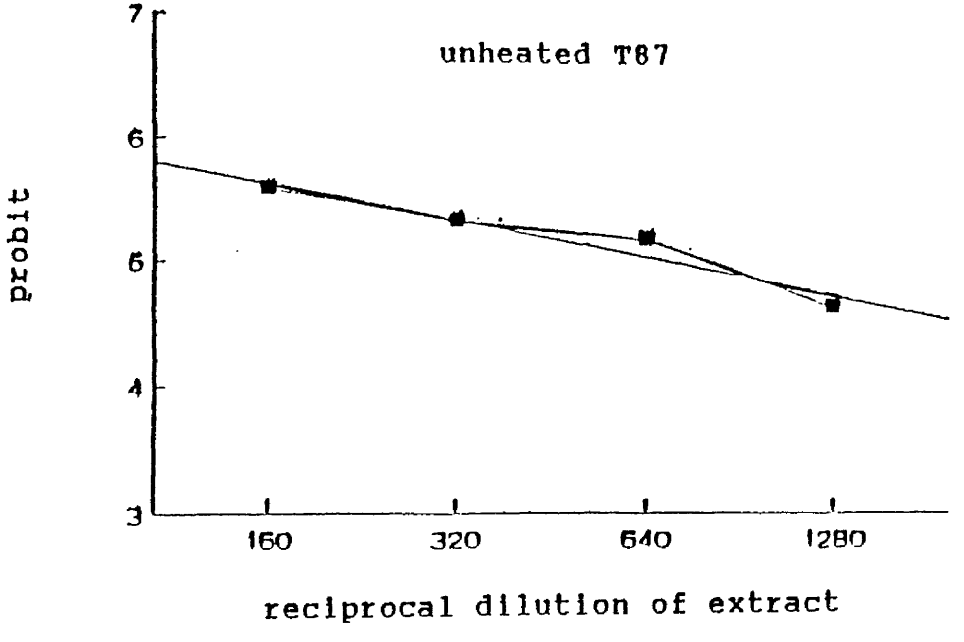
* Calculated by MAFF

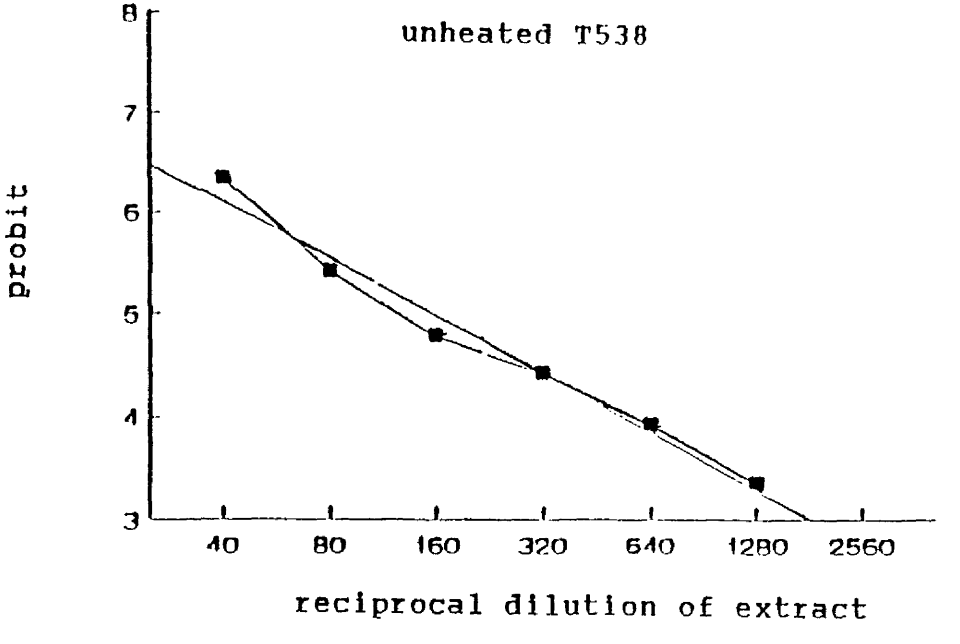
tcu: tissue culture units; reciprocal dilution of extract at 50 % SCB activity.

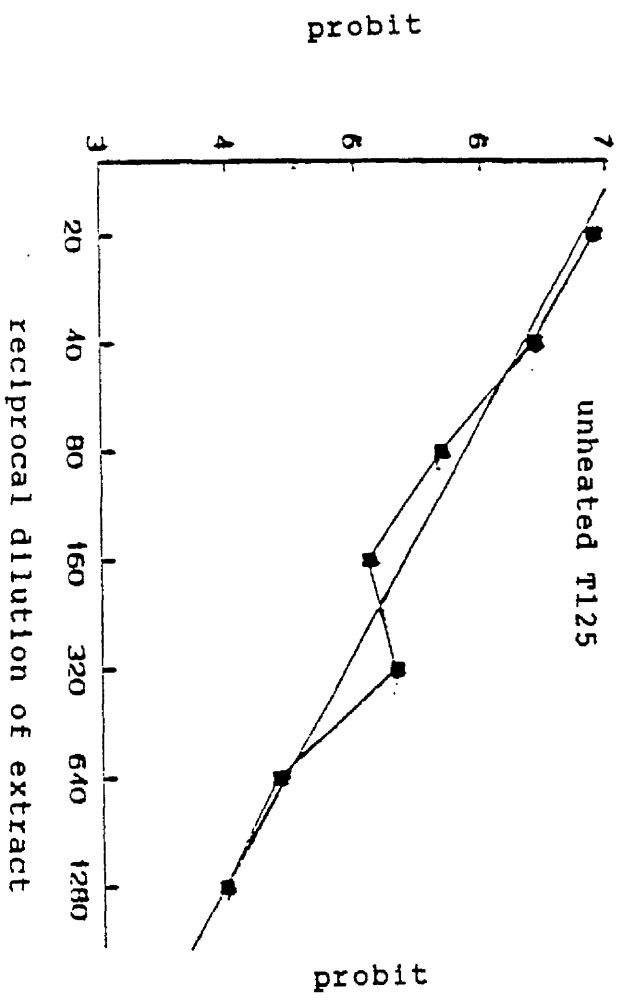
FIGURE 33 Probit conversion of SCB activity obtained from 5 unheated or heated mussel extracts.


heated T87
probit
7
6
5
4
3
20
40
80
160
320
640
1280
2560
5120
10240
reciprocal dilution of extract

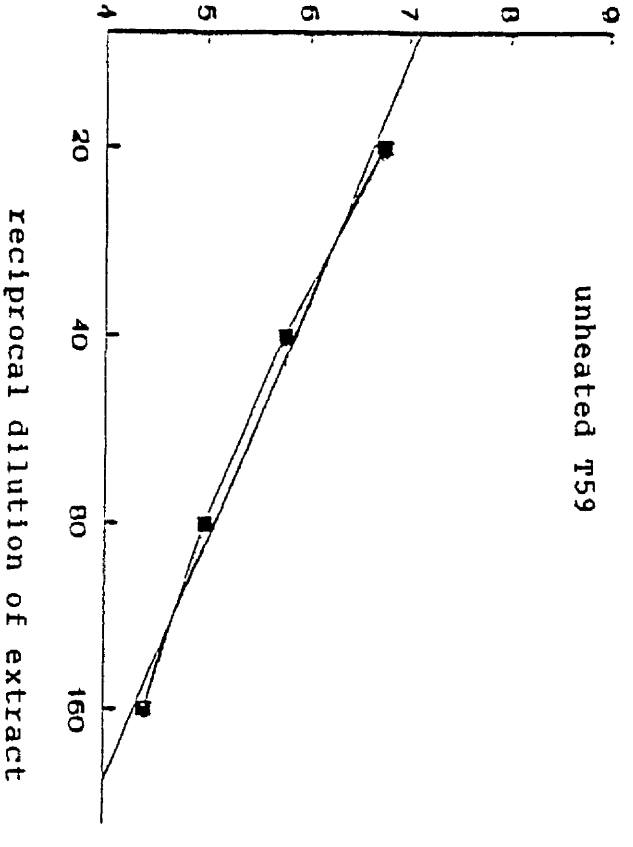

heated T538
probit
8
7
6
5
4
3
40
80
160
320
640
1280
reciprocal dilution of extract


unheated T87
probit
7
6
5
4
3
160
320
640
1280
reciprocal dilution of extract

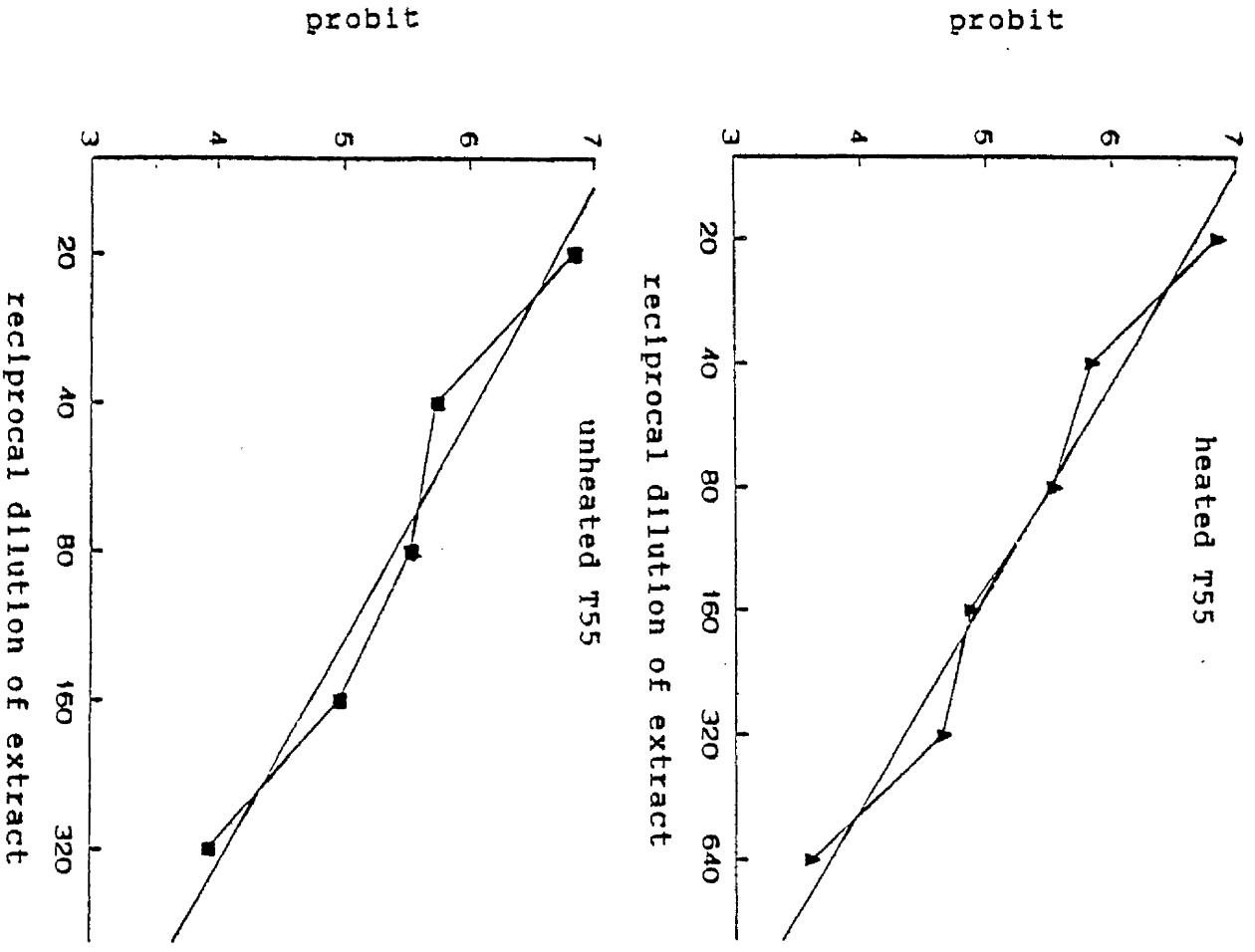

unheated T538
probit
8
7
6
5
4
3
40
80
160
320
640
1280
2560
reciprocal dilution of extract


probit
heated T125
reciprocal dilution of extract

probit
unheated T125
reciprocal dilution of extract


probit
heated T59
reciprocal dilution of extract

probit
unheated T59
reciprocal dilution of extract


probit
7
6
5
4
3
heated T55
20
40
80
160
320
640
reciprocal dilution of extract
probit
7
6
5
4
3
unheated T55
20
40
80
160
320
reciprocal dilution of extract

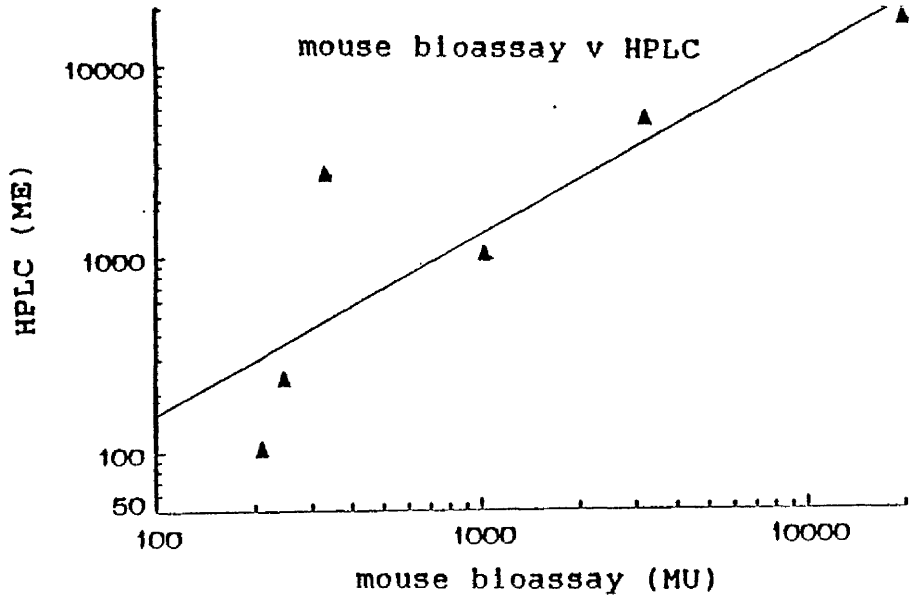
response curves and obtain 50 % end-points (defined as the dilution of extract which exhibited 50 % SCB activity; tissue culture units, tcu).

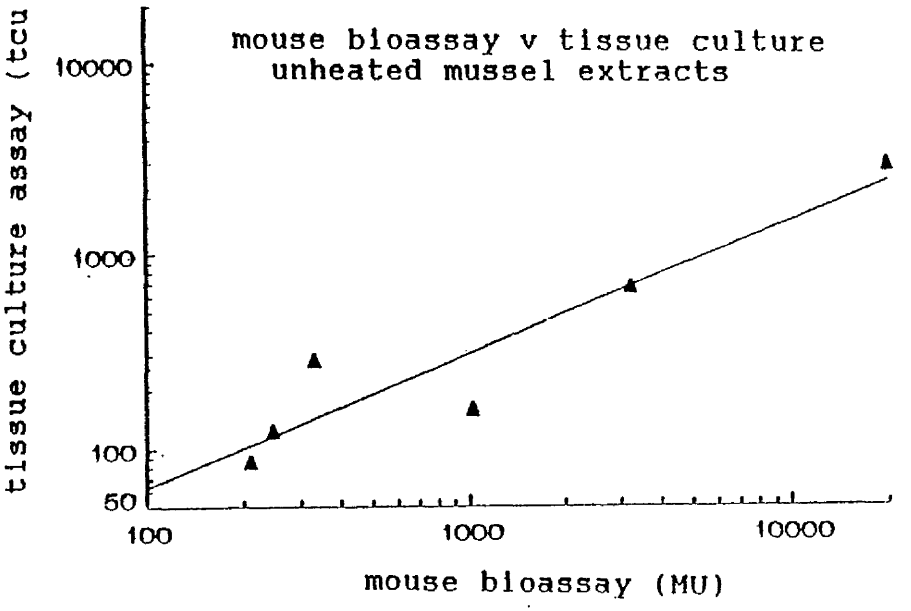
The results obtained for both the unheated and heated exracts, analysed by tissue culture, decreased, in general, in the same order as the values obtained by the mouse bioassay and from HPLC.

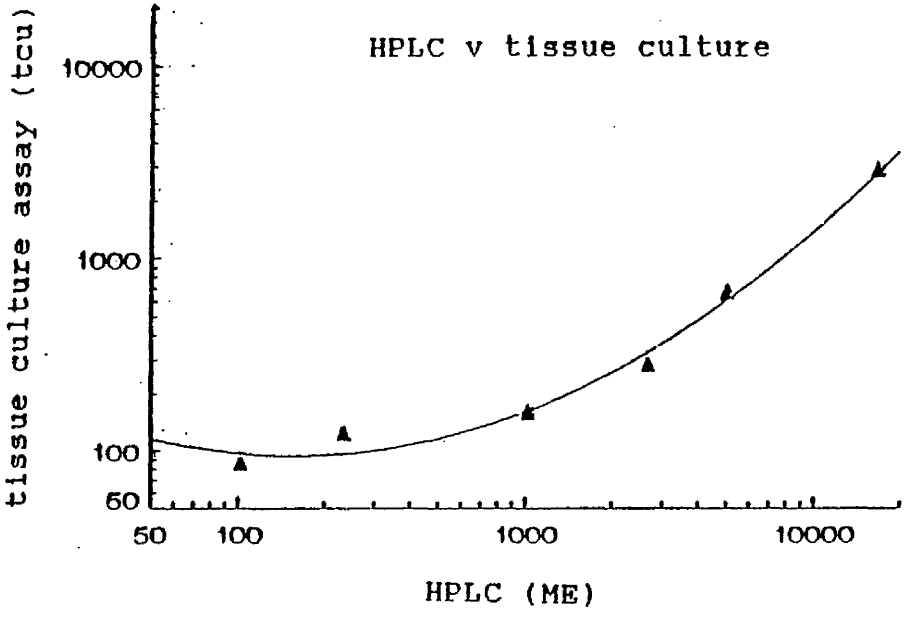
When the values obtained from the three assays were compared (Fig. 34 & Table 21) a high correlation coefficient was found between the tissue culture assay and both HPLC and the mouse bioassay, when unheated mussel extracts were examined. This correlation decreased with the heated mussel extracts.

The sensitivity of the assay could be determined by comparing the results found by MAFF, to the dilution of the extract which gave 50 % SCB activity by tissue culture. In the unheated mussel extracts this was equivalent to between 0.45 and 0.66 µg STX equiv./100 ml (15 and 22 nM STX).

FIGURE 34 A comparison of the mouse bioassay, HPLC and the tissue culture assay for the analysis of toxic mussel extracts.


mouse bioassay v HPLC
HPLC (ME)
10000
1000
100
50
100
1000
10000
mouse bioassay (MU)


mouse bioassay v tissue culture
unheated mussel extracts
tissue culture assay (tcu
10000
1000
100
50
100
1000
10000
mouse bioassay (MU)


HPLC v tissue culture
tissue culture assay (tcu)
10000
1000
100
50
50
100
1000
10000
HPLC (ME)

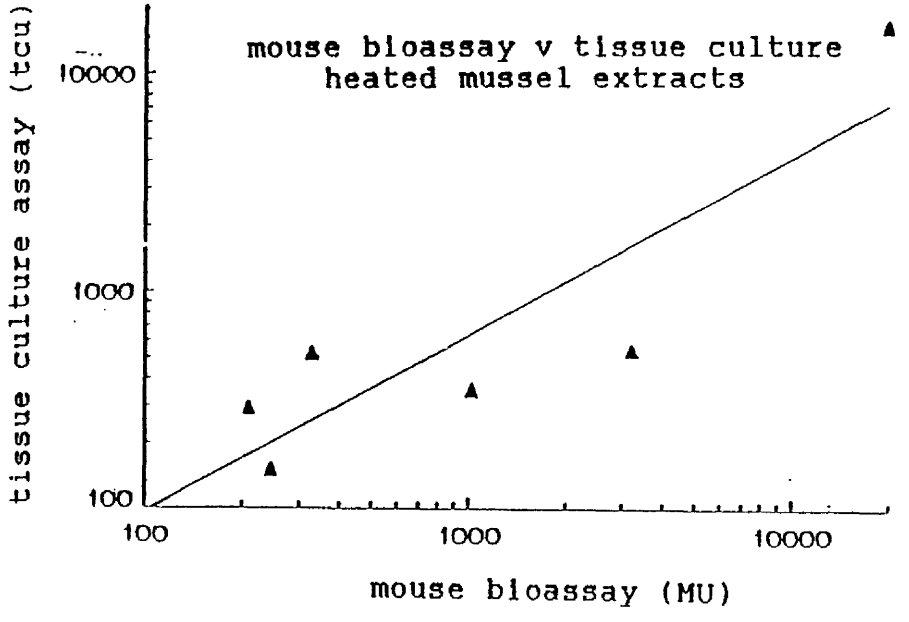

mouse bioassay v tissue culture
heated mussel extracts
tissue culture assay (tcu)
10000
1000
100
100
1000
10000
mouse bioassay (MU)

Table 21 Comparison of the correlations obtained between the mouse bioassay, HPLC and the tissue culture assay for the analysis of toxic mussel extracts.

Assay		Correlation coefficient
Mouse bioaasay v HPLC		0.86
Mouse bioassay v tissue culture	unheated	0.94
	heated	0.88
HPLC v tissue culture		0.93

LOW MOLECULAR WEIGHT TOXINS PRODUCED BY BACTERIA ISOLATED FROM SCOTTISH COASTAL WATERS

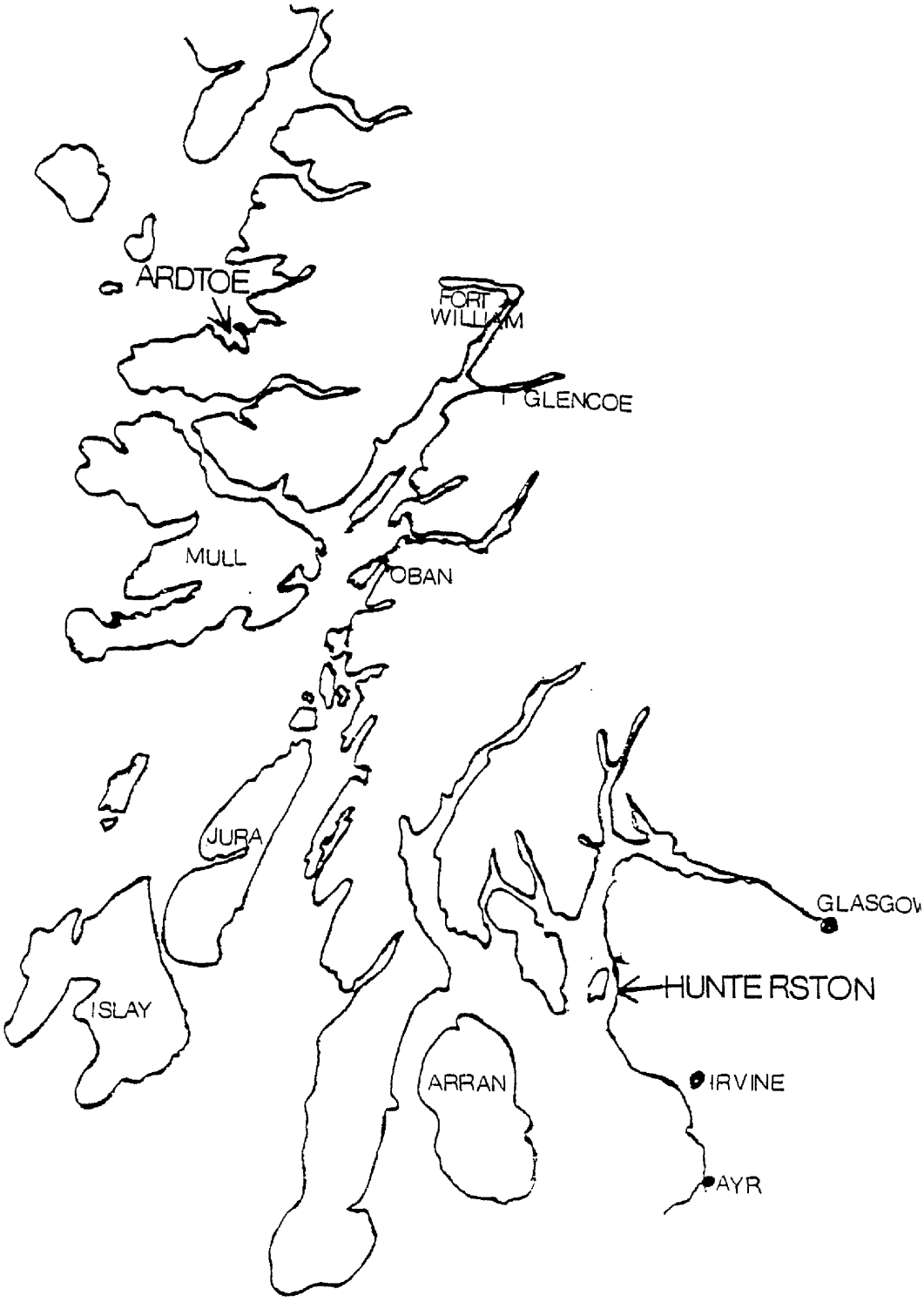
An investigation was made into the production of low molecular weight toxins by marine bacteria isolated from two fish farms, at Hunterston and at Ardtoe. The study involved measuring the temperature, viable count, presumptive vibrio count and the number of bacteria producing low molecular weight toxin in monthly water samples from two 1-year surveys over the period October 1987 to September 1990.

Both fish farms are situated on the west coast of Scotland, with Hunterston, at which turbot are cultured, located on the Clyde estuary, adjacent to the nuclear power station. Ardtoe, at which halibut are cultured, is situated at an unpolluted sealoch, remote from any industrial activity, or indeed human habitation (Fig. 35). The water samples for both locations were obtained at their intake source of seawater.

Preliminary Studies of Toxin-Producing Bacteria

In a preliminary survey of the two fish farms, between October 1987 and September 1988, the total viable count was measured by colony formation on marine agar and the presumptive vibrio count by

FIGURE 35 The location of Hunterston and Ardtoe on the West coast of Scotland.


ARDTOE
FORT WILLIAM
GLENCOE
MULL
OBAN
JURA
ISLAY
ARRAN
GLASGOW
HUNTERSTON
IRVINE
AYR

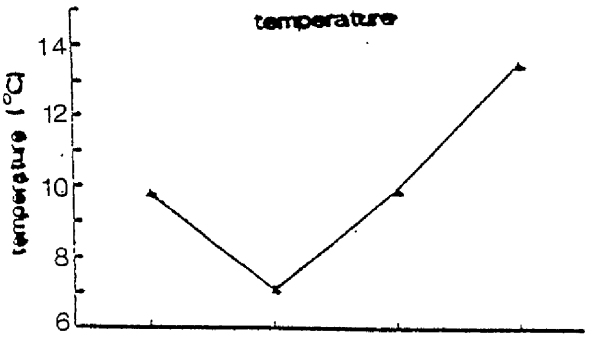
colony formation on TCBS agar. The water temperature at the time of sampling was recorded.

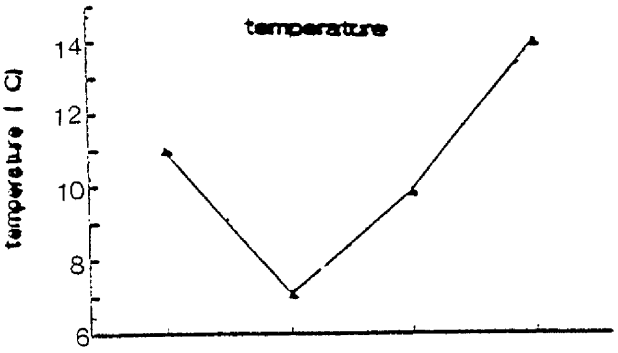
For each month, the bacterial isolates which were dominant on the marine agar plate, from which the viable count had been obtained, were isolated in pure culture. In total, 43 isolates from Hunterston and 54 from Ardtoe were collected over the 12 month period. The culture supernate of each of the bacterial isolates was later examined for ciliostatic toxin (CT) production.

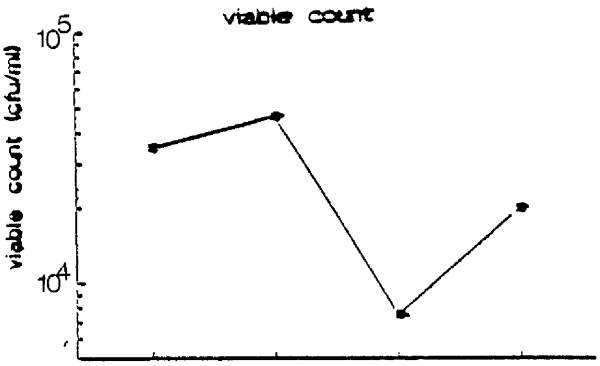
The data obtained are summarised as a series of charts (Fig. 36) showing water temperature, viable count, presumptive vibrio count and CT-producing bacteria. The presentation is on a quarterly basis, with the individual results in Appendix 7.

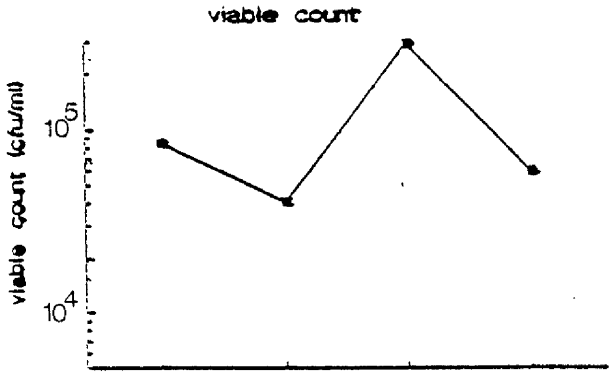
At Hunterston, the trends in the numbers of CT-producing bacteria were similiar to those of both the viable count and the presumptive vibrio count and, to a lesser extent, with the water temperature. Ardtoe was more complex in that the viable count did not seem to be related to any other variable. However, the presumptive vibrio count, the temperature and the number of CT-producers did change in unison, but not with the same regularity as at Hunterston.

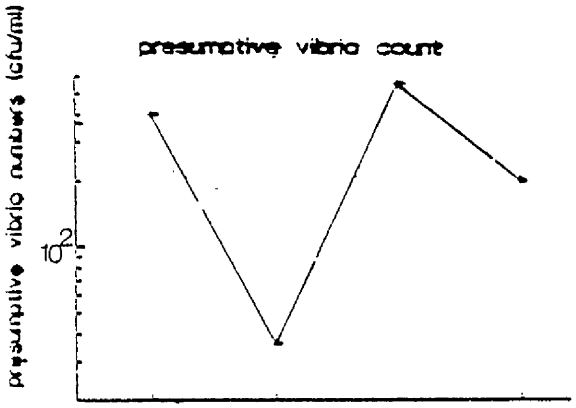
Figure 36 Viable count and presumptive vibrio count of bacteria isolated from seawater from Ardtoe and Hunterston over the period Oct. 1987 to Sept. 1988, expressed per quarter, compared to the water temperature and the quantity of bacteria capable of producing CT.

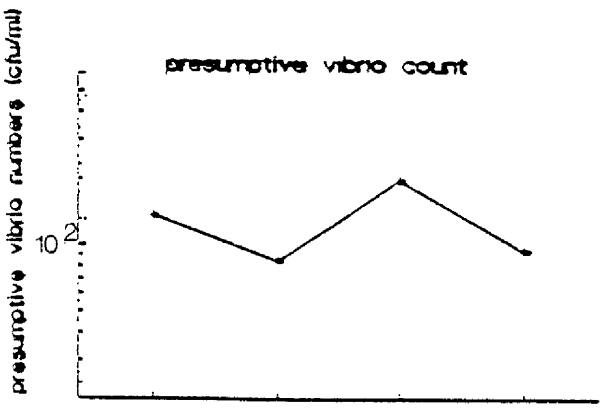

temperature
temperature (°C)
14
12
10
8
6

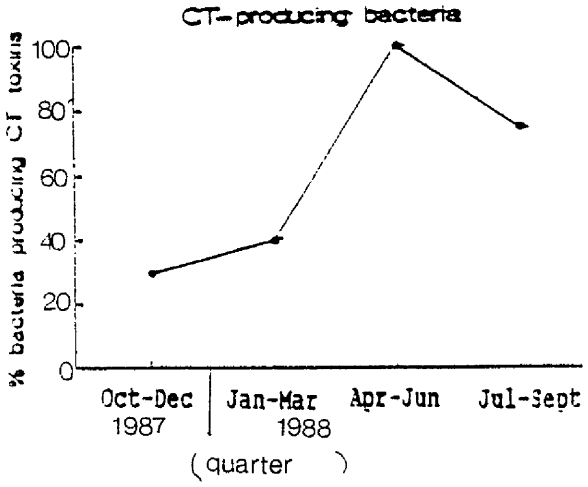

temperature
temperature (°C)
14
12
10
8
6

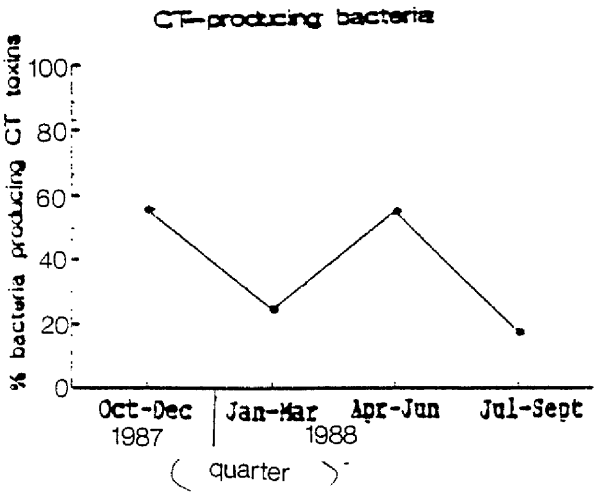

viable count
viable count (cfu/ml)
10^5
10^4


viable count
viable count (cfu/ml)
10^5
10^4


presumptive vibrio count
presumptive vibrio numbers (cfu/ml)
10^2


presumptive vibrio count
presumptive vibrio numbers (cfu/ml)
10^2


CT-producing bacteria
% bacteria producing CT toxins
100
80
60
40
20
0
Oct-Dec
1987
Jan-Mar
1988
Apr-Jun
Jul-Sept
(quarter)


CT-producing bacteria
% bacteria producing CT toxins
100
80
60
40
20
0
Oct-Dec
1987
Jan-Mar
1988
Apr-Jun
Jul-Sept
(quarter)

More Detailed Survey .

Commencing in September 1989, a more extensive survey was done at Hunterston and Ardtoe. Some improvements in methodology were incorporated, namely when the monthly water samples were cultured on marine agar plates at half-logarithmic dilutions up to 1/100, all of the bacteria on whichever plate contained approximately 50 colonies, were isolated in pure culture. With Ardtoe samples this yielded 546 bacterial isolates over the period September 1989 to September 1990, while from the Hunterston samples, 313 isolates were obtained over the period September 1989 to August 1990.

As with the previous survey, the temperature, viable count, presumptive vibrio count and CT-producing activity from each of these locations was obtained; these are summarized in Figs. 36 & 37, from the original results in Appendix 7. Isolates from Ardtoe were studied also for SCB toxin production.

Hunterston

At Hunterston, the viable count and presumptive vibrio count followed a similiar pattern, with a general increase in counts from January to July, followed by a sharp drop in August. This coincided

with the increase in temperature between January and July which also showed a slight decrease in August. The CT-producing bacteria demonstrated a decline from September to December 1989 which followed that of the temperature. However, the gradual increase from January to July seen with the other parameters was not reflected with the CT producers, whose numbers did not steadily increase until July (Fig. 37).

When the data from each parameter were compared to one another using Kendall's rank correlation coefficient, the visual observations were confirmed. The number of CT-producing bacteria was not significantly correlated to any other variable, but the temperature against presumptive vibrios demonstrated significant correlation, whilst the correlation between the viable count and the presumptive vibrio numbers was highly significant (Table 22).

Ardtoe

In general, the pattern of decline from September to December 1989 followed by an increase during March to September 1990 was reflected in all of the parameters, excluding SCB activity. The viable count, presumptive vibrio count and the CT-producers increased slightly over the Jan-Mar period when the

Figure 37 Viable count and presumptive vibrio count of bacteria isolated from seawater from Hunterston over the period Sept. 1989 to Aug. 1990 compared to the water temperature and the quantity of bacteria capable of producing CT and SCB toxins.

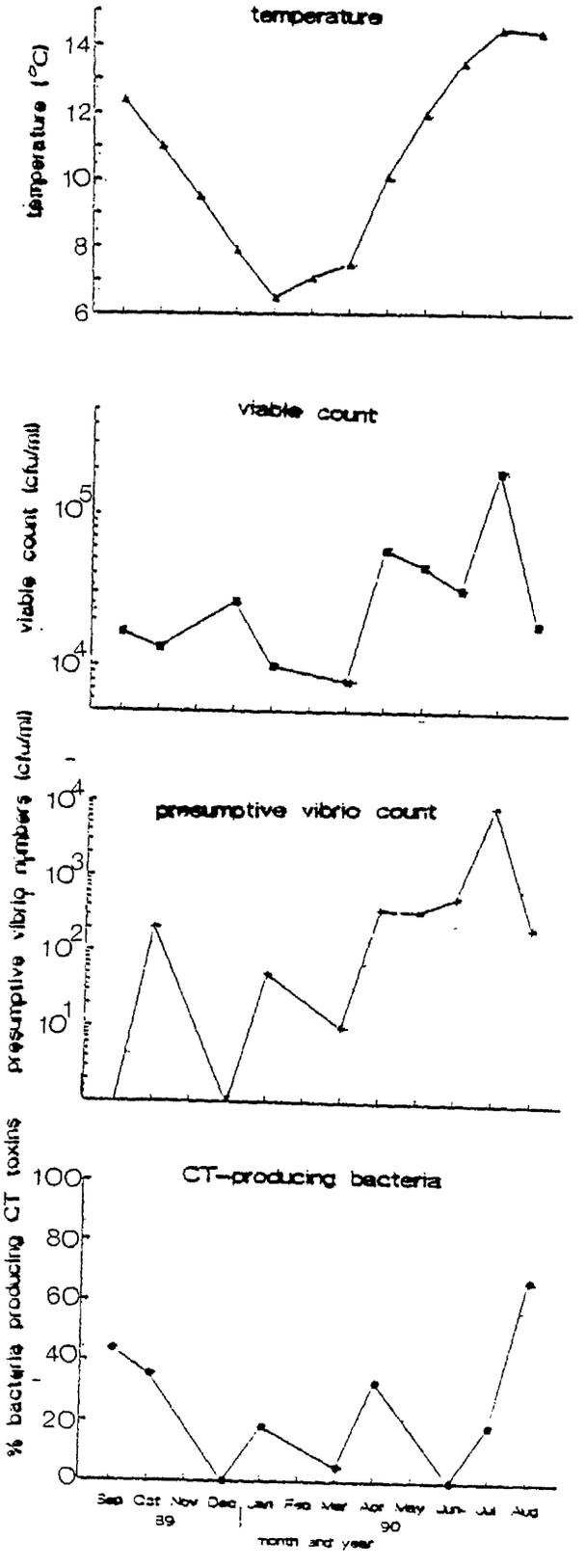

temperature
temperature (°C)
14
12
10
8
6
viable count
viable count (cfu/ml)
10^5
10^4
presumptive vibrio count
presumptive vibrio numbers (cfu/ml)
10^4
10^3
10^2
10^1
CT-producing bacteria
% bacteria producing CT toxins
100
80
60
40
20
0
Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug
89
90
month and year

TABLE 22 Kendall correlations of different variables in the data from Hunterston

Variables	Kendall correlation	
	statistic	significance
Temp v VC	0.378	0.064 NS
Temp v vibrios	0.472	0.030 S
Temp v CT	0.394	0.071 NS
vibrios v CT	0.083	0.057 NS
VC v CT	0.056	0.417 NS
VC v Vibrios	0.607	0.008 HS

VC is the viable count

P > 0.05 not significant NS

P < 0.05 significant S

P < 0. 01 highly significant HS

water temperature was at the lowest. However the number of CT-producers then remained low until July whereas the bacterial count fluctuated. The SCB toxin-producing bacteria fluctuated throughout the year with a general increase at September in both 1989 and 1990 coinciding with the highest temperatures (Fig. 38).

Examining the significance of the Kendall's correlation of the data indicated that the temperature was related to the number of SCB toxin and CT-producing bacteria but not the bacterial numbers. The viable count and presumptive vibrio count were both related to the number of CT producers but not the SCB producers. On the other hand, significant correlation existed between the numbers of CT-producers and SCB toxin-producing bacteria (Table 23).

Isolates producing SCB

The bacterial isolates producing SCB toxin were analysed in more detail, the numbers of which fluctuated between 20-40 % of October 1989 to August 1990, until at September a large increase in numbers, which coincided with the highest water temperature, was recorded.

In total, 36.8 % (177/481) of the bacterial

Figure 38 Viable count and presumptive vibrio count of bacteria isolated from seawater from Ardtoe over the period Sept. 1989 to Sept. 1990, compared to the water temperature and the quantity of bacteria capable of producing CT and SCB toxin.

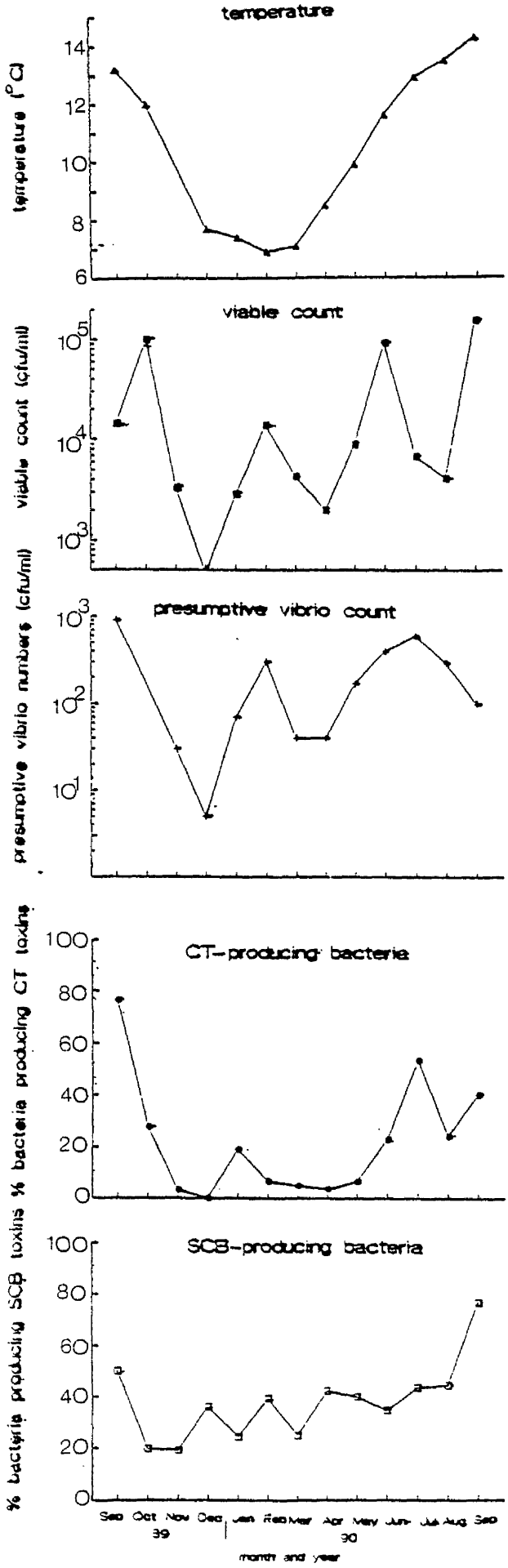

temperature
temperature (°C)
14
12
10
8
6
viable count
viable count (cfu/ml)
10^5
10^4
10^3
presumptive vibrio count
presumptive vibrio numbers (cfu/ml)
10^3
10^2
10^1
CT-producing bacteria
% bacteria producing CT toxins
100
80
60
40
20
0
SCB-producing bacteria
% bacteria producing SCB toxins
100
80
60
40
20
0
Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
89
90
month and year

TABLE 23 Kendall correlations of different variables in the data from Ardtoe

Variables	Kendall correlation statistic	significance
Temp v VC	0.305	0.085 NS
Temp v vibrios	0.418	0.069 NS
Temp v CT	0.611	0.003 HS
Temp v SCB	0.485	0.014 S
vibrios v CT	0.769	0.005 HS
vibrios v SCB	0.336	0.065 NS
VC v SCB	0.154	0.232 NS
VC v Vibrios	0.550	0.007 HS
VC v CT	0.542	0.005 HS
CT v SCB	0.361	0.044 S

VC is the viable count

P > 0.05 not significant NS

P < 0.05 significant S

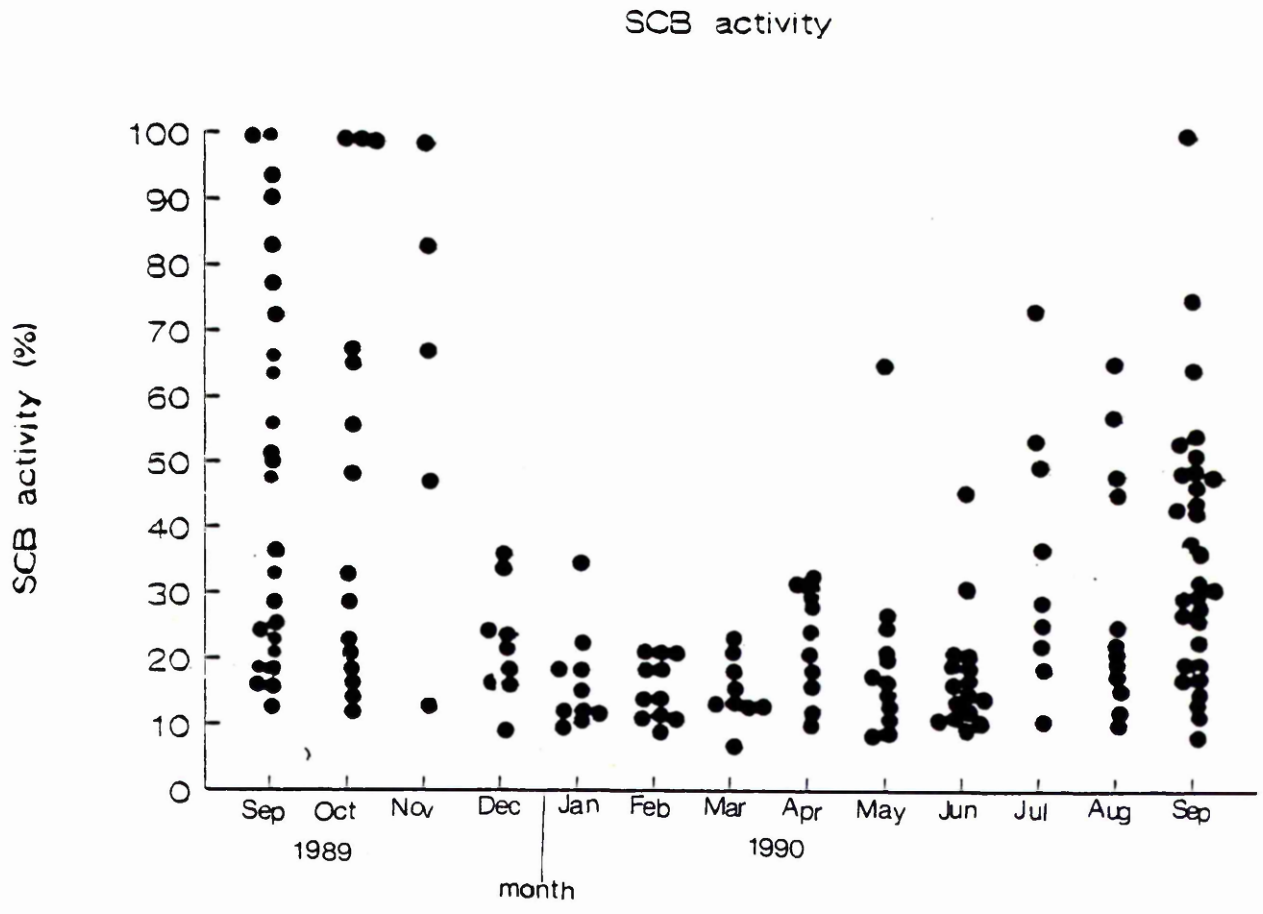
P < 0. 01 highly significant HS

isolates produced SCB toxins. When the quantity of SCB activity produced by each isolate was compared on a monthly basis and the results expressed as a scatter graph (Fig. 39), it can be seen that the greatest number of low SCB toxin-producing isolates occurred over the late winter and early spring months, whereas, the greatest number of high SCB toxin-producing isolates were found in the summer and autumn.

Ciliostatic activity in SCB toxin producing bacterial isolates.

Of the total number of isolates from Ardtoe, 26 % (144/546) produced CT, 36 % (177/481) produced SCB toxins, and 12 % (60/481) produced both CT and SCB toxins. Of the SCB-toxin-producing isolates 32 % (57/177) also produced CT. When the 6 standard SCB-toxin-producing strains used in earlier work e.g GFC etc., were examined, they were all capable of producing CT. Therefore, bacterial isolates are capable of producing both toxins.

FIGURE 39 Monthly variation in SCB activity of the SCB toxin-producing bacteria from Ardtoe.


SCB activity
SCB activity (%)
100
90
80
70
60
50
40
30
20
10
0
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
1989
1990
month

DISCUSSION

DISCUSSION

For convenience, the results of this thesis are discussed in two main sections: sodium channel-blocking (SCB) toxins are dealt with first, since the major section of the results consists of this topic. Ciliostatic toxins (CT) are dealt with subsequently although the two topics are related in that some of the bacterial isolates, from fish farms, produced both toxins.

THE PRODUCTION OF SCB TOXINS BY BACTERIA

In the interval between 1986, when Narita *et al.* first reported that bacteria, from a xanthid crab produced TTX, and the beginning of 1989 when the present work was started, there were 7 other reports of the production of TTX by marine bacteria from various sources. The majority of these articles were from Narita's group with two originating from Yasumoto's laboratory. Both groups were based in Japan and the work remained unconfirmed, with the exception of a short report in the Lancet, by Tamplin *et al.*, (1987), who found that *V. cholerae* produced SCB toxins. However, Tamplin's work lacked detail and also remains to be confirmed. Meanwhile,

the authors concerned have since published other work.

Of the Japanese publications up to 1989, only one or two isolates per report were identified as TTX producers. This seems very low when, for example, the intestinal content of a puffer fish, which has been reported to have 5×10^7 bacterial cells per gram (Noguchi *et al.*, 1987), is considered. Also, cultured puffer fish containing similiar microflora to the above were non-toxic (Sugita *et al.*, 1988).

The major tests used to detect TTX in these Japanese investigations were the mouse bioassay and HPLC, both of which are problematic as discussed later. Also, in many of the reports, particularly that of Noguchi *et al.* (1987), the published HPLC chromatograms were of poor quality and generally the results were not confirmed by testing for biological activity.

Since marine vibrios produce many toxins, it was decided to investigate these Japanese reports in greater detail. The initial problem was to find a suitable detection method for SCB toxins. Interest was therefore raised by the report of Kogure *et al.*, (1988a) on the use of a tissue culture assay, based on the differentiated properties of mouse neuroblastoma (MNB) cells, for detecting TTX. Kogure stated that the method was more sensitive

than both the mouse bioasay and HPLC and later reported its use to detect TTX in marine sediments (Kogure *et al.*, 1988b).

DEVELOPING THE TISSUE CULTURE ASSAY

Setting up a tissue culture assay following Kogure's report proved extremely difficult, due mainly to the lack of detail in the described methodology.

The method was based on that developed by Catterall who used clones of the MNB cell line C1300 to study the mode of action of several neurotoxins on the sodium channel (Catterall and Nirenberg, 1973; Catterall, 1975 a,b; Catterall and Ray, 1976; Catterall, 1977, 1981). Catterall's original method was relatively rapid and efficient. However, its major disadvantage lay in the use of the isotope ^{22}Na whose short half life is inconvenient for routine use.

Kogure circumvented the need for ^{22}Na by visually detecting changes in the cells. At least 200 cells per well in a microtire (MT) plate were randomly selected for microscopic observation. In my experience, this was too laborious and impractical for large-scale screening of bacterial samples for SCB toxins. It was also subjective, in that changes

in cell morphology were difficult to detect reliably within the 8 h observation time used by Kogure. Therefore, my initial work was directed at other methods to quantify cell viability.

Use of neutral red in a dye-uptake method (Finter, 1969) was considered, the principle being that only live cells incorporate the dye. The bound dye could then be released by citrate-buffered ethanol and measured spectrophotometrically. Greaves and co-workers (1971) used this method for titration of diphtheria toxin in cell cultures and found the results an improvement on existing cytotoxicity methods.

A logical extension of the use of vital dyes was the introduction of semi-automated colorimetric systems for the quantitation of the binding of various vital stains to the cultured cells. This was based on staining a controlled number of cells seeded onto MT plates and measuring the previously cell bound stain, after its release by an appropriate solvent. The absorbance could then be read and recorded automatically with a microtitre plate reader coupled to a microcomputer. This semi-automated system was adapted for use with neutral red (Frampton *et al.*, 1991) and forms the basis of the technique developed in this work.

In this method, MNB cells were seeded on to MT plates and treated with neurotoxins which, after the appropriate incubation period and washes, were stained with neutral red, treated with citrate-buffered ethanol and the resultant pink colour measured by using a microplate reader at 540 nm.

To achieve a good range of absorbance readings in wells containing different proportions of live cells, it was desirable to use appropiate cells numbers which would give the highest absorbance reading in the linear range defined by the Beer-Lambert Law. With cell densities of 10^5 cells/ml as controls, as used by Kogure, only a sparse monolayer at the bottom of the well was formed and an absorbance reading of <0.2 was obtained. This was too low and larger cell numbers was therefore examined.

After the initial 24 h seeding period with $3x10^7$ MNB cells/ml, a satisfactory monolayer could be observed. However, after treatment with toxins and subsequent washes, even with the controls it was obvious that cells were not adhering sufficiently to the MT plates.

This problem was largely due to preheating the serum component of the culture medium, thereby adversely effecting the heat-sensitive proteins involved in adhesion to non-cellular surfaces. These

heat-sensitive proteins are also responsible for promoting the spreading of many types of cultured cells (Yamada and Olden, 1978). In fact, it had been reported that limiting the concentration of serum protein weakened the attachment of cells to the tissue culture plastic surface (McKeehan and Ham, 1976) and the cells frequently came loose during mild mechanical disturbances such as removal of media and staining. Therefore, the loss of MNB cells was not suprising and was solved simply by not heating the serum.

Investigation of this problem had other benefits. In particular, the incorporation of CMC (carboxymethyl cellulose) into the suspension medium and pretreating the MT plates with poly-l-lysine both gave useful increases in cell adhesion. These findings have application for studies in which the serum concentration in the media is reduced.

A further benefit was the investigation of the dye MTT (3-(4,5- dimethylthiazol-2-y l) -2,5- diphenyl tetrazolium bromide) as a replacement for neutral red. However, this substance generated its own problems, relating to the insolubility of the crystals formed after incubation with live cells. Possible amendments to the system could be considered.

A major problem with Kogure's method was the lack of information on the diluents used, both in the assay and in the preparation of the neurotoxins. This is essential information as Garritsen *et al.*, (1988), in experiments with TTX and rat brain synaptosomes, stated that: "the composition of the incubation medium has a marked effect on the results obtained and the state of the sodium channel can be altered by the composition of the medium. The apparent affinity of neurotoxins when tested under these conditions was directly influenced by this change, it is therefore of great importance to specify accurately the composition of the media". This statement was found to be applicable to the tissue culture assay.

Since the method was based on Catterall's, it could be presumed that Kogure followed his protocol; however, Catterall used several different diluents. In the assay developed here, the solutions were initially prepared in saline, but as this had an adverse effect on cell survival, RPMI 1640 was subsequently used.

At this point, the assay was established in as far as MNB cells on MT plates survived over a 48 h period with no loss in viability. The cell viability could be determined, with reproducible results, with an ELISA plate reader measuring the quantity of

neutral red absorbed by viable cells.

The next step in developing the assay was to establish the concentrations of oub and ver which, individually, did not cause MNB cell death but combined were lethal, thereby providing a threshold of dead cells at which cell protection, due to the quantity of SCB toxin present in a sample could be determined.

Use of ouabain and veratridine

The initial experiment with 1.0 mM oub and 0.05 mM ver, as recommended by Kogure *et al.*, (1988), caused 70 % cell death within 24 h. In subsequent experiments, the concentration of oub varied between 0.02 and 0.5 mM and that of ver between 0.02 to 0.1 mM; these gave a range of between 20 and 80 % in cell death.

In Kogure's original paper, the regulation of neurotoxin and serum batches was not stated. However, I found that standardising the system, with the same batches of serum and oub/ver, allowed the concentration of the latter to be fixed at 0.4-0.5 mM and 0.1 mM respectively. Despite these precautions, the % cell death still varied more than was desirable, possibly due to variations in culture conditions, specifically the duration of

cultivation. Previous studies (Kimhi, 1981) showed that cultures of most MNB clonal lines contain small, electrically-passive neuroblast-like cells, as well as large neuro-like cells with well developed processes and electrically excitable membranes. Populations of passive cells or populations enriched with excitable membranes can be obtained by choice of growth condition (Peacock et al., 1972; Catterall and Nirenberg, 1973). Catterall used MNB in the stationary phase, usually 6 to 8 days after sub-culture (Catterall and Nirenberg, 1973), while Kogure did not state the incubation time used. In my assay, the initial incubation times varied according to the demand for cells but was never less than 3 days. However, in later work, the incubation time was standardised at 4 days.

The final concentrations of oub was therefore half that recomended by Kogure and twice that for ver. Taking the above factors into account, differences in the neurotoxin concentrations between the two assays may have arisen, as Kogure *et al.*, (1988a) did not clearly specify whether toxin concentrations were the final concentrations in each well as the quantities of the toxins added were not stated. In this thesis, the concentrations of these toxins are always stated as the final concentration.

Other differences between my method and Kogure's

are that here, CO_2/ air mixtures were not used in the culture of the MNB cells and the serum level in maintenance medium was eventually decreased to 5 % as compared to the 10 - 13.5 % used by Kogure *et al.* (1988a, 1989).

In summary, for the assay to work reliably, it is essential to maintain consistent culture conditions with unchanging batches of components, especially with serum, otherwise reproducible results will not be obtained.

Dose response curve for TTX

In establishing a dose response curve for TTX the results from this work were similiar to those of both Catterall and Nirenberg (1973) and Kogure *et al.* (1988), in that the effect of oub/ver on cell death could be inhibited by TTX, and a linear relationship established. Catterall found, with ^{22}Na as the detection system, that 1 nM TTX exhibited <20% MNB cell protection compared to >90% cell protection at 100 nM TTX. (Catterall and Nirenberg 1973). Kogure, by observing microscopically the protective effect of TTX on cell viability of MNB cells in the presence of oub/ver, constructed a dose-response curve in the range 0 to 100 nM TTX. He found that 50 % cell protection occurred with 50 nM

TTX after 6 h incubation and 55 nM TTX at 18 h.

A linear standard curve of TTX concentration against cell protection over the range 0-100 nM was also obtained in this assay, with a 50 % end point of 50 nM TTX after 24 h incubation, which was very similiar to Kogure's result of 55 nM after 18 h incubation. This system was therefore used as a quantitative assay.

Time course of the assay

The above experiments were done over a 24 h incubation period, however, in the presence of oub/ver, Kogure detected morphological differences in the MNB cells within 3 h and considered that at 8 h, 80-90 % of the MNB cells were dead.

In my work, however, using the uptake of vital stain as the detection system, the cells appeared 100 % viable at 8 h, and the time required for maximum cell death was 24 h. These differences may be due to the subjectivity of microscopic observations as cell rounding precedes death. Thus, although the cells after 8 h may have lost morphological rigidity, they might still be viable.

The assay at this stage was ready for use to study SCB toxin production by bacteria. Nevertheless, additional investigations were made into methods by

which the sensitivity of the assay could be increased.

Differentiating agents

MNB cells were suitable for the tissue culture assay because of their differentiated state. This involves the development of an electrically excitable membrane, the synthesis of enzymes to metabolize the various neurotransmitters and special mechanisms that control their release. However, while some workers state that neuroblastoma cells are differentiated only when they attain their final functional characteristics, in an irreversible undirectional process composed of many steps (Kimhi, 1981), others view differentiation as an expression of any specialized property or function for which the cell has become adapted, during development or maturation. (Kimhi, 1981).

For instance, Giovanni *et al.* (1981), regard the acquisition of an excitable membrane as neuronal differentiation, whereas others regard the outgrowth of any axon-like process (neurite) from a nerve cell as an expression of a differentiated property which, however, is not necessarily followed by an increase in electrical excitability (Kimhi, 1981).

The clone used in this assay, C1300 neuro N2a, grew in suspension as round neuriteless cells rapidly extending neurites on transfer to dishes to which they could adhere and give cultures of morphologically non-differentated and differentated cells (Kimhi, 1981). However, although differentation could be achieved to some extent when cells reached confluency and they may exhibit large resting potentials and well developed excitable properties (Spector, 1981), they do not exhibit contact inhibition and are therefore difficult to maintain in good condition for more than a few days (Peacock *et al.*, 1972). Subsequently, the culture may become overgrown, heterogenous and as a rule poorly excitable (Spector, 1981).

The latter author states that "in order to achieve a further expression of electrical excitability and a stable population of differentiated cells it was necessary to change the culture conditions." Therefore although the MNB cells were judged to be differentiated upon reaching confluency, processes by which they could be increased were sought.

Glial-conditioned media (Monard *et al.*, 1973), serum deprivation and chemicals such as DMSO, valinomycin, HMBA, dcAMP, prostaglandin E, and theophylline have been reported to induce differentiation in clonal lines derived from C1300.

However, although morphological differentiation was induced with all of these compounds, they had varying effects on the biochemical and electrophysiological properties of the cells (Baumgold and Spector, 1987). In this study the effect of some of these agents on MNB cells C1300 clone N-2a was investigated.

C6 medium

The first experiments investigated the effect on the sensitivity of MNB cells to oub/ver on incubating cells in the C6 medium (glial conditioned medium) which contained supernate in which rat glioma cells had previously been grown. The same clone of MNB cells cultured in a similiar medium, by Monard *et al.*, (1973), produced a higher percentage of morphologically differentiated cells. My study confirmed these results and also showed that the cells increased in electrical excitibility as indicated by an increase in sensitivity to oub/ver/TTX. However, this method was considered inconvenient for routine use as it involved the maintenance and consequent cost of another cell line. In addition, in order to obtain consistent results, large volumes of the glioma supernate would have been needed.

This led to investigations into reports of the effect of other differentiating agents, on MNB cells. The results of these studies, while forming useful guidelines, are not directly comparable, as the clones of neuroblastoma C1300 were different from the Neuro-2a. Kimhi (1981) stated that "since different clones differ in morphology, level of biosynthetic enzymes and electrophysiological properties, it is probable that they differ in their response to differentiating agents." The differentiating agents investigated here were HMBA (N, N'Hexamethylene-bis-acetamide), DMSO (Dimethyl-sulphoxide), valinomycin, aminopterin, nerve growth factor (NGF) and serum limitation.

Other differentiating agents

Aminopterin was toxic to MNB cells, and contrary to the results of Spector *et al.* (1975), valinomycin did not increase cell excitability. With NGF, there are conflicting reports of its differentiating activity (Kimhi, 1981). In my work, it did not induce neurite formation nor increase the sensitivity of the MNB cells to oub/ver. However, DMSO, serum limitation and HMBA did induce responses in the MNB cells and these are discussed individually below.

DMSO

When treated with DMSO (2 % v/v), certain clones have been reported to extend neurites and develop a higher proportion of excitable membranes when compared to untreated cells, and these were stable for several weeks (Kimhi, 1976; Baumgold and Spector, 1987). The increased electrical excitability of MNB cells cultured in DMSO was thought to be due to an increase in sodium channel density (Spector and Baumgold, 1982) because cells cultured for 8 days with 2 % DMSO gave a three-fold increase in the binding of scorpion toxin (Table 5) to the MNB cells and was accompanied by a two-fold increase in Na^+ ion influx. Subsequent work by Baumgold and Spector (1987) demonstrated an increase in scorpion toxin binding after 6 days and a two-fold increase in ^{3}H-STX binding.

Kimhi (1981) reported that medium containing DMSO induced both morphological and electrophysiological maturation of MNB cells clone NIE-115 which was both time and concentration dependent, i.e. 1 % DMSO partially inhibited cell multiplication whereas 2 % completly inhibited it and both increased electrical excitability only in cells cultured in its presence for at least 6 days. Kimhi found that the cells became opposed in a linear array and produced much

less acid, thereby allowing less frequent changes of medium. However, in my work with clone N-2a, the MNB cells did form linear arrays but continued dividing and the medium turned yellow overnight, thereby causing some difficulty in maintaining the cells in good condition. Kimhi *et al.* (1976) obtained similiar results in clone NB60. Also, contrary to what was expected by Kimhi's findings, the sensitivity of clone N-2a to oub/ver decreased in the presence of 2 % DMSO. This may have been due to one of the following:

1. The concentration of DMSO may have been too high; results obtained with clone NIE-115 indicated that when treated with 4 % DMSO the cells exhibited poorly developed excitability, and remained a homogenous population of round cells without neurites. However, this explanation seems unlikely, since in these experiments morphological differentiation was observed.

2. The incubation period of the cells may have been insufficient; the MNB cells used by Kimhi had been maintained for 12-18 days in a confluent state prior to DMSO treatment. He found that the longer the period in which the cells were maintained in the confluent state prior to replating in DMSO, the higher the % of differentiated cells and the shorter the lag in process formation (Kimhi *et al.*,

1976). In these experiments, the cells were DMSO treated after one day of reaching confluency and thereafter maintained for 7 days in DMSO medium; it is probable that an extension of this incubation period might increase sensitivity to oub/ver.

3. Clone N-2a may be insensitive to DMSO; it is possible that MNB clone N-2a cells may be insensitive to DMSO since the response to this chemical does vary between clones, with clones NB-60 and N-1a-103 being insensitive as is the human neuroblastoma cell line IMR-32 (Kimhi et al., 1976). However, this seems unlikely since a decrease in sensitivity occurred.

Of the 3 possibilities above, the most likely explanation for the drop in sensitivity to oub/vet in DMSO-treated cells was that the incubation period in DMSO was insufficient. Spector (1981), suggested that different types of treatment might be required for each clone, and this, therefore, may be the case with N-2a. However, maintaining cells in the confluent state or in DMSO media involved a high turnover of media which was both time consuming and expensive. If conditions which increased sensitivity by using DMSO were found, they would be considered impractical unless they halted proliferation.

Serum limitation

As serum is required for growth and maintenance of MNB cells in culture, Kimhi (1981) stated that "it is reasonable to assume that many unknown factors that suppress or induce changes in the phenotype of the cell are present." The fraction of differentiated cells in the population, as defined by the extension of neurites, varied as a function of serum concentration (Schubert *et al.*, 1971). This was assumed to be due to a reduction in possible differentiation suppression factors from the serum (Kimhi, 1981).

MNB cells grown in this experiment, in medium containing low serum concentrations, exhibited the formation of neurites, which previously has been reported for clones other than N2a (Kimhi *et al.*, 1976; Kimhi, 1981; Baumgold and Spector, 1987). However, the only report on the effect of serum on electrical excitability is that of Baumgold and Spector (1987) who found that serum deprivation did not enhance electrical excitability and that cells only survived under these conditions for a few days. For these reasons, serum limitiation, as opposed to deprivation, was adopted, whereby MNB cells were maintained in 2 % serum, in which they survived in a healthy state for 7 days, with only very slow cell growth occurring. This effectively allowed the

maintenance of the confluent state and a reduction of possible differentiation suppression factors.

In this work, MNB cells maintained under serum limited conditions exhibited morphological differentiation as noted by the formation of neurites and confirmed reports of this occurrence for other clones (Kimhi *et al.*, 1976; Kimhi, 1981; Baumgold and Spector, 1987). An increase in sensitivity to oub/ver also took place indicating that some factor was present in the serum which depressed the density of Na channels and hence the action of oub/ver. The only other agent examined which increased the sensitivity of the MNB cells to oub/ver was HMBA.

HMBA

When cells were cultured in 10 mM HMBA, but not in 2.5 mM HMBA, morphological differentiation and an increase in cell sensitivity to oub/ver occurred, similiar to that found with serum limitation. However, HMBA increased the sensitivity of the MNB cells at lower concentrations of oub/ver.

HMBA has previously been shown to increase electrical excitability in clone N-1E-115 after 6-9 days incubation. This was demonstated by an increase in scorpion toxin binding, ^{3}H-STX binding and Na+

ion uptake, which was attributed to a significant increase in sodium channel density and was manifested well after cells assume the morphologically differentiated state (Baumgold and Spector, 1987). It is probable that the increase in cell sensitivity to oub/ver found in these experiments was due to an increase in Na+ channel density. Therefore, the sensitivity of the MNB C1300 clone N-2a cells to the neurotoxins oub/ver could be increased by changing the components of the culture medium and is probably indicative of an increase in Na^+ channel density.

These agents, however, were not routinely incorporated into the assay, pending further work which would include investigating the effect of varying the incubation times of MNB cells cultured in media containing these agents on both cell death due to oub/ver and cell protection due to TTX.

With serum limitation, although the cells had been cultured in 2 % serum, they were seeded on to MT plates with suspension medium containing 10 % serum. It would have been desirable to reduce this to 2 %, but this would have provoked problems with insufficient cell adhesion, a problem which could be solved by pre-coating the MT plates with polylysine; this was shown to be effective here and by McKeehan and Ham (1976), who reported that

treatment of the culture surface with PLL efficiently overcame the problem of detachment of cells and permited the serum concentration to be reduced to the point where it became nutritionally limiting. Another alternative would be to solve the problems associated with the dye MTT, so that it would not be necessary for the cells to adhere.

Nevertheless, despite possible alterations to the method, it was sufficiently sensitive to detect TTX in solution and hence was used to investigate the possible production of TTX in bacteria. However, it is important to note that while the tissue culture assay is highly specific for SCB toxins, TTX is not the only toxin capable of this function, since the PSP (paralytic shellfish poisons) act in the same manner. This work was therefore extended to include PSP which are of equal, if not more, interest than TTX. Hence, toxins detected by the tissue culture assay, unless otherwise specified, are called SCB toxins which includes both TTX and PSP. Speculation on bacterial involvement in PSP production is discussed later.

DETECTION OF SCB TOXINS IN BACTERIA BY THE TISSUE CULTURE ASSAY

For the investigation of the production of SCB toxins by bacteria, 6 isolates were examined. Four strains, GFB, GFC, OK1 and OK2 were provided by Professor Simidu as TTX-producing bacteria. However, there was no information on their origin and identity which were therefore unknown, until recently, with the emergence of a paper by Simidu *et al.* (1990). Strain OK1 originated from a red calcareous alga (Kotaki et al., 1985) and was identified by Simidu *et al.* (1990) as *Shewanella alga*. Previously, the bacterium had been identified tentatively as *Pseudomonas* strain by Yasumoto *et al.*(1986). These authors provided details of its TTX-producing ability.

Strain OK2 was identified as *Vibrio pelagia* biovar II (Simidu *et al.*, 1990), and this also originated from the surface of a red alga (Kotaki *et al.*,1985). No further information on its toxin-generating abilities has been published. However, both OK1 and OK2 have been reported to convert GTX II and III (STX derivatives; Fig 4) to STX (Kotaki *et al.*, 1985).

Strains GFB and GFC were identified as *Vibrio pelagia* biovar II and *Alteromonas tetraodonis sp. nov.* respectively (Simidu *et al.*, 1990), both

originating from skin slime of a pufferfish and having been reported to produce TTX (Yotsu *et al.*, 1987).

Strains OK1 and GFC were classified as type strains by Simidu *et al.*, (1990) and were deposited in the Culture Collection of the Institute of Applied Microbiology, University of Tokyo, Japan. The two other strains investigated here were *Vibrio alginolyticus* NCIMB 1903 and *Vibrio anguillarum* NCIMB 829, from the National collection of Industrial and Marine Bacteria in Aberdeen. Both of these strains have been reported to produce anhydro-TTX (Simidu *et al* ., 1987).

The cell extracts and supernates of each strain cultured in a variety of media were subjected to the tissue culture assay; in all cases, SCB activity was detected. A notable advantage of this technique is that the extensive extraction procedures required for instrumental analysis are unneccessary as samples can be analysed directly. There was however, the problem with cell extracts of a heat-labile inhibitory factor (CIF), which blocked the detection of SCB activity at low dilutions but was removed upon further dilution. The nature of the CIF is unknown and time was not spent on its investigation, although similiar work to that described for culture

supernates could have been done.

The presence of an inhibitory factor in cell extracts of PSP-containing bacteria was also suggested by Kodama *et al.*, (1990), although they did not provide experimental evidence in support, nor did they speculate on its nature.

Heating destroyed the CIF, and the SCB activity also decreased. This was unexpected and difficult to explain since the SCB toxins are reportedly heat-stable. Possibly, if a mixture of toxins were present, there might be changes to less toxic derivatives upon heating. Instrumental analysis could be done to examine this.

These observations are relevant to the published reports of TTX activity in which, with a few exceptions, the cell extracts had been preheated for 20 min prior to use; in view of the above findings, this means that the amount of toxin found may have been underestimated.

Investigating the culture supernates for SCB activity indicated that a greater quantity of toxin was present in 7-day culture supernates then in 1-day cell extracts, but an inhibitory factor was also present (SIF), which, unlike that of the cell extracts, was heat stable (100°C for up to 1 h).

For convenience, the subsequent work concentrated on analysis of the culture supernates. Efforts were

made to identify and remove the SIF, which was subsequently found to be due to the high salt concentrations in the culture medium. In retrospect, this was not surprising, since the interchange of ions through the Na^+ channel is highly sensitive, and culture media, such as MB contain extremely hypertonic concentrations of NaCl.

A review of the literature confirmed these findings since it was reported that Na+ ions, at high concentrations, compete with TTX (Catterall, 1975) and STX (Weigele and Barchi, 1978) for binding at the Na+ channel. Also, Henderson *et al.*, (1974), showed that the binding of TTX to solubilized membrane extracts, decreased by approximately 36 % in the presence 200 mM NaCl.

Divalent cations (e.g. Ca^{2+}) also have an inhibitory effect on the binding of SCB toxins to the sodium channel (Henderson *et al.*, 1974; Weigele and Barchi, 1978), with STX being the most sensitive (Strichartz *et al.*, 1986).

Increased salt levels also affect ver toxicity, since an increase in the extracellular divalent cation (e.g. Ca^{2+}) concentration inhibits ver-dependent sodium uptake. These divalent cations inhibited activation at concentration several-fold less than that at which they affected TTX

(Catterall, 1975). The effect of excess salt, due to the medium, on ver was observed in this system, since if MB was present in the diluent, the number of cells killed by oub/vet decreased, with reduction in assay sensitivity.

Overall, the addition of MB to the tissue culture assay had an adverse effect. It is probable that the inverted dose response curve obtained with culture supernates was largely due to elevated Na^+ ion concentrations in the media. It is therefore necessary to incorporate the medium into the oub/ver control, at the same dilution as in the sample.

The effect of salts in the culture medium could be circumvented by charcoal extraction, since charcoal effectively binds organic materials, which could then be retrieved, with the appropriate solvent. However, the procedure was inefficient, with approximately 50 % of the SCB activity being lost. Also, the SCB activity obtained from untreated samples at 1/8 or 1/16 dilution was similar to charcoal-extracted samples at 1/2 or 1/4 dilution. Therefore, although the efficiency of the charcoal extraction could probably be improved by changing some of the conditions, the method without charcoal was sufficiently sensitive, since samples at 1/16 dilution still had detectable SCB activities corresponding to at least 10 nM.

Relatively simple improvements to the system could be made in terms of the overall salt concentration, in that media containing a NaCl concentration equivalent to 1/2-strength seawater, for example M2 which most marine bacteria should be able to tolerate, could be used. Also, the initial dilution of the sample could be made in deionised water followed by RPMI.

Since the SIF factor was present in bacterial culture supernates, it was expected to be present when MB containing added TTX was tested. However, this was not the case. Maybe these samples contained TTX at much higher concentrations than the SCB activity in bacterial culture supernates, to such an extent that the inhibition due to salts was suppressed.

Another unexpected result from these experiments, with both culture supernate and MB containing added TTX, was that upon heating the culture supernates, a 40 % decrease in SCB activity occurred in the first 20 min, after which activity returned to that of the control. Further experiments, involving heating the sample for up to 1 h, reproduced this effect, with the activity after 1 h being equivalent to that of the unheated sample. This may also explain the drop in SCB activity when cell extracts were heated.

Nevertheless, the above results indicate that, as with cell extracts, workers such as Narita *et al.*, (1987), through preheating their samples, may have underestimated the values obtained.

To my knowledge, there have been no studies on the heat stability of SCB toxins within bacteria, although investigations have been made into the toxicity of heated liver containing TTX. On heating acidic toxic liver extracts (100°C for up to 30 min); Fuchi *et al.*, (1986a) observed a small decrease in TTX activity whereas, with neutral extracts, heating at 100°C for up to 30 min caused a 40 % decrease in activity, which declined even further on continued heating.

Both the supernate and the cell extracts tested in my experiments were at a pH close to 7.5 and, like the liver extracts, declined in activity on heating, although with supernates the level of activity recovered on further heating.

With a standard TTX preparation heated over a period of 0-90 mins, Fuchi *et al.*, (1986a) found that activity of TTX declined with time which was the opposite to what was found here.

Two possible explanations may be considered: a) TTX molecules form relatively unstable, less toxic complexes upon initial heating; b) derivatives of TTX may exist, so that a less toxic derivative forms

on initial heating. The latter suggestion could easily be tested by HPLC, by monitoring of the toxin profiles of both culture supernate and pure TTX, obtained after heating for various periods.

Another unexpected finding was that neither the SCB activity in the bacterial culture supernate nor the TTX in MB, both with molecular weights of <500, passed through the 500 dal Amicon filter, which supports the view that some sort of complex may be formed.

In summary, SCB activity could be detected directly in both bacterial cell extracts, which contained a heat-labile inhibitory factor, and in culture supernates, which at low dilutions contained interfering salts. Also, the SCB activity in both the cell extracts and supernates decreased upon heating for up to 20 min.

In view of the above findings, the results obtained by Kogure, on testing bacteria in the tissue culture assay (Kogure *et al.*, 1988, 1989; Thuesen and Kogure, 1989; Do *et al.*, 1990, 1991), require re-evaluation. In particular, none of these reports mentioned the conditions in which cell extracts or supernates, or even sediments, were processed for the assay, although a brief mention was made by Do *et al.*, (1990) that some extracts did

damage the MNB cells.

Quantitative evaluation of SCB activity

Among the several reports of TTX-producing bacteria it is only that of Thuesen and Kogure (1989), who used the tissue culture assay, which was sufficiently quantitative for comparison with this work. Supernates of 24 h cultures of strains GFC, OK1 and 829, at 1/16 dilution, all exhibited 100 % SCB activity, therefore, without further dilution of the supernate only the minimum level of SCB activity, in terms of TTX equivalent could be obtained by comparison with the standard dose-response curve. Such interpolation yielded 510 ng TTX equiv/ml (1600 nM TTX equiv). However, strains NCIMB 1903 and A1096 demonstrated maximum activity of 26 and 45 % equal to 102 ng TTX equiv/ml (320 nM TTX equiv) and 408 ng TTX equiv/ml (1280 nM TTX equiv). These values were similiar to those of Thuesen and Kogure (1989) who found SCB activity in the range of 280-790 ng/ml for 15 bacterial strains from arrowworms on application of Kogure's tissue culture assay.

The levels of SCB activity in the above experiments are difficult to compare with other reports due to variations in culture conditions, extraction procedures, detection methods, and the

use of cells instead of supernate. This is reflected in Table 3 where the reported values are compared to my results and are found to be substantially lower. This was further emphasized when the results obtained here, for strain OK1, were compared to those of Yasumoto *et al.*, (1986) who extracted 16.6 ng/ml TTX from the cells (detected by the mouse bioassay), after 12 days culture in static conditions. Results obtained in these experiments by tissue culture revealed a concentration of 755 ng TTX equiv/ml in the supernate after 7 days in shaking culture. Note, that the concentration of SCB activity was less on incubation of the culture for 7 days as compared with 1 day indicating that for this strain the activity was not stable, this may also have contributed to the lower activity found by Yasumoto.

Yotsu *et al.*, (1987) identified strains GFC and GFB as TTX-producers, but did not quantify the amount of toxin produced, therefore the toxicities of the two strains could not be compared.

The tissue culture assay developed in my studies proved to be suitable for detecting SCB activity from bacteria. Its major advantage over instrumental analysis is that tedious and inefficient extraction procedures are unneccessary since the cell extracts

and supernates can be tested directly, provided proper care and attention is taken with the controls. This permitted confirmation that certain bacterial strains such as GFC, previously reported to produce TTX, do indeed contain SCB activity.

SCB production by a range of marine bacteria and speculation on their involvement with red tides

Simidu *et al.*, (1987) previously tested a selection of *Vibrio, Aeromonas, Alteromonas and Photobacterium* strains along with *E. coli* from type culture collections, for TTX and anhydro-TTX activity, using HPLC and GC-MS. In some of these strains only anhydro-TTX was identified by HPLC and even those, in many instances, were listed as difficult to detect. Only one strain was reported as having biological activity, as tested by the mouse bioassay.

Experiments in this work involved testing by tissue culture a series of strains, pathogenic to both fish and shellfish, for SCB activity. It was found that 8 out of the 11 *Vibrio* strains examined (excluding the Japanese strains) produced SCB activity in varying degrees, as did *Aeromonas* and *Pseudomonas* species. Non-marine strains were also examined but did not produce SCB activity. This study also confirmed that two of the strains, *V.*

alginolyticus NCIMB 1903 and *V. anguillarum* NCIMB 829, which were reported to produce anhydro-TTX by Simidu *et al.*, (1987), do indeed produce SCB toxin.

As several of the strains producing SCB toxins are known to be fish and shellfish pathogens, this raises the question of whether the Na^+ channels of these animals are sensitive to SCB toxins and, if so, whether the toxins might be involved in fish or shellfish disease. It is of interest to note that Nottage *et al.*, (1989) had examined 7 of these vibrio strains for ciliostatic toxin (CT; Table 15), implicated in the pathogenesis of vibriosis. Amongst these 5 out of 7 were SCB producers. Also in the survey of environmental isolates (discussed later) 32 % of SCB producing isolates also produced CT. Therefore, further speculation arises as to the involvement of both toxins in vibriosis. However, on the other hand, the shellfish pathogen, *V. alginolyticus* NCIMB 1339, a potent producer of vibrio ciliostatic toxin, did not produce SCB toxins. These findings could be profitably extended to a study of the effect of both purified toxin and SCB-producing bacteria on fish and shellfish larvae and adults.

Since SCB toxin activity was common in the above isolates, further work was done in these studies to

determine the number of bacteria, from the environment, capable of this function.

SCB toxin-producing bacteria were relatively common amongst isolates obtained from seawater at a fish farm at Ardtoe in Scotland. Of the bacteria tested, 36 % produced SCB toxins, a figure which did not fall below 20 %, even in the winter months.

Although it was SCB activity in general which was identified, it seems unlikely that the theory put forward by Do *et al.*, (1990), that "TTX is synthesized solely by bacteria in the sediments", is the full story. Although the water-associated bacteria obtained in this work may have originated in sediments and released by turbulence, it is likely that a more complex situation exists than the statement above describes.

As mentioned previously, the tissue culture assay can detect PSP as well as TTX. Therefore, an isolate could only be labelled as a SCB-producer. This is enough for screening purposes but, if further identification of the toxin is required, instrumental techniques must be applied. PSP is of interest because of its association with toxic dinoflagellates and the uptake of these organisms by filter feeding animals, a route of human intoxication.

However, the possibility of bacterial production

of PSP is currently controversial as reported earlier, with only Kodama's group having isolated bacteria capable of this function. Nevertheless, it does have a precedent, in that, it is generally accepted that some strains of cynobacteria do produce STX (Table 3). It is of interest that, while PSP has been isolated in animals in which TTX has also been present (Table 2) and TTX-producing bacteria have been isolated from such, no-one has examined these isolates for PSP, nor is there published work on analysis of dinoflagellates for TTX.

It was to this effect that MAFF, Burnham on Crouch, analysed the toxic components in the supernates of GFC, 829 and two Ardtoe isolates, A1096 and A862, for PSP. Neo-saxtoxin was tentatively identified in the supernates of the four strains and the results of the two separate experiments were reproducible. The toxicity of the strains ranged between 2 to 26 mouse units/g supernate, with strain GFC being the most toxic, by HPLC. However, the chromatograms of these samples showed interference from other components in the supernate. Further analysis would require purification of the supernates, involving perhaps charcoal extraction, or ultrafiltration, or both. Confirmation of these results would be the first

report of free-living bacteria, other than cyanobacteria, capable of producing PSP. This would be of particular importance since if bacteria do produce PSP, there are practical consequences. On the view that dinoflagellates are the sole route of intoxication of filter feeders, there is the question of the route by which the dinoflagellates become toxin-laden, a process whose mechanism is currently unknown.

Particles thought to be bacteria from the water column capable of producing SCB toxins have been reported by Kodama et al., (1990a). He found thatPSP toxins could be obtained from extracts of particles of <0.45 µM obtained by filtering 100 L of seawater obtained from the vicinity of toxic scallops. The quantity of toxin present in the bacterial fractions was calculated to be 6.18 MU/100 L by HPLC, or 50 MU/100 L by Kogure's tissue culture assay. However, as with Kogure's work, that of Kodama suffers from the shortcoming of not stating the methodology of the tissue culture assay. Nevertheless, Kodama associated SCB-producing bacteria with dinoflagellates and suggested that "a bloom of toxic dinoflagellates could induce an increase of toxin- producing bacteria in the environment, which could be responsible for a signficant increase in bivalve toxicity."

This may well be the case, as Romalde *et al.*, (1990a) proposed that algae blooms produced a "phycosphere" under the influence of which the microbial activity could be altered and selective stimultion of certain bacteria made possible. The results from my work indicate that there exists a base population of SCB toxin-producing bacteria in Scottish waters. It is therefore possible that a toxic bloom could stimulate a proportion of this SCB toxin-producing section of the bacterial population. This idea is supported by microalgae being known to produce allelopathic substances (Chrost, 1975), to which perhaps, the SCB producing bacteria are resistant.

This question requires further study as the role of extracellular bacteria in PSP production is controversial, since most reports have found that bacteria isolated from dinoflagellate cultures are non-toxic. However, it is possible that the methods used were not sufficiently sensitive to detect the quantities involved. Therefore, the use of the tissue culture assay for this purpose may clarify the role of bacteria in PSP production.

There is also the question of the possible role of intracellular bacteria in the toxication of dinoflagellates and subsequently, filter-feeding

animals. This is the theory favoured by Kodama, who claims to have isolated a bacterium capable of this function (Table 3). However, his work has not so far been confirmed by other workers.

Research into this area is important since blooms of toxic dinoflagellates are increasingly frequent and their effect on both human health and the economy of the shellfish industry is significant. This was recently emphasized by the banning of all sales of Scottish shellfish in Europe in 1991 due to traces of PSP in their flesh. Understanding the mechanism of toxification of dinoflagellates and the possible role of bacteria is therefore essential. It would be of interest to examine the bacteria from the culture media of the dinoflagellates, their associated external bacterial population, and any intracellular bacteria for SCB toxins with the more sensitive tissue culture assay. The suggestion that an increase in SCB-producing bacteria may coincide with a toxic algal bloom should be investigated by collecting water samples at such times and studying the associated bacteria. Trials could also be arranged whereby the effect of SCB-producing bacteria on both toxic and non-toxic dinoflagellates could be examined.

If SCB activity was found in these samples a priority would be identification of the toxins

responsible, as TTX or STX or a mixture of both with their derivatives, could be present. Any bacterial isolates obtained should also be examined for their ability to convert STX derivatives between forms, since OK1 has been reported by Kotaki *et al.*, (1985a,b) to be capable of this function.

Filter feeders have also been found to be toxic on occasions when no obvious source could be found. It is therefore possible that this is due to toxic bacteria in the water column, as found in this work. It was demonstrated that with the bivalve *M. edulis*, maintained in seawater containing the SCB-producing bacterium GFC, the tissue of the animal accumulated the toxin produced by the bacterium. This proved that mussels can be intoxicated by bacteria which excrete SCB toxins. It should be noted that the bacterial numbers used here were higher than would normally occur in seawater, although this may not be the case during a toxic bloom.

In fact, the topic of bacterial association with the production of PSP is in its infancy and much remains to be investigated. The tissue culture assay, due to its high sensitivity, is ideal for such investigations. However, it must be emphasized that the specific identification of these toxins is essential and future work should incorporate

instrumental analysis. Since the HPLC technique is problematic largely due to the post-column derivatisation, effort should be made to incorporate other technology. The use of mass spectrometry used as a detection system with HPLC holds potential (Waldock; personal communication).

CULTURAL CONDITIONS FOR SCB PRODUCTION BY BACTERIA

There are few reports on the cultural conditions for SCB activity, and exploratory investigations were therefore undertaken. In nutrient-rich medium e.g. MB, maximum SCB activity was present in the culture supernate only after the bacteria had entered stationary phase; maximum activity occurred at 24 h. This is similiar to the results of Thuesen and Kogure (1989) who, using Kogure's tissue culture assay, detected TTX activity from bacteria at 24 h. However, Narita *et al.*, (1987) did not detect TTX activity in bacterial cell extracts until the cells had been cultured for 72 h. But, this could have been due to the use of the less sensitive mouse bioassay.

Kodama *et al.*, (1990) also used Kogure's tissue culture assay in investigations of the STX-producing

Moraxella sp.. They found that SCB activity could be detected in cell extracts at 168 h, but not at 64 h, when cultured in MB under shaking conditions. A feature of these results was the extremely low absorbance value of 0.2 from the cell culture at 64 h, the viability of which was not stated. The conditions used for testing the cell extracts in the tissue culture assay were also not given. Elsewhere, it was stated that "the amount of toxins produced by *Moraxella species* varied even when grown under the same conditions, indicating the presence of unknown factors regulating toxin production" (Ogata *et al.*, 1990). This could actually have been due to inconsistencies in the methodology of the tissue culture assay as Kodama may not have been aware of the variation in MNB cell sensitivity, with changes in culture conditions. Also the heat-labile inhibitory factor (CIF) produced by cell extracts may not have been recognised.

Nevertheless, the results reported here indicate that SCB toxins are not products of cell lysis, but are secondary metabolites excreted from the cell. A secondary metabolite can be defined as "a substance which is produced in circumstances where the actively growing cell is unable to sustain balanced growth, it is a natural product with no necessary or obvious function in the growth of the producing

organism" (McGillivray, 1972). These substances are both unusual in structure and metabolic activity (Drew and Demain, 1977); they also tend to generate remarkably diverse families of products (Vining, 1990), such as compounds which function as antibiotics, insecticidal agents, iron chelators and macrophage activators (Vining, 1990). Some bacterial toxins which may be considered secondary metabolites are staphylococcal enterotoxin, diphtheria, botulism and tetanus toxins (Demain, 1972).

The ability to produce secondary metabolites is not universal but has a rather restricted distribution among microorganisms. Wild-type strains belonging to the same genus or species, may produce several secondary metabolites with different chemical strutures. Conversely, the same metabolite may be produced by strains of different species (Malik, 1980).

It was proposed by Malik (1980) that "depletion of an essential nutrient in the medium limits growth, primary metabolites and intermediates then accumulate because their use in many pathways is reduced and their synthesis is only poorly controlled. Elevated concentrations of these metabolites are the starter units and inducers of pathways leading to secondary metabolites. In

addition, ATP could accumulate since the cells do not need energy-requiring processes". Such an essential nutrient could be phosphate which was found to be depleted at the beginning of the stationary phase in all strains tested in this study.

Several workers have investigated the effect of phosphate limitation on the synthesis of bacterial products. Early studies showed that the quantity of the pigment pyocyanine, produced by *P. aeruginosa*, was inversly proportional to the concentration of PO_4 in the medium and therefore increased when phosphate was lowered. (Grossowicz *et al.*, 1957; MacDonald, 1966).

Boyer *et al.*, (1985) and Anderson *et al.*, (1990b) both found that PO_4-limitation was associated with a dramatic increase in the rate of toxin production by dinoflagellates and proposed that nutritional imbalance might inhibit dinoflagellate cell division and cause exotoxin production.

Also, many secondary metabolic processes are inhibited by inorganic phosphate concentrations that are optimal or are at least not inhibitory, to growth. The end products of these processes include chlortetracycline, candicidin, ergot alkaloids, streptomycin and neomycin (Drew and Demain, 1977).

It was therefore decided to examine the role of

phosphate in SCB toxin production in this system. Preliminary investigations involved the addition of phosphate at different concentrations to the culture medium and determining the SCB activity produced by strain GFC after 24 h. However, there were problems with the protocol of this experiment, in that addition of the phosphate solution (K_2HPO_4/KH_2PO_4) to the medium caused an increase in the total salts, which meant that as the PO_4 concentration increased the cell death produced by oub/ver decreased, effectively decreasing the sensitivity of the assay. This was probably due to two factors; firstly, at concentration of 220 mM, K^+ ions competitively block Na^+ channels. Secondly, high concentrations of extracellular K^+ make difficult the inhibition of the Na^+/K^+ ATPase with ouabain or other cardiac glycosides, because K^+ is a potent inhibitor of glycoside binding (Catterall, 1981). The situation would not have been significantly improved if Na^+ salts of PO_4 had been used instead of K^+, because of the inhibitory effects of Na^+ as discussed earlier. Therefore, in order to circumvent this problem the culture supernates were charcoal extracted.

Analysis of charcoal extracted supernates showed that as the phosphate concentration in the medium increased, the SCB activity decreased. These results

were similiar to those of McGillivray (1972) with *P. aeruginosa*, where production of pyocyanine decreased with increasing phosphate concentration. Further work demonstrated that adding extra phosphate to the culture medium late in the growth cycle also inhibited SCB toxin production.

The mechanism by which phosphate limitation induces the production of secondary metabolites has not been fully elucidated, although, with streptomycin, in the presence of excess phosphate, the intermediate streptomycin phosphate accumulates. However, in the chlortetracycline pathway no phosphorylated intermediates are known, but, it has been speculated that phosphate inhibition involves regulation of energy charges (Demain, 1972). In any event, the mechanism by which phosphate levels regulate the production of SCB toxins is unknown. It is also not known if the production of SCB toxins in stationary phase is controlled solely by phosphate levels, or if limitations of other substances such as nitrates, amino acids or even cations such as iron, as in the diphtheria toxin system, might be involved.

The formation of secondary metabolites is subject to a general physiological control that responds to environmental factors. A large body of evidence shows that secondary metabolism has a lower priority

than growth in the hierachy of regulation of bacteria cultured in a rich, balanced nutrient medium. Thus wild-type organisms either do not make secondary metabolites or do so at well below their potential. The genetic information for the process is fully expressed only when growth is restricted and the nature of the growth limitation usually has a strong influence on the level of expression (Vining, 1990). Thus, the production of SCB toxins by strains capable of this function in commercial media, was compared to the activity produced when bacteria where cultured in seawater, which is nutrient-limited and more imitative of the environment from which the organisms originated.

Similiar work had previously been done by Kodama who investigated the effect of culturing a *Moraxella sp.* in seawater. Measuring SCB activity of the cell extracts by HPLC, he detected PSP toxins within 24 h, but only at very low concentration, which did not increase significantly until the 10th day. He concluded that, "toxin content per cell grown in nutrient deficient conditions is much higher than that in commercial media and supports the conclusion that *Moraxella sp.* produces SCB toxin under starvation conditions" (Kodama *et al.*, 1990).

However, the results of Kodama's experiments in

seawater and commercial media were not directly comparable for two reasons. In particular, he based his conclusion on the difference in cell numbers by visual comparison of cloudiness of the seawater and commercial media. Confirmation using cell counts would be required to enable him to state that a lower number of cells grew in seawater. Secondly, the tissue culture assay was used to determine toxicity of cells in the commercial media, while HPLC was the preferred method for the seawater cell extracts. Although the tissue culture is reported to be the more sensitive of the two, a calibration between the two methods has not been done. Therefore, reports of toxin levels by the two different detection techniques cannot be accurately compared.

It was found from this work that in seawater SCB activity could be determined within 24 h, during the period when the viable cell number had increased 2000-fold, after which the toxin concentration remained stable for up to 7 days. The maximum SCB activity obtained upon culturing cells, for 7 days, in seawater compared to the nutrient-rich medium was calculated as 245 and 1820 ng TTX equiv/ml respectively. However, these are not directly comparable since a greater number of cells developed in the commercial medium. If the figures above were

adjusted to a per cell basis, they reveal that 4×10^{-5} ng TTX equiv was produced per cell in 1 ml of seawater compared with 5×10^{-7} ng TTX equiv/cell in MB. Hence, much more toxin was produced on a cell to cell basis in the seawater.

Later investigations into the effect of cell number on SCB activity in seawater reproduced this result; however, the quantity of toxin declined as the number of cells increased. This was also found when sucrose or glucose were added to the seawater as carbon sources. This decline in SCB activity produced as the cell numbers increased may have been due to the exhaustion of the relatively low nutrient concentrations available. It would be desirable to investigate further whether more SCB activity would be produced under nutrient-limited conditions, as the commercial medium gave a high cell density; a similiar effect to that which occurred in seawater at high cell densities may be replicated in commercial media. Production of SCB activity under nutrient-limited conditions should also be examined in strains other than GFC.

It is possible that some trace factor is present in both seawater and MB, which when depleted, beyond a certain threshhold level, limits the quantity of

SCB toxin produced irrespective of the cell numbers present.

THE TISSUE CULTURE ASSAY AS A REPLACEMENT FOR THE MOUSE BIOASSAY

The current official method for detection of PSP (paralytic shellfish poisons) in tissue extracts of filter feeding animals is the mouse bioassay, which detects a minimum of 40 µg STX/100 g of mussel flesh (Fileman, 1988). However, this method has several drawbacks, notably, subjectivity, inaccuracy at low concentrations and mouse-strain dependency. Also, current opinion favours the avoidance of live animals, and encourages alternative techniques where available.

The best alternative to date has been HPLC, based on the method of Sullivan and Wekell (1986) which was reported to detect 10-30 µg STX equiv/100 g.

Mussel extracts have been examined using the two assays by 3 groups, and the comparison of the results is listed in table 7. The correlations improve with increasing sample size and range from 0.79 to 0.9. However, there are several drawbacks to the HPLC method (Waldock *et al.*, 1991), primarily:

1. Possible co-elution of compounds with different toxicities.

2. The apparatus is difficult to maintain and allows only 20 samples per day to be processed.
3. Very low correlations were obtained between HPLC and the mouse bioassay with samples from scallop and lobster tissue.
4. Lack of easily available standards. However, this is a universal problem.
5. The detection limit, in terms of STX equivalent, depends on the toxins which are present because the potency and the detection limit of each toxin is different (Fileman, 1988).
6. Correlation of the comparitive potency of the various PSP (calculated by Sullivan and Wekell, 1986) to the HPLC results is a potential source of inaccuracy due to the use of different mouse strains (Fileman, 1988).

The tissue culture assay, described in this work, has potential as an alternative method to the mouse bioassay. Six samples of tissue extracts from mussels, which had been exposed to a toxic bloom, on the north-east coast of England in 1990, were analysed by this method. The results were compared to those supplied by MAFF (Burnham-on-Crouch), obtained by applying the HPLC method and the mouse bioassay.

SCB toxins were detected in all six samples and

the titre expressed as the dilution of sample which gave 50 % cell protection (Table 20). The samples, when arranged in order of toxicity, were similiar to those obtained by HPLC and the mouse bioassay. Extract T64, which was the most toxic, produced an inhibitory factor, which was gradually destroyed by heating (100°C for up to 60 min), with a subsequent 8-fold increase in titre. In the other samples, this inhibitory factor was only detectable in T87, but to a lesser extent than in T64. Also, upon heating a two-fold increase in the titre occurred in some, but not all, of the samples.

The nature of the inhibitory factor is unknown and requires further investigation; if the sample had been tested at a low dilution, a false negative result would have been obtained. Since the mechanism of the mouse bioassay is essentially the same as the tissue culture assay, it is feasible that, with samples containing an inhibitory factor, false negative results may occur in the mouse bioassay. Also, the observation that the titre increased upon heating is not surprising since Hall, (1985) reported that sulphamates could be converted to the more toxic carbamates, on heating. This, therefore, underlines the potential importance of the less toxic derivatives. It would be of interest to compare the HPLC chromatograms of unheated and

heated extracts.

When the values from the three assays were compared high correlation coefficients were found between the tissue culture assay and both HPLC and the mouse bioassay, the latter not surprisingly giving the highest correlation. Also, the tissue culture assay was the most sensitive of the three with a detection limit in the range of 0.4 to 0.7 µg STX equiv/100 g mussel extract. Another major advantage of the tissue culture assay over the HPLC method is that a larger quantity of samples could be processed daily than with HPLC.

Further, confirmatory work on the suitability of the tissue culture assay as a replacement for the mouse bioassay is required, since only six samples were examined. These studies should include examining more mussel extracts and also the investigation of scallop and lobster extracts, which Waldock *et al.*, (1991) stated gave relatively low correlations between the mouse bioassay and the HPLC system.

Analysis of each individual toxin component including STX by the tissue culture assay is also necessary. At the moment, however, this is hampered by the lack of the necessary standards.

The tissue culture assay in its present form,while capable of greater throughput of samples than HPLC

and its greater sensitivity, has two disadvantages:

1. As with the mouse bioassay, it measures only the total biological activity, unlike HPLC. Therefore, the toxins profiles are not obtained.

2. The total time for processing a batch of samples by tissue culture assay is 2 1/2 days; while not excessive, a shorter time is desirable. This may be possible by several methods:

a) A dye such as MTT, although posing problems, might be useable if the crystals could be dissolved without removing the medium. Possibilities include the use of other solvent systems, or heating the samples in the current solvent. This would allow the cells to be added at the same time as the toxins, thereby avoiding the initial 24 h seeding incubation and reducing the assay time to 1 day.

b) By use of a bactometer, which measures changes in ion conductivity. In this instance, the cell seeding step could again be excluded, by designing the method along the lines of Catterall's original work, but with the change in ion conductivity as a detection system instead of the Na+ isotope, or a vital dye. Even without these amendments, the work described here affirms the value of the tissue culture assay as an alternative to the mouse bioassay.

LOW MOLECULAR WEIGHT TOXINS PRODUCED BY BACTERIA ISOLATED FROM SCOTTISH COASTAL WATERS

It has been reported that seasonal mortalities of oyster larvae and spat are often associated with an increase in sea water temperature and a bloom of microorganisms (Utting, 1986). Also, a low molecular weight ciliostatic toxin (CT) has been implicated as a major virulence factor in the toxicity of *V. alginolyticus* culture supernates towards bivalve larvae. (Nottage *et al.*, 1989).

The present study was formulated to investigate the relationship between temperature, viable bacterial counts, presumptive vibrio numbers and the quantity of bacteria capable of producing CT. The work was later extended to incorporate SCB toxin-producing bacteria. The samples were obtained from the seawater source at two fish farms, Hunterston and Ardtoe. The former is located on the heavily populated, Clyde Estuary, close to Hunterston Nuclear Power Station, whereas, Ardtoe is on anunpolluted sealoch in a sparsely inhabited part of Scotland.

Preliminary investigation involved monthly sampling of seawater, from the inlet source at each fish farm and analysis for the various parameters

listed. The main drawback of this preliminary survey was that the data could only be analysed on a quarterly basis, as the numbers of bacterial isolates were too low for monthly tabu lation. Nevertheless, there were parallel trends in temperature, presumptive vibrio count and CT producers, more so at Hunterston than at Ardtoe. Also, higher values of the variables were found during the summer months.

A second survey, in which a greater number of bacterial isolates were collected, allowed the data to be analysed on a monthly basis. These results confirmed the seasonal variation in the parameters, however, more fluctuation in the data existed than was obvious in the preliminary survey.

At Hunterston, the trend in the temperature compared to the presumptive vibrio numbers, and the trend in the viable counts compared to vibrio numbers were significant whereas, the number of CT-producing bacteria was not significantly correlated to any variable.

At Ardtoe the number of CT and SCB toxin-producing bacteria were related to each other and the temperature. The number of CT-producers was also related to the viable count and presumptive vibrio count.

The relationships found at Ardtoe are of interest, as periodic mass mortalities of shellfish due to vibriosis occur in the summer months (Elston, 1984). Also, a greater number of presumptive vibrios has been reported at higher temperatures (Baron and Liston, 1970; Bartley and Slanze, 1971; Kaneko and Colwell, 1973; Leibovitz, 1978; Larsen, 1984; Lodeiros, 1987) and Elston (1984) found that higher temperatures and vibrio numbers correlated well the with reported mass mortalities of shellfish.

The present work, at Ardtoe, therefore, relates the higher incidence of bacteria capable of producing a toxin of potential significance in oyster mortality with both temperature and bacterial numbers. However, the isolates were not actually tested for pathogenicity against oyster larvae, which clearly would be of interest. A further point is that the isolates were not identified, so it is probable from the data reported by Nottage *et al.*, (1989) that not all of the CT-producing bacteria would necessarily be vibrios.

Of the total number of isolates examined, 26% (144/546) from Ardtoe, and 28 % (89/313) from Hunterston, were CT-producing bacteria which was similiar to the 28 % (15/53) obtained by Nottage *et al.*, (1989), from random sampling of waters at

Hunterston.

The numbers of SCB-producing bacterial isolates from Ardtoe fluctuated throughout the year but with 2 large peaks at September in 2 consecutive years and was also significantly correlated to the water temperature.When the data was examined in more detail, the greatest number of high SCB toxin-producing isolates occurred during the summer and autumn.

The association of SCB toxin-producing bacteria with toxic dinoflagellates was discussed previously. The above results indicate that bacteria capable of producing high levels of SCB toxins are present in the water column, in the period when these toxic algae blooms are likely to occur.

The changeable nature of the sea limits the value of a survey such as this, in that sampling is at only 1 instant each month. It is known that the numbers of viable bacteria, for instance, vary from hour to hour. Therefore, in order to establish trends for a particular area, it would be desirable to sample at the same time and date over a number of years. This however, was not possible in the current work.

Another point is that the bacterial viable counts obtained are only representive of the bacterial

population from the seawater that are capable of growing in marine agar and do not necessarily represent the bacterial population as a whole. Similiarly, while TCBS is the most commonly used selective medium for vibrios, some strains grow poorly on it. Therefore, it only captures the proportion of vibrios capable of growth on this medium. In addition, some non-vibrio strains growon TCBS.

Nevertheless, these initial findings do indicate that bacteria capable of producing high levels of SCB toxins and higher numbers of CT-producers are present in the summer months when toxic algae blooms and bivalve mortalities are most likely to occur.

PERSPECTIVE

PERSPECTIVE

The Tissue Culture Assay

The tissue culture system as described in this work is an efficient detection system for SCB toxins, providing care is taken in maintaining exact culture conditions for the MNB cells. The control for oub/ver, namely the medium used in the sample, must be at the correct concentration, in order to maintain comparable salt levels in the sample and control.

Further investigations into increasing the sensitivity of the assay could follow several lines including:

1. The use of other clones of C1300, specifically those on which more information has been published, or the use of human neuroblastoma cell lines which have been reported to be genetically more stable. Kogure previously tried a human cell line, ATCC HTB10, and found that while oub/ver caused cellular swelling and death, TTX did not suppress this process (Kogure *et al.*, 1988a). However, Littauer *et al.*, (1979), reported that the properties of a human cell line were modulated to a high degree in relation to time in culture, even without external inducing agents such as DMSO and HMBA.

2. The use of toxins, which stimulate Na+ ion influx, other than ver could also be examined (Table 5), as ver only stimulates 8 % of the Na channels present. Aconitine was tried by Kogure *et al.*, (1988a), and was found to be less effective than ver. This is not surprising since it only activates 2 % of the Na+ channels, whereas, batrachotoxin activates all of the Na+ channels at action potentials 200-fold lower than ver (Catteral, 1981). However, the action of ver is instantaneous, while many of the other toxins are slower. Nevertheless, it would be of interest to examine the effect of batrachotoxin.

3. The effects of serum limitiation and HMBA on the MNB cells could be further investigated, as discussed elsewhere. Also, differentiating agents could be tried on other clones of the MNB C1300 cells.

4. The method as described could be used to quantify the amount of TTX present in a sample by direct comparison with the standard dose-response curve. However, Wardlaw stated that "a difficulty in any biological assay is the inherent variability of the biological material, which is influenced by the sum total of all the environmental factors in the culture". Therefore, the quantification of the assay may be improved by the use of a 4 point or 6

point assay as described by Wardlaw (1985), where standard TTX samples are incorporated into each assay. However, a problem with quantifying the concentration of toxin present is that it is not known which of the SCB toxins are present. This assay only measures the total biological activity, requiring the concentration of toxins to be expressed as the equivalent units of TTX or STX.

5. MTT, or other dyes, such as fluorescent dyes, could be investigated as replacements for neutral red, in efforts to decrease the total time of the assay.

6. The principle of the tissue culture could be adapted for use with a detection system involving a bacterometer, where changes in the ion conductivity are measured. However, this would depend on the sensitivity of the bacterometer.

Bacterial Production of SCB Toxins

This work confirmed reports of bacterial production of SCB toxins as secondary metabolites and the presence of such bacteria in the environment. This raises the question of the possible role, if any, that SCB toxins play in bacteria. The fact that SCB toxins regulate the transfer of Na+ ions through biological membranes may have some relevance to the

function of the toxins in marine bacterial cells, which have a requirement for Na+ ions for cellular functions and is a subject which could be investigated further.

Several other interesting questions can also be raised with regards to culture requirments for SCB production and the role of bacteria in PSP production, thereby offering a great deal of scope for future investigations.

Culture conditions

Only preliminary work was carried out on the nutrient conditions required by cells to produce SCB. This demonstated that production of the toxin was inhibited by addition of phosphate. The work with phosphate could be extended by examining the effects of transfering bacteria at different stages of the growth cycle to phosphate—limited media. Further experiments examining the role of nitrates, carbon, vitamins, amino acids or cations such as iron could also be done, preferably using a chemostat.

SCB toxins and toxic dinoflagellates

The association of SCB toxins, PSP and toxic dinoflagellates offers a vast area of inquiries as discussed earlier, with the principal objective of establishing if bacteria do produce PSP and if so,

what is the relationship of these bacteria to toxic dinoflagellates.

Other toxins

This work could be extended to cover investigations into other toxins which accumulate in bivalves and are responsible for human intoxication.

In the past decade, three other groups of marine toxins have been identified, e.g. amnesic shellfish poison (ASP), neurotoxic shellfish poison (NSP) and diarrheic shellfish poison (DSP), some of which are associated with phytoplankton and cause human intoxication through the consumption of contaminated bivalves and fish. Similiar to TTX and STX, most of these toxins seem to consist of a group of compounds, of which, the pharmacology and chemistry in some instances are poorly understood and their association with bacteria is speculative. However, in light of the bacterial association of the neurotoxins TTX and STX, it could be feasible that some of these toxins originate from bacteria. In fact, palytoxin from the Coelaterate *palythoae* has been reported to be produced by a *Vibrio*-like bacterium (Mosher and Furham, 1984).

However, the work in the field of bacterial involvement in PSP, TTX and other phytotoxins is in

its infancy; there are many questions to be answered which can only be solved if suitable techniques are available to identify these toxins. Tissue culture seems to be a useful tool for this purpose. For instance, okadaic acid, which is involved in DSP, stimulates the entry of Ca^{2+} into a cell; using a neuroblastoma cell line which possesses active Ca^{2+} channels, the Ca^{2+} influx may be detected with a fluoresent dye specific for Ca^{2+} and the resultant fluorescence measured by a microplate reader fitted with a fluorescence detector.

The overiding question is: do bacteria produce these toxins and what is their relationship to the phytoplankton? Is it possible that dinoflagellates are merely vectors for toxin-producing bacteria as rats are for *Yersinia pestis*, the plague causing bacterium?

This work confirms that SCB toxins are produced by bacteria. The role of these bacteria in the production of toxins provides a new line of investigation which is of increasing importance in the shellfish industry and for public health.

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APPENDICES

APPENDICES

APPENDIX 1. BACTERIA

Japanese strains

Shewanella alga	OK1	a)
Vibrio pelagia biovar II	OK2	
Vibrio pelagia biovar II	GFC	b)
Alteromonas tetradonis sp. nov.	GFC	

Fish pathogens

V. anguillarum	NCMIB 91079	
"	" 829	
V. alginolyticus	" 1903	
A. salmonicida	3.10	c)
A. salmonicida	80628	d)

Bivalve pathogens

V. anguillarum	" 2981	
"	" 4979	
V. anguillarum	" 5679	
V. alginolyticus	NCMIB 1339	
Vibrio sp.	B55	
"	B2	
"	T61	e)
"	B1	

Seawater isolates

Pseudomonas 1-1-1 f)

Isolates from Ardtoe and Hunterston

Non-marine bacteria

Escherichia coli	NCTC	10537
Bacillus subtilis	"	3610
Staphylococcus aureus	"	7121

a) Isolated from the surface of red alga.

b) " " skin slime of pufferfish.

both a) b) were supplied by Prof Simidu, Japan and identified in Simidu *et al.* (1990).

c) supplied by R.C. Cipriano.

d) supplied by D.Cox of Marine Harvest Ltd.

e) supplied by C.D. Garland (Garland *et al.*, 1983).

f) supplied by Unkles and Wardlaw (1978).

All NCIMB strains were obtained from the National Collection of Marine and Industrial Bacteria, Torry, Aberdeen.

APPENDIX 2

Bacterial Culture Media

Medium 1 (M1) (Hwang et al., 1989)

Component	*Quantity (g/L)*
Protease peptone No.3 (Difco)	2
Bacto yeast extracts (Difco)	2
Phytone peptone (BBL)*	1
Sodium thiosulphate	0.4
Sodium sulphate	1
Iron citrate	0.08
Seawater**	750

(* BBL: Becton Dickinson, MD 21020, USA; **seawater from Hunterston)

Adjust to pH 7.6 (1 M NaOH) and to 1 L with distilled water, autoclave at 15 psi for 15 min.

Medium 2 (M2) (Noguchi et al., 1987)

Component	*Quantity (g/L)*
Phytone peptone (BBL)	10
NaCl	10

Adjust to pH 7.2 (1 M NaOH) and to 1 L, with distilled water, autoclave at 15 psi for 15 min.

Medium 3 (M3) (Simidu et al., 1987)

Component	*Quantity (g/L)*
Protease peptone No. 3 (Difco)	2
Bacto yeast extract (Difco)	2
Phytone peptone (BBL)	1
*Seawater	750

Adjust to pH 7.8 (1 M NaOH) and to 1 L with distilled water autoclave at 15 psi for 15 min.

Marine Broth (MB; Difco)

37.4 g of powder was dissolved in 1000 ml distilled water and autoclaved for 15 min at 15 psi.

Different strengths of marine broth

Different strengths of MB were prepared as follows: x g of powder was added to 100 ml of distilled water and autoclaved at 15 psi for 15 min.

Quantity of powder (x g)	Strength of MB
3.74	Full
1.87	1/2
1.25	1/3
0.94	1/4

Artificial seawater

25 g of Tropic Martin seawater salts was added to 1000 ml distilled water to prepare a solution of 25 ‰ seawater and autoclaved at 15 psi for 15 min.

Preparation of seawater with added carbon sources

Glucose

A 20 mM glucose solution was prepared by adding 0.72 g to 200 ml of 25 ‰ a.s.w, the pH was adjusted to 7.2 with 1 M NaoH followed by autoclaving at 15 psi for 15 min.

Sucrose

A 20 mM sucrose solution was prepared by adding 1.36 g of sucrose to 200 ml of 25 ‰ a.s.w, adjusting the pH to 7.2 with 1 M NaoH and autoclaving at 15 psi for 15 min.

Seawater and peptone

Component	*Quantity (g/L)*
Artificial seawater (Tropic Martin)	25
Oxoid peptone L37	x

x represents 10 g for 1 % solution, 5 g for 0.5 % solution and 1 g for 0.1 % solution.
Adjust to pH 7.4 (1 M NaOH) with 1 L distilled water autoclave at 15 psi for 15 min.

Preparation of phosphate media

Media containing added PO_4 at concentrations of 10, 20, 30, 40, 50, 60 and 100 µg/ml P (for details on phosphate solutions see Appendix 5) were prepared by mixing the following:

Volume of PO_4 Stock (ml)	*Volume of peptone medium (ml)*
0.1	31.9
0.2	31.8
0.3	31.7
0.4	31.6
0.5	31.5
0.6	31.4
1.0	32.0

Marine agar (Difco)

55.1 g of dehydrated marine agar powder was dissolved in 1000 ml of distilled water and autoclaved for 15 min at 15 psi.

Thiosulphate citrate bile salts sucrose agar (TCBS; Difco)

89 g of dehydrated TCBS powder was dissolved in 1000 ml of deionised water and heated in a microwave oven at low heat for 20 min, or until dissolved.

Nutrient Broth No. 2 (Oxoid)

25 g of dehydrated nutrient broth powder was dissolved in 1000 ml of distilled water and autoclaved for 15 min at 15 psi.

Nutrient agar (Oxoid)

28 g of dehydrated nutrient agar powder was dissolved in 1000 ml of distilled water and autoclaved for 15 min at 15 psi.

APPENDIX 3. TISSUE CULTURE MEDIA

Maintenance medium (modified RPMI medium)

Component	*Quantity (ml)*	
	formula 1	formula 2
RPMI 1640 (Gibco)	93	88
Foetal calf serum (Flow)	5	10
Streptomycin - penicillin (Gibco)	2	2

C6 medium

Component	*Quantity (ml)*
RPMI	44
C6 Supernate	44
Foetal Calf Serum	10
Streptomycin- Penicillin	2

Preparation of media containing differentiating chemicals

All of the media contained a base formulation of :

Material	*Quantity (ml)*
Foetal calf serum	5
Streptomycin - pencillin	2
Differentiating agent	x

and made up to 100 ml volume with RPMI 1640, where x is equal to the volume of differentiating agent added, previously filter-sterilised. All of the differentiating agents were obtained from Sigma (except for foetal calf serum (fcs) which was from Flow Laboratories).

N N' Hexamethylene-bis-acetamide (HMBA; 2.5 mM and 10 mM).

A stock solution was prepared by adding 20.03 g to 100 ml of deionised water, 1 ml of which was added to 99 ml of the maintenance medium, for the 10 mM HMBA solution, and 0.25 ml of the stock solution added to 99.75 ml of the maintenance media to produce the 2.5 mM HMBA solution.

Valinomycin (1 µM and 0.01 µM)

A 100 µM stock solution was prepared by adding 1.1 mg valinomycin to 10 ml of 10 % ethanol in water, 1 ml of which was added to 99 ml of the maintenance medium to produce a 1 µM solution, which was diluted a further one-hundred fold by adding 1 ml of the 1 µM solution to the maintenance medium to obtain 0.01 µM valinomycin.

Aminopterin (10 µM and 1 µM)

A stock solution of 1000 µM was prepared by adding 4.4 mg of aminopterin to 10 ml deionised water, 1 ml of which, was added to the maintenance medium to produce a 10 µM solution from which 10 ml was removed and added to 90 ml of maintenance medium to produce a 1 µM solution.

Nerve Growth Factor (50 ng/ml and 100 ng/ml)

Nerve growth factor (NGF) was provided by Sigma in 0.1 mg quantities which were dissolved in 1 ml of RPMI to provide a stock solution of 100,000 ng/ml; 0.1 ml of this stock was added to 100 ml of maintenance medium to provide 100 ng/ml solution and this was diluted 1:1 with maintenance medium to provide a 50 ng/ml solution.

2 % DMSO

2 ml of dimethyl sulphoxide (DMSO) was added to 98 ml of maintenance medium to provide a 2 % (v/v) DMSO solution.

Serum limited

The foetal calf serum concentration was decreased from 5 % to 2 % to provide the serum limited medium.

Suspension medium

component	*Quantity (ml)*
RPMI 1640	78
Foetal calf serum	10
Streptomycin - penicillin	2
CMC solution	10

RPMI 1640

Powdered RPMI 1640 containing 25 mM Hepes and L-glutamine was supplied by Gibco in 10 L batches of powder which were dissolved in 850 ml of deionsed water. 8.5 g of $NaHCO_3$ dissolved in 50 ml of water, was added and the pH adjusted to pH 7.4 by the addition of 1 M NaOH after which the solution was made up to 10 L with deionised water. This was filter-sterilised through a 0.22 µm filter and stored in 500 ml bottles at 4°C.

APPENDIX 4 SOLUTIONS FOR TISSUE CULTURE

Penicillin-streptomycin solution

Penicillin-streptomycin solution contained 1000 iu/ml penicillin and 1000 µg/ml streptomycin, as supplied by Gibco.

Trypsin - EDTA solution

Trypsin-EDTA was supplied as a 1x solution in modified Puck's saline, by Gibco. It was used in quantities of 2 ml for an 80 cm^2 tissue culture flask and 5 ml for 175 cm^2 flasks to detach cells.

Hank's buffered salt solution (HBSS)

Component	*Quantity (mg/L)*
$CaCl_2$	140.0
D-Glucose	1600.0
KCl	400.0
KH_2 PO4	60.0
$MgSO_4.7H_2O$	200.0
Na_2HPO_4	47.5
NaCl	8000.0

OUABAIN

Ouabain (0.058 g), supplied by Sigma, was added to 20 ml of RPMI and heated for 5 min at 100°C to produce a stock solution of 4 mM, freshly prepared before use. This stock solution was adjusted to the required concentration by dilution with RPMI.

Veratridine

Veratridine (ver) was purchased from Sigma, in 100 mg amounts which were dissolved in 148 ml ethanol to produce a solution of 10 mM ver, subsequently stored at 4°C. This was diluted a further 1/25 in RPMI to produce a 0.4 mM stock solution, freshly prepared before use. The stock solution was adjusted to the required concentration by dilution with RPMI.

Tetrodotoxin

Tetrodotoxin was supplied from Sigma in bottles containing 1 mg TTX in acetate buffer at pH 4.8. On receipt this was dissolved in 5 ml sterile deionised water and split into 1 ml aliquots of concentration 200 µg/ml (616 µM) and stored at 4°C. Before use, TTX was mixed in a 1:1 ratio with 1 M Hepes buffer to adjust the pH to 7.4, producing a stock solution of 100 µg/ml (313 µM).

Preparation of TTX solutions for standard curve

The stock solution 313 µM was diluted 1/100 to give a solution of 3130 nM which was further diluted to give a range of final concentrations of 10-1500 nM TTX.

Citrate-buffered alcohol

Tri-Na citrate (7.35 g) was added to 500 ml of distilled water and the pH adjusted to 4.6 with acetic acid, after which 500 ml of ethanol was added; this solution was stored at 4°C.

Neutral Red (0.05 % w/v)

Neutral red (0.5 g) was dissolved in 1000 ml HBSS, filtered through Whatman no.1 filter paper. It was then stored frozen until use, when the temperature was allowed to equilibrate to 37°C.

CMC solution (0.75 % w/v)

Carboxymethylcellulose (0.75 g) supplied by BDH was added to 10 ml of RPMI and autoclaved at 15 psi for 15 min. This formed the base for the suspension medium (Appendix 3) at which it was therefore used at a final concentration of 0.75 % (w/v).

Poly-l-lysine

A stock solution of 4 mg/ml was prepared, by dissolving 25 mg of poly-l-lysine (Sigma) in 6.25 ml of deionised water, followed by filter sterilization. A solution of 1.2 mg/ml was prepared by dissolving 3 ml of stock in 10 ml of RPMI and a further ten-fold dilution gave a solution of 0.1 mg/ml.

MTT

MMT (3-(4,5-dimethylthiazol-2-y-1)-2,5-diphenyl-tetrazolium bromide), supplied by Sigma was dissolved in RPMI at 5 mg/ml and filter sterilized, to prepare a stock solution which was subsequently used at 10 µl per 100 µl medium.

0.04 M HCl in isopropanol

Concentrated HCl (0.18 ml) adjusted to a 50 ml volume with isopropanol.

APPENDIX 5. SOLUTIONS FOR PHOSPHATE EXPERIMENTS

Solutions for phosphate buffer

0.2 M K_2HPO_4

K_2HPO_4 (3.48 g) was dissolved in 100 ml of deionised water.

0.2 M KH_2PO_4

KH_2PO_4 (2.72 g) was dissolved in 100 ml of deionised water.

Phosphate Buffer

Phosphate buffer was freshly prepared by adding 0.2 M K_2HPO_4 (81 ml) to 19 ml of 0.2 M KH_2PO_4 and adjusting to 200 ml with deionised water. The pH was checked, adjusted if necessary to pH 7.5 and filter sterilised. This gave a stock solution of 3200 P µg/ml.

Preparation of standard PO_4 solution

KH_2PO_4 (87.8 mg) was dissolved in 100 ml of deionised water. This was diluted a further 1/20 with deionised water in order to obtain a stock solution of 10 µg/ml P.

Colour reagent for phosphate analysis

Solutions for colour reagent

6 N H_2SO_4

Conc.H_2SO_4 (168 ml) was added to 832 ml of deionised water.

Ammonium molybdate (2.5 % w/v)

Ammonium molybdate (2.5 g) was added to 100 ml of deionised water.

Ascorbic acid (10 % w/v)

Ascorbic Acid (10 g) was added to 100 ml of deionised water.

Colour reagent

Colour reagent was freshly prepared before use by mixing the following:-

6N	H_2SO_4	1 Volume
	H_2O	2 volumes
2.5 %	ammonium molybdate	1 volume
10 %	ascorbic Acid	1 volume

APPENDIX 6: CALCULATION OF PSP CONCENTRATIONS FROM HPLC DATA AND THE ANALYSIS OF MUSSEL EXTRACTS

Calculation of PSP concentrations as done by MAFF
a) µg toxin/L:

$$\frac{\text{HPLC}}{\text{STX standard peak area}} = x$$

$$\frac{x}{\text{toxin response factor}} = \mu g \text{ toxin}$$

where the toxin response factor is the relative toxicity of each compound as follows;

Toxin	Toxin factor
C1/C2	47
B2	27
GTX 4	346
GTX 1	346
GTX 3	291
GTX 2	182
neo-STX	364
STX	364

b)STX equivalent µg/L

$$\frac{\mu g \text{ toxin}}{\text{toxin factor}}$$

this is calculated for each toxin present and then totalled to give the STX equivalent per sample

c) Mouse eqivalents/ 100 g

$$\frac{\mu g \text{ STX equivalents}}{0.2} = Y$$

$$\frac{Y}{0.18} = \text{mouse equivalents}$$

APPENDIX 7: TABLES FROM THE ENVIRONMENTAL SURVEY

TABLE 24 Hunterston data: Viable count and presumptive vibrio count of bacteria isolated from Hunterston seawater over the period Oct. 1987 to Sept. 1988 compared to the water temperature and the quantity of bacteria capable of producing CT.

Period quartly	average temperature (°C)	viable count (cfu/ml)	presumptive vibrio (cfu/ml)	Total ciliostatic toxins ratio	(%)
Oct-Dec/87	11.0	8.50×10^4	1.37×10^2	5/9	55.6
Jan-Mar/88	7.1	4.06×10^4	8.50×10^1	2/8	25.0
Apr-Jun/88	9.9	2.95×10^5	2.00×10^2	5/9	55.6
Jul-Sep/88	14.1	6.00×10^4	9.50×10^1	3/17	17.6

TABLE 25 Ardtoe data: Viable count and presumptive vibrio count of bacteria isolated from Ardtoe seawater over the period Oct. 1987 to Sept 1988 compared to the water temperature and the quantity of bacteria capable of producing CT.

Period quartly	average temperature (°C)	viable count (cfu/ml)	presumptive vibrio (cfu/ml)	Total ciliostatic toxins ratio	(%)
Oct-Dec/87	9.8	3.50×10^4	4.00×10^2	6/21	29.6
Jan-Mar/88	7.1	4.66×10^4	3.60×10^1	6/15	40.0
Apr-Jun/88	9.9	7.50×10^3	5.50×10^2	10/10	100.0
Jul-Sep/88	13.4	2.00×10^4	2.00×10^2	6/8	75.0

TABLE 26 Hunterston data: Viable count and presumptive vibrio count of bacteria isolated from Hunterston seawater over the period Sept. 1989 - Aug. 1990 compared to the water temperature and the quantity of bacteria capable of producing CT and SCB toxins.

Month and year	temperature (°C)	viable count (cfu/ml)	presumptive vibrios (cfu/ml)	Total number of bacteria producing CT ratio	%
Sep/89	14.4	1.94×10^4	2.30×10^2	23/34	67.6
Oct/89	12.5	1.66×10^4	C	25/57	43.8
Nov/89	11.0	1.32×10^4	2.10×10^2	16/45	35.5
Dec/89	9.5	-	-	-	-
Jan/90	7.5	2.66×10^4	-	0/26	0
Feb/90	6.8	1.00×10^4	5.00×10^1	4/22	18.2
Mar/90	7.1	-	-	-	-
Apr/90	7.6	8.00×10^3	1.00×10^1	2/42	4.8
May/90	10.1	5.90×10^4	3.80×10^2	16/48	33.3
Jun/90	12.0	4.60×10^4	3.60×10^2		-
Jul/90	13.5	3.20×10^4	5.60×10^2	0/23	0
Aug/90	14.5	2.00×10^5	9.20×10^3	3/16	18.8

FIGURE 27 Ardtoe data: Viable count and presumptive vibrio count of bacteria isolated from Ardtoe seawater over the period Sept. 1989 - Sept. 1990 compared to the water temperature and the quantity of bacteria capable of producing CT and SCB toxins.

Month and year	temperature (°C)	viable count (cfu/ml)	presumptive vibrios (cfu/ml)	Total number of bacteria producing CT ratio	CT %	SCB toxins ratio	SCB toxins %
Sep/89	13.2	1.43×10^4	9.00×10^2	39/51	76.4	24/48	50.0
Oct/89	12.0	1.00×10^5	-	21/76	27.6	15/76	19.7
Nov/89	-	3.28×10^3	3.00×10^1	1/30	3.3	5/26	19.2
Dec/89	7.7	4.10×10^2	0	0/29	0	9/25	36
Jan/90	7.4	2.90×10^3	7.00×10^1	9/47	19.1	11/45	24.4
Feb/90	6.9	1.40×10^4	3.00×10^2	2/30	6.7	11/28	39.3
Mar/90	7.1	4.30×10^3	4.00×10^1	2/41	4.9	9/36	25.0
Apr/90	8.5	2.00×10^3	4.00×10^1	1/27	3.7	11/26	42.3
May/90	9.9	9.10×10^3	1.70×10^2	2/30	6.7	12/30	40.0
Jun/90	11.6	9.50×10^4	4.00×10^2	12/52	23.1	18/52	34.6
Jul/90	12.9	6.90×10^3	6.00×10^2	29/54	53.7	10/23	43.5
Aug/90	13.5	4.20×10^3	2.90×10^2	9/37	24.3	12/27	44.4
Sep/90	14.3	1.62×10^5	1.00×10^2	17/42	40.4	30/39	76.9