DETECTION OF PHYCOTOXINS ALONG THE SOUTH AFRICAN COAST: A COMPARISON OF THREE DIFFERENT BIOLOGICAL ASSAY SYSTEMS AND INSTRUMENTAL ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

By

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This thesis is dedicated to my parents, for their support, financially, emotionally and spiritually

CONTENTS

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LIST OF ABBREVIATIONS

Abbreviation	In full
Chemicals/toxins:	
AcOH	Acetic acid
KCl	Potassium chloride
NaCl	Sodium chloride
DMF	N'N-Dimethylformamide
STX	Saxitoxin
OA	Okadaic acid
DTX	Dinophysistoxin
GTX	Gonyautoxin
Species:	
V. fischeri	*Vibrio fischeri*
A. minutum	*Alexandrium minutum*
A. catenella	*Alexandrium catenella*
P. micans	*Prorocentrum micans*
S. trochoidea	*Scrippsiella trochoidea*
D. acuminata	*Dinophysis acuminata*
G. breve	*Gymnodinium breve*
P. angulosus	*Parechinus angulosus*
Units:	
h	hour/s
min	minute/s
sec	second/s
g	gram/s
µg	microgram/s
ml	millilitres

l	litre/s
M	Mol
Poisoning:	
ASP	Amnesic Shellfish Poisoning
NSP	Neurotoxic Shellfish Poisoning
Other:	
SFRI	Sea Fisheries Research Institute
HPLC	High Performance Liquid Chromatography
NRC	National Research Council (Canada)

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ABSTRACT

In recent years, harmful algal blooms have been of growing concern in many parts of the world. These blooms often result in deterioration in water quality, large-scale mortalities in marine life, and shellfish poisonings which can adversely affect local mariculture industries, coastal tourism and fisheries. Because of problems related to some of the methods that are currently used to detect phycotoxins (e.g. false positives), it has become necessary to explore new approaches, especially for routine monitoring. In this study, High Performance Liquid Chromatography was used as an instrumental method for detecting Paralytic Shellfish Poisoning in mussel samples. Three bioassays were also examined, viz. the luminescent bacteria assay (LUMIStox kit), the *Artemia* larvae assay (Artox kit), and the sea urchin gamete test. The bioassays were assessed as to their suitability, reliability and practicality in detecting phycotoxins in water and mussel samples. HPLC was used to establish toxin profiles of mussel samples from South Africa, where *Alexandrium catenella* was present in the water. The three bioassays were found to be unsuitable for detection of toxins in mussel samples. However, based on the results obtained in this study, the Artox kit seems to be the most suitable, sensitive and practical bioassay for the detection of PSP toxins in water samples.

1. GENERAL INTRODUCTION

1.1 HARMFUL ALGAL BLOOMS

Algae-related seafood poisonings have increasingly attracted medical and scientific attention, and not only humans have been affected. Typically consumers at the end of the pelagic food chain, including baleen whales and porpoises, birds and certain seals are also affected (Anderson 1994, Work *et al.* 1993, Hernandez *et al.* 1998). Even the benthic carnivorous gastropod *Babylonia areolata* has been found to accumulate certain seafood toxins of dinoflagellate origin via the food chain (Chou 1998). About 2 000 cases of human poisoning are reported globally each year, mainly as a result of fish or shellfish consumption. These seafood poisonings are usually associated with harmful phytoplankton (dinoflagellates and/or diatoms), which may bloom to form "red tides". However, not all red tides are harmful or toxic and some may merely discolour the water. Blooms of phytoplankton that cause seafood poisonings or harm to the surrounding fauna are called harmful algal blooms (HAB). Algal blooms can be divided into two major categories, namely toxic or non-toxic.

1.1.1 NON-TOXIC SPECIES

The water discolourations referred to as red tides are often caused by non-toxic dinoflagellates, including *Prorocentrum micans, Scrippsiella trochoidea, Ceratium furca* and *Mesodinium rubrum* (Pitcher 1998). Apart from the fact that these blooms are aesthetically unpleasant, there are certain other problems related to blooms of non-toxic species:

1.1.1.1 Low oxygen events

The inshore decay of red tides after the depletion of nutrients has often been responsible for marine mortalities, as a consequence of oxygen depletion (Horstman 1981, Matthews and Pitcher 1996)

1.1.1.2 Mechanical damage

Fish are susceptible to physical damage of their gills by certain phytoplankton. Diatoms may for example lodge between gill tissue and trigger the release of massive amounts of mucous and death may result from reduced oxygen exchange, as found by Brown *et al.* (1979), when a *Gymnodinium* species resulted in large fish mortalities in False Bay due to gill clogging.

1.1.1.3 Alteration of the food-web

Impacts of HAB can include more subtle effects on trophic structure that can alter or destroy ecosystems over extended periods of time (Pitcher 1998). For example, in 1997/98, a bloom occurred in Saldanha Bay (on the west coast of South Africa) which persisted for several weeks and had detrimental impacts on the mariculture industry, causing growth arrest in both oysters and mussels. According to Pitcher (1998), "a very similar bloom occurred in 1985 along the U.S. mid-Atlantic coast which caused mortalities, recruitment failure, growth inhibition of some bivalves, and decimation of eelgrass beds as a consequence of severe light attenuation caused by the blooms".

1.1.2 TOXIC SPECIES

There are two main problems associated with toxic species, viz: human shellfish poisonings and

faunal mortalities.

1.1.2.1 Shellfish poisoning

1.1.2.1a) Paralytic Shellfish Poisoning (PSP):

PSP is caused by the compound Saxitoxin and/or its ~21 derivatives (Oshima 1995). PSP symptoms result primarily from the blockage of neuronal and muscular sodium channels. Binding of Saxitoxin to the sodium channel prevents the propagation of action potentials which are essential to the conduction of nerve impulse and muscle contraction. In mild cases, the symptoms are usually limited to tingling sensations or to a feeling of numbness around the lips, which can spread to the face and neck. Prickly sensations may be experienced in the fingertips and toes, and headaches, dizziness, nausea, vomiting, and diarrhoea may develop. In severe cases, the symptoms include muscular paralysis, a pronounced difficulty in breathing and even choking sensations. Death through respiratory paralysis may occur within 2 - 24 hours after ingestion (Sims 1987, Sakamoto *et al.* 1987, Cembella *et al.* 1995a). Among the major phycotoxin syndromes, PSP has the longest recorded history and it has accounted for the majority of human fatalities. The predominant dinoflagellate species that cause PSP are *Alexandrium catenella, A. fundyense, A. tamarense, A. minutum, Pryodinium bahamense* and *Gymnodinium catenatum* (Plumley 1997).

1.1.2.1b) Diarrhetic Shellfish Poisoning (DSP):

DSP toxins comprise several compounds, including Okadaic acid (OA) and its derivatives, Dinophysistoxins (DTX), Pectenotoxins and Yessotoxins (Yasumoto *et al.* 1985). Of these, only some components of the DSP toxin-complex cause severe gastrointestinal disturbances when

delivered orally. OA and DTX1 are potent inhibitors of at least two protein phosphatase enzymes (PP1 and PP2A) (Boland *et al.* 1993, Luu *et al.* 1993) and this inhibition property is linked to inflammation of the intestinal tract. This is caused by hyperphosphorylation of proteins that control sodium secretion by intestinal cells (Cohen *et al.* 1990), or by increased phosphorylation of cytoskeletal or junctional moieties (halves) that regulate solute permeability, resulting in passive fluid loss (Dho *et al.* 1990). Symptoms of DSP include diarrhoea, nausea, vomiting, and abdominal pain, which can be attributed to Okadaic acid (OA) and the Dinophysistoxins (DTX1 and 2). The symptoms of DSP can be confused with an allergic reaction to seafood, because the symptoms are very similar (Zinn *et al.* 1997, Potter and Lopata 1997). Chronic exposure to these toxins may also promote tumour formation in the digestive system (Suganuma *et al.* 1988, Amzil *et al.* 1992, Chin *et al.* 1995). Examples of the dinoflagellates that cause DSP include *Dinophysis acuminata, D. fortii, D. norvegica, D. rotundata* and *Prorocentrum lima.*

1.1.2.1c) Amnesic Shellfish Poisoning (ASP):

Domoic acid (DA) is the primary toxin associated with this type of poisoning. The toxin reacts with central nervous system receptors (Baden *et al.* 1995). Nausea, vomiting, diarrhoea and abdominal cramps may occur 3 - 5 hours after ingestion. In extreme cases, there is a decreased reaction to pain, dizziness, hallucinations, confusion, short-term memory loss and seizures (Perl *et al.* 1990). The term "Amnesic" Shellfish Poisoning was given as a result of the short-term memory loss that can be associated with this poisoning (Cembella *et al.* 1995a). The diatom *Pseudo-nitzschia multiseris* is often the cause of ASP.

1.1.2.1d) Neurotoxic Shellfish Poisoning (NSP):

The toxins implicated in NSP are collectively known as brevetoxins. This type of toxin is known to interact with specific receptors (associated with voltage-sensitive sodium channels) located on nerve membrane glycoproteins (Baden *et al.* 1995). There are two types of contact with toxin, namely by aerosol and via ingestion. In the first case, the symptoms include respiratory distress, as well as eye and nasal membrane irritation, caused principally by exposure to sea-spray aerosols and by direct contact with toxic blooms while swimming (Tester *et al.* 1995). In the second case, when shellfish (which contain the accumulated toxins) are consumed, some of the symptoms are similar to those of PSP and include chills, headaches, diarrhoea, muscle weakness, muscle and joint pain, nausea and vomiting. In severe cases, patients may suffer an altered perception of hot and cold, experience difficulty in breathing, talking and swallowing, and double-vision may occur (Sakamoto *et al.* 1987, Sims 1987). The dinoflagellate *Gymnodinium breve* is responsible for NSP.

1.1.2.1e) Ciguatera Fish Poisoning:

The major toxin implicated in this type of poisoning is ciguatoxin, which becomes concentrated through the food chain in certain reef fishes (e.g. surgeon fish, and amberjack) throughout the Pacific. The mode of action is very similar to that of the brevetoxins, where the toxin interacts with specific receptors located on nerve membrane glycoproteins (Baden *et al.* 1995). Ciguatoxins and brevetoxins share a common binding site on voltage-dependent sodium channels, and this may account for similarities in the neuropathological symptoms. The symptoms include a numbness and tingling of the hands and feet, temperature reversals, and hypersensitivity to cold. Difficulty with balance, low heart rate and blood pressure, and rashes

can also be experienced. In extreme cases, death can occur through respiratory failure. Neurological symptoms may last for months and in some cases, years (Hokama 1993). *Gambierdiscus toxicus* is a common cause of ciguatera poisoning.

1.1.2.2 Faunal mortalities

Mortalities of fauna as a consequence of poisoning can occur either directly or indirectly. For example, white and black mussel mortalities have occurred as a consequence of direct poisoning by *Alexandrium catenella*, while indirect poisonings have resulted in several cases of paralysed or dead seabirds following consumption of contaminated shellfish (Horstman 1981).

1.2 INCREASE OF ALGAL BLOOMS

While harmful algal blooms are natural phenomena that have occurred throughout recorded history, there seems to have been an increase in their frequency, intensity and geographic distribution over recent time (Hallegraeff 1995). A number of different explanations have been proposed for this apparent increase.

An increased scientific awareness could provide one explanation. Reports of harmful algal blooms, human illnesses or damage to aquaculture operations seem to be receiving attention in the media and in the scientific literature. This results in an increased effort by researchers to survey their local waters for causative organisms. For example, *Alexandrium minutum* (a dinoflagellate that causes PSP) was only known in Egypt until 1988. However, it has since been reported from Australia, Ireland, France, Spain, Portugal, Italy, Turkey, the east coast of North

America, Thailand, New Zealand, and Taiwan (Hallegraeff *et al.* 1991, Yuki 1994).

Together with this awareness, increased pressure on usable marine resources has led to more and more countries looking towards aquaculture as an alternative to terrestrial farming. Aquaculture operations act as sensitive "bioassay systems" for harmful algal species, and highlight the presence of problem organisms not previously known. Shellfish farming is leading to more reports of PSP, DSP, NSP, and ASP, while fin-fish farming is drawing attention to algal species that cause fish mortalities (Hallegraeff 1995).

Eutrophication due to domestic, industrial and agricultural waste is another possible explanation for the increase in harmful algal blooms. For example, between 1976 and 1986 there was an 8-fold increase in the number of red tides per year in Hong Kong Harbour. This seems to have been related to an increase in human population density and concurrent increase in untreated domestic and industrial waste (Lam and Ho 1989). A similar experience was noted in the Seto Inland Sea, one of the major fish farm areas in Japan, where there was a progressive increase in the number of confirmed red tide outbreaks, concurrent with an increase in untreated sewage and industrial waste (Okaichi 1989). In South Africa, industrial development along the west coast during the last few decades has led to a population increase, and greater efforts are therefore being directed towards monitoring harmful algal blooms (Horstman 1981). The nutrient composition of treated wastewater is not the same as that of the coastal waters in which it is being discharged, and there is considerable concern that such altered nutrient ratios in coastal waters may favour harmful algal species (Smayda 1990).

Ballast water from cargo vessels serves worldwide as a carrier of non-indigenous marine plankton. This problem of introduced species gained considerable interest during the 1980's when non-indigenous toxic dinoflagellates were introduced into Australia, with disastrous consequences for commercial shellfish operations (Hallegraeff and Bolch 1992). At least fourteen phytoplankton species have become established in Australia following their introduction via ballast water, although not all of these are toxic species (Rigby *et al.* 1993). In Scotland, a similar situation has been observed by Macdonald and Davidson (1998), where non-indigenous toxic dinoflagellate species were found in ballast water and tank sediments. The resistant cysts of some dinoflagellates are well suited to survival during the long voyage in dark ballast tanks (Rigby and Hallegraeff 1994). Although the International Maritime Organisation (IMO) has taken steps to try and rectify the situation (Hallegraeff 1995), the transport of dinoflagellates in ballast water remains a problem in many parts of the world.

1.3 GUIDELINES AND REGULATIONS FOR SHELL FISHERIES

Most countries implement some kind of restriction on their shell fisheries when concentrations of DSP in shellfish reaches ~20 $\mu g.100\ g^{-1}$ (Quilliam and Wright 1995), or when concentrations of dinoflagellates causing DSP reach ~1 000 $cells.l^{-1}$ (Andersen 1996), approximately $3x10^{-5}$ $\mu g.ml$ okadaic acid (OA) (Cembella 1989). When shellfish concentrations of PSP reach ~ 80 $\mu g.100g^{-1}$,which is approximately equivalent to 0.4 $\mu g.ml^{-1}$ saxitoxin (STX) (Park *et al.* 1986), many countries implement some kind of restriction on their shell fisheries (Andersen 1996). The presence of some dinoflagellates in water samples, for example, *Alexandrium* minutum, at concentrations of 10^3 $cells.l^{-1}$ require closing of areas for harvesting of shellfish, whereas

concentrations of *Alexandrium catenella* can reach 10^4 cells.l^{-1} before closures are initiated (Andersen 1996).

1.4 HARMFUL ALGAL BLOOMS ALONG THE SOUTH AFRICAN COAST

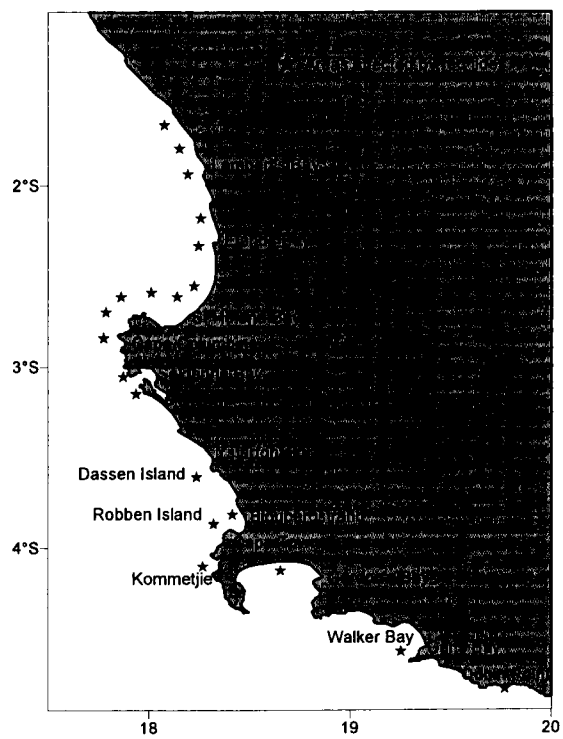


Figure 1.1 Occurrence of red tides along part of the South African coast (unpub. data - SFRI)

Upwelling is a feature of oceanic circulation along the entire west coast of South Africa. The longshore, equatorward winds responsible for upwelling are determined by the South Atlantic Anticyclone, the pressure field over the adjacent continent and by eastward moving cyclones (Shannon 1985). This upwelling, caused by strong south-easterly winds lasting between 2 to 10 days, is often followed by 2 to 3 days of calm weather during which stabilization of the water column occurs. This allows for the formation of red tides (Horstman 1981). Dinoflagellate-

dominated red tides are most common during late summer when the South Atlantic Anticyclone recedes and the pressure gradients responsible for equatorward winds weaken. This results in diminished upwelling (Pitcher *et al.* 1993a), and the creation of a low turbulence, nutrient deficient environment, which is well suited to dinoflagellates (Holligan 1979). It has been suggested that wind plays a principal role in the formation and dissipation of red tides, through the action of turbulence and advection (Pitcher *et al.* 1993a; Pitcher and Boyd 1996).

Some of the red tides along the South African coast are caused by toxic dinoflagellate species, which can be responsible for shellfish poisoning and mass mortalities of marine life. The first confirmed case of PSP along the South African coast was in 1948, when people became ill after eating mussels (Sapeika, 1948). Later, in December 1967 around Elands Bay, PSP was caused by the toxic dinoflagellate *Alexandrium (= Gonyaulax) catenella* (Horstman 1981). In 1980 at Elands Bay on the West Coast, large numbers of mussels were also killed by this species (Horstman 1981). DSP along the west and south coasts of South Africa was first identified in 1991 and attributed to the dinoflagellate *Dinophysis acuminata* (Pitcher *et al.* 1993b). Since then, toxic dinoflagellates causing both DSP and PSP have been regularly recorded along the coast of South Africa during red tide events [unpub. data - Sea Fisheries Research Institute (SFRI)]. In March 1994, St Helena Bay was the site of mass marine mortality which included more than 1 500 tons of dead fish and invertebrates. The event was caused by the entrapment and subsequent decay of an expansive red tide which contained various dinoflagellates, including the toxic species *A. catenella* and *D. acuminata* (Matthews and Pitcher 1996). Toxic red tides caused by a *Gymnodinium* species (a dinoflagellate associated with NSP) were first reported in False Bay in April/May 1988 and again in March 1989. Approximately 30 tons of abalone were

washed ashore at Betty's Bay on the South coast and various other faunal mortalities were reported (Horstman *et al.* 1991, Matthews and Pitcher 1996).

The cultivation of shellfish is presently the most successful mariculture activity conducted on the South African coast and a section of the South African Fisheries policy is devoted to "developing a wider range of mariculture and/or fish-farming activities" (Stuttaford 1997). The culture of black mussels (*Mytilus galloprovincialis*) takes place essentially in Saldanha Bay, and the dynamics of Saldanha Bay have resulted in this system being relatively free from harmful algal blooms. However, the regular occurrence of *Dinophysis acuminata* and *Alexandrium catenella* along the west coast presents a significant toxicological danger to a substantial number of people who fall victim to shellfish poisonings as a result of eating wild shellfish.

1.5 TOXIN ANALYSIS

It is essential that methods used in monitoring programs to detect phycotoxins in shellfish and water samples are effective, reliable and practical. Following is an overview of methods of analysis used worldwide.

1.5.1 MAMMALIAN ASSAYS

One method used to analyse shellfish for DSP and PSP is the standard mouse bioassay, recommended by the Association of Official Analytical Chemists International. This reference method is the only procedure [apart from High Performance Liquid Chromatography (HPLC)]

that is recognized internationally for quantifying PSP and DSP toxicity and is used worldwide in monitoring programs (Fernandez and Cembella 1995). The mouse bioassay was first applied in 1937 and the general procedure has been subsequently standardized and validated in a series of intercollaborative studies (AOAC 1990). Although the mouse bioassay is the most commonly used assay method for detection of DSP and PSP toxins, a suckling mouse assay (Hamano *et al.* 1985) and a rat bioassay (Kat 1983) are also used for DSP detection.

1.5.2 *IN VITRO* TECHNIQUES AND QUICK TEST KITS

There are a number of *in vitro* techniques for detecting shellfish toxins. These include immunoassays, enzyme assays and cytotoxicity tests. The development of these *in vitro* techniques offers an alternative to the mammalian bioassays for the detection of phycotoxins in complex organic matrices such as shellfish extracts and toxic microalgae. Relatively crude extracts may be assayed without resorting to the extensive clean-up procedures required for instrumental analysis (e.g. HPLC) (Cembella *et al.* 1995b). Rapid assays can be employed routinely in monitoring programs to provide an early warning before significant toxin accumulation occurs in shellfish.

A number of different test kits are available for the detection of different toxins. These include two immunoassays, the "RIDASCREEN" for the detection of STX in shellfish (Van Egmond *et al.* 1994) and the "DSP-Check ELISA" for the detection of OA and DTX1 in phytoplankton and shellfish samples (Chin *et al.* 1995). An enzymatic assay that is based on the inhibition of protein phosphatase-1 enzyme by OA (Baden *et al.* 1995), and the "Ciguatect" for detection of

ciguatoxins (Park 1995) are also available commercially. A tissue culture technique using an established mouse neuroblastoma cell line has been developed for the assay of toxins which block Na^+ channels (Kogure *et al.* 1988), and is now available in kit-form as the "MIST" test (Jellet *et al.* 1998).

1.5.3 INVERTEBRATE AND MICROBIAL ASSAYS

Some authors have used crustaceans such as *Artemia salina* (Demaret *et al.* 1995) or *Daphnia magna* (Vernoux *et al.* 1993), and luminescent bacteria (Bulich *et al.* 1980) for the detection of toxins. A summary of some of the methods used throughout the world for analysis of the different types of seafood poisoning is given in Table 1.1

Table 1.1 Some methods used for analysis of seafood toxins (from Hokama 1993, Andersen 1996)

TYPE OF POISONING	SEAFOOD TOXIN	METHODS
Diarrhetic Shellfish Poisoning (DSP)	Okadaic acid and derivatives	Mouse assay; suckling mouse assay; rat bioassay; *Daphnia* assay; HPLC-fluorometric; Enzyme-linked immunosorbent assay (ELISA); radioimmunoassay; pNPP inhibition assay; solid-phase immunobead assay
Paralytic Shellfish Poisoning (PSP)	Saxitoxin and derivatives	Mouse assay; ELISA; HPLC-fluorometric; radioimmunoassay; *Artemia* larvae
Amnesic Shellfish Poisoning (ASP)	Domoic acid	HPLC
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxins	Mouse assay; "guppy" *Gambusia* bioassay; C-18 HPLC UV; radioimmunoassay; ELISA
Ciguatera Fish Poisoning (CFP)	Ciguatoxin	Mouse assay; radioimmunoassay; ELISA; solid-phase immunobead assay; HPLC-fluorometric; guinea pig atrium

1.5.4 METHODS OF ANALYSIS USED IN SOUTH AFRICA

The method currently used in South Africa to analyse shellfish for PSP is the standard mouse bioassay. The assay involves the acidic aqueous extraction of the shellfish tissue, followed by an intraperitoneal injection of 1 ml of the extract into each of three mice (weighing ~ 20 g). The mice are observed for classical PSP symptoms, such as jumping in the early stages, followed by death in less than 15 minutes by respiratory arrest. The time from initial injection to mouse death is recorded and the toxicity is determined in mouse units (MU). One mouse unit is defined as

the amount of PSP toxin required to kill a 20 g mouse within 15 minutes. Mouse units are then converted to toxicity units (μgSTXeq) per 100 g of soft tissue. The tolerance level agreed on by most countries (including South Africa) is 80 μgSTXeq.100 g^{-1} tissue (400 MU.100 g^{-1}) (Shumway *et al.* 1995).

There are two methods which are currently employed in South Africa to test for DSP (Pitcher *et al.* 1993b). An unofficial (yet routinely used) method, namely an ELISA assay known as the "DSP Quick Test Kit" (marketed by UBE Industries Ltd. in Japan) detects okadaic acid using a monoclonal antibody specific for the toxin. By addition of the extracted mussel sample to an enzyme-labelled antibody, a competitive antigen-antibody reaction is advanced on an immunoplate. The quantity of the fixed antibody is determined in terms of enzyme activity, and measured calorimetrically through a substrate reaction. This method has a detection limit of 2 ng.g^{-1} meat (UBE Industries Ltd. 1988) and is used for routine monitoring. The mouse bioassay of Yasumoto *et al.* (1980) is the official method used to detect DSP. In this case, three successive extractions from 30 g of mussel hepatopancreas are obtained using acetone. After a number of evaporations and extractions, the residue is suspended in Tween solution and injected intraperitoneally into mice. The minimum amount of toxin required to kill a mouse after 24 h is defined as 1 mouse unit. The maximum allowable level of toxin currently employed for shellfish monitoring in most European countries (and South Africa) is 0.5 MU.g^{-1} hepatopancreas, which corresponds to 2 μg OA.g^{-1} hepatopancreas (Lee *et al.* 1987).

Although not used routinely, the sea-urchin bioassay has also been used to analyse samples for NSP. The gametes of the sea urchin are exposed to the toxin and the percentage of fertilized

eggs is used as a measure of toxicity. This sea urchin gamete bioassay has been used to test for *Gymnodinium* sp. causing NSP (Horstman *et al.* 1991), but work has yet to be done using this test on other dinoflagellate species, which produce toxins with different modes of actions.

1.6 OBJECTIVES OF THIS STUDY

Shellfish poisoning can affect not only consumer health, but also impact severely on the growth of the local mariculture and shellfish industry and may limit the harvesting of wild shellfish populations by coastal communities. If the exposure to marine toxins is not controlled, considerable economic damage will be caused through a reduction in the local consumption and export of seafood products. In spite of increasing ethical considerations, most countries rely primarily or exclusively upon mammalian bioassays for detection of phycotoxins (Fernandez and Cembella 1995).

Although the mouse bioassay is the internationally recommended and accepted method for testing for phycotoxins, mammalian bioassays have numerous inherent and operational deficiencies when used to accurately quantify toxicity, and these artifacts can bias the validity of the results. False reactions (positive or negative) can also occur due to interference by substances co-extracted during the sample preparation (Lee *et al.* 1987). Furthermore, because high capital investment and maintenance costs are often associated with the operation of bioassays involving live animals, their use in routine monitoring work is problematic. In comparison to instrumental analytical methods (eg. HPLC) mammalian bioassays are often much less sensitive and precise, and there is little margin for technical error (Fernandez and Cembella

1995).

Typically, ELISA tests configured for OA show some cross reactivity with certain other DSP toxins, although the affinity for other analogues varies greatly. The monoclonal antibody in the DSP-Check kit cross-reacts with DTX1 at a level comparable to OA, but pectenotoxins and yessotoxin are unreactive (Usagawa *et al.* 1989). This method is therefore not toxin-specific. Many investigators have also reported inconsistencies including false positive responses when applied to either phytoplankton or shellfish samples (Cembella *et al.* 1995b).

Because of the problems related to the mouse bioassay and ELISA check kit, there is a need to explore new approaches, especially for routine monitoring. The aim of this study was to evaluate four methods of analysis for the detection of phycotoxins in water and shellfish samples. The specific objectives were:

1. To analyse South African mussels for PSP and establish which toxic components were present. HPLC was used as an instrumental, physico-chemical method for detecting PSP toxins in these mussel samples. Although HPLC is not often used routinely in monitoring as it is expensive and requires technical expertise to run, it can be used to identify and quantify different toxic components in samples. No HPLC work has been done on South African mussels contaminated with PSP. This study was conducted under the guidance of Dr. Y. Shimizu at the University of Rhode Island, USA.

2. To evaluate the potential in terms of reliability, practicality and suitability of three

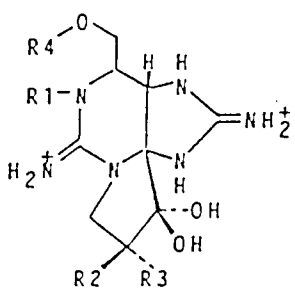
bioassays, namely the luminescent bacteria test (LUMIStox kit), the *Artemia* larvae assay (Artox kit), and the sea urchin gamete test for the detection of phycotoxins in water and mussel samples. The luminescent bacteria test, as well as the *Artemia* larvae test have been used to detect PSP toxins in dinoflagellate extracts (Bruno *et al.* 1990, Demaret *et al.* 1995), the sea urchin gamete test has been used to detect NSP toxins in water samples (Horstman *et al.* 1991), but specific methods of extraction, tests-kits, and dinoflagellate species with different modes of actions have been used for previous work, and therefore relevant results obtained on these bioassays has been limited.

A full discussion on the strengths and weaknesses of the methods of the above assay systems is included in the relevant chapters that follow.

2. ANALYSIS OF PSP TOXINS BY HPLC

2.1 INTRODUCTION

Paralytic shellfish Poisoning (PSP) toxins comprise at least 20 different compounds, plus their derivatives, which can be recognized by HPLC (Oshima *et al.* 1993, Oshima 1995b, Cembella *et al.* 1995). The first PSP component to be chemically characterized was saxitoxin (STX) which is a hygroscopic, solid substance, soluble in water and methanol, but almost insoluble in most non-polar solvents (Schantz *et al.* 1975). Subsequently, several other toxins of the PSP group have been characterized chemically, including 1-hydroxysaxitoxin (neoSTX), and the epimeres of 11-hydroxysaxitoxin sulphate and 11-hydroxyneosaxitoxin sulphate. The latter compounds [gonyautoxins (GTX) 2,3,1 and 4] are less basic, but otherwise their properties are similar to those of STX. PSP compounds with a sulphocarbamoyl group and decarbamoyl toxins (dc-STX) have also been isolated (Oshima *et al.* 1987, Sullivan *et al.* 1983). Referring to their chemical structure, three classes of PSP toxins (N-sulphocarbamoyl, carbamate and decarbamoyl) are known (Figure 2.1).



R1	R2	R3	Carbamate Toxins R4:$CONH_2$	N-sulfo-carbamoyl Toxins R4:$CONHSO_3^-$	Decarbamoyl Toxins R4: H
H	H	H	STX	GTX5	dcSTX
OH	H	H	neoSTX	GTX6	dcneoSTX
OH	H	OSO_3^-	GTX1	C3	dcGTX1
H	H	OSO_3^-	GTX2	epiGTX8	dcGTX2
H	OSO_3^-	H	GTX3	GTX8	dcGTX3
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Figure 2.1 Structures of paralytic shellfish toxins (Oshima *et al.* 1989)

A number of species within the dinoflagellate genus *Alexandrium* (*Gonyaulax*) produce saxitoxins (Shimizu 1987). Dinoflagellate toxicity varies, both between different isolates of a species and for individual isolates under varying growth conditions (Shimizu 1979, Boyer *et al.* 1986, Ogata *et al.* 1987). Differences in toxin composition (combinations of saxitoxin and its derivatives) can be responsible for toxin variability among isolates of a species (Shimizu 1979, Cembella *et al.* 1987). These differences have important implications due to the differences in potency between the saxitoxins (Hall and Reichardt 1984), and have also proved useful in linking certain dinoflagellates to toxin profiles in shellfish (Anderson *et al.* 1989). Variability in the toxicity of a single isolate is generally attributed to differences in the rate of toxin production or accumulation under different growth conditions, and not to differences in toxin composition, as found with individual isolates of *A. tamarense* and *A. catenella* (Boczar *et al.* 1988, Anderson *et al.* 1990).

Although the "mouse assay" remains the standard test procedure for the assessment of toxins in shellfish (AOAC 1984), emphasis has recently been placed on the development of a standard HPLC procedure for the assessment of toxins causing PSP. Although HPLC cannot at this stage be used routinely as a method of analysis for PSP determination, as it is expensive and needs a relatively high level of technical expertise to run, it nevertheless has advantages over other assays in that it can be used to identify and quantify the different components of PSP toxins (Hokama 1993).

An HPLC method proposed by Sullivan and Wekel (1984), which makes use of post column fluorometric detection, has been found to be effective in monitoring the major toxin groups,

gonyautoxins, saxitoxin and neosaxitoxin. Subsequent investigations on contaminated shellfish from Spain and Australia (Rodriquez-Vazquez *et al.* 1988, Oshima *et al.* 1987), have indicated the need for a further improvement in the resolution of chromatographic separation. To facilitate analysis of samples having complicated PSP toxin profiles, Oshima *et al.* (1989) have divided the toxins into three groups according to their basicity, and the application of three chromatographic runs was proposed for the determination of the PSP toxins. The three toxin groups are:

Group A: - STX, neoSTX, decSTX and decneoSTX. The overall charge on the toxins in this group is ($^{++}$).

Group B: - GTX 4,1,3,2; decGTX 4,1,3,2 and GTX 5,6. The overall charge on the toxins in this group is ($^{+}$).

Group C: - C1,2,3 and 4. The overall charge on toxins in this group is ($^{-}$).

The sample is run three times, each time using a different mobile phase to analyse the sample for a specific toxin group. This method produces reliable results (Rodriguez-Vazquez *et al.* 1988) and has been used for the analysis of the samples in this study.

The dinoflagellate *Alexandrium catenella* responsible for PSP on the South African coast occurs regularly along the West Coast during the latter part of the upwelling season (late summer to early spring), (Horstman 1981, Pitcher *et al.* 1993, 1995, 1998). Contaminated mussels are usually tested for PSP, using the mouse-test. Although this method provides an indication of toxicity, it does not indicate which toxic compounds are present in the shellfish. The main aim of this study was to analyse contaminated mussel samples from the West Coast of South Africa using the HPLC in order to establish which individual PSP toxin components were present, and

to record their profiles. These profiles were compared with profiles from other PSP producing species, as well as other isolates of *Alexandrium catenella*, from various parts of the world.

2.2 METHODS AND MATERIALS

2.2.1 APPARATUS

The conditions for the HPLC setup and methods were similar to those described by Oshima *et al.* (1989). The HPLC system consisted of a high pressure pump (Hitachi L-6200) with a syringe injector for loading samples. An Inertsil C8 (Alltech) stainless steel column (reverse phase), and a double head reaction pump (Hitachi) for delivering both oxidizing and acidifying reagents were used. Teflon tubing of 0.5 mm in diameter, immersed in an 80 ^{0}C water bath for temperature controlled reactions was attached to the column, as well as a fluoromonitor (Hitachi F-1000) equipped with 150 W xenon lamp and 12 µl flow cell, and a chromato-integrator for recording peaks. Mobile phases and other reagents [see also Appendix (2.4) for details], together with the operating conditions are described in Table 2.1.

Table 2.1 Operating conditions for HPLC analysis of PSP toxins

i. Column: Inertsil C8 (4.6x150 mm)
ii. Mobile Phase: flow rate at 0.8 $ml.min^{-1}$
a) for GTX2 and GTX3: - sodium 1-heptanesulfonate in ammonium phosphate [made up as described in Appendix (2.4)]
b) for STX and neoSTX: - sodium 1-heptanesulfonate in ammonium phosphate with acetonitrile added [made up as described in Appendix (2.4)].
iii. Oxidizing reagent: flow rate at 0.1 $ml.min^{-1}$
7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9)
iv. Reaction: at 80 °C
v. Acidifying reagent: flow rate at 0.1 $ml.min^{-1}$
0.5 M acetic acid
vi. Detection: excitation wavelength 330 nm; emission wavelength 390 nm
vii. Injection volume: 10 µl

2.2.2 PREPARATION OF STANDARD TOXINS

Commercial toxin standards of STX, neoSTX, GTX3 and GTX2 were purchased from the National Research Council (Canada) and stored in 0.1% Acetic Acid (AcOH) at -20 °C. Before injection into the HPLC, they were made up to the following concentrations:

STX (4.4 µM): 1.4 $\mu g.ml^{-1}$

neoSTX (4.8 µM): 1.4 $\mu g.ml^{-1}$

GTX3 (1.46 µM): 0.58 $\mu g.ml^{-1}$

GTX2 (6 μM): 2.4 $\mu g.ml^{-1}$

An "in-house" standard was also prepared for the STX- group by mixing 0.1 g of dried *Aphanizomenon flos-aquae* (a blue-green alga) with 10 ml of 0.03 M AcOH, and sonicating for 1 min. This was then centrifuged at 2 000 rpm for 10 min and the supernatant removed and retained. The pellet was resuspended in 10 ml of 0.03 M AcOH and centrifuged again in the same manner. The combined supernatants (from the first and second extraction) were made up to 40 ml with distilled water. A 6 ml aliquot was mixed with 200 μl of 12 N HCl and boiled for 5 min in a water bath. This solution was then ready for injection into the HPLC.

2.2.3 MUSSEL SAMPLES

According to monitoring data available from the SFRI, the red tides that occurred in the late summer and autumn of 1995/1996 were dominated by the non-toxic dinoflagellate *Ceratium furca*. Towards the end of March the toxic dinoflagellate, *Alexandrium catenella*, was observed as a minor component of these blooms in samples collected at various localities around the Cape Peninsula.

Mussel samples were collected for HPLC analysis during these red tide events. Mouse-tests were performed (tests were performed according to the AOAC protocols (AOAC 1984) at the Fishing Industry Research Institute, Cape Town) on samples collected at similar times to those collected for HPLC analysis. The sites where these samples were collected were Kommetjie (April 1996), Robben Island and Bloubergstrand (May 1996) (Figure 1.1). The mussels from

Robben Island and Kommetjie were frozen immediately following collection. To establish how long toxins remains in mussel tissue and also to establish differences in toxin composition in mussels over a depuration period of four days, the mussels from Bloubergstrand were placed in an aquarium which was supplied with continuously flowing, filtered (2 μm) seawater. Mussel samples were removed from the aquarium on each of the four days and frozen for subsequent analysis. Mussels from an uncontaminated area (False Bay) which had been kept in filtered seawater for two weeks to depurate were used as a control.

2.2.4 EXTRACTION OF MUSSEL SAMPLES

Mussel tissue (1 g whole mussel) was homogenized with 4 ml of 0.5 M AcOH. A few drops of 6 N HCl were added to lower the pH to 2. Samples were then heated in a water bath at 100 °C for 5 min and 2 ml aliquots of material were centrifuged at 2 000 rpm for 15 min. The supernatant was filtered through a 0.45 μm glass fibre filter and again through a 5 000 MW ultra-filter (Ultrafree, Millipore), prior to injection in the HPLC system.

A 10 μl sample of each of the "in-house" and commercial standards were first subjected to HPLC analysis, in order to establish the retention time of the peaks as well as to determine the peak areas (Figure 2.2). 10 μl of each mussel sample was then subjected to HPLC analysis. Each mussel sample was analysed twice, once to detect the STX-group of toxins and once to detect the GTX-group of toxins, using different mobile phases [see appendix (2.4)] for each toxin group (Figure 2.3).

2.2.5 CALCULATION OF TOXICITY

The concentration of toxin in the samples was determined by comparing the area under each toxin peak with that of the commercial standards.

Toxicity was calculated according to Oshima (1995b):

$$Ct = \frac{ToxSt}{ArSt} \times \frac{ArSm}{VSm} \times 1000$$

where: Ct = concentration of each toxin (pmole.ml^{-1})

ToxSt = the amount of standard toxin injected (pmole, MU* or ngSTX eq**.)

ArSt = area or peak height of standard toxin in the nearest chromatogram (mm^2)

ArSm = area or peak height of the toxin in sample (mm^2)

VSm = volume of extract injected (ml).

All Ct values for each toxin were summed to obtain the total toxin concentration of the extract (Cts). The toxicity of the sample (Tox) is given as follows:

Tox (nmole.g^{-1}) = Cts x 0.002 x dilution factor (of extract before injection) or,

Tox (MU.g^{-1}) = Cts (Mu.ml^{-1}) x 2 x dilution factor or,

Tox (µgSTXeq.100 g^{-1}) = Cts (ngSTXeq.ml^{-1}) x 0.2 x dilution factor.

* MU = mouse units (the amount of PSP toxin required to kill a 20 g mouse in 15 min)
** ngSTXeq = nanograms of saxitoxin equivalent

2.3 RESULTS AND DISCUSSION

The NCR standards had peaks at retention times of 9.77 and 14.16 min for neoSTX and STX respectively, and at 15.5 and 19.8 min for GTX3 and GTX2 respectively (Figure 2.2). Because the "in-house standard" exhibited peaks at the same times as the NCR standard for neoSTX and STX, it was assumed that these two toxins were present in the "in-house standard"(Figure 2.2). The retention times of peaks obtained in the mussel samples were compared to those of the standards to establish which toxins were present, and the concentrations of the toxins were calculated from the NCR standards.

The maximum concentration of PSP that is legally permitted in shellfish flesh in South Africa is 0.8 $\mu g.g^{-1}$ or 4 $MU.g^{-1}$ of mussel tissue. All toxin concentrations in samples examined here exceeded the legal limit (Table 2.2). The toxin concentrations determined by means of HPLC were higher than those of the mouse bioassay, at all three sampling sites. These findings were in accordance with those of Oshima (1995b), who found that the mouse bioassay underestimated the toxin content in shellfish. However, the samples in this study for the mouse bioassay and those for HPLC were not taken at precisely the same place and the results are therefore not directly comparable. The mouse-test in this study, therefore, should be used only as an indication as to whether toxins were present at these times.

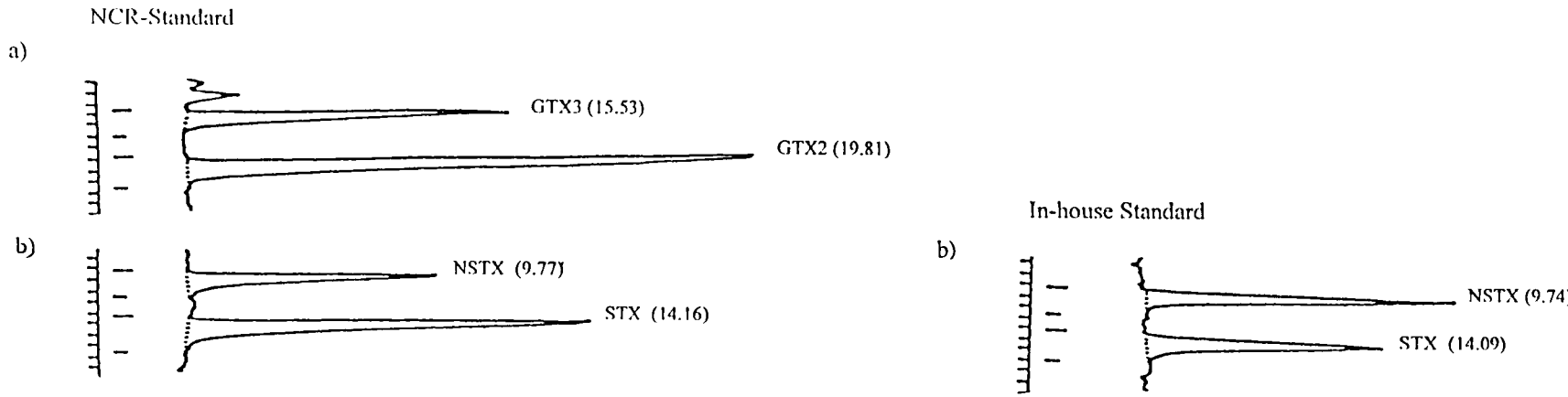


Figure 2.2 HPLC chromatograms of Standard toxins
a) and b) correspond to the mobile phases specified in Table 2.1. Retention Time in parentheses

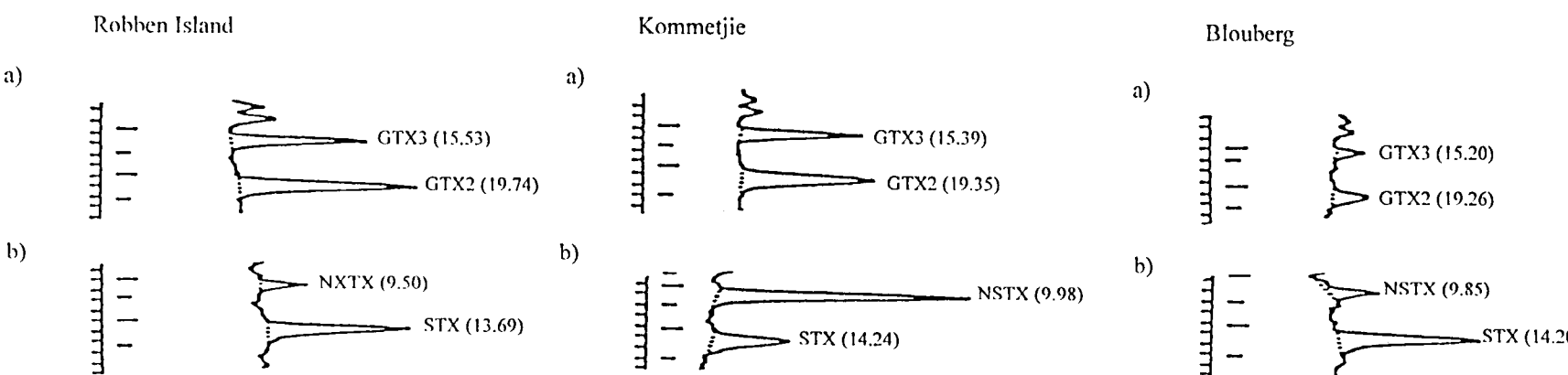


Figure 2.3 HPLC chromatograms of toxins in mussels from three different sites along the South African coast
a) and b) correspond to the mobile phases specified in Table 2.1. Retention Time in parentheses

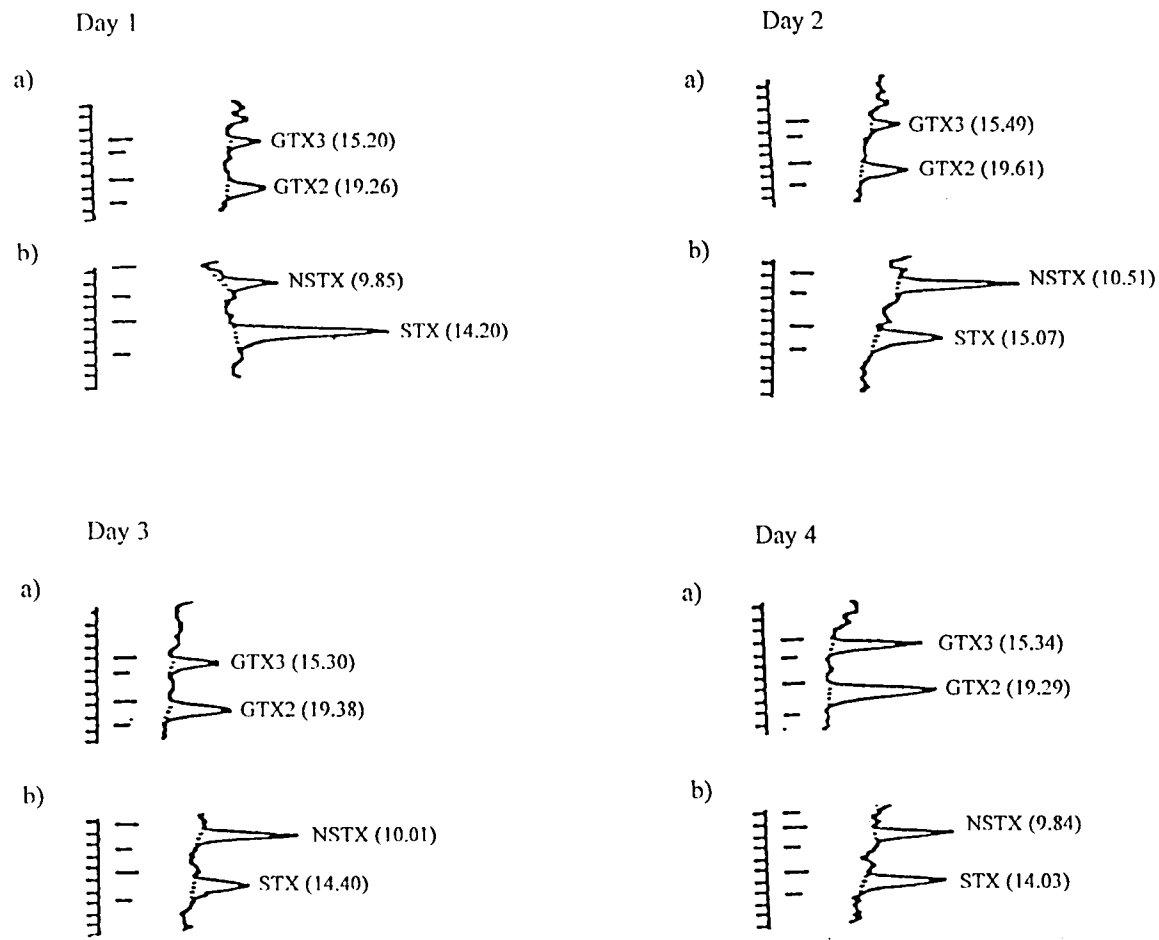


Figure 2.4 HPLC chromatograms of toxins in mussels from Blouberg. Chromatograms show toxin profiles of day 1 to 4 of a depuration experiment. a) and b) correspond to the mobile phases specified in Table 2.1. Retention Time in parentheses

Table 2.2: Concentrations of toxins in standards (NRC = National Research Council of Canada and "in-house" = blue-green algal extract) and mussel samples from three different locations on the South African coast. (Legal limit for PSP is 0.8 μgSTX.g^{-1} or 4 MU.g^{-1})

Sample name	Toxin group	μgSTX.g^{-1}	MU.g^{-1} (HPLC)	STX's:GTX's	MU.g^{-1} (mouse test)
STD (NRC)	neoSTX	10.12	50.6		
	STX	11.90	59.5		
	GTX3	2.30	11.5		
	GTX2	5.35	26.8		
	Total PSP	29.67	148.4		
STD ("in-house")	neoSTX	15.04	75.2		
	STX	8.23	41.2		
	Total PSP	23.27	116.4		
Robben Island	neoSTX	0.184	0.92		
	STX	0.474	2.37		
	GTX3	0.160	0.80		
	GTX2	0.155	0.78		
	Total PSP	0.973	4.87	2:1	2.4
Kommetjie	neoSTX	1.272	6.36		
	STX	0.308	1.54		
	GTX3	0.135	0.68		
	GTX2	0.120	0.60		
	Total PSP	1.835	9.18	6:1	6.7
Bloubergstrand	neoSTX	0.315	1.58		
	STX	0531	2.66		
	GTX3	0.020	0.10		
	GTX2	0.030	0.15		
	Total PSP	0.896	4.49	17:1	2.3

All the mussel samples analysed for PSP contained STX, neoSTX, GTX3 and GTX2 epimeres (Table 2.2)(Figures 2.3 and 2.4). No GTX1 and GTX4 epimeres were found. Unfortunately, a standard was unavailable for the C-group toxins, so it was not possible to establish if the mussel samples contained any C-toxins. In the samples that were analysed using both the mouse-test and the HPLC, the highest combined toxin concentrations were found in material collected from Kommetjie, and the lowest were recorded from material collected at Bloubergstrand. In all three areas, the concentration of neoSTX and STX was higher than that of GTX3 and GTX2 (see ratios in Table 2.2).

A summary of toxic components (major and minor) in other PSP producing species around the world is presented in Table 2.3.

Table 2.3 A summary of toxin components in various PSP producing species

ORIGIN	SPECIES	MAJOR COMPONENT	MINOR COMPONENT	REFERENCE
South Africa	*A. catenella*	STX, neoSTX	GTX2/3	this study
Chile	*A. catenella*	GTX1/2/3/4	C1/2, STX, neoSTX, dcSTX	Lagos *et al.* 1996
USA (N.Y.)	*A. fundyense*	GTX3, STX	C1/2, GTX1/2/4, neoSTX	Bricelj *et al.* 1990
Korea	*Alexandrium*	C1/2, GTX1/4, neoSTX	GTX3/4/5, dcSTX	Kim and Lee 1996
Korea	*A. tamarense*	GTX1/4	GTX2/3, C1/2, dcGTX2	Jeon *et al.* 1996
Japan	*A. tamarense*	GTX3/4, C2	GTX1/2, STX, neoSTX, C1	Oshima *et al.* 1992
France	*A. tamarense*	GTX2/3	neoSTX, STX	Lassus *et al.* 1989
Argentina	*A .tamarense*	GTX1/2/3/4, C1/2	dcSTX, STX	Reyero *et al.* 1998
Adriatic Sea	*A. minutum*	GTX2/3	STX, C's	Honsell *et al.* 1996
France	*A. minutum*	GTX2/3	STX, C's	Ledoux *et al.* 1993
Mediterranean	*A. minutum*	GTX1/4	GTX2/3, C's	Forteza *et al.* 1998

Due to the fact that results on the C-group toxins were not obtained for this study, only rough comparisons can be made. Therefore, focusing on the other components present in the samples, two types of comparisons can be done. Firstly, a comparison of the components in the South African mussels to components found in other PSP producing species, and secondly, a comparison of the South African components to those found in the same species, but found in a different country. For example, a comparison to other PSP producing species (Table 2.3) reveals that, although the toxin composition is not altogether different, the major components of those from South Africa are different to the major components in other species around the world. For example, results obtained from samples collected France (Le doux *et al.* 1996) reveal that the major components were GTX2/3 and the minor components were STX and C toxins, whereas the South African samples had major components of STX/neoSTX and minor components of GTX2/3.

A comparison of the components of the South African samples to samples where the same species (*A. catenella*) was present, but in a different country (e.g. Chile) reveals that the concentrations of individual toxic components and ratios are different, despite the fact that *Alexandrium catenella* was present in the water at both locations. In the Chilean mussels, the concentrations of gonyautoxins (GTX 1,2,3 and 4) were highest, and STX and neoSTX concentrations fluctuated, whereas the major toxin in South African mussels were neoSTX and STX. The profiles observed in mussels collected on the South African coast suggest that *Alexandrium catenella,* found along this coastline is possibly a different strain to the strain found in for example Chile. Lagos *et al.* (1996) noted that toxin profiles were distinctive for different regions, indicating the occurrence of diverse strains of *A. catenella.* Results obtained by Lassus

et al. (1989), corroborate the noticeable differences observed worldwide on different strains of *A. tamarense* species. The differences in toxin composition were also observed in different isolates of *A. tamarense* and *A. catenella* by Oshima *et al.* (1990), as well as by Kim and Lee (1996).

Examination of the Bloubergstrand mussel samples following depuration (Table 2.4), revealed a drop in the concentration of total PSP toxins from day 1 to day 4. Interestingly, however, there was a lower concentration of toxin in the mussels collected on day 3 than those harvested on day 4 (Figure 2.5). This could be explained by the fact that the mussels initially collected were not necessarily all equally toxic. There is a certain amount of variability in the toxin concentration of individual mussels. This can be caused by differences in the orientation of the mussel in the water and by the concentration of mussels in the area, or by the fact that individual mussels might filter water at slightly different rates. This inherent variability could therefore have contributed to the very low toxin concentration on day 3 of the depuration experiment. Unfortunately there was a limited number of samples collected for this study and, therefore, a large amount of replication within this experiment was not possible, although this would have been desirable.

Table 2.4 Concentrations of individual toxin components in mussels (collected from Bloubergstrand) on the 1st, 2nd, 3rd and 4th day of a depuration experiment.

Sample name	Day	Toxin group	ugSTX.g^{-1}	MU.g^{-1}	% decline from day 1
Bloubergstrand	1	neoSTX	0.315	1.58	
		STX	0.531	2.66	
		GTX3	0.020	0.10	
		GTX2	0.030	0.15	
		Total PSP	0.896	4.49	
Bloubergstrand	2	neoSTX	0.504	2.52	
		STX	0.228	1.14	
		GTX3	0.015	0.08	
		GTX2	0.030	0.15	
		Total PSP	0.777	3.89	13.36
Bloubergstrand	3	neoSTX	0.400	2.00	
		STX	0.032	0.16	
		GTX3	0.030	0.15	
		GTX2	0.040	0.20	
		Total PSP	0.502	2.51	44.09
Bloubergstrand	4	neoSTX	0.206	1.53	
		STX	0.228	1.14	
		GTX3	0.05	0.25	
		GTX2	0.080	0.40	
		Total PSP	0.564	3.32	26.06

Examination of the individual toxin groups shows that, neoSTX decreases from day 2 onwards, and STX also decreases, but STX has an unusually low concentration on day 3 (Figure 2.6). This variability could be attributed to the inherent variability between individual mussels, as explained

in the previous paragraph.

Both the GTX3 and GTX2 groups of toxins seemed to show a slight increase in concentration from day 1 to day 4. This could be explained by two possibilities. Firstly, during the extraction process, the mussel extracts were boiled at a low pH (1 - 2). The C- toxins (sulfamate toxins) would therefore have been converted to GTX3 and GTX2 (carbamate toxins) by mild hydrolysis of the N-21 sulphate group (Hall 1982, Ledoux *et al.* 1993). Although there is evidence of an increase in GTX3 and 2, this theory cannot unfortunately be demonstrated conclusively, as the concentration of C- group toxins was not initially established. Indeed, it is not even certain if C- toxins were present in the samples.

Secondly, Oshima (1995a) has observed a chemical transformation during depuration of the Scallop *Patinopecten yessoensis*. This reductive process is accompanied by an increase in GTX3 and GTX2. The author stated that the chemical transformation seems to be common in the natural environment, especially when toxins are transmitted through the food chain. This chemical transformation could therefore also have occurred in the mussels analysed from Bloubergstrand.

The above observations, combined with the fact that the carbamate toxins are more toxic than the sulfamate toxins (Oshima *et al.* 1992), could explain the slight increase in total toxin concentration on the last day of the experiment.

A more comprehensive depuration experiment would have to be run to obtain more complete

results. *In vitro* studies would have to be performed under standardized conditions. The study would have to include HPLC analysis for the sulfamate toxins (C1-4) as well as an analysis of an extract of the causative dinoflagellate, *Alexandrium catenella*. Caution would however need to be exercised when comparing toxin profiles in the shellfish with those from dinoflagellate cultures. Natural environmental variability can result in dinoflagellate (and therefore shellfish) toxin profiles which differ significantly from those of the same species grown in batch cultures (Anderson *et al.* 1990). Enzymatic transformation can also take place in dinoflagellates, which can cause their profiles to differ from those found in the mussels (Oshima 1995a).

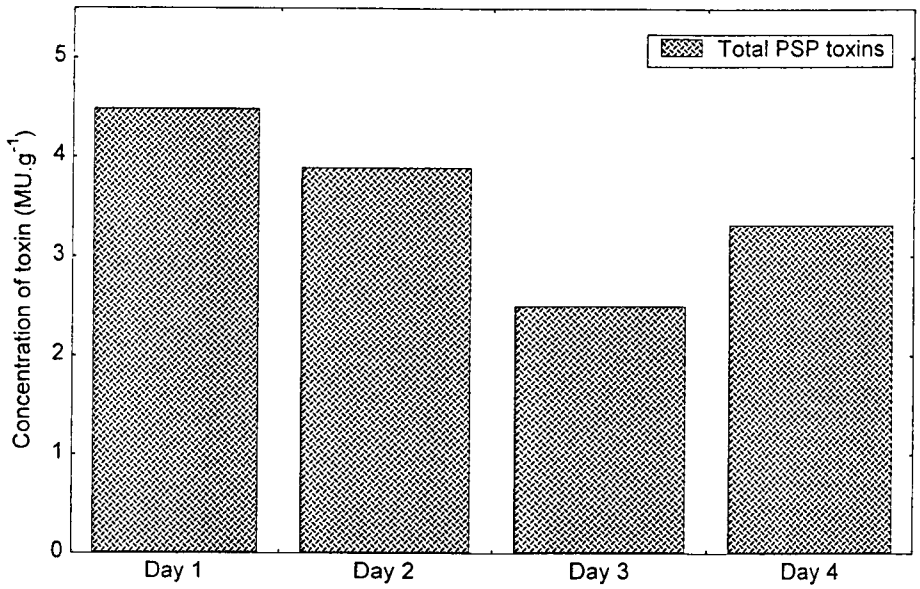


Figure 2.5 Total concentration of PSP toxin in mussels (collected from Bloubergstrand) on four consecutive days of a depuration experiment

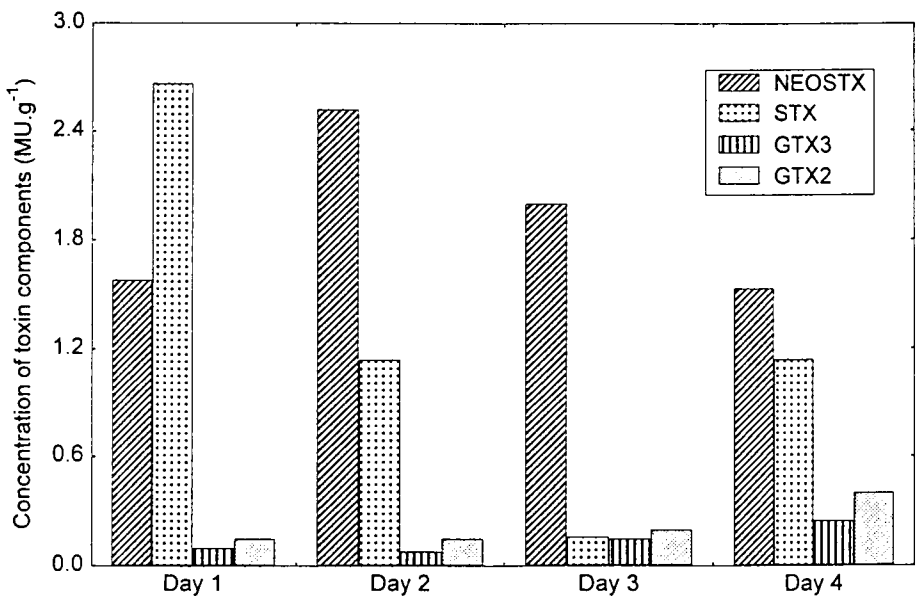


Figure 2.6 Concentration of individual PSP toxin components in mussels (collected from Bloubergstrand) on four consecutive days of a depuration experiment

2.4 APPENDIX

2.4.1 PREPARATION OF STOCK SOLUTIONS FOR USE IN MOBILE PHASE:

The stock solutions (A - E), which were used in the preparation of the mobile phase below, can be kept refrigerated indefinitely.

Solution A was 100 mM 1-heptanesulfonic acid, which was made up by adding 2.1208 g of 1-heptanesulfonic acid, sodium salt (Aldrich) to 104.8 ml distilled water.

Solution B (500 mM phosphoric acid) was made up by adding 100 ml distilled water to 3.37 ml phosphoric acid.

Solution C was a 1:4 dilution of ammonium hydroxide (25 ml NH_4OH + 75 ml distilled water).

Solution D was a 70 mM periodic acid solution, made up by adding 1.6101 g of periodic acid (Aldrich, lot no. 02727Px) to 100 ml of distilled water.

Solution E was 1 N KOH (5.611 g KOH +100 ml distilled water).

2.4.2 PREPARATION OF MOBILE PHASE FOR "STX"-GROUP ANALYSIS:

Solution A (10 ml) and 30 ml of solution B were mixed in a beaker with 400 ml of filtered,

distilled water. The pH of the mixture was adjusted to 7.1 by adding solution C, drop by drop. This volume was made up to 500 ml with filtered, distilled water and 25 ml of acetonitrile was then added. This solution was degassed.

2.4.3 PREPARATION OF MOBILE PHASE FOR "GTX"-GROUP ANALYSIS:

Solution A (10 ml) was mixed with 10 ml of solution B and added to 400 ml of filtered, distilled water. The pH of the solution was adjusted to 7.1 with solution C. This mixture was then filtered and made up to 500 ml with filtered, distilled water. This solution was then degassed.

2.4.4 PREPARATION OF ACIDIFYING REAGENT FOR "STX" AND "GTX"-GROUPS:

Acetic acid (15 ml) was made up to 500 ml with distilled water to form a 0.5 N solution. This solution was filtered and degassed.

2.4.5 PREPARATION OF OXIDISING REAGENT FOR "STX" AND "GTX"-GROUPS:

4.36 g of K_2HPO_4 was added to 350 ml of filtered distilled water. 50 ml of a 70 mM periodic acid solution was added to this. The pH was adjusted to 9 with 1 N KOH and then made up to 500 ml with filtered distilled water. The solution was then degassed.

3. THE LUMINESCENT BACTERIA ASSAY FOR THE DETECTION OF PHYCOTOXINS

3.1 INTRODUCTION

Microorganisms have long been used to evaluate various toxicological concerns in aquatic systems. For example, Calder and Lader (1976) described the effects of a number of hydrocarbons on the growth of marine bacteria in culture, and Hertkorn-Obst and Frank (1979) have used physiological measurements of bacteria to monitor toxic materials in water. In South Africa the Ames Test has been used to detect mutagens in waste-water, drinking water and surface water. In this assay, histidine-requiring bacteria revert to histidine-independence when exposed to mutagens (Grabow *et al.* 1980). Assays for detection of toxic chemicals in water, based on physiological functions of microorganisms (e.g. oxygen uptake, growth and colony formation) have been investigated at South Africa's National Institute for Water Research for a number of years (Slabbert and Morgan 1982, Slabbert 1988).

Bioluminescence is the light produced by certain organisms as a result of the enzyme luciferase - the primary source of energy for the light supplied by the oxidation and conversion of a long-chain aldehyde to the corresponding fatty acid (Dunn *et al.* 1973). Numerous marine and terrestrial organisms are bioluminescent. Apart from the common requirement for oxygen, a feature that was recognized by Robert Boyle as long ago as 1668, for most, the biochemistry and molecular biology of the underlying processes occurring during production of luminescence by

organisms remains unstudied (Bronstein *et al.* 1994). Luminescent marine bacteria are common throughout the world's oceans. Although the luminescent reaction appears to be controlled by a complex set of regulatory mechanisms its function is unknown (Hastings and Nealson 1977). Bacterial luciferase, which catalyses light emission, is in many cases an inducible enzyme. The inducers, referred to as autoinducers, are small molecules produced by the bacteria themselves, and excreted into the growth medium where they accumulate (Friedrich and Greenberg 1983, Kaplan and Greenberg 1985). When the autoinducer reaches a critical extracellular concentration, induction of luciferase commences. Several additional factors can also affect the luminescence. These include the types and concentrations of carbohydrates, amino acids, salts and cyclic nucleotides in the growth medium. It is also influenced by temperature and oxygen tension, as well as certain toxic substances (Eberhard *et al.* 1981).

A test was developed by Tchan *et al.* (1975) that employed both a marine alga and a luminous bacterium to screen for the presence of toxic chemicals in sea-water samples. In 1979 the Microtox Toxicity Analyser (Beckman Instruments, Inc.) was placed on the market (Bulich *et al.*1980). This instrument employed the luminescent bacterium, *Photobacterium phosporeum*, (later *Vibrio fischeri*). This test system has been widely used in many studies on chemical toxicants since 1979 (Dutka and Kwan 1982, Persoone *et al.* 1984, Vasseur *et al.* 1984 and McFeters *et al.* 1983). A similar system was later developed by Dr Lange (Dusseldorf) call the LUMIStox system. The system uses a standardized luminometer and luminescent bacterial kits (*V. fischeri*).

Table 3.1 Summary of substances tested using luminescent bacteria kits

SUBSTANCE TESTED	REFERENCE
Pesticides	Somasundaram *et al.* 1990
Industrial waste chemicals	Svenson *et al.* 1992
Sediments pore water	Hoke *et al.* 1992
Benthic assemblages	Becker *et al.* 1990
Heavy metals	Blaise *et al.* 1994; Lau-Wong 1990
Mussel and clam depuration	Lau-Wong 1990
Genotoxins (DNA-damaging)	Thomas-Johnson 1991
Extracts from microalgae	Arzul *et al.* 1995; Bruno *et al.* 1990
Sediment toxicity	Matthiesson *et al.* 1998

Luminescent bacteria have also been used to study the effect of toxicants. Arzul *et al.* (1995) evaluated the effect of lipids, extracted from a certain dinoflagellate causing NSP, on luminescent bacteria . Bruno *et al.* (1990) established the presence of saxitoxin in *Gonyaulax (Alexandrium) polyedra* using the luminescent bacteria assay. Depuration efficiency of mussels and clams were tested with the luminescent bacteria assay (Lau-Wong 1990). Table 3.1 summarises some of the uses of luminescent bacteria tests.

The above mentioned studies have shown that luminescent bacteria can be used to test mussel and dinoflagellate extracts for various toxic substances. In South Africa, the luminescent bacteria assay has not yet been used as a method to detect toxins. In this thesis it was therefore assessed as to its suitability and sensitivity in detecting the main toxic components of PSP and DSP, as well as mussel and water samples containing PSP and DSP toxins. This chapter also aims to establish the feasibility of using a laboratory-grown culture of the luminescent bacterium *V.*

fischeri to detect these phycotoxins, and to compare these results with those obtained with the commercially available test-kit, LUMIStox.

3.2 METHODS AND MATERIALS

3.2.1 TEST MATERIAL

3.2.1.1 Reference chemical

3,5-Dichlorophenol was purchased from Sigma-Aldrich Co. Ltd. As stipulated in the LUMIStox kit manual, 3,5-Dichlorophenol was used as a reference chemical to test the sensitivity of the kit. Different dilutions were made up using filtered (0.2 μm) seawater, ranging from 0.75 $mg.l^{-1}$ to 24 $mg.l^{-1}$. The EC_{50} (effective concentration at which a 50% inhibition of luminescence occurs) was calculated and compared to the EC_{50} stipulated for this chemical on the verification certificate supplied with the LUMIStox kit.

3.2.1.2 Okadaic acid standard

Okadaic acid, dissolved in the carrier N'N-Dimethylformamide (DMF), was obtained from Calbiochem Biochemical Company, USA. For this study, a range of Okadaic acid concentrations spanning $2.3x10^{-1}$ - $1.38x10^{1}$ $\mu g.ml^{-1}$ were made up with filtered seawater. DMF was made up in similar dilutions to the Okadaic acid solutions and used as a control.

Okadaic acid in free acid form without DMF was also obtained from Calbiochem Biochemical Company, USA. A range of concentrations of this free acid were also made up with filtered

seawater, ranging from $2.5x10^{-1}$ $\mu g.ml^{-1}$ to $1.0x10^{2}$ $\mu g.ml^{-1}$.

3.2.1.3 Saxitoxin (STX) standard

Saxitoxin Diacetate standard toxin was purchased from Calbiochem, USA. This Saxitoxin standard was transported in a 0.1 M solution of AcOH, as it is unstable at neutral and high pH's.

A stock solution of 100 $\mu gSTX.ml^{-1}$ was made up for this study using 0.2 μm filtered seawater. Serial dilutions were then made using filtered seawater (maximum concentration of 10 $\mu gSTX.ml^{-1}$). A 0.1 M solution of AcOH was made up in the same way and used as a control. Each control and STX dilution was tested in triplicate.

3.2.1.4 Algal extracts

A culture of the toxic dinoflagellate *Alexandrium minutum* was purchased from CSIRO Marine Laboratories, Tasmania, Australia. This culture, together with a local isolate of the toxic species *Alexandrium catenella*, and two non-toxic dinoflagellates, *Prorocentrum micans* and *Scrippsiella trochoidea*, were grown in Keller medium [enrichment basal salt mixture without silicates, (Sigma Chemical Company K1630)].

All four dinoflagellate species were grown in batch cultures at 17 ^{0}C on a 12hr/12hr light:dark cycle. When the cultures grew dense ($\sim 10^{5}$ cells.l^{-1}), subsamples were removed and cell counts were performed using the Utermohl counting method and an inverted microscope (Hasle 1978).

Extracts were made of the cultures in the following way:

The cells were concentrated on a fine mesh (15 μm) and then rinsed into a 20 ml beaker with filtered seawater. The cells were sonicated with an ultrasonicator at maximum intensity (i.e. 10 x 10 second bursts) using a microtip probe. The bottom of the beaker containing the cells was emersed in ice for cooling. Cellular disruption was confirmed by visual inspection and the suspension was centrifuged at 2 000 rpm for 10 min. The supernatant was then frozen (-20 ^{0}C) until needed. Two different extracts (Extract A and B) were made from each of the dinoflagellate species (except *S. trochoidea*, where only Extract A was made). The cell concentration in Extract A was higher than in Extract B for all the dinoflagellate species. For Extract B of *A. minutum, A. catenella* and *P. micans*, the filtered medium (after the cells had been concentrated and removed) was retained for further experimentation.

These cell extracts, and their respective filtered media were tested undiluted (neat), and also tested after a dilution series of each extract was made by adding seawater. Each dilution was tested in triplicate. The undiluted sample of the two toxic extracts (*A. minutum and A. catenella*) were allocated a "Saxitoxin equivalent"(STXeq.) value, by counting the number of cells in each extract and multiplying this by an estimated value of toxin per cell (100 pg STX.cell^{-1} (Dale *et al.* 1978)).

The cell concentrations of Extracts A and B and toxin concentrations are shown in Table 3.2.

Table 3.2 Cell concentrations and estimated toxin concentrations of dinoflagellate extracts

SPECIES	EXTRACT A		EXTRACT B	
	cells.l^{-1}	µ.ml^{-1}STXeq.	cells.l^{-1}	µ.ml^{-1}STXeq.
Alexandrium minutum	$3x10^7$	30	$1.9x10^7$	19
Alexandrium catenella	$1.1x10^7$	11	$0.7x10^7$	7
Prorocentrum micans	$3.6x10^6$	none	$8.1x10^5$	none
Scrippsiella trochoidea	$1.9x10^7$	none		

3.2.1.5 Mussel extracts

Clean samples of the mussel *Mytilus galloprovincialis*, which had been left to depurate in through-flowing 0.2 µm filtered seawater for 2 weeks, were used to make extracts. To prevent certian factors like age, size and sex from having an influence on the result, the mussels samples were all pooled before being treated in one of the following three different ways.

The first method was performed according to the AOAC protocols (AOAC 1984) for the mouse bioassay, in which 100 ml of 0.1 N HCl was added to 100 g of mussel tissue. This was homogenized with an electric blender, after which the mixture was boiled for 5 min and allowed to cool. The mixture was then centrifuged for 5 min at 3 000 rpm, and the supernatant was used for the bioassay.

The second method extracted the mussel tissue as described above, but the supernatant was neutralized to pH7 by adding sodium hydroxide (NaOH), prior to use in the bioassay.

A similar procedure was used for the third method, but in this case the HCl was replaced with filtered seawater. The supernatant was not neutralized.

Dilutions were made of the supernatants using filtered seawater, and each dilution was tested in triplicate.

3.2.2 *VIBRIO FISCHERI* ("IN-HOUSE" BACTERIAL TEST)

A culture of *Vibrio fischeri* was ordered from American Type Culture Collection (ATCC no. 7744), Maryland, USA. This culture had been freeze-dried in a sterile, sealed ampoule for transportation. "Seawater Complete" broth (SWC) was used as growth medium because it results in maximal luminescence (Eberhard *et al.* 1981). See appendix 3.5 for recipe (Nealson 1978).

The freeze-dried pellet of *V. fischeri* was reconstituted by adding growth medium to the ampoule containing the bacteria. Agar plates were prepared in sterile petri-dishes by adding 1-2% agar to the growth medium. The reconstituted bacteria were then streaked out on these plates which were maintained at 17 ^{0}C until colonies began to develop. The plates were visually examined for luminescent colonies. Luminescing colonies were transferred into sterile SWC broth using an inoculation needle, and grown at 17 ^{0}C to an absorbency (A_{600}) of 0.30 prior to use. Alternatively, a luminescence reading was taken on the LUMIStox photometer (see section 3.2.3 for use), and when the culture started to luminesce, it was used for experimentation. [I]

[I] Frequent reisolation of this bacterium on agar plates was required in order to maintain a culture with a high level of luminescence as it was prone to reverting to a "dark" form.

The luminescing bacteria culture (0.5 ml) was pipetted into glass vials and incubated for 15 min at 15 °C. Equal amounts (0.5 ml) of test substances were pipetted into other vials which were also incubated at 15 °C. A 2% NaCl solution was used as a control substance. After 15 min, luminescent readings were taken of each vial containing bacteria using the LUMIStox photometer. The test substance was then added to the vials of bacteria and incubated for a further 30 min. A second luminescence reading was then taken of each vial. The decrease in luminescence from the initial reading to the second reading was calculated as a percentage (% inhibition).

For calculation of % inhibition the following formulae were used (LUMIStox test manual, Dr Lange):

$$f_{k30} = \frac{I_{k30}}{I_0} \qquad (1)$$

where I_{k30} is the control's reading after 30 minutes, and I_0 is the sample's initial reading

$$I_{c30} = I_0 \times F_{k30} \qquad (2)$$

where F_{k30} is the mean of the three f_{k30}'s (1)

$$H_{30} = \frac{I_{c30} - I_{t30}}{I_{c30}} \times 100 \qquad (3)$$

where H_{30} is % inhibition

3.2.3 COMMERCIAL "LUMISTOX" BACTERIAL TEST

The luminescent bacteria and reconstitution solution were ordered from Dr Lange, Dusseldorf, Germany. The bacteria had been freeze-dried, and the reconstitution medium was frozen. The LUMIStox photometer and LUMIStherm, which both maintained the bacteria at a temperature of 15 ^{0}C, were also kindly supplied by Dr Lange.

No preparation of the kit bacteria was necessary, because they were kept frozen and were only reactivated immediately before experimentation.

The LUMIStox measuring instrument is the photometer, which contains a photomultiplier. The photomultiplier detects the relatively weak primary signals from the luminescent bacteria and electronically amplifies them. The measurements are performed in a measuring well which is equipped with light traps so that no external light can reach the photomultiplier during a measuring cycle. The entire measuring well is kept at 15 ^{0}C in order to avoid temperature differences between measuring and incubation, which could cause the light signals to fluctuate.

A vial of reactivation solution was allowed to thaw in a water bath at room temperature. The bottle was mixed thoroughly and put into a storage shaft in the LUMIStherm for 15 min. A vial of luminescent bacteria was thawed quickly in a beaker with tap water, and the contents were suspended in 0.5 ml of the reactivation solution. The bacteria were left to stand for 15 min in the second LUMIStherm storage shaft before being suspended into the remaining reactivation solution and allowed to stand for a further 15 min. Meanwhile, 0.5 ml of the sample/control

substances were pipetted into vials and put into the wells of the LUMIStherm.

When the bacteria, control (2% NaCl solution) and test solutions had adjusted to the correct temperature, the experiment was initiated. Luminescence readings were taken of the vials containing bacteria (using the photometer), and then the control and sample dilutions were added to these vials. After an incubation time of 30 min, a second luminescence reading was taken. The % inhibition was calculated as for the "In-house" bacteria. A sample subjected to the LUMIStox bacteria test is considered by the manufacturers to be non-toxic if, in its undiluted state, it causes less then 20% inhibition of luminescence after 30 min incubation.

3.2.4 STATISTICAL ANALYSIS

To determine if there was a significant difference in inhibition of luminescence between the different treatments, or between the treatment and the control at any one dilution, data were analysed using Student T tests (Zar 1983). The results of these tests are indicated (**indicating significant difference) on the appropriate figures.

3.3 RESULTS

3.3.1 *VIBRIO FISCHERI* ("IN-HOUSE" BACTERIAL TEST)

Reference chemical: 3,5 Dichlorophenol

3,5 Dichlorophenol caused the highest inhibition of luminescence (80%) at 24 $mg.l^{-1}$ with a

linear decrease as toxin concentrations decreased (Figure 3.1). An EC_{50} was observed at a concentration of ~9 $mg.l^{-1}$.

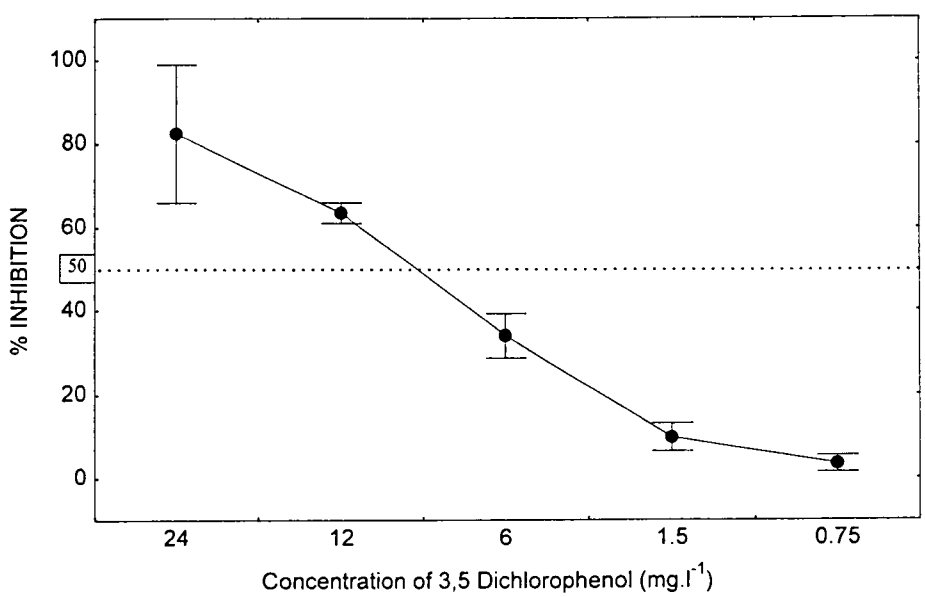


Figure 3.1 In-house bacteria: Inhibition of luminescence caused by different concentrations of the reference chemical 3,5 Dichlorophenol (each point represents the mean of 6 replicates ±SE)

Although the initial luminescence readings obtained from the in-house bacteria before toxicants were added equalled those of the LUMIStox kit bacteria, the results obtained when testing the dinoflagellate extracts and mussel samples were, however, too varied and were often not repeatable (Table 3.3). The results on the reference chemical also differ widely between the in-house and LUMIStox bacteria (Figures 3.1 and 3.2). It was therefore not possible to compare the results from these bacteria to those obtained with the LUMIStox kit.

Table 3.3 % Inhibition of luminescence caused by an extract of *A. minutum*, using in-house bacteria. Examples demonstrate the inconsistency of results

DILUTION	% INHIBITION Experiment 1	% INHIBITION Experiment 2	% INHIBITION Experiment 3
neat	4	0	0
1:2	48	0	25
1:6	2	0	0
1:10	5	0	17
1:100	26	0	22

3.3.2 LUMIStox BACTERIAL TEST

Reference chemical: 3,5 Dichlorophenol

3,5 Dichlorophenol caused a 100% inhibition at concentrations >3 $mg.l^{-1}$, with a linear decrease in inhibition with decreasing concentrations (Figure 3.2). The EC_{50} was observed at a concentration of ~1.5 $mg.l^{-1}$.

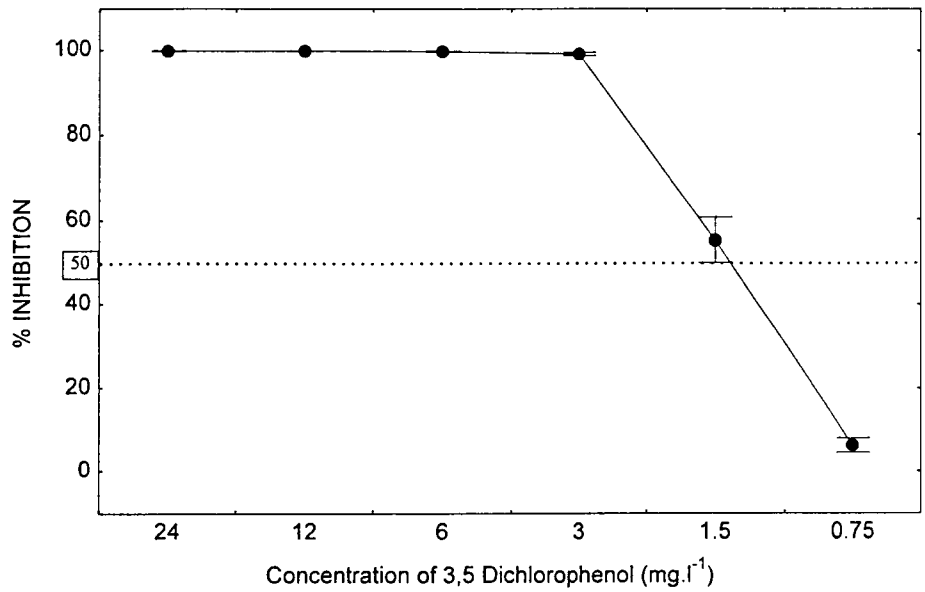


Figure 3.2 LUMIStox kit: Inhibition of luminescence caused by different concentrations of the reference chemical 3,5 Dichlorophenol (each point represents the mean of 4 replicates ±SE)

Okadaic acid standard:

Okadaic acid (OA) dissolved in DMF caused a 100% inhibition of luminescence at the highest concentration, with reduced inhibition at lower concentrations. Similar results were obtained using DMF as a control, indicating the possibility that the inhibition was caused by DMF and not by OA (Figure 3.3). Pure OA was therefore tested, and despite the use of much higher OA concentrations, very little inhibition of luminescence was observed (~12%) (Figure 3.4).

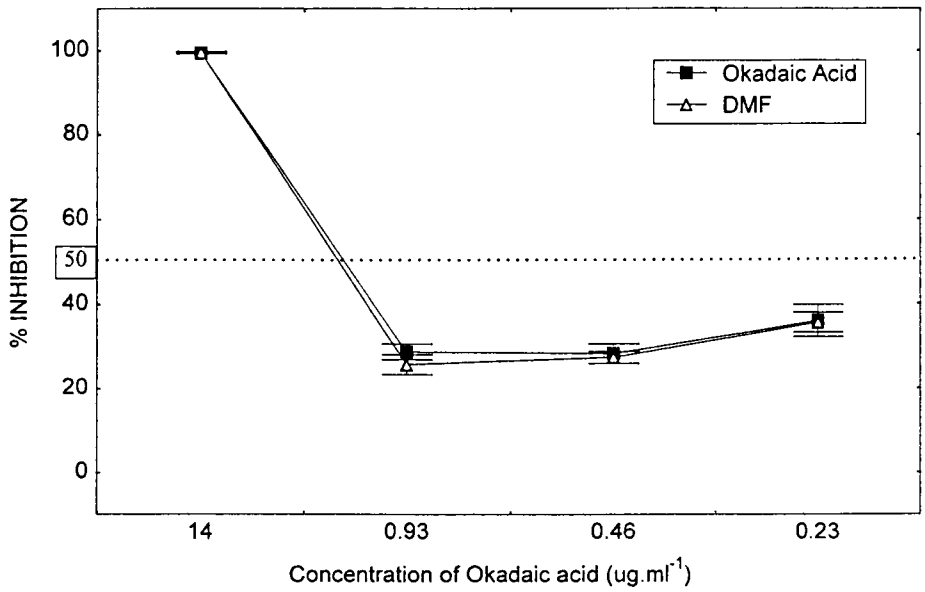


Figure 3.3 LUMIStox kit: Inhibition of luminescence caused by OA and DMF (control) (each point represents the mean of 3 replicates ±SE)

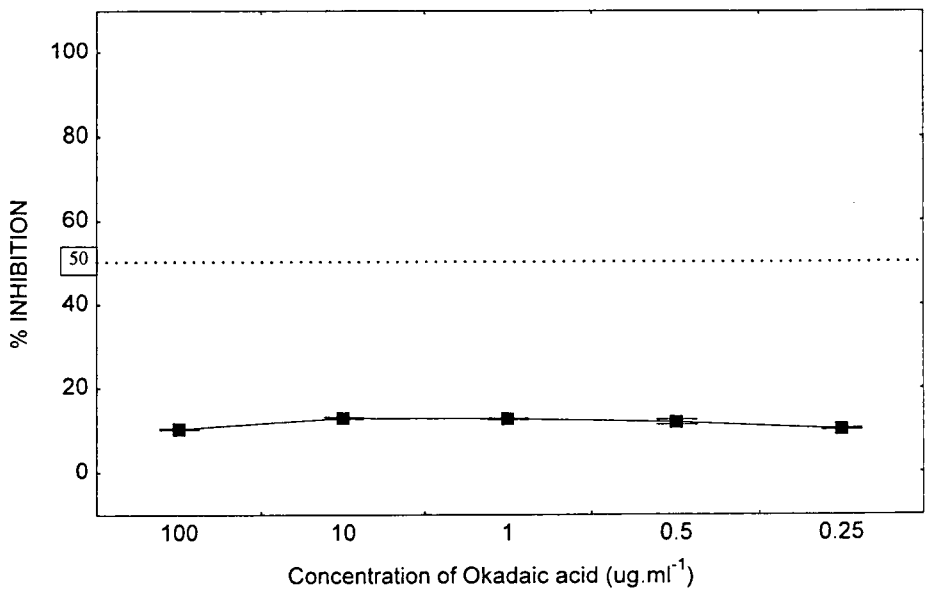


Figure 3.4 LUMIStox kit: Inhibition of luminescence caused by pure OA (each point represents the mean of 3 replicates ±SE)

Saxitoxin standard:

At a concentration of 10 $\mu g.ml^{-1}$, the STX standard caused a significantly higher inhibition of luminescence than the AcOH (** $p<0.01$) (Figure 3.5). At lower concentrations, there was no significant difference between the effect of the STX and the control (AcOH).

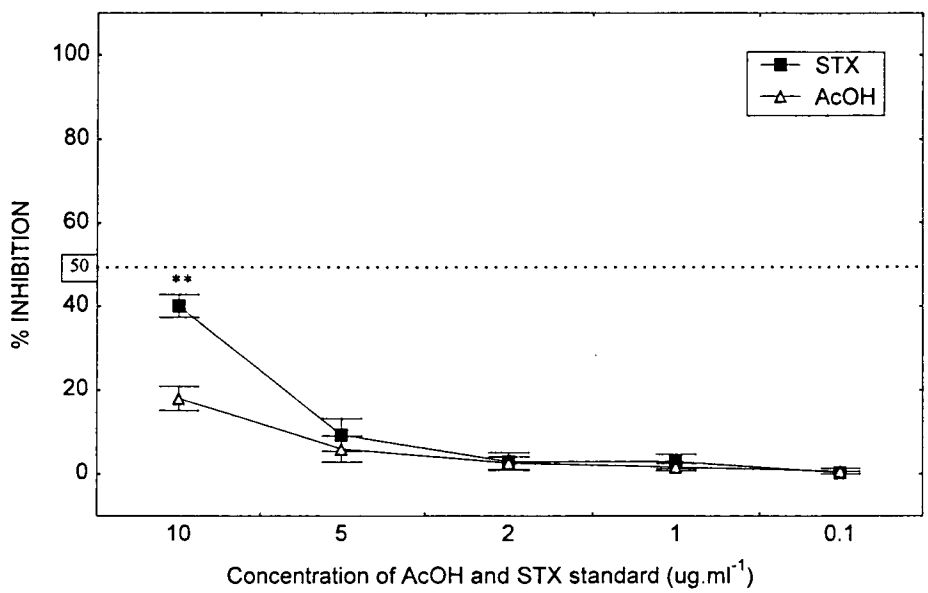


Figure 3.5 LUMIStox kit: Inhibition in luminescence caused by STX standard and AcOH (control) ("neat" = 10 $\mu g.ml^{-1}$, ** $p<0.01$; each point represents the mean of 3 replicates ±SE)

Algal extracts:

Alexandrium minutum:

At a "neat" concentration of Extract A, inhibition of luminescence ranged between 30 and 40% (Figure 3.6). There was little decrease in the inhibition of luminescence as the toxin concentration decreased, except for the final dilution. Variation between replicates was high.

Extract B at a "neat" concentration caused inhibition of luminescence similar to that obtained with Extract A, although the variation between the replicates was smaller (Figure 3.7). Interestingly, the filtered medium of Extract B at "neat" and 1:2 dilutions effected greater inhibition of luminescence than did the cells themselves, though no difference between the extracts were obtained as the concentration decreased.

In both Extracts A and B, and the filtered medium of Extract B, the inhibition of luminescence was above 20 % at dilutions greater than 1:10, and would be considered toxic.

Alexandrium catenella:

Extract A and B of *A. catenella* both caused an inhibition of between 20 and 40% (Figures 3.8 and 3.9). The filtered medium from Extract B caused significantly lower inhibition at all dilutions except the lowest concentrations (Figure 3.9). Only the neat and 1:2 dilutions of both extracts caused greater than 20% inhibition of luminescence and all weaker dilutions caused little inhibition.

Prorocentrum micans:

Both Extracts A and B caused inhibition of between 20 and 30% (Figures 3.10 and 3.11). The cells of Extract B caused a significantly higher inhibition of luminescence than the filtered medium (Figure 3.11).

Scrippsiella trochoidea:

Extract A caused an inhibition of ~ 90% at a neat concentration, with a linear decrease in inhibition as the concentration decreased (Figure 3.12). All dilutions except 1:100 caused inhibition of luminescence above 40 percent.

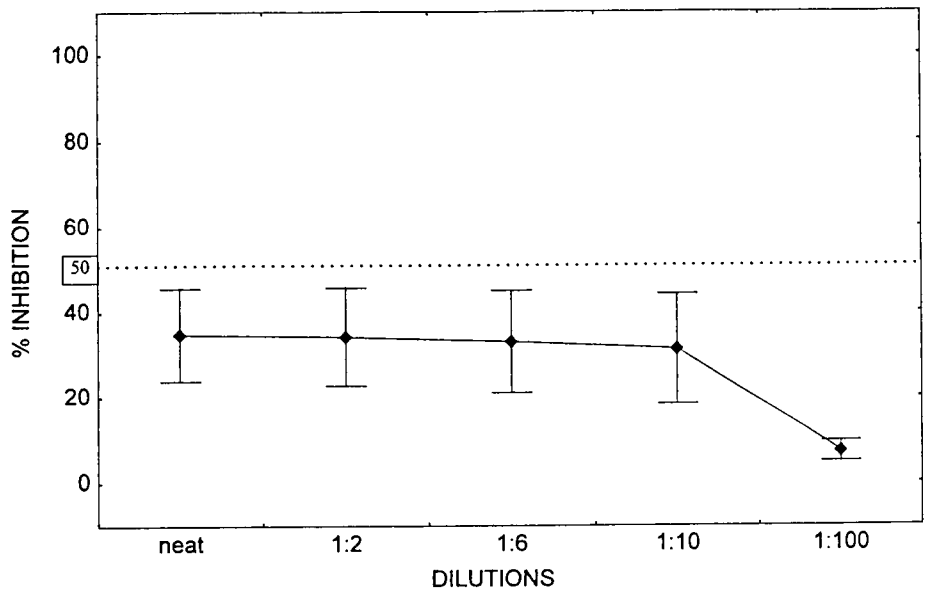


Figure 3.6 LUMIStox kit: Inhibition of luminescence caused by *Alexandrium minutum* Extract A ("neat" = $3x10^7$ cells.l^{-1}; ~30 µg.ml^{-1} STXeq.; each point represents the mean of 4 replicates ±SE)

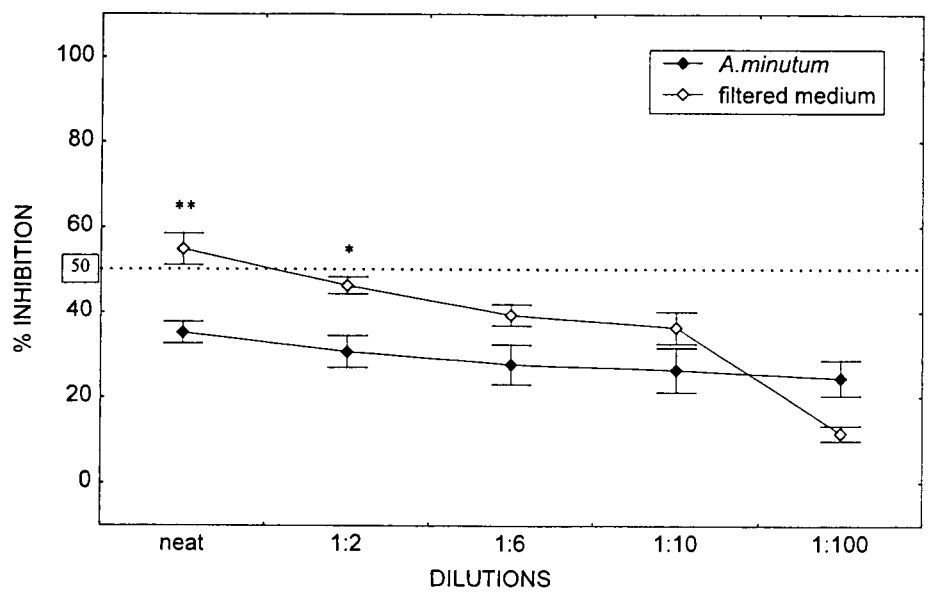


Figure 3.7 LUMIStox kit: Inhibition of luminescence caused by *Alexandrium minutum* Extract B and filtered medium ("neat" = $1.9x10^7$ cells.l^{-1}; ~ 19 µg.ml^{-1} STXeq.; ** $p<0.01$; * $p<0.05$; each point represents the mean of 8 replicates ±SE)

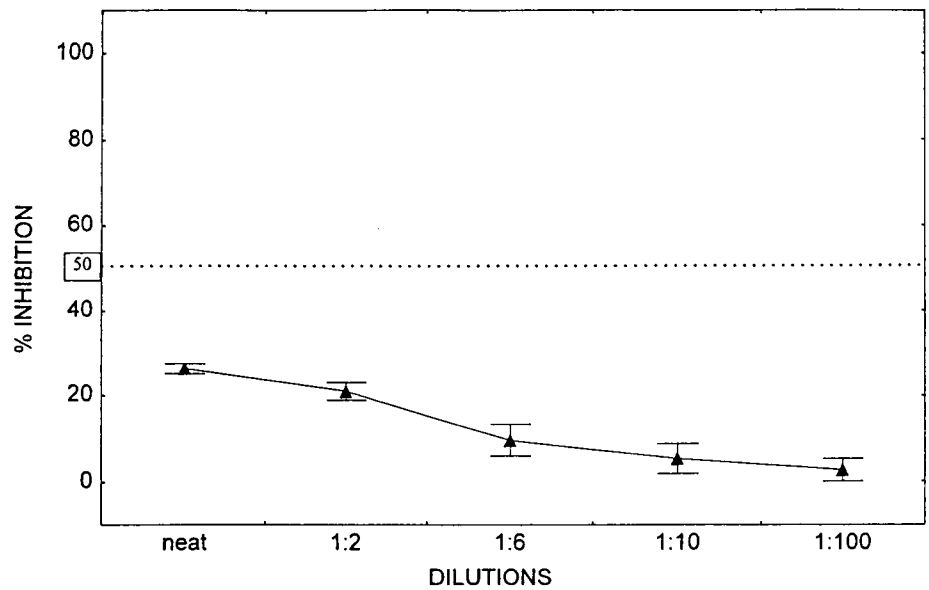


Figure 3.8 LUMIStox kit: Inhibition of luminescence caused by *Alexandrium catenella* Extract A ("neat" = $1.1x10^7$ cells.l^{-1}; ~ 11 µg.ml^{-1} STXeq.; each point represent the mean of 3 replicates ±SE)

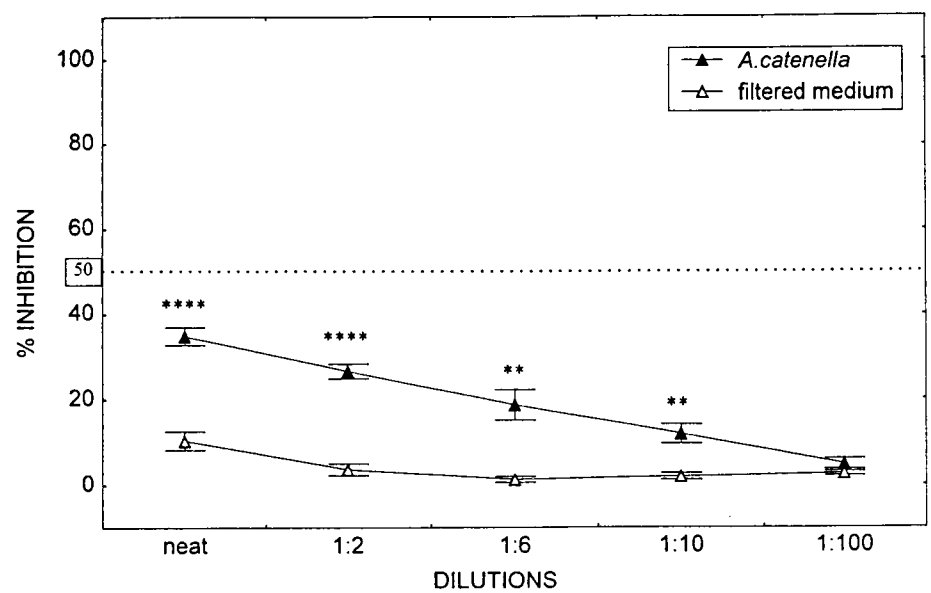


Figure 3.9 LUMIStox kit: Inhibition caused by *Alexandrium catenella* Extract B and filtered medium ("neat" = $0.7x10^7$ cells.l^{-1}; ~ 7 µg.ml^{-1} STXeq.; **** $p<0.0001$; ** $p<0.01$; each point represents the mean of 9 replicates ±SE)

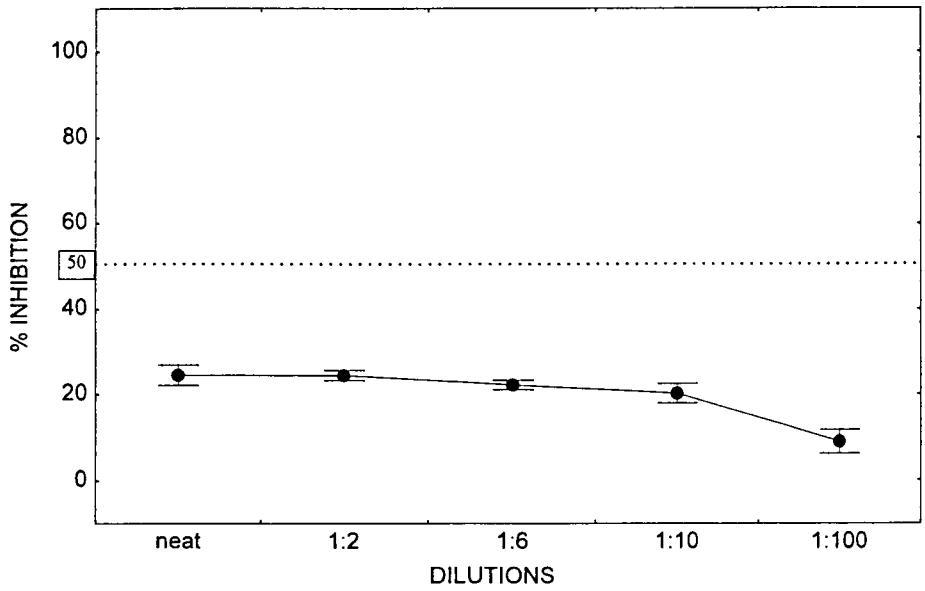


Figure 3.10 LUMIStox kit: Inhibition of luminescence caused by *Prorocentrum micans* Extract A ("neat" = $3.6x10^6$ cells.l^{-1}; each point represents the mean of 4 replicates ±SE)

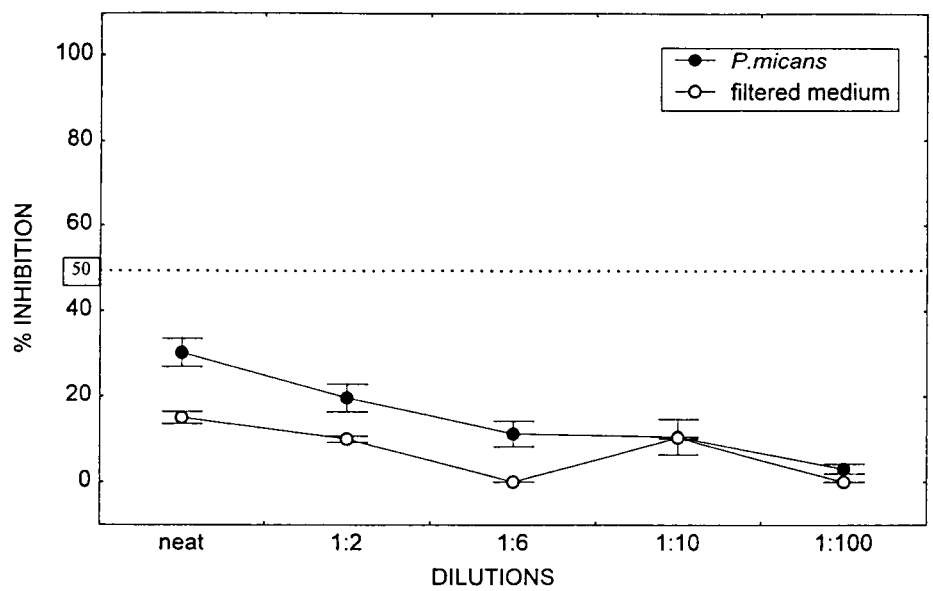


Figure 3.11 LUMIStox kit: Inhibition caused by *Prorocentrum micans* Extract B and filtered medium ("neat" = $8.1x10^5$ cells.l^{-1}; ** $p<0.01$; each point represents the mean of 5 replicates ±SE)

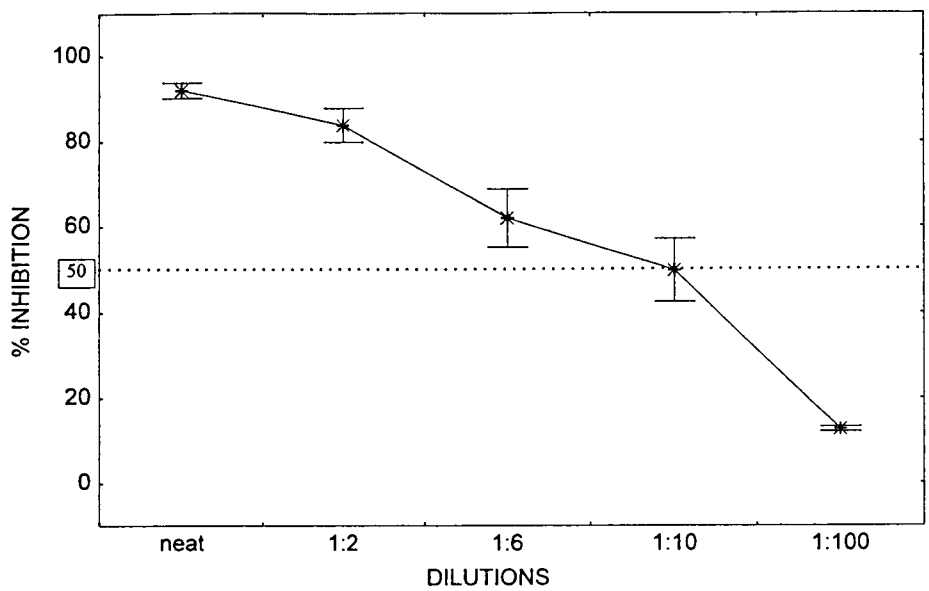


Figure 3.12 LUMIStox kit: Inhibition of luminescence caused by *Scrippsiella trochoidea* Extract A ("neat" = $1.9x10^7$ cells.l^{-1}; each point represents the mean of 3 replicates ±SE)

Mussel extracts: (Figure 3.13)

a) with HCl: all dilutions of this mussel extract caused complete inhibition of luminescence.

b) with HCl (neutralized with NaOH): the neat extract caused an inhibition of 75%, and there was a linear decrease in inhibition with decreasing extract concentration. The lowest concentration of mussel extract still caused an inhibition of luminescence greater than 45%.

c) with sea water: the neat extract caused a 70% inhibition of luminescence. The lowest extract concentration caused an inhibition of ˜ 45%.

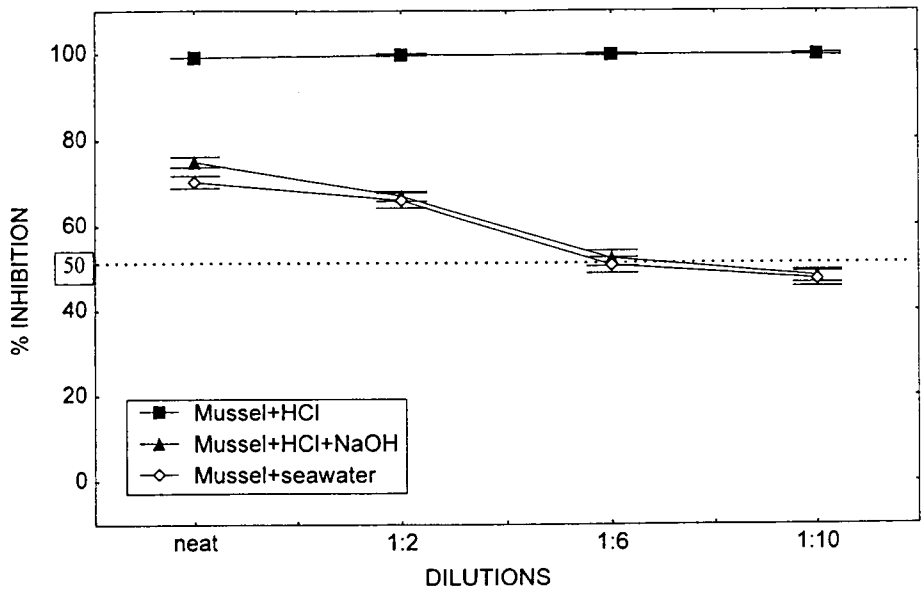


Figure 3.13 LUMIStox kit: Inhibition caused by three different mussel extracts (the points represent the mean of 3 replicates ±SE)

3.4 DISCUSSION

The purpose of the reference chemical is to verify the validity of the bacteria test. At a certain range of concentrations specified in the LUMIStox manual, the chemical will cause inhibition of luminescence of between 20 and 80%. When the LUMIStox bacteria were tested in this study, the concentration of the reference chemical at which 20 to 80% inhibition occurred was slightly

lower than prescribed on the verification sheet, but was nevertheless within the same range. It was therefore accepted as valid. If anything, this would mean that the test was slightly more sensitive than predicted. A comparison of the EC_{50} values with those of the in-house bacteria reveals that the latter were much less sensitive. McFeters *et al.* (1983) found similar results in their comparison of the Microtox kit and an in-house bacterium, where the Microtox kit bacteria detected several test compounds at lower concentrations than their in-house counterparts. These authors suggested that damage caused by the drying procedure of the kit bacteria might render them more susceptible to the subsequent effects of toxic test chemicals than the freshly-cultured cell suspensions of the in-house bacteria. Although the LUMIStox bacteria are preserved by means of a "very mild drying procedure" (LUMIStox manual), some damage may occur during this process which could render the bacteria more sensitive than the in-house bacteria.

It is difficult to directly compare results of this study to results obtained in previous studies, as the methods used by previous authors were not always similar, test kits were not similar (e.g. Microtox versus LUMIStox), or the mode of action of the toxin tested was different. For example, Arzul *et al.* (1995) extracted and tested fatty acids, and Lau Wong (1990) extracted and tested heavy metals, each of which have different modes of action to the the okadaic acid, saxitoxin, dinoflagellate and mussel extracts tested in this study. Nevertheless, certain observation can be made.

The effect of Okadaic acid on eukaryotic cells is relatively well understood (Bialojan and Takai 1988, MacKintosh *et al.* 1990, Baden *et al.* 1995), but there is no comparative information on its effect on bacteria. The mechanisms of action on mammalian, yeast and even protozoan cells

do not necessarily apply to bacteria. Okadaic acid influences the enzymes inside eukaryotic cells by inhibiting the protein phosphatase enzymes (Luu *et al.* 1993). Although it is not known why OA did not have any major effect on the bacterial luminescence, a number of possible explanations can be put forward. One possibility is that OA cannot penetrate the cell wall of bacteria. Similar results have been observed for some eukaryotic cells (Cohen *et al.* 1990). Alternatively, OA did penetrate the bacterium cell, but bacteria might not have any of the enzymes that OA is thought to act upon (e.g. protein phosphatases), and therefore the luminescence is not affected. It could be argued that OA may act more slowly than the 30 min incubation time tested here. However, this explanation can be discounted because preliminary experiments (using bacteria without adding a toxicant) had established that inhibition of luminescence was maximal after 30 min of incubation, and was lower at 10 min and 60 min incubation time. The concentrations of OA that were used in this experiment were very high compared to the concentrations that would need to be detected occurring naturally in a red tide for restrictions to be imposed on shell fisheries (refer to chapter 1, section 1.3). Although no conclusion can be reached as to why even these high concentrations of OA did not inhibit bacterial luminescence, the results indicate that this assay is unsuitable as a test for detecting OA.

In a previous study of the effect of Saxitoxin on luminescent bacteria (using the Microtox kit), an EC_{50} was establish at 1.28 $\mu g.ml^{-1}$ STX (Bruno *et al.* 1990). It was therefore assumed that a concentration of 10 $\mu g.ml^{-1}$ here would allow both the establishment of a standard curve and the determination of an EC_{50}. Unfortunately, the inhibition of luminescence even at this concentration was not sufficiently high to establish an EC_{50}, although the neat sample did

indicate toxicity (with 40% inhibition of luminescence). However, at all concentrations less than 10 $\mu g.ml^{-1}$, there was no difference between the influence of STX on luminescence and the effect of AcOH and it was impossible therefore to discriminate between the effects of AcOH and STX. It is difficult to explain the results observed here, given that Bruno *et al.* (1990) obtained a reduction of luminescence at much lower concentrations. The discrepancy may have resulted from different preparation methods of the bacteria for the two different commercial test kits.

The results obtained from both toxic species of dinoflagellates were as expected. If one looks at the STX-equivalent estimated for all of the extracts (Table 3.2), one can see that their concentrations are all above 10 $\mu g.ml^{-1}$ STX eq. (except Extract B of *A. catenella).* As 10 $\mu g.ml^{-1}$ pure STX elicited a toxic response, one would expect that the toxic extracts would elicit similar responses, which they did. Although *A. minutum* caused higher inhibition of luminescence than *A. catenella*, it should be realized that the cell concentrations in the extracts of *A. minutum* were higher than those of *A. catenella.* The results obtained with the filtered media agree with previous toxicity studies performed on these species, although neither of the studies had been performed on luminescent bacteria. Ogata and Kodama (1986) observed that the medium from which the *A. catenella* cells had been filtered was not toxic, whereas the medium from which *A .minutum* cells had been filtered was found to be toxic by Lush and Hallegraeff (1996). The results obtained with the luminescent bacteria test therefore supported the previous results. Extract A of *A. minutum* did not seem to cause greater inhibition than Extract B of *A. minutum.* This was true for both *A. catenella* extracts as well, and can possibly be explained in terms of variation in the toxin content of dinoflagellates at various growth stages of culture (Anderson *et al.* 1990).

The results obtained for *P. micans* and *S. trochoidea* were contrary to expectation. Although the filtered medium of *P. micans* caused less than 20% inhibition of luminescence (and was therefore not considered toxic), the cell extracts of *P. micans* and *S. trochoidea* caused inhibition above 20% (above 80% for *S. trochoidea*). These dinoflagellate species are both considered to be non-toxic (Demaret *et al.* 1995), so it is likely that something else was causing the inhibition of luminescence. As these cells were sonicated, it was possible that some other substances from the dinoflagellate cells (e.g. proteins) were having some negative effect on the bacteria.

The LUMIStox bacterial test could be useful as a method to detect PSP toxins in water samples if certain conditions were met. Firstly, the problem with false negatives would have to be overcome, and secondly, the cell concentrations would have to be sufficiently high to elicit a response from the LUMIStox bacteria. Results observed by Bruno *et al.* (1990) seem to support this. The cell concentration with which these authors were working was ~ $2x10^7$ cells.l^{-1}, and this was also concentrated by centrifuging the cells. The algal cell concentrations used in this study (0.7 - $3x10^7$ cells.l^{-1}) were in the same range as those used by Bruno *et al.* (1990). Although this cell concentration is high compared to the concentration needed to cause restrictions on shell fisheries, this problem can be overcome by concentrating water samples containing low cell concentrations. Although the LUMIStox kit is not a very sensitive method, if the above mentioned problems are overcome, it could still be useful in detecting PSP toxins in water samples.

The preliminary studies performed on mussel samples were to establish if this test would be suitable to detect toxins in mussels, specifically PSP toxins, as it had already been established that this test was unsuitable for detecting OA. The method that is normally used to extract PSP

toxins from mussel tissue makes use of an acid, yet the acidification process itself causes a high level of inhibition of luminescence. One of the prerequisites for the LUMIStox test is that the pH of the sample must be similar to that of the marine environment. Unfortunately, saxitoxin is inactivated at an alkaline pH (Adams and Miescier 1980), so it was not advisable to neutralise the samples and thereby possibly decrease the toxicity of the extract. Nevertheless, the neutralization of the acid extract with sodium hydroxide, (even at the lowest concentration) still caused an inhibition of 45%, implying that it was not necessarily the acid that was responsible for this inhibition. Indeed, the extract made with sea water alone caused a 45% inhibition at its lowest concentration. All three extractions had been boiled, so proteins would have been destroyed. Some other substance in the mussel extract was causing inhibition of luminescence. This factor could be one or more of any number of things, e.g. particulate matter and colouring (Vasseur *et al.* 1984), nutrients, catabolite, and oxygen levels (Nealson and Hastings 1979, Grabert and Kossler 1996), or fatty acids (Arzul *et al.* 1995).

In hindsite, the extracts could have been further diluted until the extract did not cause inhibition anymore, but at the time it was thought that, if these uncontaminated mussel extract had to be diluted out too much, the method would become unsuitable, as any toxin present in contaminated samples would not be detected in very dilute extracts as the toxin would then only be present in very minute amounts.

To definitively conclude that the luminescent bacteria assay is unsuitable as a test method for PSP toxins in mussel tissue would require extensive further studies. Therefore the LUMIStox kit is at this stage unsuitable for detecting PSP toxins in mussel samples. Regarding the

sensitivity of the LUMIStox kit, Hoke *et al.* (1992) have stated that the rationale for the use of bacterial assays is based on the fact that the toxin to be tested has a non-specific mode of action. Okadaic acid and Saxitoxin both have relatively specific modes of action and therefore the LUMIStox kit would appear to be unsuitable for the purpose of detecting these pure toxins. However, as a method for detecting PSP toxins in water samples, the LUMIStox kit is able to detect toxicity, although the results are unspecific and the reduction in luminescence can be caused by substances and factors other than dinoflagellate toxins, as seen with non-toxic samples of *S. trochoidea*. This makes the method inappropriate, as these sorts of factors will always be present in unknown samples.

3.5 APPENDIX

Recipe for Seawater Complete Broth (SWC):

Seawater	750 ml
Distilled water	250 ml
Glycerol	3 ml
Peptone	5.0 g
Yeast extract	0.5 g
Agar (if required)	2% (2 $g.ml^{-1}$)

4. THE ARTOX KIT (*ARTEMIA* LARVA) AS A BIOASSAY FOR THE DETECTION OF PSP TOXINS

4.1 INTRODUCTION

Research involving the use of the brine shrimp *Artemia salina* in toxicological studies was first reported in detail by Michael *et al.* (1956). The potential of *Artemia* for aquatic toxicological research and applications has been explored since 1975 at the Laboratory for Biological Research in Aquatic Pollution at the State University of Ghent in Belgium (Sorgeloos *et al.* 1978). In 1980 an experimental protocol was established for a short-term toxicity test using *Artemia* nauplii, and in 1981 this Artemia Reference Centre -test (ARC-test) was commercialised (Persoone and Wells 1987). The ARC-test was rigorously investigated during an extensive intercalibration exercise involving 80 European and American laboratories, and found to be satisfactory (Persoone and Vanhaecke 1981). It is based on the determination of the 24 h LC_{50} (concentration where 50% of the nauplii die) on instar II - III nauplii of a specific *Artemia* strain (Vanhaecke and Persoone 1984).

For practical reasons, species like *Artemia,* which have a cryptobiatic (cyst) stage during their life cycle are most suited for the development of a standard bioassay. The permanent availability of resting eggs from which larvae can be obtained, means that there is no need for the maintenance of live stock cultures, the test can be carried out wherever and whenever needed, and sufficient organisms of the same age and physiological condition are always available

(Vanhaecke and Persoone 1984). These characteristics make *Artemia* ideal for reliable and simple testing on a routine basis (Vanhaecke *et al.* 1980), and it has been used to test a wide variety of substances (Table 4.1).

Table 4.1 Substances that have been tested using the brine shrimp assay

SUBSTANCE TESTED	REFERENCE
waste water and marine pollutants	Foster and Tullis 1985
mycotoxins	Bijl *et al.* 1981
petroleum products	Foster and Tullis 1984
food dyes and compounds in aquatic herbicides	Browne 1980
antifouling paints for ships	Miyauti 1986
medicinal plants	Lewis 1995
marine flagellates	Edvardsen *et al.* 1996
dinoflagellates	Medlyn 1980; Lush and Hallegraeff 1996; Demaret *et al.* 1995
pharmacological drugs	Du Plooy 1997

Artemia larvae have been used to detect various phycotoxins. Toxicity levels in cells of the marine flagellate *Chrysochromulina polylepis* were used as a means of distinguishing between two cell types of the same strain (Edvardsen *et al.* 1996), and Medlyn (1980) studied the susceptibility of adult *Artemia* (not nauplii, as used in this study) to breve toxins in the dinoflagellate *G. breve.* The *Artemia* test has also successfully been used to detect toxins in the PSP producing dinoflagellates, *A. minutum* and *A. tamarense* (Demaret *et al.* 1995, Lush and Hallegraeff 1996).

A research team headed by Prof. Persoone at the State University of Ghent, Belgium, has developed a screening bioassay for marine and estuarine waters, called the "Artoxkit". It is in quick-kit form and contains *Artemia* cysts which are hatched on demand within 24 h.

This study examined the feasibility of using the Artoxkit to detect toxic phytoplankton in water samples, as well as phycotoxins in mussels. Two PSP producing dinoflagellates, *A. minutum* and *A. catenella,* and two non toxic dinoflagellates *P. micans* and *S. trochoidea,* as well as three different mussel extracts were used to assess the suitability of this test kit.

4.2 METHODS AND MATERIALS

The method was performed according to the manual provided with the "Artoxkit".

4.2.1 HATCHING THE *ARTEMIA* CYSTS

The brine shrimp cysts that were provided in the kit, were emptied into a petri dish, and immersed in 12 ml of standard seawater. The dish was then covered, exposed to light for 1 h, and incubated at 25 ^{0}C in darkness for a further 24 h.

The condition of the larvae was examined under a dissecting microscope (10x magnification) and the healthy hatched larvae (instar I) were transferred using a micropipet into a second petri dish containing 12 ml of standard seawater, and then covered. Since the nauplii are positively phototactic, they could be more easily harvested by focusing a light source on one side of the

petridish. The nauplii collect around the light, and could therefore be picked out more easily. The second petri dish was incubated for a further 24 hours in the dark at 25 ^{0}C.

4.2.2 PREPARATION OF THE TEST MATERIAL

The algal extracts, Saxitoxin standard, 3,5 Dichlorophenol and mussel extracts were prepared in the same way as outlined in chapter 3 (section 3.2.1). Dilution series were made of the test material, and each dilution was tested in triplicate. Tests were carried out in a 24 well test plate (Figure 4.1)

4.2.3 ADDING THE LARVAE (see Figure 4.1)

A 1 ml aliquot of seawater was transferred into the first column of the test plate, and 1 ml of each test substance was added to the remaining five columns, progressing from low to high concentrations in columns 2 to 6. Approximately 50 second or third stage larvae were transferred with a micropipet from the petri dish to row D of the test plate. This was to rinse the animals to prevent dilution of the substances in the subsequent wells. Ten larvae were then transferred from the wells in row D to each well in the corresponding column above it. The test plate was covered with Parafilm and incubated at 25 ^{0}C in the dark. After 24 h, the dead larvae were counted in each test well and the percentage mortality calculated. Larvae were considered dead if they did not exhibit any internal or external movement in 10 sec of observation. If the mortality in the control column (seawater) exceeded 10%, the test was considered invalid.

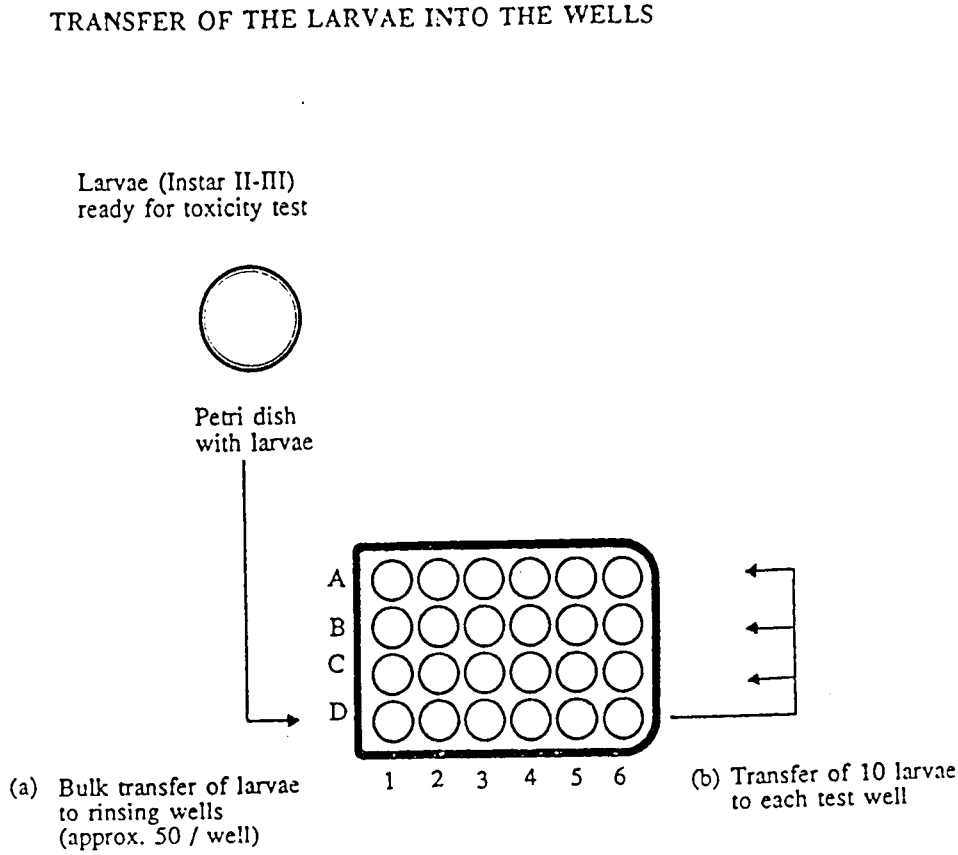


Figure 4.1 Diagram showing transfer of nauplii into test wells

4.2.4 CONTROL TEST

A control test using potassium dichromate ($K_2Cr_2O_7$) was carried out to assess the test sensitivity as specified in the kit manual. The $K_2Cr_2O_7$ was dissolved in distilled water to make a 1 $g.l^{-1}$

stock solution. A 5 stage dilution series was then made with standard seawater, ranging from 10 $mg.l^{-1}$ to 100 $mg.l^{-1}$. These dilutions should cause between 20 and 80% mortality for the kit to be valid. Each dilution was tested in triplicate. The EC_{50} (concentration where 50% mortality occurs) should be within 95% of the limits stipulated on the specification sheet of the test kit.

4.2.5 STATISTICAL ANALYSIS

To determine if there was a significant difference in mortality between the different treatments, or between the treatment and the control at any one dilution, data were analysed using Student T tests (Zar 1983). The results of these tests are indicated (**indicating significant difference) on the appropriate figures.

4.3 RESULTS

Control test: $K_2Cr_2O_7$

Mortalities in the control test ranged from 80% at the highest concentration to 20% at the lowest concentration (Figure 4.2). The LC_{50} approximated 40 $mg.l^{-1}$, which was in the range stipulated on the specification sheet for the kit.

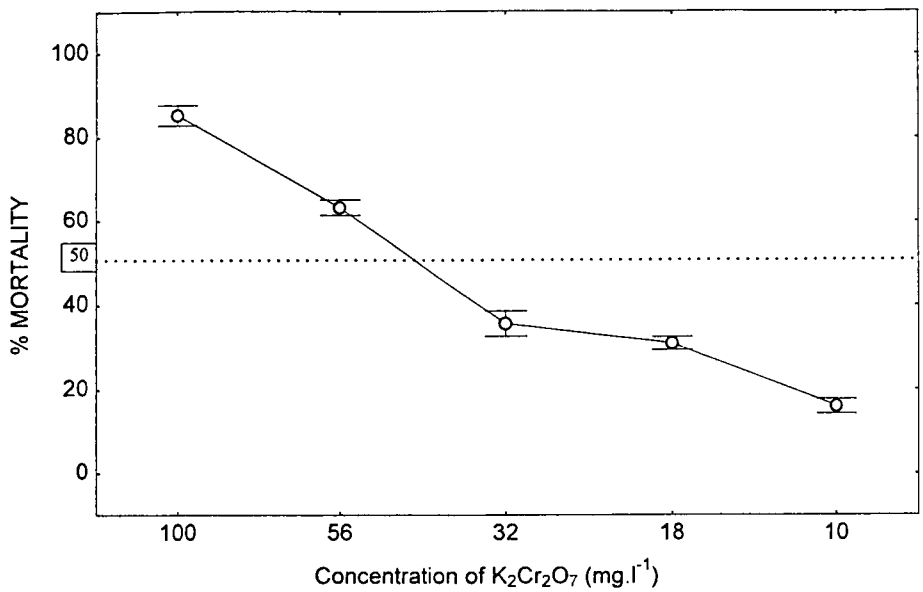


Figure 4.2 Artox kit: Mortality caused by $K_2Cr_2O_7$ (each point represents the mean of 3 replicates ±SE)

Reference chemical: 3,5 Dichlorophenol

This reference chemical is considered hazardous and it was therefore used as an additional control test on the *Artemia* larvae. There was 100% mortality at the highest concentration of 24 mg.l^{-1}, and thereafter a decrease in mortality was noted with a decrease in concentration. The EC_{50} was observed at ~2 mg.l^{-1} (Figure 4.3).

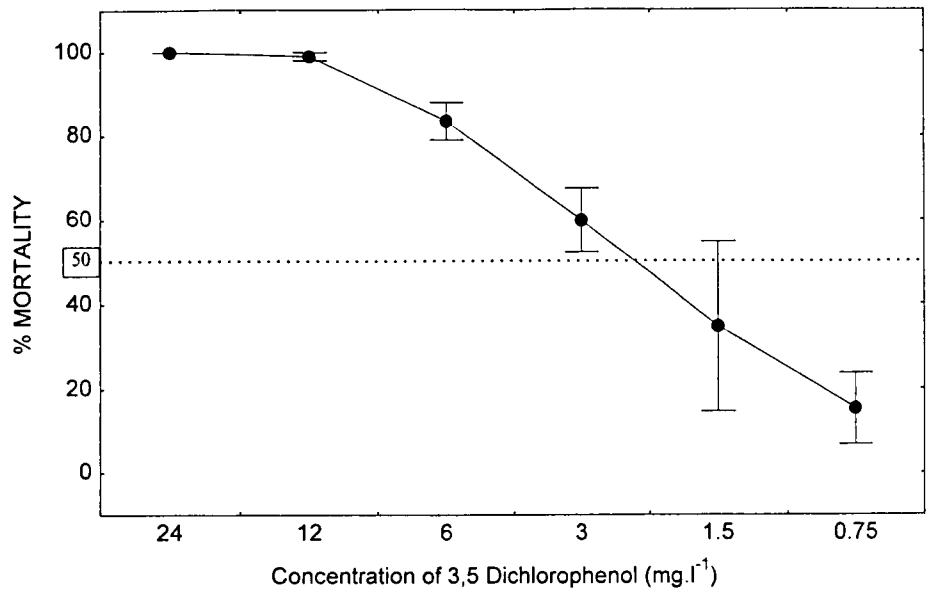


Figure 4.3 Artox kit: Mortality caused by 3,5 Dichlorophenol (each point represents the mean of 3 replicates ±SE)

Saxitoxin standard:

Although the STX caused 70% mortality at 20 $\mu g.ml^{-1}$, the AcOH control caused even greater mortality (Figure 4.4). The percentage mortality caused by STX and AcOH at concentrations < 10 $\mu g.ml^{-1}$ was considerably reduced (~10%) and values were not significantly different from each other. It seems that mortality of the *Artemia* was the result of the AcOH and not necessarily of the STX. These results show that *Artemia* larva are sensitive to acid, and therefore any mortality that was possibly being caused by STX was masked.

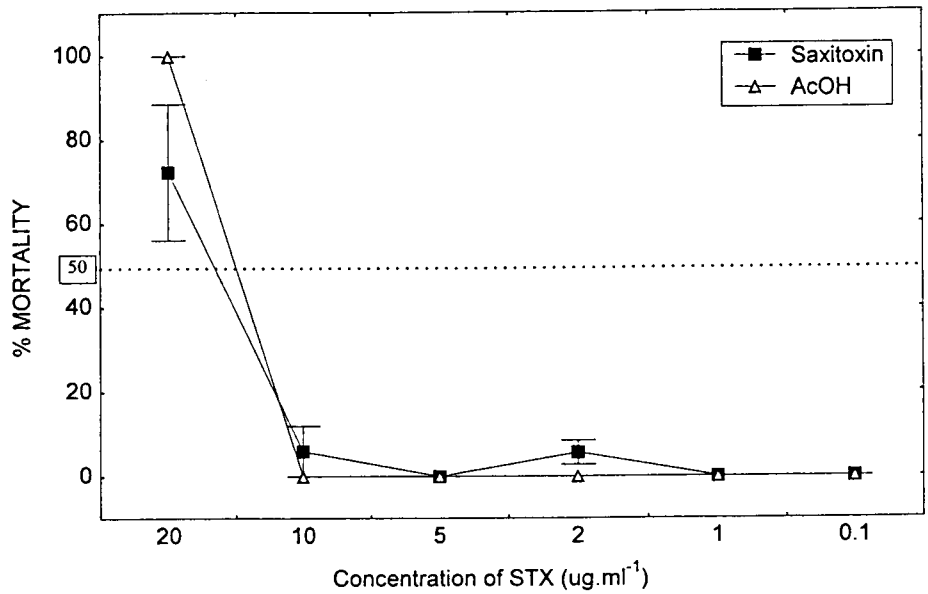


Figure 4.4 Artox kit: Mortality caused by STX standard and AcOH (control) (each point represents the mean of 3 replicates ±SE)

Algal extracts:

Alexandrium minutum:

Extract A ($3x10^7$cells.l^{-1}; ~ 30 µg.ml^{-1}STXeq.) caused 90% mortality, which decreased as the concentration decreased (Figure 4.5). Extract B ($1.9x10^7$cells.l^{-1} ; ~ 19 µg.ml^{-1} STXeq.) caused a slightly lower mortality of *Artemia* (~ 80%) which decreased considerably with the decrease in concentration (Figure 4.6). The filtered medium initially seemed to cause a higher mortality than the cell extract, which would support the theory that *A. minutum* excretes toxins into the

culture medium (Lush and Hallegraeff 1996). Having said that, it was established that there was no significant difference between mortalities caused by the cell extract of Extract B and the filtered medium.

Alexandrium catenella:

Extract A ($1.1x10^7$cells.l^{-1}; ~ 11 μg.ml^{-1} STXeq.) caused 40% mortality (Figure 4.7), whereas Extract B ($0.7x10^7$cells.l^{-1}; ~ 7 μg.ml^{-1} STXeq.). caused a lower mortality of 20% (Figure 4.8). Variation between replicates was large. Although the filtered medium appeared to have less of an affect on *Artemia* survival than the cell extract, the difference was not significant. Both extracts A and B of *A. catenella* caused a lower mortality than observed for the *A. minutum* extracts, which was expected, given that the estimated toxin concentration owing to the lower cell concentration was lower in both *A. catenella* cultures.

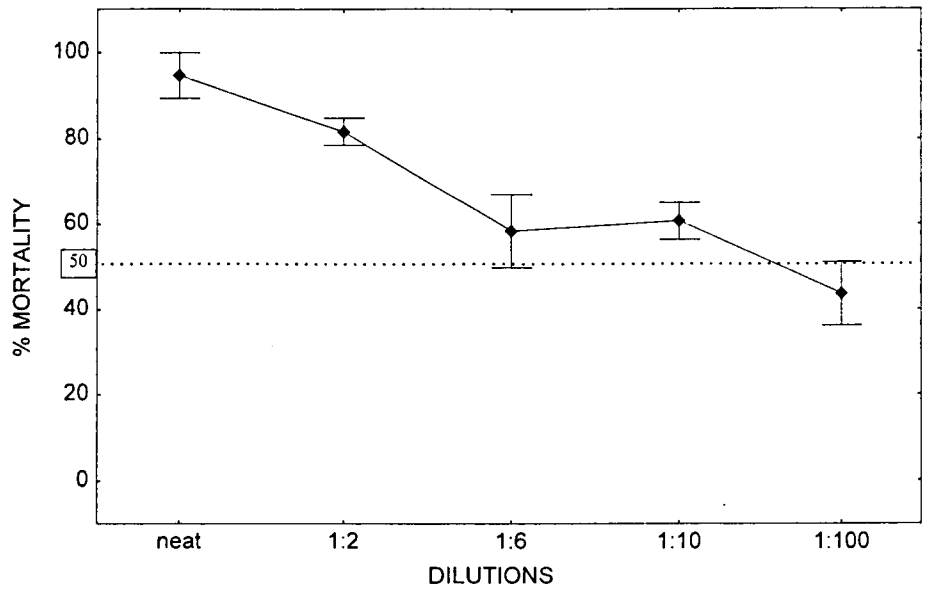


Figure 4.5 Artox kit: Mortality caused by *Alexandrium minutum* Extract A (neat" = $3x10^7$ cells.l^{-1}; "~ 30 $\mu g.ml^{-1}$ STXeq.; each point represents the mean of 3 replicates ±SE)

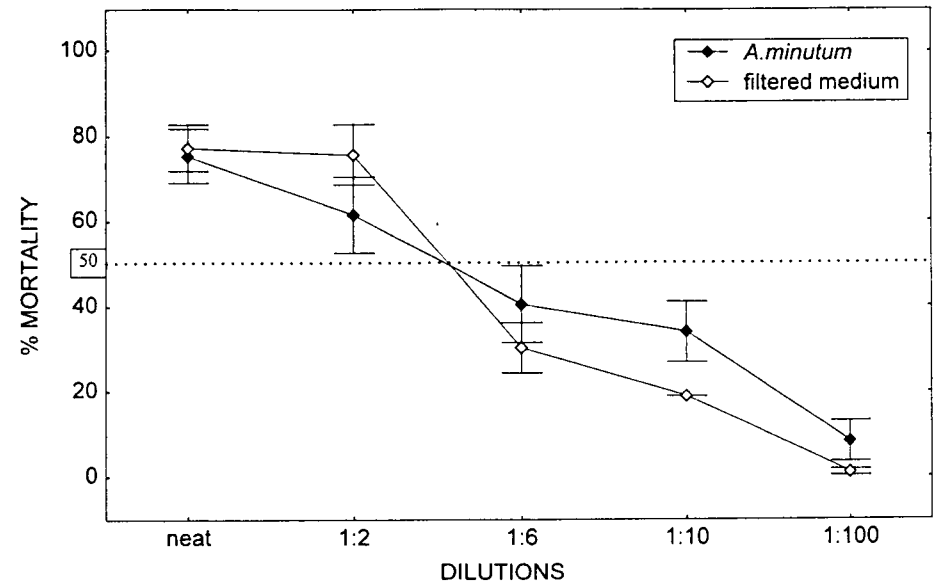


Figure 4.6 Artox kit: Mortality caused by *Alexandrium minutum* Extract B and filtered medium ("neat"= $1.9x10^7$ cells.l^{-1}; ~ 19 $\mu g.ml^{-1}$ STXeq.; each point represent the mean of 4 replicates ±SE)

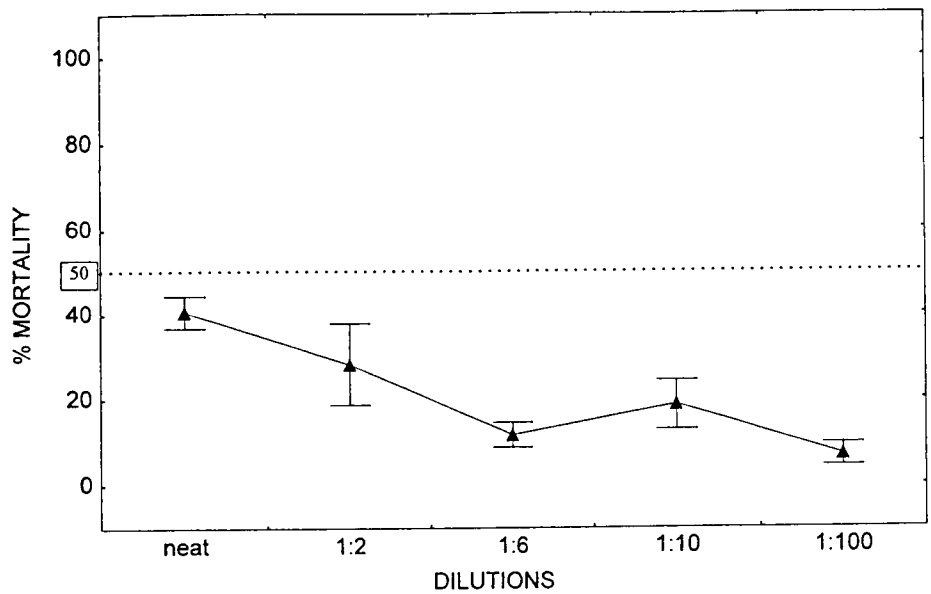


Figure 4.7 Artox kit: Mortality caused by *Alexandrium catenella* Extract A ("neat" = $1.1x10^7$cells.l^{-1}; ~ 11 µg.ml^{-1} STXeq.; each point represents the mean of 3 replicates ±SE)

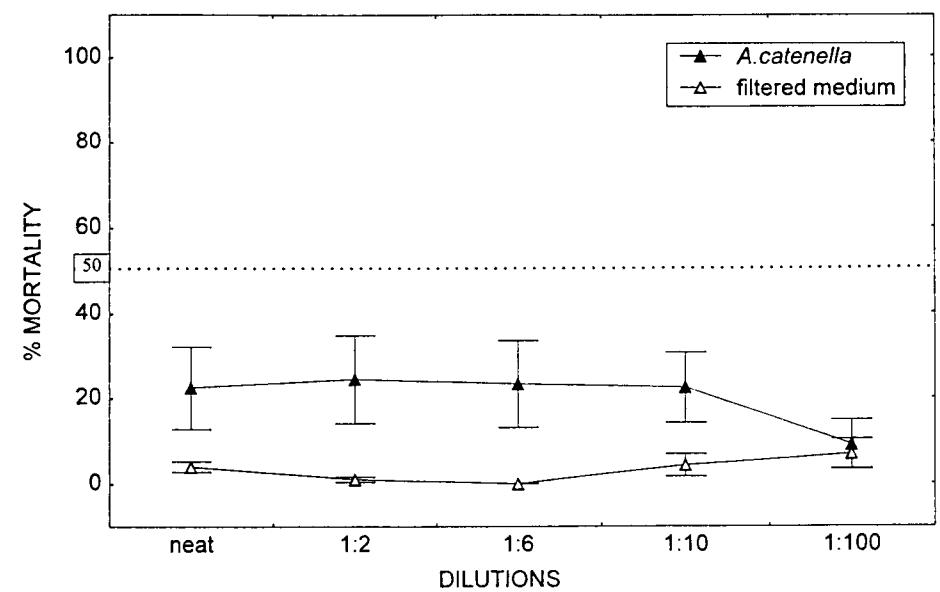


Figure 4.8 Artox kit: Mortality caused by *Alexandrium catenella* Extract B and filtered medium ("neat" = $0.7x10^7$cells.l^{-1}; ~ 7 µg.ml^{-1} STXeq.; each point represents the mean of 3 replicates ±SE)

Prorocentrum micans:

The percentage mortality caused by Extract A ($3.6x10^6$cells.l^{-1}) and Extract B ($8.1x10^5$cells.l^{-1}) of *P. micans* was relatively low, and the highest mortality observed was ~10% (Figures 4.9 and 4.10). No mortality was caused by the filtered medium, and this was not significantly different (at all dilutions) to results obtained from the cell extract (Figure 4.10). Both extracts of *P. micans* therefore were considered non-toxic to the *Artemia* larvae.

Scrippsiella trochoidea:

Mortality caused by Extract A ($1.9x10^7$cells.l^{-1}) of this species was below 5% at all dilutions (Figure 4.11). The extract was therefore considered to be non-toxic to the *Artemia* larvae.

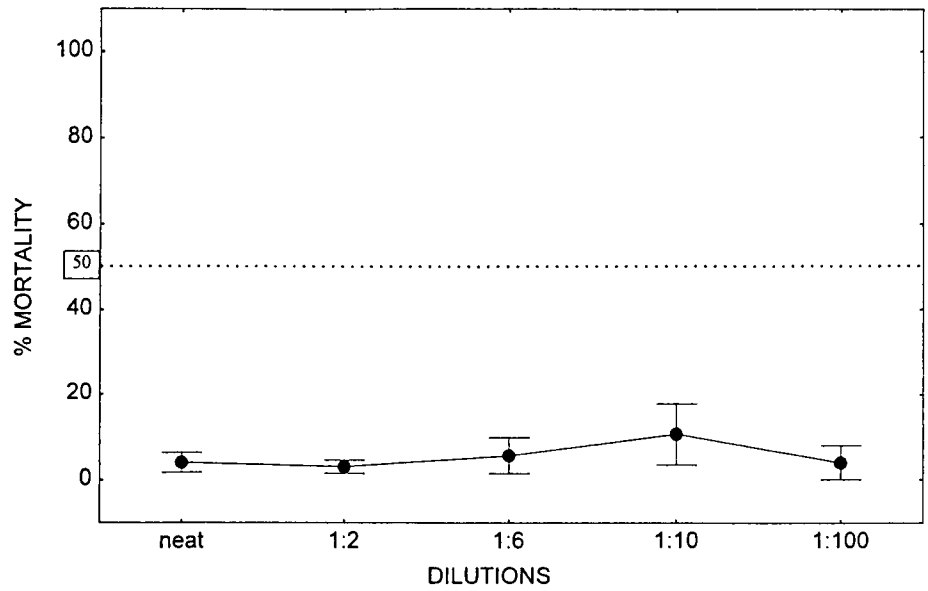


Figure 4.9 Artox kit: Mortality caused by *Prorocentrum micans* Extract A ("neat" = $3.6x10^6$cells.l^{-1} ; each point represent the mean of 3 replicates ±SE)

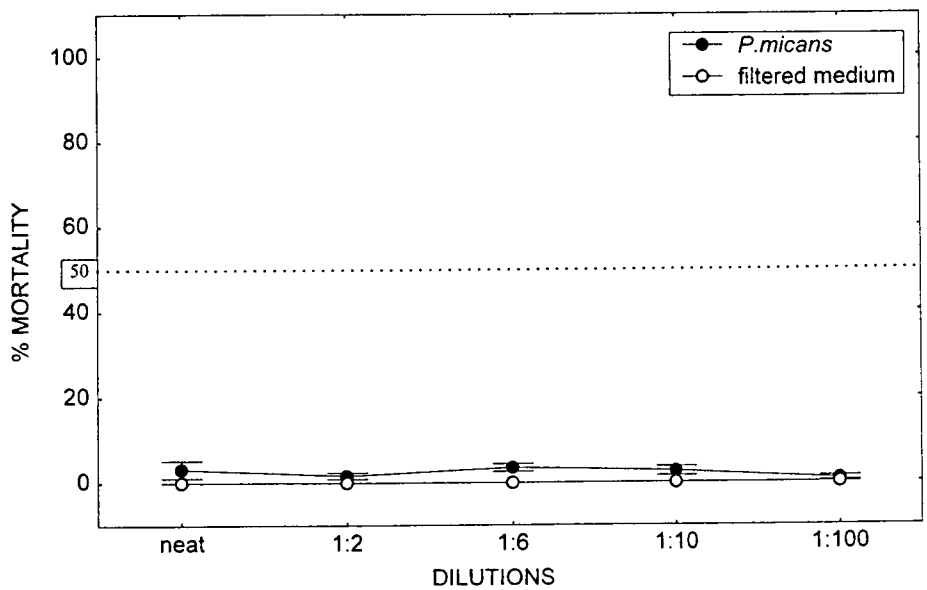


Figure 4.10 Artox kit: Mortality caused by *Prorocentrum micans* Extract B and filtered medium ("neat" = $8.1x10^5$cells.l^{-1}; each point represents 6 replicates ±SE)

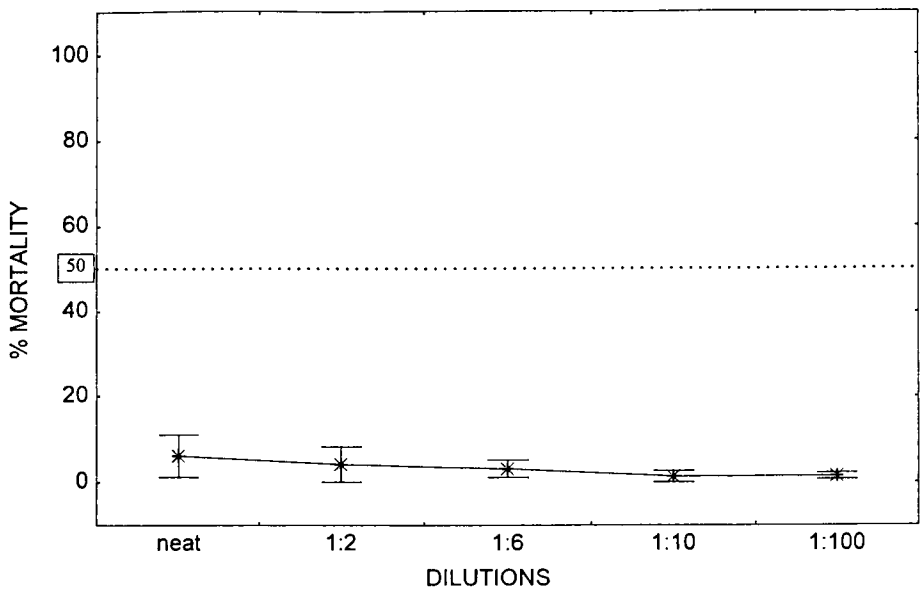


Figure 4.11 Artox kit: Mortality caused by *Scrippsiella trochoidea* Extract A ("neat" = $1.9x10^7$cells.l^{-1}; each point represents the mean of 3 replicates ±SE)

Mussel extracts: (Figure 4.12)

a) with HCl: This extract caused a 100% mortality at all dilutions. As established in a previous experiment, *Artemia* larvae seem to be sensitive to acidic solutions, so it was not surprising that such a high mortality occurred with this extract.

b) with HCl (neutralized with NaOH): This extract caused varying degrees of mortality, ranging from 0% to 40%, although there did not seem to be a trend in these results.

c) with sea water: The neat extract caused 90% mortality, which decreased sigmoidaly with decreasing concentrations.

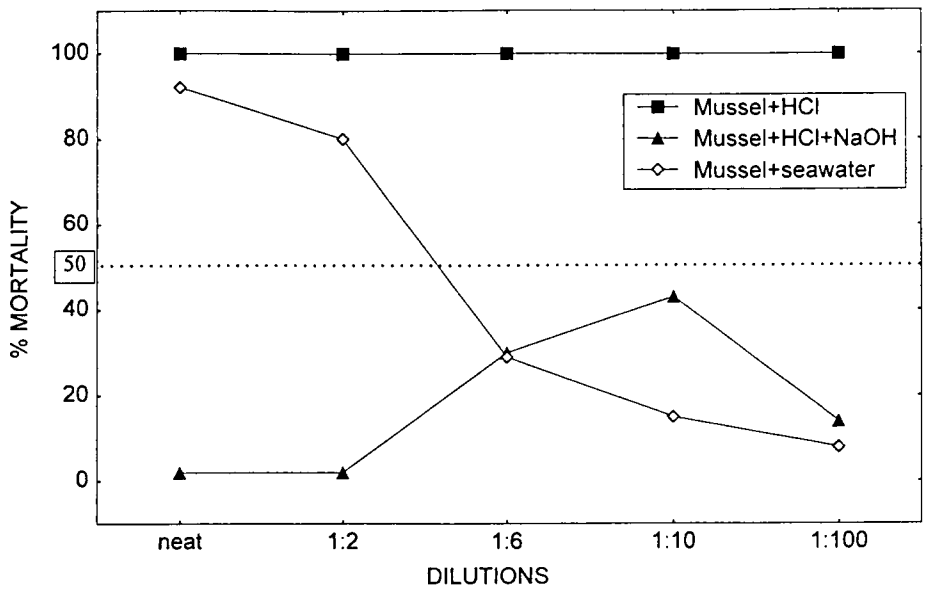


Figure 4.12 Artox kit: Mortality caused by three different mussel extracts (n = 1)

4.4 DISCUSSION

Although $K_2Cr_20_7$ is stipulated in the Artox manual as the substance to be used for a control test, 3,5 Dichlorophenol was also used in this study as a second reference chemical. The results are used in a later comparative study between the three bioassays (chapter 6).

Results obtained with the purified Saxitoxin standard seem surprising. One would expect STX

to induce high mortality, considering that all of the toxic algal extracts caused some mortality. Even Extract B of *A. catenella*, with the lowest cell concentration and therefore lowest approximate toxin concentration (7 $\mu g.ml^{-1}$ STXeq.) caused a mortality of 20%. The STX at a concentration of 10 $\mu g.ml^{-1}$ caused very low mortality at less than 10%. However, the results observed in this test using pure STX were similar to those observed by Lush and Hallegraeff (1996). They found that *Artemia* died within 24 hrs when exposed to PSP dinoflagellates (*A. minutum;* $10^6 cells.l^{-1}$) as well as the cell-free medium. When exposed to pure PSP fractions (GTX 1-4 equivalent to concentrations found in culture) however, no significant mortality was observed when compared to that in the whole cell or cell-free experiments, although the amount of GTX in their study was estimated at lower amounts per cell than STXeq. per cell estimated in this study. Lush and Hallegraeff (1996) suggested that the death of *Artemia* in their study was a consequence of a fast acting toxin distinct from PSP, as pure fractions produced no significant mortalities. Dinoflagellates are not generally known to excrete PSP toxins into their surrounding medium, although *A. tamarense* has been implicated in producing toxins other than the typical PSP neurotoxins (Simonsen *et al.* 1995), and this could also be true of *A. minutum*. Results observed with *A. minutum's* filtered medium in this study would seem to support this suggestion.

The results obtained with the *P. micans* and *S. trochoidea* extracts were similar to results obtained by Demaret *et al.* (1995). The authors also used these species as control (non-toxic) species, and found that, as in this study, neither species induces any mortality of *Artemia* larvae, although the dinoflagellates were not sonicated but used as whole cells in their study.

As expected, the mussels extracted with HCl caused high mortalities owing to the acid extraction.

Mortalities in the neutralized extract and seawater extract were, however, surprising as nothing in the sample should have been toxic to the larvae. A film was observed to have formed over each well in the test plates (possibly caused by fatty substances in the mussel tissue), which could have had something to do with the high mortality rates in each of these experiments. Curtis *et al.* (1974) found that foodstuffs suspected of mycotoxin contamination contained several other substances which interfered with test results, particularly fatty acids which are toxic for *Artemia*. The *Artemia* could alternatively have died due to oxygen starvation, caused by the breakdown of the mussel tissue by bacteria. Whatever the reasons, the Artox kit is obviously not suitable for detecting PSP toxins in mussel samples, unless the samples are treated in some way to remove the substances interfering with the assay.

However, for the detection of PSP toxins in water samples, the Artox test would be more useful, provided the concentration of dinoflagellates in the water samples was sufficiently high.

5. THE SEA URCHIN GAMETE TEST AS A BIOASSAY FOR THE DETECTION OF PSP TOXINS

5.1 INTRODUCTION

Since 1892 a large number of experimental observations have been made on the effects of various substances on the survival and development of the germ cells and embryos of echinoderms (Stearns 1974). Table 5.1 lists some substances that have been tested on the sea urchin.

Table 5.1 Substances that have been tested by means of the sea urchin gamete test

SUBSTANCE TESTED	REFERENCE
Heavy metals	Kobayashi and Fujinaga 1976
Copper sulphate and ammonia products	Kobayashi 1980
Municipal effluents and anthropogenic pollutants	Dinnel *et al.* 1981; Oshida *et al.* 1981
Metabolites synthesized by some green macro algae	Paul and Fenical 1986; Lemee *et al.* 1993
DDT and Dieldrin	Wynberg *et al.* 1989
Effluents from herbicide, textile and fertilizer plants	McGibbon and Moldan 1986
Anti-mitotic cytotoxicity of benthic organisms	Martin and Uriz 1993
Dinoflagellates	Horstman *et al.* 1991

The sea urchin test has a number of important advantages over other bioassays. It is simple and easy to use, and it is fast and highly sensitive. The results are also unambiguous, uniform and highly accurate (Kobayashi 1984).

A number of different aspects of the biology of sea urchin gametes and embryos have been used to quantify environmental stress. These include the degree of binding between the eggs and sperm (Vacquier and Payne 1973), the disturbance of cell proliferation during embryogenesis (Lemee *et al.* 1993), abnormal larval development (Kobayashi 1980), and loss of sperm motility (Paul and Fenical 1986). However, the measurement of fertilization success (as defined by the elevation of a fertilization membrane around the egg) appears to be the simplest analytical technique (McGibbon and Moldan 1986).

Although the sea urchin has been used in toxicity tests of a large variety of substances (Table 5.1), no work has been done on toxic dinoflagellates, except by Horstman *et al.* (1991). The latter study tested water samples that contained a *Gymnodinium* species (associated with NSP) using the sea urchin gamete test. Breve toxins (found in NSP) are neurotoxic, and work in a similar way to the Paralytic shellfish toxins, in that they act on sodium channels. As this test was used to establish toxicity of water samples containing NSP, the sea urchin gamete test was assessed as to its suitability to detect PSP toxins in this study, as the actions of saxitoxin on sea urchin gamets has not yet been studied.

In South Africa, gravid sea urchins can be easily obtained along the western Cape coastline, and this makes the test convenient to use. To establish if the sea urchin gamete test was suitable to detect PSP toxins in water samples, the toxic dinoflagellate species *A. minutum* and *A. catenella* were used. *P. micans* and *S. trochoidea* were used as non-toxic controls. A Saxitoxin standard, the reference chemical 3,5 Dichlorophenol and 3 different mussel extracts were also tested.

5.2 METHODS AND MATERIALS

The substances to be tested were prepared according to the protocols outlined in chapter 3 (section 3.2.1).

Sexually mature sea urchins, *Parechinus angulosus,* were collected between Cape Town and Bloubergstrand on the west coast of South Africa. Animals were transported in seawater to the laboratory where they were maintained in continuously flowing seawater, at an ambient temperature of ~15 ^{0}C. Animals were left to acclimatise for two weeks prior to experimentation. Animals were returned to the sea after three weeks.

Spawning was induced by injecting each of six animals with a 1 ml solution of 0.75 M potassium chloride (KCl) through the peristomial membrane (McFadzen and Bussarawit 1995). Males and females were placed, oral side up, on separate petridishes. The sperm was collected straight from the petridish, and the eggs were washed with fresh seawater onto a 125 µm mesh to remove any debris. Each animal was only spawned once and the gametes were used within 2 h of production.

The test was conducted according to the method of Dinnel and Stober (1985). For experimental purposes, the sperm was diluted (one in ten) with filtered seawater and the eggs were used without dilution. For each toxic extract tested, 1 ml of toxin was put into each of two 20 ml containers together with 9 ml of seawater, and then 20 µl of either sperm or eggs were added. The containers were mixed on a rotary mixer for 10 min, before the toxin and egg mixtures were

combined with toxin and sperm mixtures, and further agitated on the rotary mixer for 10 min. Control containers were made up of seawater and either sperm solution, or eggs. All dilutions were made up in triplicate. Four millilitres of a 5% solution of formalin in seawater was then added to the sperm/egg mixture to fix and preserve the sample prior to analysis.

The presence or absence of a fertilization membrane around the eggs was noted for each of 200 eggs at x100 magnification using a dissecting microscope. The number of successfully fertilized eggs was converted to a percentage and plotted against toxin concentration. The toxin concentration causing 50% inhibition of fertilization is termed the Fertilization Inhibition Dose (FID_{50}) (McGibbon and Moldan 1986). If the degree of fertilization in the control was less than 95%, the experiment was rejected.

STATISTICAL ANALYSIS

To determine if there was a significant difference in fertilization caused by the different treatments, or between the treatment and the control at any one dilution, data were analysed using Student-T tests (Zar 1983). The results of these tests are indicated (**indicating significant difference) on the appropriate figures.

5.3 RESULTS

Reference chemical: 3,5 Dichlorophenol

At concentrations of < 6 $mg.l^{-1}$ of 3,5 Dichlorophenol, the fertilization of eggs was less than 10%

(Figure 5.1). As the concentration of toxicant decreased, the fertilization increased, and the FID_{50} was observed at ~ 3.5 $mg.l^{-1}$.

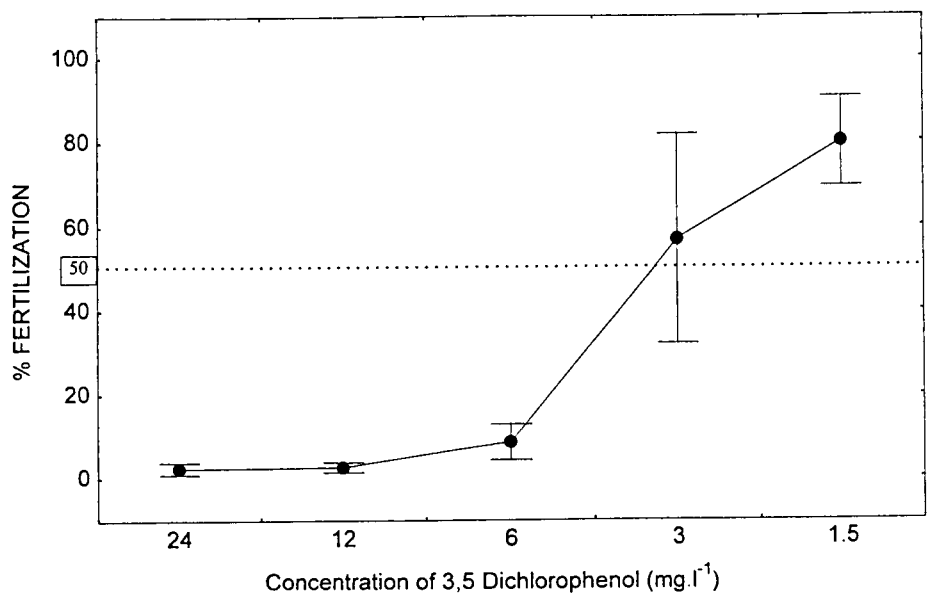


Figure 5.1. Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of 3,5 Dichlorophenol (each point represents the mean of 3 replicates ±SE)

Saxitoxin standard:

The control of AcOH caused no noticeable inhibition of fertilization, with almost 100% fertilization at all dilutions (Figure 5.2). The percentage fertilization when testing the STX standard was significantly lower than the AcOH at all dilutions, and no eggs were fertilized at 10 $\mu g.ml^{-1}$ of STX. The FID_{50} was observed to occur at ~3 $\mu g.ml^{-1}$.

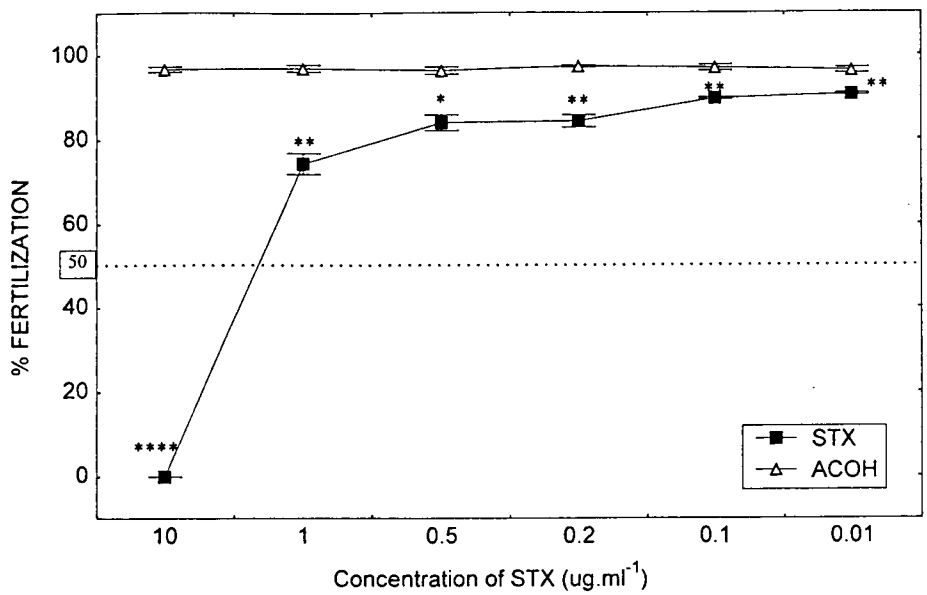


Figure 5.2 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of Saxitoxin standard and AcOH (control)(**** p<0.0001; ** p<0.01; * p<0.05; each point represents the mean of 3 replicates ±SE)

Algal extracts:

Alexandrium minutum:

Extract A ($3x10^7$cells.l^{-1}) and at a 1:2 dilution were relatively toxic to the sea urchin gametes resulting in ~20% fertilization (Figure 5.3). The fertilization increased as the concentration decreased. With Extract B ($1.9x10^7$cells.l^{-1}) the fertilization was higher at around 65% (Figure 5.4). Although the filtered medium of Extract B seemed to cause less fertilization than the cell

extract, there was no significant difference between the two at any of the dilutions tested. At the lowest concentration, fertilization was approximately 90 % and did not have a negative affect on the gametes.

Alexandrium catenella:

Both Extract A ($1.1x10^7 cells.l^{-1}$) and B ($0.7x10^7 cells.l^{-1}$) of *A. catenella* were toxic to the sea urchin gametes as fertilization was reduced to 65 - 70% (Figure 5.5 and 5.6). The filtered medium of Extract B caused only a slight inhibition of fertilization, but when the results were evaluated, there was no significant difference between the cell extract and the filtered medium (Figure 5.6). In both cell extracts, the variation between the replicates was high and was no considered to be reliable.

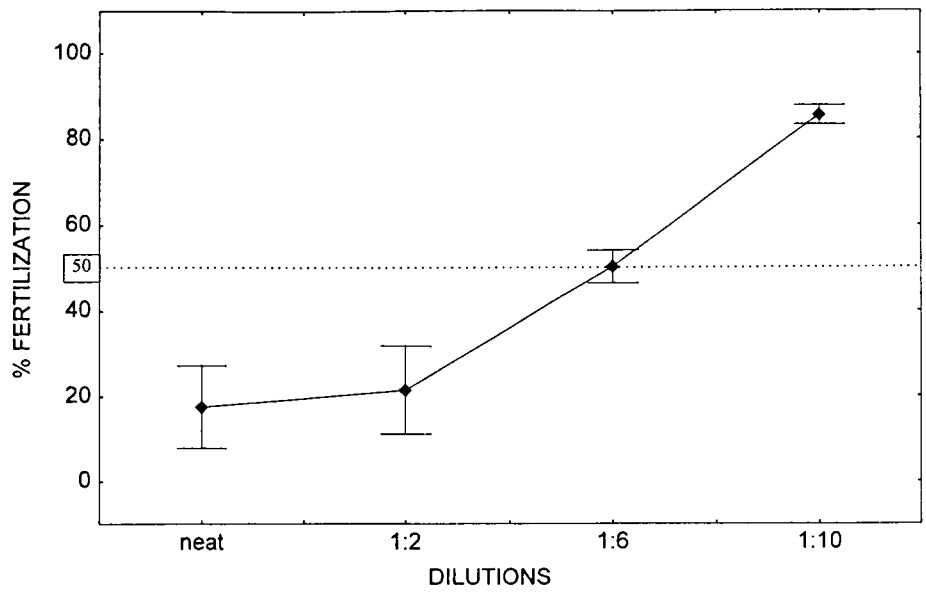


Figure 5.3 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Alexandrium minutum* Extract A ("neat" = $3x10^7$cells.l^{-1}; ~ 30 µg.ml^{-1} STXeq.; each point represents the mean of 6 replicates ±SE)

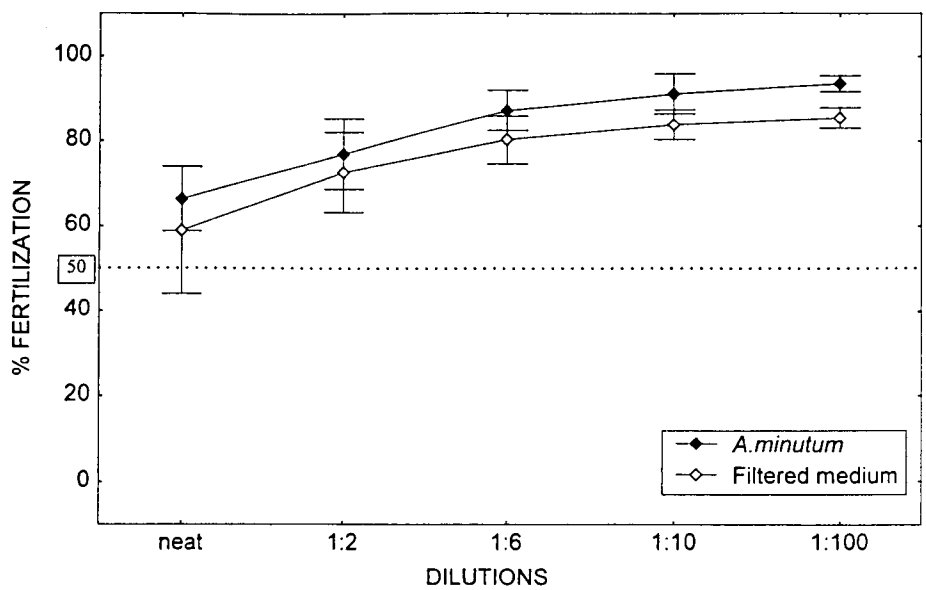


Figure 5.4 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Alexandrium minutum* Extract B ("neat" = $1.9x10^7$cells.l^{-1}; ~ 19 µg.ml^{-1} STXeq.; each point represents the mean of 3 replicates ±SE)

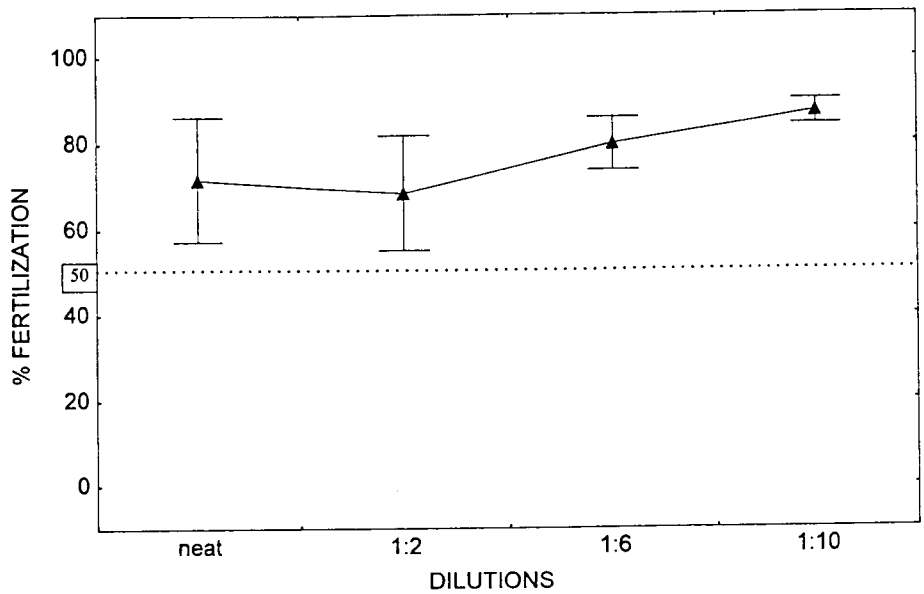


Figure 5.5 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Alexndrium catenella* Extract A ("neat" = $1.1x10^7$cells.l^{-1}; ~ 11 µg.ml^{-1} STXeq.; each point represents the mean of 6 replicates ±SE)

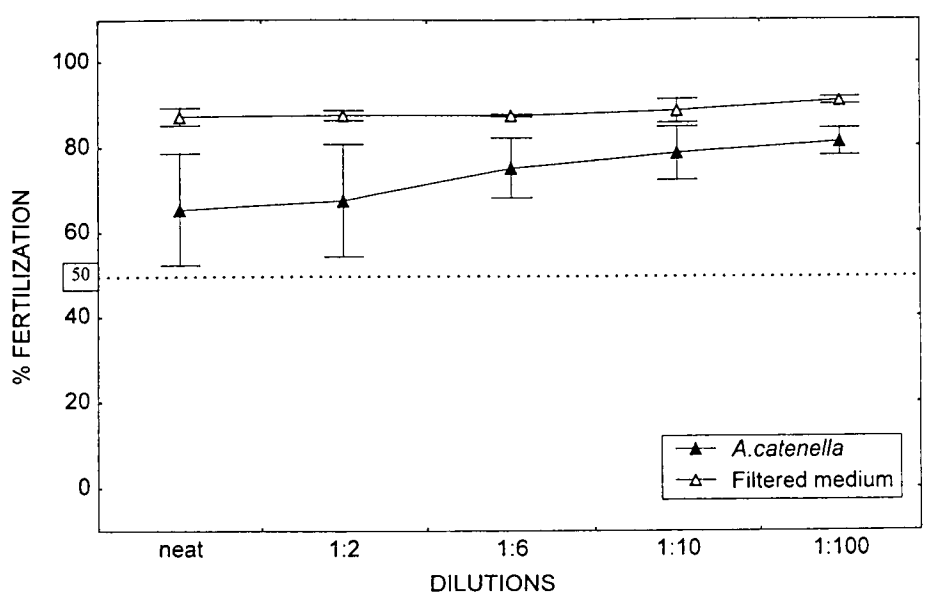


Figure 5.6 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Alexandrium catenella* Extract B ("neat" = $0.7x10^7$cells.l^{-1}; ~ 7 µg.ml^{-1} STXeq.; each point represents the mean of 3 replicates ±SE)

Prorocentrum micans:

Extract A ($3.6x10^6$cells.l^{-1}) of *P. micans* seemed to cause some inhibition of fertilization (20 - 30 % inhibition) at all concentrations, although the variation between replicates was great (Figure 5.7). The results obtained here were surprising, as *P. micans* is considered to be non-toxic (Demaret *et al.* 1995). As the variation between replicates was very high, the results were not considered to be reliable. Extract B ($8.1x10^5$cells.l^{-1}) caused much less inhibition of fertilization, with less variation between replicates. There was no significant difference between the filtered medium and the cell extract and neither were considered to be toxic to the sea urchin gametes at this concentration (Figure 5.8).

Scrippsiella trochoidea:

Extract A had almost no effect on the gametes and 90% fertilization was observed at all dilutions of the extract (Figure 5.9). It was not considered to be toxic to the sea urchin gametes.

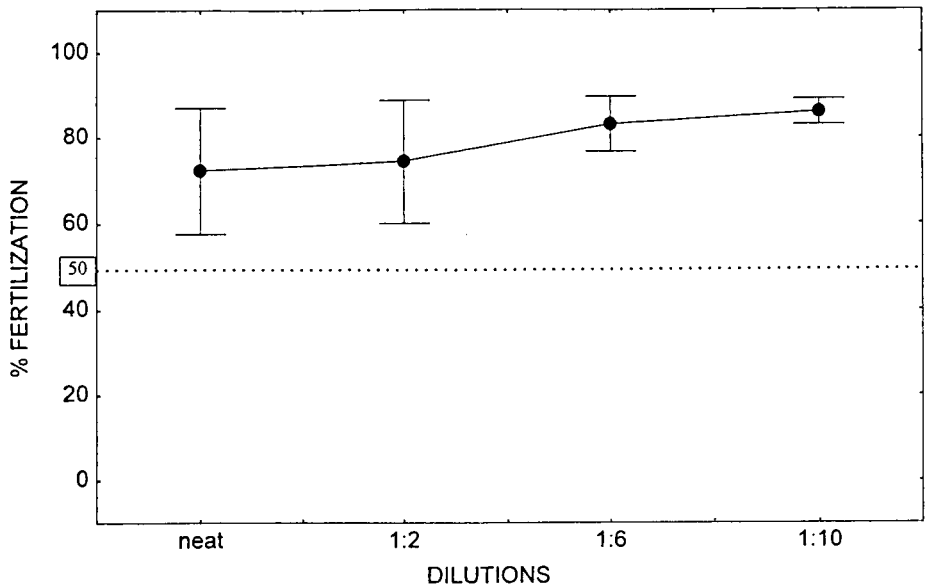


Figure 5.7 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Prorocentrum micans* Extract A ("neat" = $3.6x10^6$cells.l^{-1} ; each point represents the mean of 6 replicates ±SE)

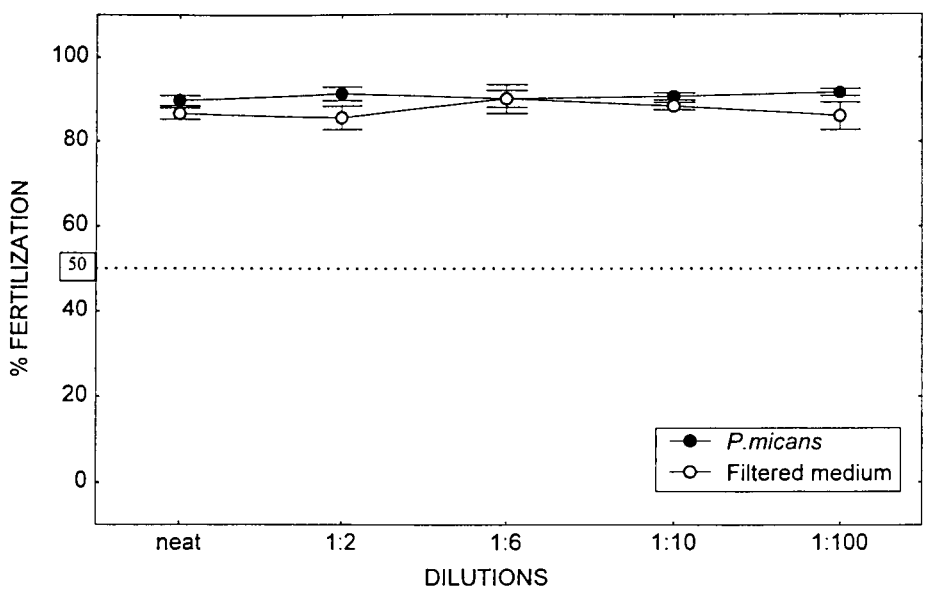


Figure 5.8 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Prorcentrum micans* Extract B and filtered medium ("neat" = $8.1x10^5$cells.l^{-1}; each point represents the mean of 3 replicates ±SE)

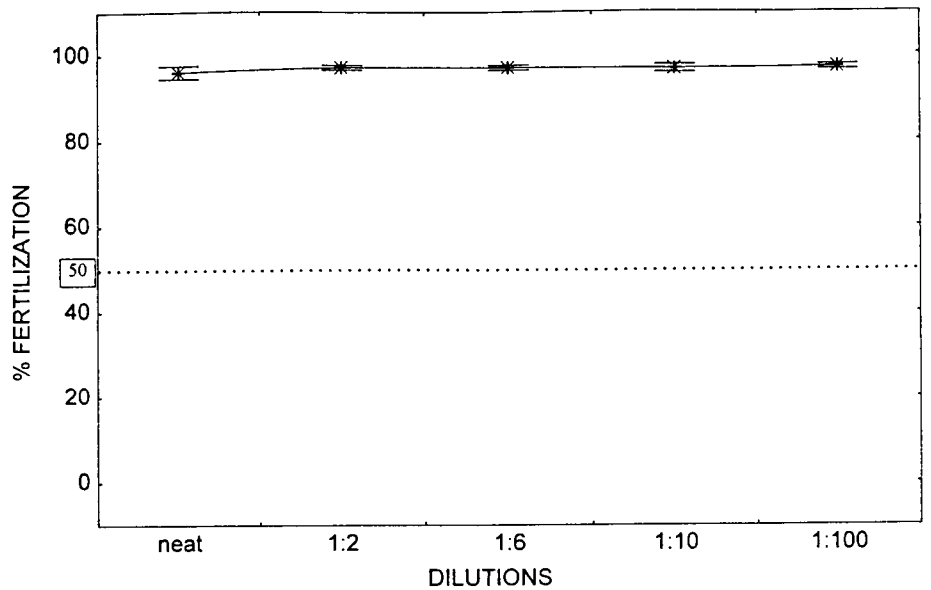


Figure 5.9 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of *Scrippsiella trochoidea* Extract A ("neat" = $1.9x10^7$cells.l^{-1}; each point represents the mean of 3 replicates ±SE)

Mussel extracts: (Figure 5.10)

a) with HCl: Fertilization remained low (~20%) for all dilutions of this extract.

b) with HCl (neutralized with NaOH): Although the acid in the extract had been neutralized, the gametes were obviously still sensitive to some substance in the extract, as the highest fertilization that occurred was only 30 % at a 1:10 dilution.

c) with sea water: When treated with the undiluted extract, fertilization was low (20%), but increased as the extract concentration decreased. Fertilization was 80% at the 1:10 dilution of the mussel extract.

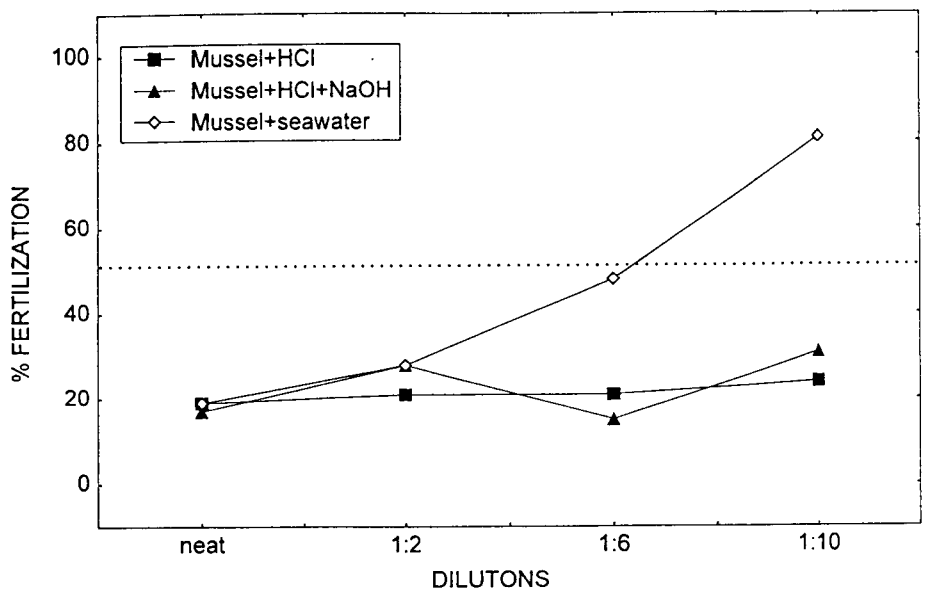


Figure 5.10 Sea urchin gamete test: % Fertilization of *Parechinus angulosus* eggs at different concentrations of three different mussel extracts (n = 1)

5.4 DISCUSSION

The sea urchin gametes proved to be very sensitive to the pure STX standard (10 $\mu g.ml^{-1}$), although the acid itself did not seem to affect them. If one looks at the estimated STX equivalent values given to the extracts of the toxic algal cultures (Table 3.2), one would expect the cultures to cause inhibition of fertilization, as all these toxin values were above 10 $\mu g.ml^{-1}$ STXeq. (except Extract B of *A. catenella*, which was 7 $\mu g.ml^{-1}$ STX eq.). As expected, all the toxic extracts, including Extract B of *A. catenella* caused some inhibition of fertilization.

Some results, however, showed great variation. The high degree of variability can possibly be explained by observations made in previous studies, where maturity of gametes (eggs as well as sperm) have been noted to affect fertilization. Kobayashi (1984) noted that in 'overmature' eggs, the elevation of the fertilization membrane may be retarded and the eggs can maintain the appearance of an unfertilized egg. McGibbon and Moldan (1986) observed that 'immature' sperm gave inconsistent FID_{50} values with a number of toxicants.

Also, in the experiments performed with *P. micans* extracts, a certain degree of toxicity was observed with one of the extracts. Although there is an inhibition of fertilization, this does not necessarily imply the presence of a toxin. Horstman *et al.* (1991) suggest that the normal sea urchin bioassay of 10 min is unsuitable because of the false negatives (i.e. apparent fertilization) that occur. They observed that unfertilized eggs added to some samples, in the absence of sperm, nevertheless developed a fertilization membrane within minutes. They state that the eggs should be observed over a longer time period to establish with certainty the toxicity of the sample. This 'apparent fertilization' could explain the "toxicity" of some of the *P. micans* extracts.

When looking at the results obtained with the mussel extracted with sea water, one would have expected fertilization to be high, as no "toxic" substances were present. However it is possible that the sea urchin gametes were sensitive to something else in the mussel tissue and this was affecting the fertilization. Martin and Uriz (1993) found when studying the effect of benthic organisms on the embryos of the sea urchin, that some extracted substances other than the toxin could contribute to the toxicity of the crude extract (particularly fatty acids). This could possibly explain the sensitivity of the sea urchin gametes to the mussel extracts.

Although the sea urchin gamete test appears unsuitable as a means to detect toxins in mussel samples, it does appear to be useful as a method of detecting PSP in water samples. The cell concentrations used in this study were high when compared to the cell concentrations at which some countries place restrictions on harvesting their shellfish (refer to chapter 1, section 1.3). It would, however be possible for water samples to be concentrated when using this method to detect cells at lower concentrations. Unfortunately the test itself seems to exhibit a few inherent problems, although these are not insurmountable. Firstly, the sea urchins would not always spawn when injected with the KCl solution, and sometimes many animals had to be injected before enough gametes were obtained for experimentation. Secondly, the experiments had to be repeated a number of times for most of the substances tested, because fertilization in the control samples frequently fell to below 95 %, although there seems to be some variation on this value, as Kobayashi (1971) used a value of 85% fertilization for the control.

6. CONCLUSIONS

6.1 AIMS AND OBJECTIVES OF A TOXICITY TEST

Although sophisticated physico-chemical methods for phycotoxin analysis have been developed, there is still a need for simple, and sensitive procedures that can be used with reliance by laboratories for detection of seafood toxicity (Fernandez and Cembella 1995). In many cases, relatively crude extracts have been assayed, without resorting to the extensive clean-up procedures required for instrumental analysis by physico-chemical methods (Cembella *et al.* 1995). Although biological toxicity tests are not always as sensitive as instrumental analysis, they have the advantage that they are often much simpler to perform.

There are two types of biological toxicity tests that each assess responses of an organism to a toxicant. They are the Sublethal Chronic test and the Acute Toxicity test. Long-term, sublethal, or chronic toxicity tests are most commonly designed to provide information on the effect of various concentrations of toxicant on the survival, growth and reproductive success of an organism. In acute toxicity tests, the response of an organism, exposed to various test concentrations for a certain time period, is measured. Response to a toxicant is usually measured as mortality or lethality, or some impairment of function (Buikema *et al.* 1982). All three bioassays used in this study are acute toxicity tests.

Although the information generated from these various tests can be used for a number of different purposes, (e.g. the effects of a toxin on the environment, or a comparison of toxins)

one toxicity test does not serve each purpose equally well. Whatever purpose the toxicity test is used for, ultimately the aim of the test is to obtain accurate, reproducible and repeatable measurements, for reliable and practical toxin detection (Hokama 1993, Boenke 1998). These criteria were used to assess the bioassays in this study.

6.2 DISCUSSION POINTS OF THIS STUDY

6.2.1 HPLC WITH REFERENCE TO THE MOUSE TEST:

The results of instrumental methods of analysis (e.g. HPLC) are difficult to compare directly with those generated from bioassays, as there are so many factors that influence living organisms. However, a comparison of some of the parameters of the tests can be made, for example, the detection limits. Compared to instrumental analytical methods like HPLC, the mouse bioassay is less sensitive (by up to five orders of magnitude) (Fernandez and Cembella 1995), although the mouse bioassay is sufficiently sensitive to detect toxins at concentrations well below the official (legal) PSP limit in shellfish (Figure 6.1).

When comparing the results of local mussel samples analysed by HPLC and the mouse bioassay, the toxin concentrations obtained by HPLC were consistently higher than

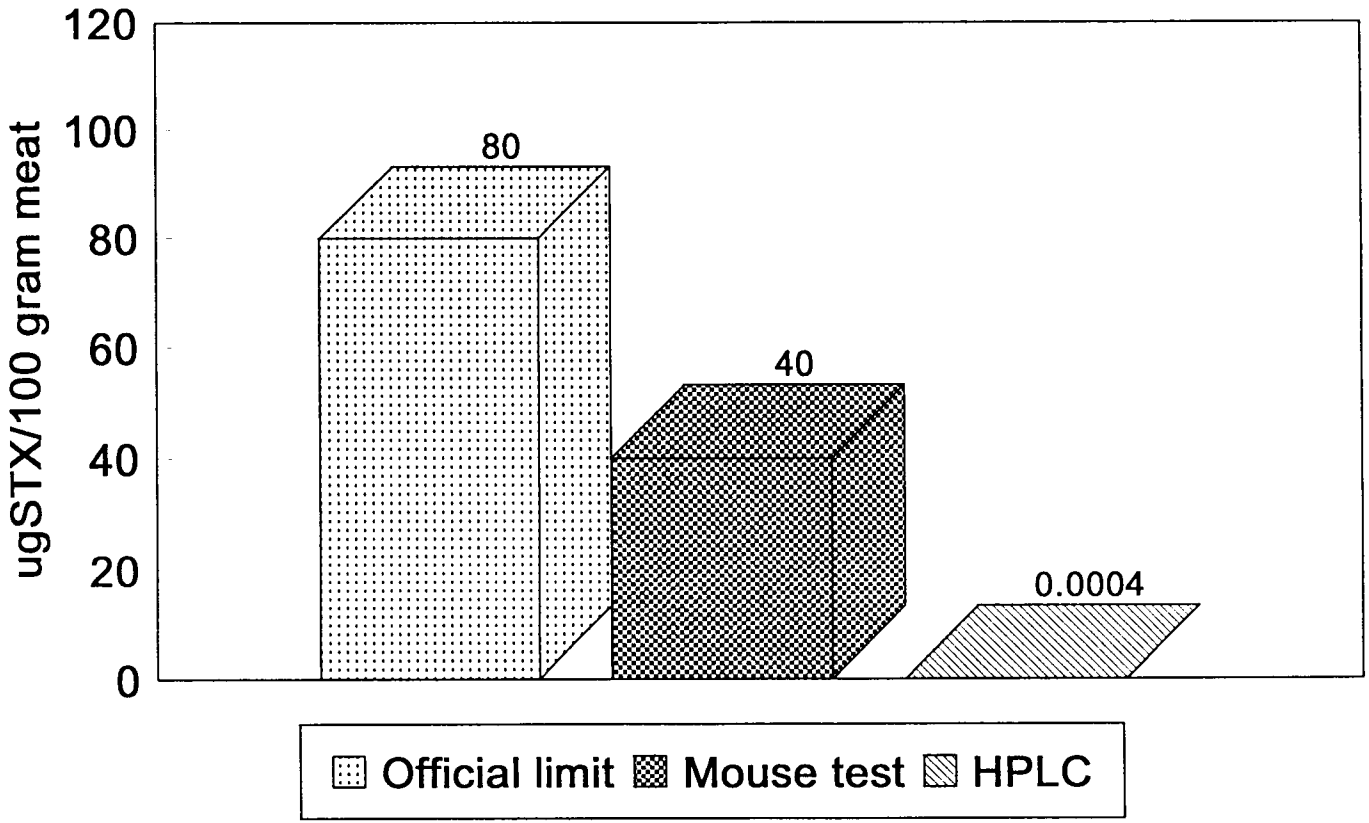


Figure 6.1 Detection limits of HPLC and mouse test for PSP

those obtained by mouse bioassay. Possible reasons for this have already been discussed in chapter 2, but these results support the fact that HPLC is more sensitive than the mouse bioassay. Individual toxic components were identified in the mussels, and components that were present were similar to those in samples from other areas in the world where the toxic dinoflagellate *A. catenella* occurs, although the individual ratios of major components were different. The difference in major and minor components would support the idea that the *A.catenella* species found in South Africa and the species found in Chile are two different strains. HPLC was only performed on toxic mussel samples in this study, and unfortunately no dinoflagellate extracts were available at this time to be tested with the HPLC samples, so no comparison was possible between mussel profiles and dinoflagellate profiles.

6.2.2 ARTOX KIT, LUMIStox KIT AND SEA URCHIN GAMETE TEST:

In order to compare all three bioassays in this chapter, the "% of fertilized eggs" in the sea urchin gamete tests have been converted to "% of unfertilized eggs". High toxicity is therefore represented by a high percentage in all three bioassays, and low toxicity by a low percentage.

A comparison of the sensitivity of the three bioassays can be made by comparing the EC_{50}'s of the three bioassays for each substance tested (Figure 6.2) . Using 3,5 Dichlorophenol the EC_{50} indicates that the sea urchin gamete test was the least sensitive of the three tests, as the EC_{50} occurs at a higher concentration (~ 3.5 $\mu g.ml^{-1}$) than in the other two tests. However, the variation which occurred around this concentration was very high, so there is not much

difference between the sea urchin and the Artox results. One could say that the sensitivity of the two tests is therefore similar at this concentration. With Saxitoxin, the sea urchin gamete test was definitely more sensitive than the others and an EC_{50} was observed at a concentration of ~ 2 $\mu g.ml^{-1}$. The Artox kit and LUMIStox did not even show an EC_{50} at 10 $\mu g.ml^{-1}$ of Saxitoxin.

The difference in sensitivity of the different bioassays to the test substances is important, as it suggests that different organisms respond differently to each substance tested. This implies that, if one needs a general bioassay, not only one substance should be used to measure the sensitivity of a test, but rather a few different substances should be used to obtain a range of responses to determine sensitivity. However, in the case of this study, a more specific bioassay is needed to detect PSP toxins in water samples, and therefore the response of these bioassays specifically to the dinoflagellate extracts is important.

It is difficult to compare the sensitivity of the three bioassays by looking at the results of the dinoflagellates as a whole, because the starting cell concentrations (and therefore toxin concentrations) of the extracts were not identical (Table 3.2). One can however, use each extract individually to compare the response of the bioassays for that particular extract (Figure 6.3). For example, when looking at the response of the bioassays to the *A. minutum* extract, one can see that the Artox kit was most sensitive, while the LUMIStox kit was the least sensitive to this extract. The response to the *A. catenella* extract was however, very similar for all three bioassays. Although the overall response to *A. catenella* was lower than for *A. minutum*, this was to be expected, as the cell concentration of *A. catenella* was lower.

The responses of the bioassays to the non-toxic *P. micans* extract were also similar to each other, although the Artox kit showed the least response. This in itself is a good result, as *P. micans* is not considered to be toxic and should not elicit a "toxic" response. One of the more interesting results is that the non-toxic dinoflagellate, *S. trochoidea*, would appear to be highly toxic if assayed using the LUMIStox kit, but elicited no response from the other two bioassays. The LUMIStox kit is obviously very sensitive to a substance in the *S. trochoidea* extract, and possible reasons for this have been explained in chapter 3, but this emphasizes the fact that the bioassays can respond very differently to different substances.

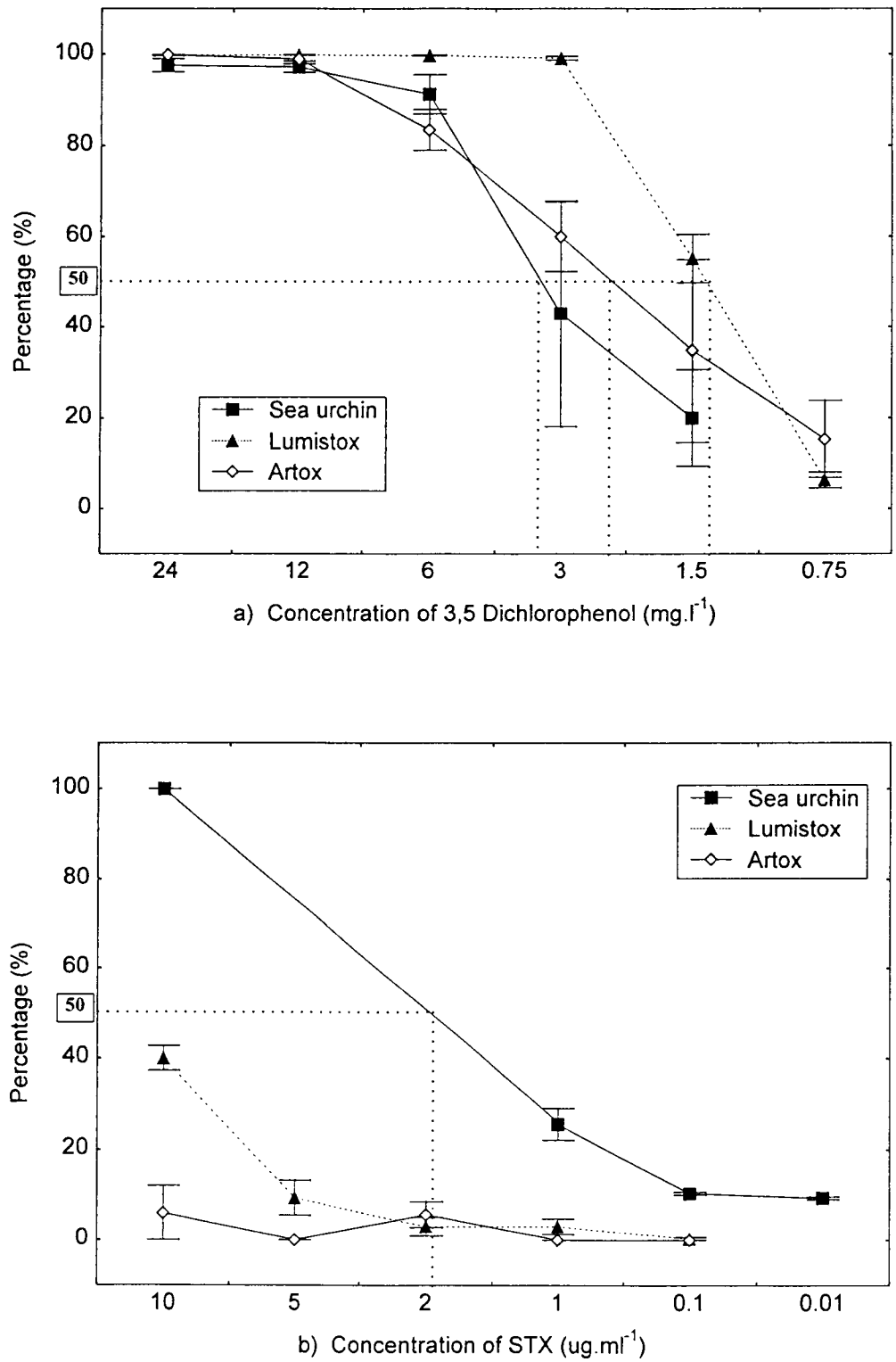


Figure 6.2 Comparison of sensitivity of bioassays using EC_{50}'s of a) 3,5 Dichlorophenol and b) Saxitoxin

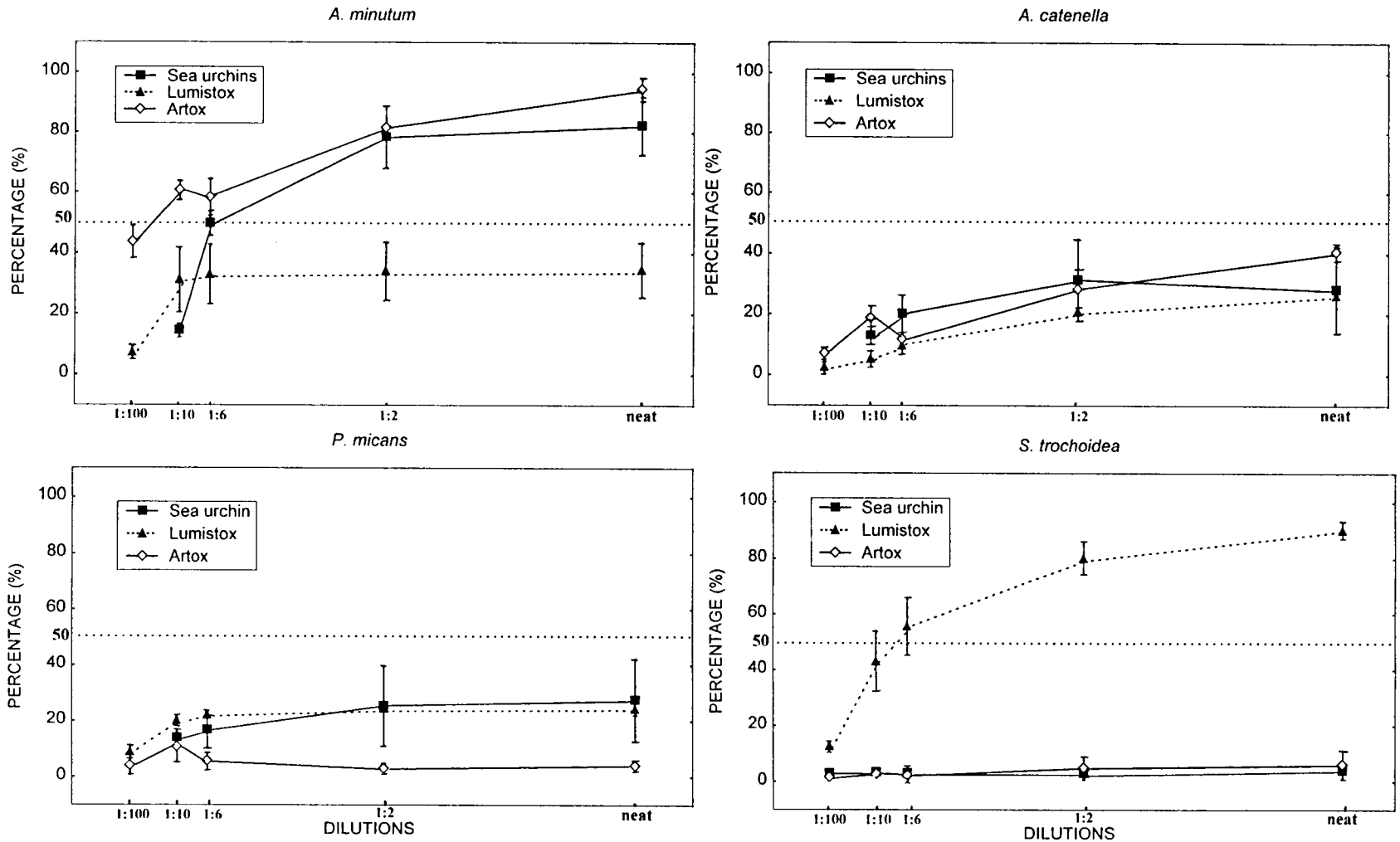


Figure 6.3 Comparison of bioassay responses to the dinoflagellate extracts

From Figure 6.3 one can see that all three bioassays successfully detect toxins in *A. minutum* and *A. catenella*. However, the concentrations of the cells in these extracts are relatively high (refer to chapter 1, section 1.3) and need to be put into perspective. Although cell concentrations this high do occur during "red tides", the cell concentrations that require detection in monitoring programmes are much lower. Detection limits for the three bioassays in this study have been estimated by examining the concentrations at which a toxic response still occurs. The lowest cell concentration at which these dinoflagellates still cause a toxic response with the three bioassays cannot definitively be determined from the results obtained in this study, as the dinoflagellate cells were harvested at different phases of growth, and it is well known that toxicity varies at different stages of a culture (Boyer *et al.* 1987, Boczar *et al.* 1986). One can however get an indication of the range of concentrations where the bioassays would be most sensitive, and an estimated value can provide an idea of the cell concentration above which the bioassays gives results that one can be confident in (Figure 6.4). These estimated detection limits were compared to the suggested cell concentrations at which restrictions are imposed, specifically on shell fisheries, in certain countries. Unfortunately, all bioassay detection limits were well above the suggested limits.

All three bioassays responded in similar ways to the mussel extracts. The bioassays were either sensitive to the acid, or some other substances extracted from the mussel tissue by the acidic treatment. This causes a problem, in that PSP toxins are only stable at low pH's, but the bioassay organisms are sensitive to acid. This makes the bioassays unsuitable for detecting PSP toxins in mussel samples, using the method of extraction specified in this study.

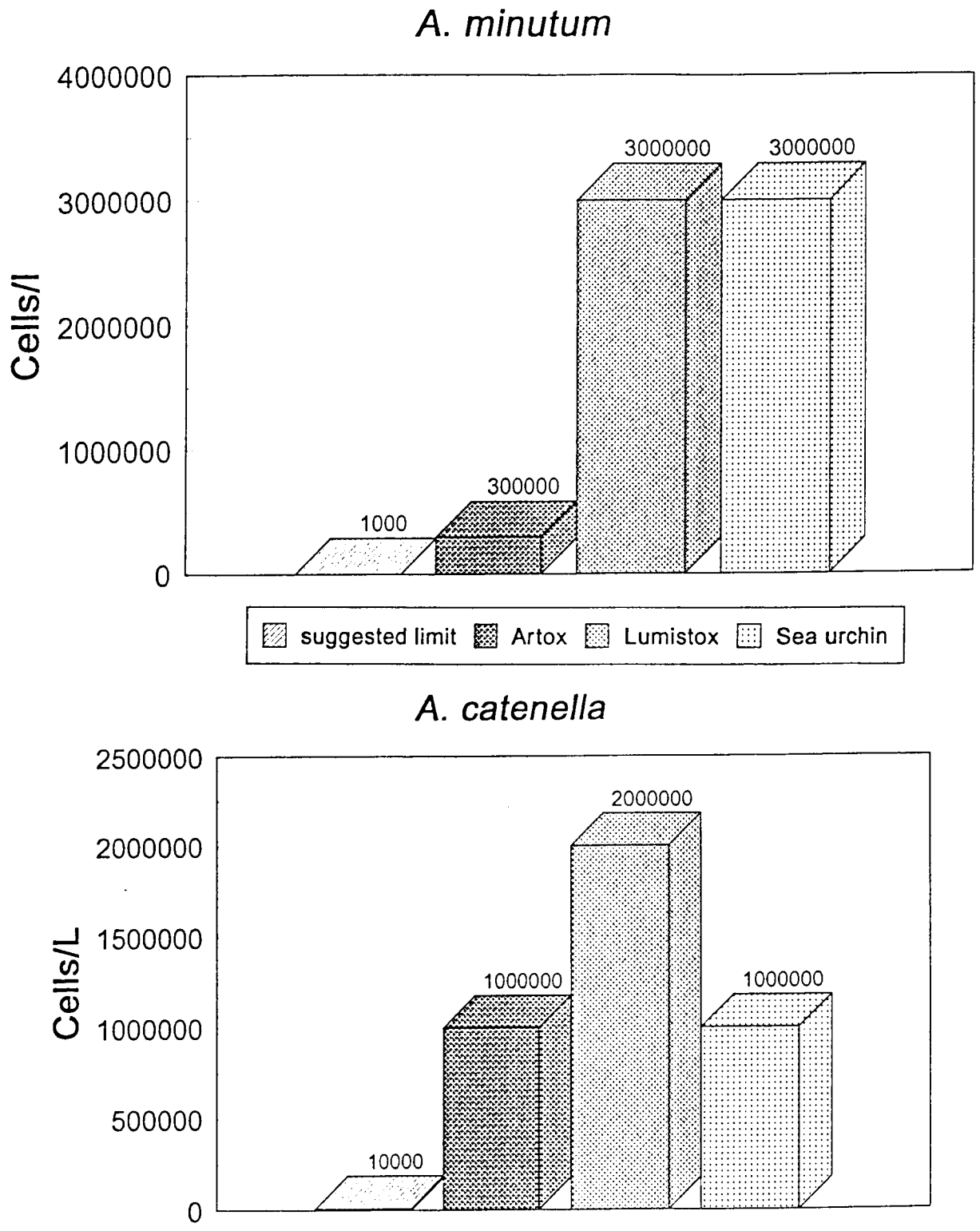


Figure 6.4 Suggested limit for shell fisheries (Andersen 1996) and estimated detection limits for bioassays

6.3 SOME FACTORS THAT CAN INFLUENCE THE RESULTS OF BIOASSAYS

1. Duration of the test: The duration of the test should be as short as possible to avoid toxicant loss, oxygen depletion, or the accumulation of metabolic waste. Both the sea urchin gamete test and the LUMIStox test can be completed within ~ 30 min, so the test duration should not have been an influencing factor on any of the results. The Artox kit, however, is performed over a 24 hour period, and the results could therefore have been influenced by factors like metabolic waste and oxygen depletion.

2. Poor estimation of toxicity: The concentration of the toxin should be verified, in order to avoid inaccurate estimations of toxicity. Quantification and verification is, however, often expensive and impractical. Unfortunately, information was not obtained in this study. The exact toxin concentration of each dinoflagellate extract was not established, and therefore the estimated concentration was not verified. In future studies, verification should be done on extracts by either testing the samples with the mouse bioassay, or by HPLC analysis.

3. Stress: Organisms can be stressed by handling, and this can result in the generation of false results. Although this is difficult to avoid, handling should be minimised where possible. Manuals included in the test kits (Artox and LUMIStox) provided definite handling instructions, which kept the amount of handling to a minimum. Sea urchin eggs were also handled as little as possible.

4. Environmental conditions: Photoperiod, light intensity, temperature, water quality, pH etc. are all important factors that should be standardized for toxicity testing. Test organisms should be cultured or acclimatised to control conditions before being tested. These conditions were all taken into consideration when performing the tests in this study, and were kept as constant as possible to individual requirements of the bioassays.

5. Test organisms: Organisms should be similar in size or age, sex, moulting stage, or health condition. The LUMIStox and Artox kit provide test organisms in the kits, although the harvesting of the *Artemia* larvae should be done with care to ensure animals are of the correct moulting stage. The maturity of the sea urchin gametes could have influenced the results of this test to some extent (as noted in chapter 5).

6. Condition of test chambers or equipment: Dirt or residues from previous substances can influence results. Most equipment used in this study was disposable and provided new with the kits (e.g. glass measuring vials for the LUMIStox kit, and measuring wells for the Artox kit). All other equipment was kept clean.

6.4 ADVANTAGES AND DISADVANTAGES OF THE DIFFERENT TEST METHODS

Table 6.1 summarizes some of the advantages and disadvantages of HPLC, the mouse test and the three bioassays used in this study.

Table 6.1 Advantages and disadvantages of the different test methods

FACTOR	HPLC	MOUSE TEST	LUMISTOX	ARTOX	SEA URCHIN
Specialized equipment needed	yes	no	yes	no	no
Qualification and quantification of individual toxin components	yes	no	no	no	no
Technical expertise needed	yes	no	no	no	no
Cost: capital investment and maintenance	yes	yes	yes	no	no
Sensitive	yes	yes	no	no	no
Possible ambiguity of results	no	yes	yes	yes?	yes?

6.5 CONCLUDING REMARKS

Biological organisms have long been used to test for the lethal and sublethal affects of toxins on life-processes. However, there are a number of inherent problems in their use that have become clear during the course of this study, not least being the fact that different organisms have different responses to toxicity itself. Also, recently the question has arisen whether bioassays can be used at all to extrapolate data when related to human response . For example, in a comparative study at the Medical University of Southern Africa, it was established that a particular dose of aspirin and paracetamol that induced a 50% mortality of brine shrimp had no effect on human cells (Du Plooy 1997). However, having said that, bioassays can still provide indications of the toxicity of certain samples.

When a detailed knowledge of toxin composition and concentration in mussel samples is required, HPLC would be the most suitable method to use. HPLC provides a very sensitive and accurate method of determining the toxicity of samples. However, as HPLC requires technical expertise, and specialized equipment, this method is not always practical or available as a method of analysis. Also, detailed information about the toxin composition is not always needed, but only information as to whether a sample is toxic or not. The Artox kit, the LUMIStox kit and sea urchin gamete test are all sensitive to the acid used during the mussel extraction process. This renders all three bioassays unsuitable for the testing of mussel toxicity.

However, information about the toxicity of water samples can be very valuable as an indication of the toxicity of mussels. As no specialized equipment is needed for either the sea urchin or Artox kit, these methods are relatively easy to perform and they could be regularly carried out at several facilities around the coast as part of a monitoring program. However, the bioassays would only be useful for PSP detection if the concentration of cells in the water was sufficiently high. This high cell concentration would either occur naturally or be accomplished by manual concentration of cells in the water samples. Depending on the initial cell concentration (establish by performing a manual cell count with a microscope), one would also have to decide if it was practical to concentrate the water sample, as concentrating very large volumes of water to obtain high enough cell concentrations could be impractical without the necessary equipment (for e.g. a vacuum pump).

Assuming the cell concentrations are high enough, all three bioassays respond to PSP toxins in water samples. However, the LUMIStox kit responded to all the dinoflagellate extracts,

including the non-toxic extracts and therefore can be excluded as a suitable test for water samples. Although the sea urchin gamete test and the Artox kit seem to be similar in sensitivity, with the sea urchin gamete test, more variation occurred at some concentrations than with the Artox kit. The sea urchin gamete test was less reliable than the Artox kit, and experiments had to be repeated a number of times before consistent data were obtained. The Artox kit also gave more reliable results with the non-toxic *P. micans* extract than the sea urchin gamete test. Although the Artox kit showed a very weak response to the pure saxitoxin, this is not relevant, as the bioassay would ultimately be used to test water samples, and not pure toxic components. Therefore, based on the results obtained in this study, the Artox kit seems to be the most suitable, sensitive and practical bioassay for the detection of PSP toxins in water samples.

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