PARALYTIC SHELLFISH TOXINS IN A CLAM AND A MUSSEL FROM MUMBAI WATERS

Thesis Submitted
in Partial Fulfilment of the Requirements
for the award of the Degree of

DOCTOR OF PHILOSOPHY
(INLAND AQUACULTURE)

BY
RUPAM SHARMA

Central Institute of Fisheries Education
(Deemed University)
Indian Council of Agricultural Research
Versova, Mumbai-400 061

FEBRUARY 2001

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FEBRUARY 2001

DEDICATED TO MY PARENTS.....

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CERTIFICATE

Certified that the thesis entitled "**PARALYTIC SHELLFISH TOXINS IN A CLAM AND A MUSSEL FROM MUMBAI WATERS**" is a record of independent *bonafide* research work carried out by **Mr. Rupam Sharma** during the period of study from 1997 to 2000 under our supervision and guidance for the degree of Doctor of Philosophy (Inland Aquaculture) and that the thesis has not previously formed the basis for the award of any other Degree, Diploma, Associateship, Fellowship or any other similar title.

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DECLARATION

I hereby declare that the thesis entitled **"PARALYTIC SHELLFISH TOXINS IN A CLAM AND A MUSSEL FROM MUMBAI WATERS"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any Degree, Diploma, Associateship, Fellowship or any other similar title.

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ABSTRACT

Bioaccumulation of paralytic shellfish toxin in the clam *Meretrix meretrix* and the mussel *Perna viridis* from Mumbai waters was studied. The level of accumulated toxin was found to be much lower than the hazardous level, which can cause intoxication to humans. The toxins present in *M. meretrix* were dcGTX/STX and C1/C2 and in *P. viridis,* GTX2/3 and C1/C2, which were characterized using HPLC post-column reaction system. The death time of the bioassayed mice was found to be one hour or more and the corresponding mouse unit was found to be less than 0.875. Histopathological studies revealed profound effect on kidney and liver and mild effect on heart of the animal. Hemolytic and hemagglutinating studies revealed potent hemolytic activities on chicken blood, but there was no hemagglutination. The toxins showed a very mild analgesic activity also. The toxins were found to be stable in acidic medium and at –80^{0}C, but unstable upon lyophilisation. The present study also dealt with the depuration of the toxin from ~~these~~ species and it was found that ozone treatment was more effective than chlorine treatment. There was no correlation between the condition factor of these species and the accumulation level of the toxin. The results are discussed in the light of published literature and the scope for future studies is outlined.

सारांश

मुंबई के पार्ना में पाए जाने वाले मीरिट्रिक्स-मीरिट्रिक्स व परणा विरिडिस में पैरालैटिक शैल फिंश विष के जैव संचयन पर एक अध्ययन किया गया । संचित विष का दर खतरे की दर से कम पाया गया जो मनुष्य के लिए हानिकारक होता । एम. मीरिट्रिक्स में विष की मात्रा dcGTx/STX तथा C1/C2 व पी. विरिडिस में GTX2/3 तथा C1/C2 पाया गया । जिसका विश्लेषण एच.पी.एल.सी. पोस्ट कोल्म प्रतिक्रिया प्रणाली का उपयोग कर किया गया । बायोएसेसड़ चूहे की मृत्यु दर एक घंटे या उससे ज्यादा था, वही पर चूहे की यूनिट में मृत्यु दर 0.875 से भी कम देखा गया । हिमैग्लूटिनेशन अध्ययन से यह पता चला कि इसका प्रभाव चूहे के गुरदे, जिगर एवं हल्का असर हृदय पर पड़ा । हिमोलैटिक तथा हिमैग्लूटिनेशन के अध्ययन से यह पता चला कि मुर्गी के रक्त पर हिमोलैटिक का प्रभाव रहा वहीं पर हिमैग्लूटिनेशन का प्रभाव नहीं पाया गया । इस विष ने थोड़ा वेदनाहर प्रवृति दिखाई । इस विष ने एसिडिक माध्यम व -80° सी में स्थिरता दिखाई, लेकिन लाथोफिलाजेशन (द्रव रार्गायन) में अस्थिरता दिखाई ।

वर्तमान अध्ययन में इस प्रजातियों के विष को निकालने का भी अध्ययन किया गया । जिसमें यह देखा गया कि ओज़ोन उपचार का प्रभाव क्लोरीन उपचार के प्रभाव से ज्यादा प्रभावी रहा । इन प्रजातियों के देखे गए विभिन्न अवस्थाओं व विष संचयन की मात्रा में आपस में काई संबंध नहीं था ।

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INTRODUCTION

1. INTRODUCTION

1.1 HARMFUL ALGAL BLOOMS

Although algae form the base of most aquatic food chains and are vital to both freshwater and marine ecosystems, certain species frequently become nuisance. Their presence, especially in high cell concentrations, may discolour water or produce unpleasant odours or flavours. Extremely high cell concentrations may produce episodes of hypoxia or anoxia in water bodies, either because of high respiratory oxygen demand during hours of darkness or because of chemical oxygen demand when the cells die and begin to decay. Algal cells may clog water filtration and purification equipment. Though all these effects cause problems, such problems rarely became life threatening to humans or domestic animals. More dangerous effects occur when algal species produce chemical compounds that are highly toxic. The terms "red tide" and "brown tide" are increasingly associated in the public mind with outbreaks of toxin-producing algae, but these are misnomers. In many cases, toxin concentration may reach dangerous levels with no apparent colour change in the water (Hashimoto, 1979).

The organisms that inhabit such environments, particularly shellfishes, accumulate the toxin within their body tissue because of their filter feeding habit, without harming themselves. But, when these organisms are consumed by man, it causes intoxication, even though for centuries mankind has been enjoying the pleasures and benefit of eating shellfish. This sickness or intoxication that sometimes may become fatal, was thought to be the result of some other factors.

Earlier approaches to reducing the risks included folklore and religious customs, which preached abstinence from eating certain shellfish at particular times of the year. Other approaches were to feed suspected food to a domestic cat or dog and observe the effects.

The shellfish themselves do not produce any of these "shellfish" toxins. The main sources of all these shellfish toxins are phytoplankton. These microscopic marine plants are essential diet for filter feeders – such as mussels, clams, scallops and oysters and it is by this feeding process, that toxins enter the shellfish. Phytoplankton are ubiquitous in the ocean and under certain favourable environmental conditions, the population of a particular species can rise from a few cells per litre of seawater to a dense "bloom" containing millions of cells per litre. These can be so dense that they discolour the sea water, which is how the Red Sea got its name and why such blooms are often called 'red tides'. Sournia (1995) found the dinoflagellates (Dinophyceae) form the greatest number of the harmful bloom-forming species. Four genera, *Alexandrium*, *Dinophysis, Gymnodinium* and *Prorocentrum*, dominate in terms of causing toxic blooms. Diatoms are the second algal class in terms of causing harmful blooms (Bates *et al.,* 1989).

The majority of phytoplankton blooms are non-toxic. Toxic blooms will only be of significance to human health if the toxin(s) find the its way through seafood into human diet. Harmful phytoplankton may contain potent neurotoxins that, when they become concentrated in, for example, fitter-feeding bivalves, can cause a serious public health threat. A number of toxins produced by

phytoplankton affect humans as also fish (White, 1977; Gosselin *et al.,* 1989; Riley *et al.,* 1989; Robineace *et al.,* 1991). It has been estimated that on a global scale, approximately 300 people die annually as a result of eating shellfish contaminated with toxic phytoplankton (Hallegraeff, 1993). Other phytoplankton species contain toxins that can induce sublethal responses in humans (diarrhoea, eye/skin irritation, breathing difficulties, etc.). Some phycotoxins appear to be carcinogenic. This, for example, is the case for some blue-green algae (cyanobacteria), which are primarily found in freshwater but which invade and can bloom in brackishwaters (Falconer, 1991; Carmichael, 1992).

Mortalities of wild or cultured fish and shellfish can occur (Shemway, 1992) as a result of phytoplankton blooms, either due to abrasion of the gills leading to gill damage and ultimately suffocation of the fish (Bill, 1961; Taylor *et al.*, 1985; Farringtan, 1988; Pensel, 1993; Kent *et al.,* 1995; Tester and Mahoney, 1995), or due to anoxia and/or bacterial infection in combination with the damaged gill tissue leading to mortality of the fish (James and Rhodes, 1994; Tester and Mahoney, 1995). Respiration or decay of dense blooms of phytoplankton can also lead to fish or shellfish kills (Steimle and Sindermann, 1978; Taylor *et al.,* 1985). However, the important reason is the production of toxins by phytoplankton blooms that cause fish mortality.

1.2 DIFFERENT TYPES OF SHELLFISH POISONINGS

Though there are several types of toxins produced by harmful algae, only a few shellfish toxins cause public health problems.

1.2.1 Amnesic Shellfish Poisoning (ASP)

In this type of poisoning, there is temporary loss of memory. Although ASP has only recently been recognized, the fact that the causative organisms have a wide geographic distribution (Douglas *et al.*,, 1993; Hallegraeff, 1993) and that commercial finfish have the potential to harbour and transmit domoic acid poisoning suggests that there is a serious risk of ASP incidents occurring in many parts of the world's oceans.

1.2.2 Neurotoxic Shellfish Poisoning (NSP)

This is another type of human poisoning that is caused by a group of toxins known as brevetoxins from the dinoflagellates *Ptychodiscus breve* (formerly *Gymnodinium breve*) and is characterized by neurological symptoms but no paralysis. Brevetoxins are potent polyether neurotoxins (Baden, 1989; Trainer *et al.*, 1990). One form of poisoning causes paraesthesia, alternating hot and cold sensations, nausea, diarrhea and ataxia; the other form is characterized by upper respiratory distress and eye irritation (WHO, 1984). Brevetoxins also affect marine organisms (Riley *et al.*,, 1989) and have been implicated in a number of mortality events for marine mammals (Hofman, 1989; O'shea *et al.*, 1991; Anderson and White, 1992). NSP is a serious problem along the southeastern coast of North America and in the Gulf of Mexico. However, the causative organism has also been recorded in other parts of the world.

1.2.3 Diarrhetic Shellfish Poisoning (DSP)

The incident of diarrhetic shellfish poisoning was first recorded in Japan in 1976 (Yasumoto *et al.*, 1978). The causative agents, dinoflagellates of the genus *Dinophysis* (Hallegraeff, 1993), are broadly distributed on a global scale and

confirmed DSP incidents have been reported from all continents except Africa and Antarctica, but may be geographically more widespread than the scientific literature would suggest. The toxins implicated here are okadoic acid and derivatives, and polyether lactones (WHO, 1984). No fatalities directly associated with DSP have ever been recorded, but some of the toxins associated with DSP may promote the development of stomach tumors (Suganuma *et al.,* 1988). Thus, prolonged or chronic exposure to DSP toxins may have long-term negative effects on public health.

1.2.4 Paralytic Shellfish Poisoning (PSP)

Paralytic shellfish poisoning is caused by certain molluscs and one or two echinoderms and arthropods, which have ingested toxic dinoflagellates and are subsequently eaten by man. PSP is a worldwide problem. It has long been known along the Pacific and Atlantic coasts of North America and Canada, and many fatal cases were recorded.

The toxins responsible are all hygroscopic, water-soluble alkaloids viz. Saxitoxin (STX), Neosaxitoxin (neoSTX), Gonyautoxins 1-8 (GTX 1-8), C 1-4, B1-2, dcGTX and dcSTX (Oshima, 1995). The initial symptoms, observed within 30 minutes of consumption of the toxic molluscs, consist of a number of neurological disturbances. Gastrointestinal symptoms like nausea, vomiting, diarrhoea and abdominal pain are less common and are variable and secondary to neurological disturbances. In the terminal stages, the victim becomes progressively worse, finally developing muscular twitching, convulsions, paralysis and death by respiratory failure. Those who survive after 12 hours or better still,

24 hours, may expect to recover with no lasting effects (Halstead and Courville, 1965; Quayle, 1969; Schantz, 1973).

1.3 DEPURATION

Depuration refers to techniques of reducing the number of pathogenic organisms or other biotoxins that may be present in shellfish to levels acceptable for human consumption. Careful use of depuration permits some shellfish to be harvested from moderately polluted waters. It can also offer additional safety assurance for molluscs taken from approved waters. Since the molluscs are filter-feeders, they pump water through their systems when feeding and have the ability to pump out of themselves any bacteria, algal toxins or the particles like sand and grit that they may have ingested. If the new water they are pumping through is sanitized, all the remainings in the gut are gradually expelled in the animals' waste matter. Using this principle, the depuration is carried out for the entire mollusc to make it safe for human consumption. In a depuration plant, sanitized water is passed over the shellfish for a period long enough to ensure that all the bioaccumulated materials like bacteria, toxins, sand, etc. are removed. Depuration is quite complex because it deals with a group of animals, which are not all identicle (Dore, 1991). Depuration only works if the shellfish are pumping actively. This requires the correct water temperature, salinity and turbidity.

1.3.1 Types of Depuration Plants

Two general types of molluscan shellfish depuration plants exist and are either (i) flow through or (ii) recirculating or closed systems. When the depuration facility is close to reliable and consistently clean source of seawater to

pass over the shellfish, flow-through systems are chosen. Recirculating or closed depuration systems require the same consistent high quality source of seawater. However, the seawater is recirculated for at least 24 hours and then, released into the environment. One advantage that the closed systems have over the flow through one is that the inadvertent introduction of toxic or any adverse chemicals that may suddenly appear in the seawater can be eliminated.

1.3.2 Importance of Depurating Water Quality

Although depuration is a natural process to molluscan shellfish, man can enhance the process if he can modify the seawater environment that the shellfish are exposed to in the depurating tanks. The physiological activity, pumping rate and behavioral responses of molluscan shellfish will vary in response to changes in their seawater environment. Successful depuration depends upon knowing how the environmental parameters affect the depuration process (Roderick and Schneider, 1994). Those factors associated with the seawater that have been shown to significantly alter the depuration process are temperature, salinity, dissolved oxygen content, turbidity and phytoplankton concentration.

1.3.3 Types of Seawater Treatment

Depuration is a process of purification in which filter feeding shellfish are placed in a clean environment and allowed to pump, thus ridding themselves of pathogens, and other bacteria and bioactive compounds. The depuration of contaminated shellfish was addressed as early as 1911 (Phelsp, 1911). Four methods exist for the disinfection of seawater used in depurating molluscan shellfish and include the use of chlorine, ultraviolet light, ozone and iodophors.

1.4 SCOPE OF PRESENT STUDY

In India, the first incidence of paralytic shellfish poisoning was recorded in 1954 along the Malabar Coast and in 1981, there was an episode of PSP due to ingestion of mussels in Tamil Nadu (Bhatt, 1981). However, no data were available on the toxins contained in the mussels. PSP was reported from Mangalore in 1983 (Karunasagar *et al.*,, 1984) and again in 1986 (Karunasagar *et al.*,, 1989a). Recently in 1997 and 1999, South Kerala experienced such poisoning (Karunasagar, personal communication). These outbreaks of PSP has opened up a new area for investigation in as much as it has far-reaching influence on public health and on the fishery in the Indian Cost. Although there are no reports of PSP from Mumbai so far, the matter needs scientific attention since consumption of shellfish in this region is very high and people accept shellfish, particularly clams and mussels as delicacy. Moreover, there is no clear method for depurating the bivalves and according to Bhatt (1981), red tides may be a regular annual feature in Indian waters. So, the possibility of PSP incidence in this coastal area cannot be ruled out. Keeping this in view, the present work was undertaken to study the levels of accumulation and storage of the paralytic shellfish toxin in two filter feeding marine molluscs *viz*. the mussel *Perna viridis* and the clam *Meretrix meretrix*, that are widely consumed in this part of the country and to evolve a suitable method of depuration which would render these organisms safe for human consumption.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 GEOGRAPHICAL DISTRIBUTION

Though the outbreak of paralytic shellfish poisoning (PSP) was reported in the American Continent only in 1793 (Meyer, 1953; Halstead, 1962), toxic dinoflagellate activities were recorded 208 BC (Phillips and Brady,1953) or even earlier, as far back as during 1491 BC (Hayes and Auston, 1951). Centuries before the white man ever reached the shores of the Pacific Coast, the Indians watched the sea at night for luminescence, a phenomenon caused by the dinoflagellates *Noctiluca* spp. that frequently accompany *Gonyaulax catenella,* the source of PSP along the Pacific Coast (Carson, 1951). In 1799, a group of Aleutian hunters had a dinner of mussels in a place presently known as Perilway, near Alaska. After that more than 100 men are said to have died in less than two hours (Dall, 1870; Petroff, 1884). The famous German Wilhelmshaven outbreak of PSP which occurred in 1885 (Virchow, 1885), helped tc attract the attention of European scientists to this toxication. Combe (1828) reported an outbreak in Scotland and discussed the various theories in vogue as to the cause of the poison. Many believed that mussel poisoning was due to the copper salts present in seawater (Brosch, 1896). Combe (1828) appears to be the first to present autopsy findings from a case of mussel poisoning. Even at this early date, it was noted that the disease occurred more frequently during the warm months of the year. In 1862, Husemann (cited by Muller, 1935) postulated that shellfish became toxic because of putrefaction during the warm summer months. Cameron (1890) was the first to suggest that the toxicity of shellfish was due to "contaminated or filthy" water, followed by Engelsen (1922) and Giunio (1948).

According to Farrar (1882), some workers felt that the toxin either arose from a secretory organ at the base of the "foot" or was in some way connected with the byssus of the mussel. The bacteriological tests conducted by Meyer *et al.,* (1928) failed to yield bacterial pathogens having toxic properties consistent with this toxication.

The series of 14 outbreaks that occurred in the vicinity of San Francisco during July 1927, was probably the most significant epidemic in terms of stimulating modern research on PSP (Meyer *et al.,* 1928). The brilliant studies of Sommer *et al.* (1937) demonstrated that the California mussel *Mytilus californianus,* which lost toxicity in filtered seawater in the laboratory, became strongly poisonous after being fed fresh, unfiltered ocean water for 16 days. The increase in toxicity could be quantitatively correlated with the number of *G. catenella* in the water. Sommer *et al.* (1937) also developed methods for the extraction and demonstration of the paralytic shellfish poison from plankton. Samples of plankton collected during thE summer months of 1935 yielded amounts of poison roughly proportional to the number of *G. catenella* present. Thus, the causative agent for mussel poisoning along the Pacific Coast of North America was finally demonstrated. The conclusions of Koch (1938, 1939), similar to those of Sommer's group, demonstrated that *Pyrodinium phonus,* a dinoflagellate, was the source of the poison present in some of the outbreaks of mussel poisoning in Belgium. Needler (1949) produced evidence indicating that the dinoflagellate *G. tamarensis* was the primary cause of paralytic shellfish poisoning along the Atlantic coast of Canada.

Extensive toxicological tests were conducted on the shellfish of the Pacific Coast of North America by Meyer *et al.* (1928), Meyer (1929, 1931), Sommer and Meyer (1935), Sommer *et al.* (1937), Pugsley (1939), Chambers and Magnusson (1950) and Furk (1950). Atlantic North American species of shellfish have been tested by Gibbard *et al.* (1939), Macht *et al.* (1941), Medcof *et al.* (1947) and Gibhard and Naubert (1948). Although in Australia no outbreaks of paralytic shellfish poisoning were reported, Le Messurier (1935) had run toxicity tests on some of the shellfish of that region. In Japan, molluscs were tested by Akiba and Hattori (1949) and Hattori and Akiba (1952) following the 12 cases of PSP in 1948 (Akiba, 1970 cited by Ray 1970). Pinto and Silva (1956) reported incidence of PSP after the consumption of mussels on the west coast of Portugal. Cases of PSP were reported in Norway in the year 1964, by Offebro (1965). From England, Ingham *et al.,* (1968) and Robinson (1968) reported occurrence of PSP.

The first reported case of PSP in Malaysia was in 1976, following an outbreak of red tide in the coastal waters (Ray, 1970) followed by outbreaks in 1980 and 1983-84 (Jothy, 1984). PSP was reported in the Philippines in 1983 (Estudillo and Gonzales, 1984). In Thailand, occurrence of red tides was reported in 1957 (Suvapepum, 1984).

From Argentina, the first report of PSP was in 1975 (Correto *et al.,* 1985) and from Norway in 1981 (Langeland *et al.,* 1984). The first recorded outbreak occurred in 1988 in Venezuela and then in 1989 (La-Barbera, 1990) and 1991 (La-Barbera,1993). Kim (1995) reported one in Korea in 1993. It was in 1987 that Canada experienced PSP (Subba Rao *et al.,* 1988). From

Guatemala, reports of PSP occurrence came in 1987, 1988 and 1989 (Rosales-Loessener, 1989).

In India the first report of mussel poisoning was by Bhat (1981) in Tamil Nadu. The outbreak of PSP in the west coast of India in which one person died and several hospitalised was investigated by Karunasagar *et al.* (1984). They collected the clams from houses of affected people and examined them. The toxin levels were found to exceed 18,000 mouse units and contained mainly Gonyautoxin 1-8 and epi-Gonyautoxin-8 (Karunasagar *et al.,* 1990). The toxin profile of shellfish involved in the Indian outbreak of PSP was similar to that of *Alexandrium cohorticula,* which was involved in the PSP outbreak in Thailand (Fukuyo *et al.,* 1989). Segar *et al.* (1988) revealed the presence of paralytic shellfish poison in some clams and oysters along the coast of Karnataka during April 1985, but the toxin levels were within the permissible limit (<400 MU/100 g). During 1986 PSP was detected again in low levels in shellfishes along the Karnataka coast (Karunasagar *et al.,* 1989) and the toxin profile of the shellfishes was identical to that obtained during the 1983 outbreak, suggesting the involvement of the same dinoflagellate species.

2.2 TOXIN IDENTIFICATION

The toxins extracted from clams and mussels are metabolic products of dinoflagellates that appear to be bound in molluscan tissues without undergoing change (Schantz *et al.,* 1966). Paralytic shellfish toxin, a non-protein substance of low molecular weight is one of the most potent human poisons known (Ray, 1970). These toxins are basic, water-soluble compounds, but less soluble in alcohols and insoluble in all lipid solvents. The toxins are a metabolic products of dinoflagellates that appear to be bound

in molluscan tissues without undergoing change (Sehantz *et al.,* 1966). The paralytic shellfish toxins, which are produced by dinoflagellates are of several types. So far, many different kinds have been identified (Genenah and Shimizu, 1981; Shimizu, 1978; Shimizu *et al.,* 1975; Shimizu, 1988) and characterized as Saxitoxin (STX), Neosaxitoxin (neoSTX), Gonyautoxins 1-8 (GTX 1-8), C 1-4, B 1-2, dcGTX and dcSTX. All these toxins with known structure are alkaloids. Shimizu (1978) found that all these toxins are very hygroscopic and thus, very water soluble. STX, GTX-2 and GTX-3are more stable in acid than in alkaline medium, even at low temperatures (Shimizu, 1978). The stability of these toxins decreases with increasing pH; thus, gradual loss in toxicity occurs at room temperature at pH 6.6; heating at this pH abolishes halt of the toxicity in a week. Ordinary cooking destroys up to 70% of shellfish toxicity; even greater loss in toxicity occurs during pan-frying (Medcof *et al.*, 1947).

The presence of the PSP toxins in both shellfish and the causative dinoflagellates has been analysed traditionally by using mouse bioassay (Arlingten, 1980). Due to the non-specific nature of the bioassay and a number of factors affecting its precision and accuracy, Krogh (1979) felt that there was a need for alternative assay procedures. More traditional analytical procedures such as gas chromatography and spectrometry have proven ineffective in assaying for the PSP toxin since these toxins lack native fluorescence, useful ultraviolet absorption or adequate volatility (Sullivan and Wekell, 1984).

High performance liquid chromatography (HPLC) is the most valuable instrumental analytical tool for toxins because it is so well suited to the

analysis of such polar, non-volatile compounds. It provides excellent quantitative precision and is easily automated. There are still a number of difficulties with the technique, however not the least of which is that most of the toxins do not possess a chromophore for sensitive UV absorption or fluorescence detection. This has necessitated the use of either pre- or post-column derivatization methods to allow detection (Quilliam *et al.,* 1992). This method is more sensitive than the Association of Official Analytical Chemists (AOAC) mouse bioassay and can be operated at a comparable cost on a routine basis. Inter-comparison of the two methods has shown that the HPLC method provides reliable results (Satler *et al.,* 1989).

During the last decade, considerable effort has been applied for developing an automated (HPLC) method for routine PSP toxins analysis. One method has emerged using reversed phase chromatography and ion-pairing reagents, which effectively separate all the important PSPs, and these are detected by oxidation to their fluorescent derivatives following elution from the HPLC column. This coupled with post-column derivatization system requires a considerable amount of skill to make it operate routinely, but a more serious drawback is the absolute requirement for PSP toxin standards in order that the data can be accurately quantified (Wright, 1992).

For the first time, a sensitive quantitative method was available in the early 1970's for non-volatile compounds, such as saxitoxin. HPLC is distinguished from the related techniques of liquid column chromatography in its use of small particle-size packing materials (<20 μm), which leads to highly efficient separations. Although there have been only a few applications of HPLC to the saxitoxins, it has proven to be one of the more useful techniques

for individual quantification of various toxins. Boyer (1980) was the pioneer in using HPLC to separate various derivatives of STX, but the detection method utilized (UV at 220 nm) provided poor sensitivity: so the method was only applicable to relatively concentrated toxin solutions. After this, Rubinson (1982) reported an HPLC separation of STX from dc-STX using a C-18 HPLC column and refractive index detector. But, sensitivity was low and the system was not amenable to dilute toxin solutions. Onoue and Nozawa (1989) described an HPLC system that uses ion-exchange resins to separate the toxins with fluorescence detection following a post-column reaction. The combination of the selective and sensitive oxidation fluorescence method of Bates and Rapoport (1975) with HPLC in a post-column reaction system provided a rapid and efficient analysis technique for saxitoxin and this method was initially proposed by Buckley *et al.* (1978). An HPLC procedure with a big merit of quantifying all PSP toxins, including latent toxins, was developed by Sullivan's group for routine analysis (Sullivan *et al.*, 1983; Sullivan and Iwaoka, 1983). Oshima *et al.* (1984) reported efficient ion exchange separation of the toxins by HPLC and substituted *t*-Butyl hydroperoxide as the oxidant in the post-column reaction system. Sullivan (1982), and Sullivan and Iwaoka (1983) tried a variety of columns for HPLC separation of PSP toxin, but incomplete resolution the toxins and poor column stability were experienced. These problems were alleviated with the application of ion interaction chromatography (Iskandarani and Pietrzyk, 1982) to the separation of the toxins (Sullivan and Wekell, 1984). In this system, the toxins are separated on a reversed phase porous polymer column with mobile phases containing alkyl sulfonates, ammonium phosphate and acetonitrile. This

system increases the efficiency of separation since a variety of factors that affect the retention times of the various toxins can be altered. This method has been utilized in a wide variety of studies on both dinoflagellates and shellfish (Sullivan *et al.,* 1985; Boyer *et al.,* 1985). The HPLC system has been developed with a primary purpose as a replacement for the mouse bioassay in shellfish toxicity monitoring programmes. A good correlation between the bioassay and HPLC has been demonstrated (Sullivan *et al.,* 1985) and in larger monitoring programs, clear cost advantages of the HPLC are evident (Sullivan *et al.,* 1986). In addition to its utility in toxin monitoring, the HPLC method provides a powerful tool for research into the chemistry and biochemistry of PSP toxins.

A major achievement in the analysis of PSP toxins was the development by Sullivan *et al.* (1988) of an HPLC method based on ion-pairing chromatography with a reversed phase polymeric column and a post-column reaction detection system. Earlier, Bates and Rapoport (1975) developed a post column reaction, which showed that saxitoxin could be oxidized to a fluorescent derivative. Quilliam *et al.* (1992) used the HPLC method given by Sullivan *et al.* (1988) for analyzing PSP toxin extracted form scallop to detect saxitoxin, neosaxitoxin, GTX-2 and GTX-3. Lee *et al.* (1987) used reversed phase HPLC with fluorescence detection that involved a pre-column derivatization to detect diarrhetic shellfish poison. Pleasance *et al.* (1990) also used this method with slight modification to characterize DSP.

Many other HPLC methods for PSP determination were based on the extraction with hydrochloric acid, followed by ion pair chromatographic separation, post-column oxidation of the PSP toxins with periodic acid, and

fluorescence detection of the PSP toxin oxidation products (Oshima *et al.*, 1984; Sullivan and Wekell, 1987; Nagashima *et al.*, 1987; Oshima *et al.*, 1988; Thielert, 1993).

But for the accurate separation of complex toxin mixtures, three different isocratic chromatographic separations are required to distinguish all 18 known toxin fractions (Oshima *et al.*, 1988). Toxin profiles derived from *Pyrodinium bahamense* appear to be relatively simple and contain only STX, neoSTX, dcSTX, GTX5 and sometimes GTX6 (Oshima, 1989). This has formed the basis for the simplified HPLC method developed by Oshima (1995), which used a 20 - minute isocratic elution with a single solvent system.

Kirschbaum *et al.*, (1995) worked on ion-exchange HPLC with post-column electrochemical oxidation and fluorescence detection to determine PSP toxins. They found STX, neoSTX and small amounts of GTX-2/3 after chromatographic separation. Anderson *et al.* (1996) found out the presence of GTX-2 and GTX-3 and toxin C1-C4 form Chinese waters for the first time using the method of Oshima *et al.* (1988). Later, Hummert *et al.* (1997) used HPLC method based on ion pairing chromatographic separation, post-column oxidation and fluorescence detection to analyse the PSP profile of *P. bahamense* and *Alexandrium* sp. They detected dcSTX, STX, GTX-2, GTX-3 and GTX-5 and neoSTX.

2.3 ANIMAL BIOASSAYS

The biological assays for the toxin are by far the most widely utilized method for their determination. A wide range of organisms are sensitive to the toxins and, therefore, are potential test organisms for bioassays, but the

mouse and housefly are the only species utilized to date (Sullivan *et al.*, 1988). The relationship between toxin concentration and death time in mice following intra- peritonial injection was first described by Sommer and Meyer (1937) and was found to follow a logarithmic relationship. It was also found that the weight of mice affected death time, with lighter mice exhibiting significantly shorter death times. The mouse bioassay was later modified by Medcof *et al.* (1947) and when pure toxin became available (Schantz *et al.*, 1958), it was subjected to a collaborative study (McFarren, 1959). Subsequently, AOAC adopted this bioassay as an official procedure (Williams, 1984) and is in use today as the primary analytical technique to support the majority of toxin monitoring programmes in shellfish.

2.4 SYMPTOMS OF PSP POISONING

2.4.1 Humans

The initial symptoms are observed within 30 minutes after consuming the toxic mollusk and consist of a tingling burning sensation and numbness in the lips, gums, tongue, face and fingertips. Then it spreads to the neck, arms and legs. Ataxia, a feeling of being chocked, incoherent speech and loss of voice are seen in severe cases. Victims often report a feeling of lightness as though floating in air. Other symptoms like weakness, dizziness, malaise, prostration, headache, salivation, rapid pulse, intense thirst, perspiration, impairment of vision or even temporary blindness are also seen as symptoms of PSP. Gastrointestinal symptoms like nausea, vomiting, diarrhoea, and abdominal pain are less common. Gastrointestinal symptoms are variable and secondary to the neurological disturbances. Mental processes do not appear to be affected. Some patients complain of feeling as it their teeth were

loose or being set on edge. In the terminal stages, the victim becomes progressively worse, finally developing muscular twitching, convulsions, paralysis and death by respiratory failure. The death due to respiratory paralysis depends on the amount of toxin ingested and death time and varies from 3 to 12 hours after intoxication. Those who survive after 12 hours or better still, 24 hours, may expect to recover with no lasting effects (Halstead and Courville, 1965; Quayle, 1969; Schantz, 1973).

2.4.2 Experimental Animals

Increased palpitation, excessive urination, vigorous jumping, dragging of the hind limbs, apathy, raising hairs, etc. are common symptoms observed in experimental animals such as mice (Halstead and Courville, 1965).

2.5 HEMOLYTIC ACTIVITY OF THE TOXINS

Hemolytic activity has been observed in many of the tissue products of aquatic organisms including fish, molluscs, algae, etc. Hemolysis in mammalian, chicken, carp, eel and other erythrocytes, as the case may be, by skin mucus of the eel - *Anguilla japonica* (Suzuki, 1985), skin secretion of puffer fishes (Malpezzi *et al.,* 1997), skin secretion of the oriental catfish *Plotosus lineatus* (Shimomi *et al.,* 1986), bile of freshwater carps (Chen *et al.,* 1984; Munilkumar, 1994; Khobragade, 1996; Talukdar, 1998) has been reported. Sakai (1981) reported that normal sera of 12 species of fish that are heat-labile possessed natural and spontaneous hemolytic functions against various heterologous red blood cells, but not against homologous red blood cells.

Among invertebrates, hemolytic activity or hemagglutinating activity or both have been reported with toxins from the nematocysts of jellyfish (Azila *et*

al., 1991; Azila and Othman, 1992; Rottini *et al.,* 1995). But, the extent of hemolysis of the extract from tentacles of *Catostylus mosaicus* was different in different erythrocytes (Azila and Othman, 1990); rat erythrocytes were most susceptible for lysis followed by those from rabbit and humans. The hemolytic activity was dependent on the concentration of crude extract protein. Comis *et al.,* (1989) reported a 2.6-fold increase in hemolytic activity of the box jellyfish (*Chironex fleckeri*) tentacle extract upon partial purification by filtering through Sep-Pak (Sub 18) Cartridge. Sea anemone is another marine invertebrate that possesses venom capable of causing potent hemolysis of erythrocytes (Macek and Lebez, 1988; Malpezzi and Freitas, 1991; Morera *et al.,* 1995). Hemolytic activity of certain secretions of horseshoe crab was also reported (Ohashi *et al.,* 1984; Quigley *et al.,* 1997). Aquatic extracts of certain marine molluscs like *Aplysia californica* (Merker and Levine, 1986) and various species of *Conus* (Ramu *et al.,* 1994; Sakthivel, 1999) also exhibit hemolytic activity on erythrocytes.

Most of the marine algae, dinoflagellates and diatoms are toxic in nature and the aqueous extracts of these flora cause hemolysis in red blood cells. Targett and Mitsui (1979) assayed three species of marine algae, *viz. Anadyomene stellata, Dictyota dichotoma* and *Wrangelia penicillate,* for their toxicity using two bioassay methods, fish mortality and fish erythrocyte hemolysis. The aqueous extracts of these three species lysed erythrocytes of sea bream, *Archosargus rhomboidallis.* Marine algae collected from Mandapam, Tamil Nadu, were studied for hemolytic and antimicrobial activities and almost all the species showed antibiosis and strong hemolytic activity (Rao *et al.,* 1991). Blooms of the marine flagellate *Chrysochromulina*

sp. have caused mortality of marine organisms in Scandinavian waters including fish in aquaculture and, therefore, Simonsen and Moestrup (1997) assayed eight species of *Chrysochromulina* (*C. aplules, C. brevifilum, C. ericina, C. hirta, C. leadbeateri, C. parva, C. polylepis and C. simplex*) for toxicity in *Artemia* sp. and also hemolytic activity. All the eight species showed hemolytic activity up to six spots but only *C. polylepis* was found to be toxic to *Artemia* sp. Toxins from dinoflagellates and other phytoplankton that cause red tides possess a potent hemolytic property. Bass *et al.* (1983) reported the hemolysis of human erythrocytes by the toxic extract of red tide dinoflagellate, *G. monilata*. Following a massive fish kill in the Norwegian coastal waters due to the *Chrysochromulina polylepis* and *Gyrodinium aureolum* bloom, Yasumoto *et al.* (1990) studied the presence of biactive components in these two phytoflagellates by hemolytic test. They found 1-Acyl-3-Digalactosyl glycerol and octadecapentaenoic acid to be present which caused hemolysis and fish killing. A toxin separated from the harmful red tides that occurred along the coast of Kagoshima Prefecture in Japan was assayed for toxicity and hemolytic activity by fish bioassay by Onoue and Nozawa (1989). The red sea bream, which was used for toxicity testing died within 4 -10 minutes and the toxin was found to possess hemolytic activity of 0.18 HU/mg toxin. It also caused hemagglutination.

Certain diatoms also cause blooms and bring in mortality of aquatic organisms. Toxin extracted from cultured *Nitzschia* sp. (Bacillariophyceae) showed hemolysis of erythrocyte suspension (Rangel *et al.*, 1997). The marine phytoflagellates *Prymnesium* spp. Are among the most harmful red tide causing microalgae. Igarashi *et al.*, (1996) isolated a potent ichthyotoxin

named prymnesin from *P. parvum* and showed its potent hemolytic property. The hemolytic activity is different in different species of *Prymnesium* and *Prymnesium parvum* extract was found to be nine times more hemolytic than *P. patelliferum extract* (Meldahl *et al.,* 1994). Meldahl *et al.,* (1994) also found two other marine algae *C. polylepis* and *C. leadbeateri* to cause hemolysis. The hemolytic property of *P. patelliferum* depends on the culture medium; extract of *P. patelliferum* collected from phosphorus-deficient medium was five times more hemolytic than when grown in phosphorus-sufficient medium (Meldahl and Fonnum, 1993). Moreover, the hemolytic rate of prymnesin, the purified toxin of *Prymnesium parvum,* declines upon being subjected to inactivation by UV irradiation, incubation at 37°C or exposure to alkaline medium (Reich *et al.,* 1965). Prymnesin-2, isolated from the red tide algae *P. parvum* by Igarashi *et al.,* (1998) caused 50% hemolysis of a suspension of dog red blood cells. The extract of toxic dinoflagellate *Amphidinus carterae,* while assayed for hemolysis against sheep and catfish blood showed the highest production of hemolysin (Nayak, 1999).

2.6. HEMAGGLUTINATING ACTIVITY OF THE TOXINS

The literature on hemagglutination assay of paralytic shellfish poisons as such is scanty. Johnson *et al.* (1964) conjugated paralytic shellfish poison to protein by formaldehyde condensation that showed passive hemagglutination of rabbit antisera. Many marine algal extracts exhibit hemagglutination. Aqueous ethanolic extract of the marine red alga *Hypnea japonica* was found to cause agglutination of human blood cells and four electrophoretically homogenous agglutinins *viz.* Hypnins A, B, C, and D, were isolated (Hori *et al.,*, 1986). Aimouz *et al.* (1992) assayed 27 species of

Brazilian marine algae (16 red, seven green and four brown) for hemagglutinating activity using human, chicken, goat, pig and rabbit erythrocytes. Human erythrocytes showed better agglutination than others. Dalton *et al.* (1995) also reported higher agglutination in human erythrocytes than sheep and rabbit erythrocytes by protein extracts of green and red marine algae (Chlorophyta and Rhodophyta). A high molecular weight hemagglutinin was purified from a phosphate buffer extract of *Gracilaria verrucosa* (Kakita *et al.,* 1997). Later, Kakita *et al.* (1999) isolated three different hemagglutinins from the phosphate buffer extract of red algae, *G. verrucosa* from Japan and found them to cause hemagglutination of horse erythrocytes. The authors also isolated a fourth hemagglutinin after partially purifying the phosphate extract by gel filtration chromatography. Aqueous ethanolic extracts from five species of the genus *Eucheuma* (Rhodophyta) *i.e. E. serra, E. amakusaensis, E. cottanii, E. gelatinae and E. denticulatum,* were examined for hemagglutinating activity by Kawakubo *et al.,* (1997). All the extracts tested agglutinated trypsin-treated sheep and rabbit erythrocytes as well as untreated sheep erythrocytes. The hemagglutinating activity was stable over a wide pH range and at a relatively high temperature, but was inhibited by a number of glycoproteins.

Compared to marine algae, there are very few reports on hemagglutination caused by toxins from freshwater blue-green algae (Cyanobacteria). The toxin produced by them besides causing death of animals, also cause illness, especially gastrointestinal disorders, in humans. Carmichael and Bent (1981) assayed the toxins produced by two important species of cyanobacteria namely, *Anabaena flosaquae and Microcystis*

aeruginosa and were found to cause agglutination of blood cells of mice, rats and humans. In China, all the species of blue-green algae collected from the Donghu Lake caused hemagglutination (Yu *et al.*, 1987).

Many workers isolated hemagglutination-causing lectins from certain molluscs. Lectin present in the American oyster *Crassostrea virginica* was shown, by utilizing a micro-hemagglutination assay, to cause distinct hemagglutination (Vasta *et al.*, 1982). Belogertseva *et al.*, (1998) purified a lectin from a mussel by affinity chromatography and gel filtration. The lectin was found to agglutinate all types of human erythrocytes as also those of mouse and rabbit. The hemagglutination activity was independent of divalent cations Ca super (2+) and Mg super (2+).

Among the other marine invertebrates, sea cucumbers, sponges, and crabs are the most important ones from point of view of hemagglutination studies. Matsui *et al.*, (1994) purified two structurally distinct lectins from the coelomic plasma of a holothurian, *Stichopus japonicus,* that showed hemagglutination of rabbit erythrocytes. Four Ca super (2+) dependent lectins were purified from a marine invertebrate, *Cucumaria echinata* (Holothuroidea), by Hatakeyama *et al.*, (1994). Two lectins were purified from the sea cucumber, *Stichopus japonicus* by affinity chromatography (Hatakeyama *et al.*, 1993). Bretting *et al.*, (1981) assayed 21 species of sponges which were found to produce agglutinins reacting with human and animal red blood cells. Toxins found in the marine sponge *Anthosigmella varians* had hemagglutinating activity on human erythrocytes irrespective of A, B, O groups (Atta *et al.*, 1992). A D-galactose specific agglutinin was isolated from a Fijian sponge by Kamiya *et al.*, (1986). Hemocytes of horse shoe crab

also has hemagglutination property which agglutinated human erythrocytes (Okino *et al.,* 1995). Murali *et al.,* (1994) detected a naturally occurring hemagglutinin in the serum of hermit crab, *Diogenes affinis,* which showed strong affinity to cause hemagglutination of rat erythrocytes. A new lectin was isolated from Japanese horseshoe crab, *Tachypleus tridentatus,* by Inamori *et al.,* (1999) which exhibited hemagglutinating activity against human - A type erythrocytes but not against B- and O-types of erythrocytes, and animal erythrocytes including those of sheep, rabbit, horse and bovine. A hemagglutinin (lectin) from the hemolymph of the blue crab *Callinectes sapidus* caused agglutination of human erythrocytes (Vella, 1987). Human A - type erythrocytes were also susceptible for agglutination by the sperm extract of sea urchin (Seike *et al.,* 1992).

Apart from all these invertebrates, many fish and prawn species also show hemagglutination of erythrocytes; these include skin mucus of the eel *Anguilla japonica* (Suzuki, 1985), mucus of the fish *Genyptenis blacodis* (Oda *et al.,* 1984), a lectin found in the ova of the Japanese trout (Ozaki *et al.,* 1983) a lectin isolated from the roe of the catfish *Silurus asotus* (Hosono *et al.,* 1993) and hemolymph of the marine shrimps *Parapenaeus longirostris* and *P. stylirostris* (Vargas-Albores *et al.,* 1992; Fragkiadakis and Stratakis, 1995).

2.7 STABILITY OF THE TOXIN

Although the stability of the paralytic shellfish toxin has been studied since 1885, it was Muller (1935) who made extensive studies on the stability aspects of PSP toxins. He noted a gradual decomposition of the toxin in the dried organs when stored, but this deterioration was stabilized in the presence

of chloroform. Storage of the dried material for a period of five weeks resulted in 50% deterioration which increased to 75% in the course of a year. But, he found the toxin stable on storage in alcohol. Boiling the toxin in acid destroyed its toxicity, but boiling in neutral solutions for short periods resulted in no appreciable destruction of the toxin. Muller (1935) found the toxin stable to treatment with methyl iodide and UV light.

Mold (1947) observed that the toxin decomposed in the presence of mercuric or gold salts. He also reported the toxin to be largely destroyed under hydrogenation in the presence of platinum catalyst, which was contradictory to the observation made by Muller (1935) who found the toxin stable for one hour under such treatment.

Sommer *et al.* (1948) also studied the effects of variations in pH and temperature conditions on the stability of the toxin. They noted complete stability of the toxin at a pH of 5.0 at 5°C. Any change involving a rise in either pH or temperature, or both represented conditions of less stability. The toxicity dropped only by 25% when heated in 3 *N* HCl for 24 hours. The toxin was stable in 3 *N* HCl at 0°C for four weeks and at 55°C for two weeks. At pH 5, the toxin was stable at 0°C for five weeks, but a loss of one-third of the toxicity was observed when kept at 55°C for one week. Heating for 24 hours at 100°C at a pH of 5.5 resulted in complete destruction of the toxin.

Medcof *et al.* (1947) determines the effects of cooking on the retention of toxic properties of the PSP toxin. He reported a loss of toxicity up to 70% by ordinary cooking and also observed that pan frying was more destructive than boiling. Concon (1988) also reported that there is a very large decrease

of toxicity in cooked mollusc, especially when higher temperatures are employed in cooking, as in pan frying.

Wiberg and Stephenson (1961) studied the stability of toxicity in the presence of various metal ions. Unfortunately, it was not possible to use the same concentration in all cases, because of the inherent toxicity of many of the ions. Only Na^+ ion had the ability to raise LD_{50} of the toxin.

Chang *et al.,* (1988) evaluated the stability of PSP toxin extracted from intoxicated sea mussel *Mytilus edulis* by the change of heating conditions and pH of the PSP solution. The extracted PSP was stable over the range of pH 2.0 to 4.0, but it was unstable above pH 4.5.

Alfonso *et al.,* (1994) analysed the stability of STX and neoSTX in acidic solutions and lyophilized samples. They demonstrated that STX was very stable in dilute acidic solution for 18 months without loss of potency. However, neoSTX was unstable, possibly due to transformation to other toxins (Alfonso *et al.,* 1994). Louzao *et al.,* (1994) studied the stability of GTX obtained from contaminated mussels and stored at different temperatures in lyophilized samples. The stability of the toxin was analysed by HPLC. These researchers concluded that the lyophilization procedure is not the safest way to process most of the GTXs.

Sako *et al.,* (1995) investigated the stability and PSP toxin composition in the dinoflagellate *Alexandrium catenella* and *A. tamarense,* and the genetic system producing it. They found that the toxin composition did not significantly change in any growth phase. Furthermore, they determined the toxin compositions of many sub progenies from crosses between algal strains having different toxin profiles. They also reported that toxin compositions were

inherited mostly in a 1:1 Mendelian pattern and sexuality (mating type) was not associated with toxin inheritance. These workers confirmed that the PSP toxin composition of *A. catenella* and *A. tamarense* are stable and that Mendelian inheritance of toxin profiles occur in both these heterothallic dinoflagellates.

2.8 ANALGESIC ACTIVITY

The use of extracts of plants and animals for medicinal purposes is a practice as old as the history of mankind. However, man's efforts have largely been confined to land plants and animals probably because of their easy accessibility. These represent only 20% of the earth's total plant and animal life. The oceans, which cover about 71% of the earth, support a very rich plant and animal life, some of which are exclusively marine and thrive under extremely diverse environmental conditions. A large number of marine organisms are known to possess bioactive substances that have tremendous pharmaceutical potential for the future (Qasim, 1998).

Bathing in the extract from the mangrove plant *Acanthus illicifolius.* in the form of a decoction obtained by boiling the leaves and young shoots, has long been used in Goa to provide relief from rheumatic pain (Qasim, 1998). The crude methanolic extract was found to exhibit analgesic and anti-inflammatory properties that are more effective in small doses than the standard analgesics pethidine hydrochloride and sodium salicylate. It did not show any addiction either (Qasim, 1998). Fiore *et al.,* (1983) reported that calcitonins produced by salmon have analgesic effect on patients suffering from chronic, intractable oncological pain. The result showed effective analgesic effect in the patients that lasted up to 12 hours. The dose was quite

lower than the dose of commonly used analgesics and there was no irritation or respiratory difficulties during the treatment. Martin *et al.* (1994) found that intraperitoneal administration of analgesic doses of salmon-calcitonin enhanced the *in vitro* effects of opioid agonists. Goicoechea (1999) reported that the analgesic effect of the opioids was significantly enhanced by pretreatment of the animals with intraperitoneally administered salmon-calcitonin. This result suggests that the joint administration of salmon-calcitonin and opioids may be useful and interesting alternative in the treatment of painful diseases resistant to other treatments. Guidobono *et al.*, (1986) administered eel-calcitonin to rats and found them to lack analgesic property that is comparable with the salmon-calcitonin.

Spampinato *et al.*, (1994) studied the effect of Omega conotoxin and verapamil on antinociceptive, behavioural and thermoregulatory responses to opioids in the rat and found that this omega conotoxin and verapamil prolonged and potentiated the effect of opioids. Cai *et al.*, (1997) showed that Tong Ke, which is composed of the extracts from the river clams *Hyriopsis cumingii* and *Raix notoginseng*, possessed a marked analgesic effect on mice as measured by the hot-plate and slow ache tests. The authors also observed an obvious anti-inflammatory effect on crofon oil-caused mice ear swelling and agar or carrageenan-caused swelling on foot in mice and rats.

A soluble fraction from shark cartilage was found to possess both anti-inflammatory and analgesic properties on rats and mice (Fontenele *et al.*, 1996). Sakthivel (1999) reported analgesic property of conotoxin extracted from two marine conus, namely, *Conus lentiginosus* and *C. mutabilis*.

Analgesic effect of medicinal plants is a widely studied subject. Several of the flavonoids have been shown to elicit significant dose-related analgesic activity (Ramaswamy *et al.,* 1980, 1985; Viswanathan, 1984). In addition to this, these compounds also possess anti-inflammatory and anti-ulcer properties, which is a rare combination (Parmar, 1977; Viswanathan *et al.,* 1984). Presently, these agents are considered as experimentally safe analgesics and clinical trials are under way (Padmakumar *et al.,* 1994). A report on *Acanthus ilicifolius* explains its analgesic effect on two different assay models (Agshikar *et al.,* 1979). Padmakumar *et al.,* (1994) assayed 17 species of marine plants for their analgesic activity; all these plants were less effective when compared to morphine. However, information pertaining to the analgesic effect of PSP toxins from marine algae, dinoflagellates, etc. is very scanty.

2.9 DEPURATION

Commercial depuration of molluscan shellfish has been practised for at least 75 years (Canzonier, 1991). The association between typhoid fever and raw mussel consumption led to the development of the first depuration facility and Dodgson (1928) was the pioneer. Specifically, seawater was disinfected by the use of hypochlorite and then neutralized by adding sodium thiosulfate to eliminate the residual chlorine. Other disinfecting methods for seawater were investigated several years later, and included ozone and ultraviolet light. Ozone was first introduced by Violle in 1929 to disinfect seawater and reported that it required 5-6 days to sterilize oysters (Roderick and Schneider, 1994). During the late 1920s, there was much discussion concerning the improvement of shellfish sanitation, as there was a series of typhoid fever

outbreaks associated with the consumption of raw oysters (Canzonier, 1991). Other shellfish purification activities during 1930's and 1940's included those of Misser in 1930, Tarbell in 1934 and Fisher in the year 1941 (Canzonier, 1991). During 1950s, a soft-shell clam depuration plant was established in Maine and also Newbury Port, Massachusetts (Canzonier, 1991).

In depuration plants, seawater quality plays a major role in depurating the shellfish. Research on the effects of seawater temperature upon bacterial reduction in depuration plants had been carried out by Arcisz and Kelly (1955), Heffernan and Cabelli (1970), Cabelli and Heffernan (1971), Souness and Fleet (1979), Rowse and Fleet (1984), Richards (1990), and Roderick and Schneider (1991).

The depuration of contaminated shellfish was addressed as early as 1911 (Phelps, 1911) and the first use of chlorine in the depuration of shellfish was by Johnstone (1914). The use of UV light for depuration is the most effective one and was first introduced in the United States by Arcisz and Kelly (1955) and in the United Kingdom by Wood (1961a,b). Many studies have been carried out to show the effectiveness of UV light for disinfecting water and subsequent use of that water for depuration of shellfish (Cortelyou, 1954; Arcisz and Kelly, 1955; Fogh, 1955; Kawabata and Harada, 1959; Hill *et al.,* 1969, 1970; Cabelli and Heferrnan, 1970; Huntly and Hammerstrom, 1971). Ozone, which is most successfully and popularly used for depurating molluscan shellfishes to get rid of bacteria, viruses, and also dinoflagellate and other biotoxins, was first used by a commercial depurating plant in France in 1963 (Fauvel, 1963). The literature contains numerous reports on ozone's ability to inactivate bacteria (Blogoslawski and Monasterio, 1982), viruses

(Englebrecht and Chain, 1985; Sproul *et al.,* 1985) and pathogenic yeasts (Combs and Blogoslawski, 1975). In addition to ozone's ability to inactivate bacteria and viruses, it also oxidizes marine dinoflagellate toxins (Thurberg, 1975) and also tetrodotoxin (Blogoslawski and Stewart, 1977). Blogoslawski *et al.,* (1979) studied the effects of ozone on potent neurotoxins produced by *G. tamarensis*, which are known as PSP. Clams exposed to a *G. tamarensis* bloom were collected and transported to a pilot-scale depuration facility. The clams were kept in the system for 72 hours, where oxidant concentration was measured at 1.2 or 0.5 mg/l. After that the toxin was extracted from the muscle and when bioassayed, showed no toxicity. Dawson *et al.,* (1976) showed ozone to be effective in detoxifying *Mya arenaria* exposed to *Gonyaulax tamarensis.* Ozone is also effective in detoxifying the toxins from *Gymnodinium breve* from the mollusc (Blogoslawski *et al.,* 1975; Thurberg, 1975). Gacutan *et al.,* (1984) detoxified *Pyrodinium*-generated toxin in *Perna viridis* using ozone, chlorine and PVP-iodine (polyvinyl pyrrorlidone-iodide-iodine). Chakrabarti and Khasim (1989) did not find much effect of chlorination during depuration of Indian cockle.

In Netherlands, the Netherlands Institute of Fisheries Research has designed a large-scale water recirculating system to depurate the imported mussels and oysters, so that no PSP intoxicated mollusc or dinoflagellate cysts enter to the natural water body after introducing the imported stock (Dijkema, 1995). In Bay of Fundy, Canada, giant scallop *Placopecten magellanicus* is contaminated with PSP toxin throughout the year. These scallops are depurated by keeping them in filtered aerated seawater at ambient temperature and are fed culture of algae, *Chaetoceros gracilis* and

Isochrysis galbana. These treated scallops, while testing for toxicity by mouse bioassay, showed reduced toxicity (Waiwood *et al.,* 1995). Lassus *et al.* (1994) suggested a relationship between initial toxicity, the concentration of nontoxic algae available and the time required for depuration. They reported that decontamination experiments on PSP toxin- contaminated mussels fed two nontoxic algal diets showed an appreciable reduction in the time needed to decrease toxin concentration below the accepted threshold for human consumption. Lassus *et al.,* (1993) reported that farmed mussels (*M. edulis*) when contaminated with a pure culture of an *Alexandrium tamarense* showed that GTX-2 formed in increasing quantities than other gonyautoxins and eliminated slowly while depurating.

Mercenaria mercenaria, which was previously reported to remain non-toxic during red tides, was investigated to see its susceptibility towards PSP toxin and depuration by Lee *et al.,* (1989). The hard clams were exposed to two strains of *Alexandrium fundyense* over two weeks and then allowed to depurate for another three weeks. HPLC analysis showed that the clam readily ingests the toxin and during depuration, it showed a decrease in GTX-1 and 4. Dijkema *et al.,* (1995) reported the depuration of PSP, DSP and ASP toxins at 10°C occurs within 3, 10 and 15 days, respectively, in clean water. The authors also found that during toxic blooms, an equilibrium between intake and excretion of phycotoxins in bivalves was generally reached within 10 days.

Depuration of other algal toxins from cyanobacteria and diatoms has also been tried. Vasconcelos (1995) depurated mussels exposed to a toxic

cyanobacterium and found a 50% decrease in the amount of detectable toxin in the mussels within two days and on the13th day, no toxin was detected.

While domoic acid, which is produced by diatoms, depurated from oyster *Crassostrea* spp. at a slow rate of depuration over a 72-hour period (Roelke et al., 1993), 50% of domoic acid was eliminated within 24 hours from mussels and after 72 hours, mussels were either clean or contained only residual levels of toxin (Novaczek *et al.,* 1992). The concentration of domoic acid, the toxin involved in amnesic shellfish poisoning, in mussel tissues did not decrease consistently over a depuration period of 48 hours (Novaczek *et al.,* 1991). Marcaillou-le-Baut *et al,* (1993) reported that the rate of DSP depuration was better in culture ponds than in the laboratory. In the culture pond, it took 20 days for depuration as against 42 days in the laboratory.

2.10 LENGTH-WEIGHT RELATIONSHIP AND CONDITION FACTOR

Length-weight relationship and Condition factor of fishes have been studied extensively, including in India. Notable reports in this regard are those of Jhingran (1952) on Indian major carps, Bhatt (1970) on *Mystus seenghala*, Devaraj (1973) on *Ophiocephalus marulius*, Basu (1975) on *Otolithus argentius*, Choudhary (1978) on *Lactarius lactarius*, Acharya and Dwivedi (1981) on *Nemipterus japonicus*, Acharya and Dwivedi (1985) on *Trypauchen vagina*, Jaiswar and Acharya (1991) on *Megalaspis cordyla* and Raje (1996) on *Nemipterus mesoprion*.

Work on the condition factor of various fishes has been conducted by many scientists. Le Cren (1951) worked on correlation between condition factor and seasonal changes in gonadal development growth, effect of stomach content on weight of fish. Bhatt (1970) has attributed the cause of

fluctuations of K value to the rate of feeding. In *N. japonicus*, Acharya (1980) noticed fluctuations in K value to be related with biological factors like fatness, suitability of environment, gonadal development, spawming and growth. In *Cirrhinus mrigala* the K value depends on physiological factors such as maturity, spawning, environmental condition and availability of food (Raizada and Raizada, 1982). Other important works on different aspects of K value include those of Hart *et al.,* (1940) on herring; Ganapati and Rao (1957) on *Sardinella gibbosa*, Basu (1975) on *Otolithus argentius*, Choudhary (1978) on *Lactarius lactarius*, Gulati (1987) on *Otolithus cuvieri*, Goswami and Devaraj (1992) on *Wallago attu*, and Shinde (1994) on *Rastrelliger kanagurta* and *Megalaspis cordyla.*

In comparison to this, there are very limited work on length-weight frequency and condition factor of molluscs, Benny and Ayyakkannu (1992) studied the length-weight relationship in *Chicoreus ramosus* collected from the Gulf of Mannar area. Regression analysis showed linear relationship between length and weight of both sexes. They also observed great variations in the length-weight relationship between males and females. Although generally positive allometric relationships exist in bivalves, Mohan (1980) found that in *P. viridis,* the relationship between length and weight was highly complicated and the conventional allometric equation $W = al^b$ could not be employed. He could not plot a single regression line for the entire range of length of samples of cockle when he plotted the logarithmic values of length and the corresponding weight. There appear to be a break in the relationship at a length of about 20 mm. Sriram *et al.* (1987) showed a linear relationship between length and weight of *Telescopium telescopium* after fitting

regression line to the length-weight data. The logarithmic transformation of the length-weight relationship formula gives a linear regression in oyster (Pota and Patel, 1987). Besides these, works on different aspects of length and weight relationship and condition factor in molluscs were done by Rao (1987), Ayyakkannu (1992), Laing and Utting (1994), Amini and Pralampita (1987), Brey and Hain (1992), Paninee and Pitiwang (1991), Sockendarsi *et al.* (1998); Vandepeer *et al.* (1999) and Rabi and Maravi (1997). But there are hardly any report on the inter-relationship between the condition factor and the accumulation of toxicity level in any organisms except that of Deo (2000) who found condition factor to affect the toxicity level of catfish mucus.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 SPECIMEN COLLECTION

Live specimens were collected from the Palghar landing centre, from where the shellfish, mainly clams and mussels, are supplied to the whole Mumbai area. Specimens of *Meretrix meretrix* and *Perna viridis* were collected once a month from January 1999 to January 2000 except September and October when there was no harvesting for mussels. They were brought to the laboratory in ice. Upon arrival at the laboratory, individul length and corresponding weight of each specimen were recorded and a few were pried open for extirpation of body parts, and the rest stored at -20^0C until further analysis.

3.2 EXTRACTION OF TOXIN

Paralytic shellfish poison was extracted from the clams and mussels following the AOAC (1995) method. Specimens were cleaned thoroughly from outside with freshwater and then, cut open by cutting the adductor muscles. The inside was washed with freshwater to remove sand and other foreign material. The meat part was removed from the shell and 100-150 g of meat was collected in a glazed dish. The meat portion was then drained for 5 minutes and ground in a house-hold type grinder until homogenised. This well-mixed material was taken in a beaker and 100 ml of 0.1 *N* HCl was added to the beaker. It was mixed thoroughly, always maintaining the pH at around 3.0. Then, the mixture was boiled gently for 5 minutes and was allowed to cool to room temperature. pH was again checked and adjusted around 3.0 by using 0.5 *N* HCl or 0.1 *N* NaOH. The mixture was then transferred to a

graduated cylinder and diluted to 200 ml. The mixture was stirred to homogeneity and centrifuged at 3000 revolution/minute for 5 minutes and the supernatant was taken as the crude toxin.

3.3 MICE BIOASSAY

The presence of paralytic shellfish toxin in the crude extract from clams and mussels was tested by the mouse bioassay technique of AOAC (1995). Clinically healthy male albino mice (Kasauli strain) of 20±2 g weight were procured from M/s.Shinde Animal Breeding Lab, Vashi, and were maintained in a healthy condition in the animal house of the Biotoxinology laboratory.

The crude toxin was injected intraperitoneally to the mouse at 1 ml/mouse. The time of injection was noted down and injected mice were kept under careful observation till the death as indicated by last gasping breath. The death time was recorded as also the clinical symptoms. For each dose, triplicate sets were maintained. A control was maintained with each set of experiment by injecting an equal volume of normal saline or 0.15 M phosphate butter saline of pH 7.5.

3.4 CALCULATION OF TOXICITY

The toxicity of the PSP toxin was carried out using AOAC (1995) method. First, the median death time was determined and from Sommer's Table (AOAC, 1995) corresponding number of mouse units were determined.

3.5 AUTOPSY

Autopsy was conducted on mice that died upon envenomation to observe symptoms such as hemorrhage, gross anatomical changes, granular appearance or discolouration of heart, liver and kidney, septicemia, dropsy and other abnormalities, if any.

3.6 HISTOPATHOLOGICAL STUDY

Kidney, liver and heart of mice that died upon envenomation were excised and fixed in 10% formalin. It was then rinsed in three changes of 70% (v/v) alcohol to remove the excess fixative. The tissues were dehydrated in ascending grades (50%, 70%, 90%, 100%) of alcohol for one hour each. The tissues were then cleaned by keeping in xylene for two hours and then embedded in paraffin wax thrice, each time for 45 minutes. Once the tissues were blocked, it was allowed to solidify and the surplus amount of paraffin wax was trimmed off. Sections of 10 µm thickness were cut using a hand rotary microtome. The ribbon thus obtained was kept with its shiny side downwards onto the surface of warm slide maintained at a temperature 2-3°C less than the melting point of paraffin wax. The best sections among them were picked up on a microscopic slide. The excess water was removed using a blotting paper. The dewaxing was done by drying the slides on a hot plate for 2-3 hours and by cleaning them in xylene. Samples were them hydrated in descending grades of alcohol. The staining was done using Delafield's haematoxylene for seven minutes, dipped once in acid alcohol and then for three minutes in Scott's tap water. These were then passed through descending grades of alcohol (3 min each), followed by eosin stain (3 min) and absolute alcohol (1 dip) and finally cleared in xylene. These were mounted on DPX. Prepared slids were examined and photographed on a phase contrast microscope.

3.7 HEMOLYTIC ASSAY

3.7.1 Preparation of Erythrocyte Suspension

Fresh chicken blood was collected from a nearby slaughter house and was added with EDTA solution (2.7 g in 100 ml distilled water) as anticoagulant at 5% of the volume of blood. The blood was centrifuged at 5000 rev/min for seven minutes at 4°C. The supernatant was discarded. The residue was washed with normal saline and centrifuged at 5,000 rev/min for 7 minutes. The supernatant was discarded and the process of washing and centrifugation was repeated again. A 1% erythrocyte suspension was prepared by adding 99 ml of normal saline to 1 ml of the packed erythrocytes (residue).

3.7.2 Assay for Hemolysis

Hemolysis assay was done following Pani Prasad and Venkateshvaran (1997) in 'V' shaped microtitre plates. The crude toxin extracts from clams and mussels collected every month were assayed. One row was used for samples from one particular month. Initially 100 µl of normal saline was added to each well of the plate. Then, 100 µl of the toxin was added to the first well of the plate and was thoroughly mixed. From the first well 100 µl was transferred to the second well and mixed thoroughly and this process was repeated till the last well and from the last well, 100 µl was discarded. Then, 100 µl of the prepared erythrocyte suspension was added to all the wells. A positive control was kept by mixing 100 µl distilled water and 100 µl 1% erythrocytes suspension, and a negative control was kept by mixing 100 µl of normal saline and 100 µl of 1% erythrocytes suspension. Formation of a fine "button cell" with regular margin indicated a negative reaction, whereas a

uniform red coloured suspension of the lysed erythrocytes indicated a positive result.The plates were incubated for two hours at room temperature and the result was read. Hemolytic activity was expressed as hemolytic unit (HU). The reciprocal of the highest dilution of the toxin showing the hemolytic pattern was taken as 1 HU.

3.7.3 Assay for Hemagglutination

Hemagglutination study was conducted following the method of Liang and Pan (1995) using U-bottomed microtitre plates. Procedure for the preparation of erythrocyte was the same as that of the hemolysis study but a 2% erythrocyte suspension was used. Initially, 25 µl of normal saline (0.9% NaCl, pH 7.2) was transferred to the wells. This was followed by mixing of 25 µl toxin in the first well. The serial dilution was carried out up to the last well from where 25 µl was discarded. After this, 25 µl of four time washed 2% erythrocyte suspension was added to all the wells. The plates were kept on a mini shaker for 1 minute and were incubated for one hour at 4°C and the results were read. A positive control was maintained by adding 25 µl of 2% erythrocyte suspension to 25 µl distilled water and a negative control by adding 25 µl of 2% erythrocyte suspension to 25 µl of normal saline. Formation of many small clumps in an irregular manner denoted positive reaction, while formation of a single smooth edged 'button' at the bottom of the well was read as negative reaction.

3.8 ANALGESIC ACTIVITY

Analgesic activity was measured according to D'Amour and Smith Test (D'Amour and Smith, 1941) using a Tail Flick Analgesia Meter (Harvard, USA, 50-9495, 230 V, and 50Hz) with a variable 150 W 24 V quartz lamp as the

heat source. The mouse was held in an improvised holder in such a way that its tail covered the photocell of the meter. The heat source was turned on using a foot switch, which started an electronic timer. When the mouse felt the pain, it flicked the tail, and therefore, uncovered the photocell. This action switched off the bulb and stopped the counter. The reaction time of the animal was then noted down. The mice injected with normal saline were used as control, while those injected with paracetamol (at 0.8 ml/20 g mouse) served as reference standards. Experimental mice in triplicate sets received 0.8 ml of the toxin and were subjected to a light intensity of four different current strengths *viz*. 3.0, 3.5, 4.0, 4.5 A. For studying the analgesic property of the crude extracts of clams and mussels, the most toxic one in each case, based on earlier mouse bioassay results, was used. Thus, October sample in the case of the clam and March sample in the case of the mussel were taken for assaying their analgesic property. Analgesic activity was expressed as a ratio of reaction time of envenomated mice against those of the control as also those of the mice injected with paracetamol.

3.9 HPLC ANALYSIS

The method followed was that of Oshima (1995) which is a modified version of the Oshima (1989). HPLC system consisted of a high pressure pump (Hitachi L6200) with syringe loading sample injector (Rheodyne 67125), a stainless steel column of reversed phase packing, a triple head reaction pump (655 A-B Hitachi), 10 m teflon tubing of 0.5 mm internal diameter immersed in oil bath (Hitachi) for reaction, a fluorescent spectrophotometer (Hitachi F-1050) with 150w xenon lamp, 12 µl flow cell and a chromato

integrator (Hitachi D-2500). The column used was a Devosil C8-5 (Nomura Chemicals, Japan). For analysis, 10µl of the crude extract was injected.

3.9.1 Reagents

A. Stock solutions

1. 100 m*M* sodium 1-heptansulfonate solution: 2.02 g of HPLC grade reagent in 100 ml of distilled water.

2. 500 m*M* phosphoric acid: 28.8 g of concentrated analytical grade (85%) phosphoric acid in distilled water and made up to 500 ml.

3. 1 *N* ammonium hydroxide: 70 ml of ammonium hydroxide made up to 1000 ml with distilled water.

4. 350 m*M* periodic acid: 7.978 g of analytical grade periodic acid in 100 ml of distilled water.

5. 250 m*M* disodium phosphate: 44.77 g of analytical grade Na_2HPO_4. $2H_2O$ in distilled water and made up to 1 l.

6. 1 *N* sodium hydroxide: 4 g of NaOH in 100 ml of distilled water.

B. Mobile Phase

Ten milliliter of stock solution-1 and 30 ml of solution-2 in 450 ml of distilled water, adjusted to pH 7.1 by adding NH_4OH (solution-3) and made up to 500 ml: 30 ml of acetonitrile was then added, mixed well and degassed by sonication.

C. Oxidising Reagent

Ten milliliter of stock solution-4 and 100 ml of solution-5 were added to 100 ml distilled water and titrated to pH 9.0 with sodium hydroxide (solution-6); then diluted to 500 ml with distilled water. This reagent was prepared freshly and used.

D. Acidifying Reagent

Acetic acid (0.5 M) was used as the acidifying reagent.

3.9.2 Reaction Conditions

Injection volume of the sample was 10 µl. The flow rate of the solvent was maintained at three different levels, 0.6 ml/min, 0.8 ml/min and 1.0 ml/min depending on the requirements for better separation of peaks. Flow rate for the oxidizing and acidifying reagents was maintained at 0.4 ml/min. The elute from the column was mixed with oxidizing reagent and the reaction was made at 65°C in 10 meter Teflon tubing (0.5 mm internal diameter) inside the oil bath. The mixture was mixed with acidifying reagent and passed into the detector (fluorescent spectrophotometer) at an isolation wavelength of 330 nm and emission wavelength 390 nm.

3.9.3 Standard Toxin

The extract of clam from which concentration of toxins have been previously determined (Karumasagar *et al.,* 1990) was used as the reference standard.

3.9.4 Preparation of Shellfish Extract for HPLC Analysis

The shellfish extracts that were prepared by AOAC method for standard mouse bioassay were further processed for HPLC analysis. The crude extracts were centrifuged at 10,000 rev/min for 10 minutes. The supernatant was passed through Sep-pak C-18 cartridge column. Prior to this, the cartridge column was washed with 10 ml of methanol and equilibrated with 10 ml of deionised (Millipore) water. After passing the supernatant through the column cartridge, the first 1.5 ml of the elute was discarded and the rest was collected. The filtered, cleaned-up elute was then passed through a

10,000 dalton cell of membrane filter (Ultra Free LGC Millipore) and the elute was ready for HPLC analysis.

3.9.5 Operation of HPLC

The HPLC pump was primed with the mobile phase and the flow rate was established between 0.6-1.0 ml/min as required and the pump was run for 15 minutes. The post-column reaction pumps were then primed with the oxidizing reagent and 0.5 *M* acetic acid separately and a flow rate of 0.4 ml/min was established then. The detector was operated at the excitation wavelength of 330 nm and emission wavelength of 390 nm. After this, the standard toxin was injected repeatedly until retention times were constant, which indicated that the column has been equilibrated with the mobile phase: 10 µl of the sample was then injected and the peak height was recorded. For calculation of toxin concentrations for the sample, the nearest standard chromatogram was used for comparison with the chromatogram of the sample.

3.10 STABILITY

3.10.1 By Lyophilisation

The extracts from the clams and mussels found to be toxic by mouse bioassay were lyophilized. The lyophilized sample was dissolved in distilled water and injected to the mice to know its toxicity. After the bioassay, the solution was run through HPLC with post-column reaction.

3.10.2 By Altering the pH

Normally, the PSP toxin is stable at pH of 2.0-4.5. To know the stability of the toxin at higher pH, the pH values of the crude toxin was raised above 5.0 using NaOH and then the toxin was assayed for toxicity by injecting it to

mice. The death time of the mice was compared with the death time of those mice that were injected with the crude toxin kept at pH between 2.0 and 4.5.

3.10.3 By Storing the Crude Toxin at – 80°C

The crude toxin was stored in a deep freezer at –80°C for a period of more than six months. After that, the toxin was thawed and injected to the mice. Also the samples were run in HPLC to know the toxin composition of the sample. Before storage, the toxin was assayed for toxicity by mouse bioassay and the results of both the experiments were compared.

3.11 DEPURATION

If the bioassay confirmed the accumulation of PSP, specimens from the collected samples were taken for depuration. Thus, clams collected during May and the mussels collected during March were taken up for depuration studies.

3.11.1 Acclimation and Detoxification

The mussels and clams were kept floating in plastic bags in 32 ppt seawater for acclimation for 30 minutes and then kept in a fiberglass tanks for detoxification. Chlorine and ozone were used for detoxification following Gacutan *et al.* (1984) using ozone and chlorine, with slight modification.

For chlorination treatment, calcium hypochlorite was added to the water to a concentration of 0.5 mg/l. For ozonation, ozone was generated from Bio-Klan-108R ozoniser unit at the rate of 25 mg O_3/h. This ozoniser takes up atmospheric air and converts part of the oxygen into ozone by electronic circuit and delivers it under pressure. A PVC pipe was connected to the ozoniser at one end and from the other end, connections were given to the depuration tank. The tanks were maintained for 24 hours and after that, the

water in each tank was replaced with a fresh solution having the same ingredients at the specified quantities for each chemical as stipulated earlier as also the same rate of O_3 discharge. Results of the previous bioassay for the same sample were taken as control for comparing the toxicity of depurated and undepurated clams and mussels.

3.11.2 Extraction of Toxin and Assay for Toxicity

The standard mouse bioassay method (AOAC, 1995) was used for the extraction of PSP from the depurated clams and mussels. Extractions were carried out after 5, 10 and 15 days. They were bioassayed to see the toxicity of those extracts. The average death time for each extract was calculated and the corresponding mouse units were drawn from Sommer's Table (AOAC, 1995).

3.12 LENGTH-WEIGHT RELATIONSHIP AND CONDITION FACTOR

The length and weight data were statistically treated by the least square method and put in logarithmic form following Hile (1936) using general formula

$$W = al^b$$

Where,

W = Total weight of clams/mussels in g

L = Length of clams/mussels in cm

a = Constant

b = An exponential value which usually lies between 2.5 and 4.0 for fishes, which maintain their specific body shape throughout their lives.

$W = al^b$ can also be expressed as

$\log w = \log a + b \log L$ or $Y = A + BX$

Where,

$A = \log a$, $B = b$, $Y = \log W$, $X = \log L$

$Y = A+BX$ gives the linear relationship between Y and X, otherwise the relationship is curvilinear.

Condition factor has been calculated using the formula

$$K = \frac{W}{L^b}$$

Where, K = Condition factor

W = Total weight of the species in g

L = Total length of the species in mm

b = an exponent

RESULTS

4. RESULTS

4.1 MICE BIOASSAY

In all the cases, the death time was more than one hour. In case of crude extract of *M. meretrix,* the minimum death time recorded was 1hour 05 minutes in the month of July and the maximum was more than 48 hours, which was not considered as lethal. Similarly in case of crude extracts of *P. viridis* also, recorded death times were always more than one hour, except in March 1999, when it was 1 hour. The mouse units calculated according to Sommer's Table given by AOAC (1995) ranged between 0 and <0.875 in all the cases. The details of the results are presented in Tables 1 and 2.

4.2 AUTOPSY

Autopsy revealed hemorrhage inside the body cavity, especially around the trachea of the mice injected with 1 ml crude toxin. Pale discoloration of the heart and dark discolouration of the liver were observed in a few cases.

4.3 HISTOPATHOLOGY

Microscopic examination of the prepared sections of heart, liver and kidney of the intoxicated mice revealed many changes when compared to the controls. In case of the kidney, moderate hemolysis was seen in some of the blood vessels. Tubular epithelium showed unaltered condition in most of the cases except in a few where the tubules contained network of proteinaceous material. Most of the glomerulii exhibited normal appearance. Mild centrilobular necrosis was seen in the liver tissue and hepatic cells revealed pyknotic nuclei and karyorexis. Small nodular foci containing pleomorphic deeply stained cells and mono-nucleus cells could be discerned at some places. Focal areas in the liver revealed hepatic cells undergoing

degenerative changes and coagulative necrosis. Mild to moderate accumulation of mono-nucleus cells as seen around the blood vessels. Mild hemolytic changes were seen in the blood vessels of the heart. The results are shown in Plates 1-6.

4.4 HEMOLYTIC AND HEMAGGLUTINATING ACTIVITIES

The results are presented in Plates 7-10 and Tables 3 and 4. The crude extracts of both the bivalves showed spontaneous hemolysis of chicken blood. Hemolytic activity was present in the extracts of both the bivalves throughout the year but the HU values were higher in *P. viridis* than in *M. meretrix*. The highest hemolytic activity of 512 HU was recorded in January 1999 in case of the mussel extracts. In case of the clam, extracts from the March sample showed the highest hemolytic activity with a HU of 64.

Extracts of neither species elicited any hemagglutinating reaction in chicken blood.

4.5 ANALGESIC ACTIVITY

Extracts from the clam and mussel showed slight analgesic activity in comparison with normal saline (control), ranging from 1.125 AU to 1.500 AU in *M. meretrix* and from 1.040 AU to 1.500 AU in *P. viridis*. However, the activity was negligible as compared to that of paracetamol. Details are presented in Tables 5 and 6.

4.6 HPLC ANALYSIS

The toxin composition of the sample, which was used as standard for the present experiment, is given in Table 7. The chromatograms obtained by running the standard at three different flow rates *i.e.* 0.6 ml/min, 0.8 ml/min and 1.0 ml/min are presented in Figs. 1-3. But the chromatogram obtained at

0.8 ml/min (Fig.3) only was used for comparing the result of our samples, the chromatogram of which is presented in Fig. 4 (*M. meretrix)* and Fig. 5 (*P. viridis*).

By comparing this chromatogram with the standard, toxin composition of the sample was found and the details are presented in Tables 8 and 9. Crude toxin extracted from *M. meretrix* contained *N*-Sulfocarbamoyl toxins C1 & C2 and decarbamoyl toxins and carbonate toxins, *i.e.* dcGTX/STX. In case of *P. viridis*, carbomate toxins GTX-2 & 3 and *N*-sulfocarbamoyl toxins C1 & C2 were present. The peak recorded at 8.56 minutes in the case of the clam, and the two peaks recorded at 5.66 minutes and 8.96 minutes in thecase of the mussel could not be identified for want of pure reference standard.

4.7 STABILITY

4.7.1 Lyophilisation

The PSP toxins in the present study were found to lose their lethality upon lyophilisation, since they showed no symptoms of toxicity when injected intra peritonealy into mice.

4.7.2 Altering the pH

The toxin showed no symptoms of intoxication when injected into mice, after the pH was raised above 5.0. Initially, slight sluggishness was shown by the injected mice but very soon they recovered, whereas the sample which was kept at pH 2.0-4.5 showed intoxication on the mice with prominent symptoms like paralysis, jerking movement, excess urination and finally death.

4.7.3 Storing at -80°C

The PSP toxins showed no loss of toxicity upon storage at -80°C even after six months.

4.8 DEPURATION

Death times by mouse bioassay were recorded to be 1 hour and 20 minutes in case of the clam extract of May and 1 hour in case of mussel extract of March. After depuration, these clam and mussel extracts showed the death time to be more than those of the corresponding crude samples, which indicated that the toxicity was reduced after depuration.

Ozone treatment is found to be more effective than the chlorine treatment. The toxin extracts of the clams and mussels which were subjected to ozone treatment showed no toxicity after the 10^{th} day of treatment. There was no death of the mouse even after the 48 hours of injection. On the other hand chlorine- treated clam and mussel extracts became nontoxic after the 15^{th} day of treatment. The details of the result are presented in Tables 10 and 11.

4.9 LENGTH-WEIGHT RELATIONSHIP AND CONDITION FACTOR

The results of length-weight relationship and condition factor of both the bivalves are presented in Tables 12 and 13. In case of mussel, the sample collected in November 1999 showed the highest condition factor of 3.90, whereas it was below 1.0 during the rest of the year. On the other hand, clams showed maximum condition in the month of February 1999 at 4.61 and the lowest in July.

TABLE 1

Toxicity of crude extract of *Meretrix meretrix* to male albino mice (20±2 g) at a dose of 1 ml/mouse

Sl. No.	Month	Death time h:min:sec	Mouse unit (MU)	Remarks
1	January 1999	--	--	Not lethal
2.	February	--	--	Not lethal
3.	March	01:30:00	<0.875	Lethal
4.	April	12:00:00	-	Sublethal
5.	May	01:20:00	<0.875	Lethal
6	June	4:00:00	-	Sub lethal
7	July	01:05:00	<0.875	Lethal
8	August	07:30:00	<0.875	Sub Lethal
9	September	-	-	Not lethal
10	October	02:00:00	<0.875	Lethal
11	November	14:00:00	-	Sublethal
12	December	20:00:00	-	Sublethal
13	January 2000	19:00:00	-	Sublethal

TABLE 2

Toxicity of crude extract of *Perna viridis* to male albino mice (20±2 g) at a dose of 1 ml/mouse

Sl. No.	Month	Death time h:min:sec	Mouse unit (MU)	Remarks
1	January 1999	--	--	Not lethal
2.	February	--	--	Not lethal
3.	March	01:00:00	0.875	Lethal
4.	April	02:00:00	<0.875	Lethal
5.	May	-	-	Not lethal
6	June	-	-	Not lethal
7	July	12:00:00	-	Sublethal
8	August	-	-	Not lethal
9	September	No sample	-	--
10	October	No sample	-	-
11	November	23:00:00	-	Sublethal
12	December	01:46:00	<0.875	Lethal
13	January 2000	01:30:00	<0.875	Lethal

TABLE 3

Hemolytic activity of crude PSP extracted from *Meretrix meretrix*

Month	Total hemolysis up to dilution	Hemolytic Unit (HU)
January 1999	3	8
Feb	3	8
March	6	64
April	3	8
May	2	4
June	3	8
July	3	8
August	4	16
September	2	4
October	2	4
November	2	4
December	1	2
January 2000	1	2

TABLE 4

Hemolytic activity of crude PSP extracted from *Perna viridis*

Month	Total hemolysis up to dilution	Hemolytic Unit (HU)
January 1999	9	512
February	5	32
March	6	64
April	6	64
May	4	16
June	4	16
July	4	16
August	4	16
November	4	16
December	5	32
January 2000	6	64

TABLE 5

Showing analgesic activity of crude extract from *Meretrix meretrix* in terms of tail flick response of 20 ± 2 g male albino mouse.

S.No	Intensity of light (lux)	Response time (sec) when injected with 0.8 ml/mouse of			Analgesic Ratio against normal saline	Analgesic Ratio against Paracetamol
		Normal	Crude	Paracetamol		
1	3.0	20	22.5	571	1.125	0.039
2	3.5	16	20.6	106	1.288	0.194
3	4.0	14	19.7	82	1.407	0.240
4	4.5	12	18.0	60	1.500	0.30

TABLE 6

Showing analgesic activity of crude extract from *Perna viridis* in terms of tail flick response of 20 ± 2 g male albino mouse

S.No	Intensity of light (lux)	Response time (sec) when injected with 0.8 ml/mouse of			Analgesic Ratio against normal saline	Analgesic Ratio against paracetamol
		Normal	Crude	Paracetamol		
1	3.0	25	27.5	260	1.100	0.105
2	3.5	22	26.0	94	1.180	0.276
3	4.0	16	25.2	60	1.500	0.420
4	4.5	12	12.5	14	1.040	0.892

TABLE 7
Showing HPLC profile of Standard Toxin

Name of the toxin	RT (min)	Area conc.	MU
GTX 2/3	508	1774761	1.1128
C1/C2	6.84	4199015	1.2796
dcGTX/STX	7.32	3399610	1.2222
GTX1-4	12.94	796631	0.1632

TABLE 8
Showing HPLC profile of crude extract of *Meretrix meretrix*

Name of the toxin	RT (min)	Area conc.	MU
C1/C2	6.58	33785	0.0103
dcGTX/STX	7.35	125230	0.0450
-	8.56	53286	-

TABLE 9
Showing HPLC profile of crude extract of *Perna viridis*

Name of the toxin	RT (min)	Area conc.	MU
GTX2/3	5.07	36157	0.02277
-	5.66	11947	-
C1/C2	6.53	16803	0.0051
-	8.96	2217	-

RT – Retention Time

TABLE 10

Showing toxicity levels, as death time of mice, upon depuration with ozone treatment

Species	Death time			
	Initial	After 5th day	After 10th day	After 15th day
Clams	1.20 h	6.20 h	> 48 h	No death
Mussels	1.00 h	5.30 h	> 48 h	No death

TABLE 11

Showing toxicity levels, as death time of mice, upon depuration with chlorine treatment

Species	Death time			
	Initial	After 5th day	After 10th day	After 15th day
Clams	1.20 h	4.20 h	24.20 h	> 48 h
Mussels	1.00 h	3.30 h	20.00 h	> 48 h

TABLE 12

Length-weight relationship and conditon factor for *Meretrix meretrix*

S No	Month	Length weight relationship $W=aL^b$	Condition factor
1	January 1999	$W=-0.035177\ L^{2.273279}$	0.93
2	February	$W=0.662509\ L^{1.181252}$	4.61
3	March	$W=0.03219\ L^{2.148927}$	1.08
4	April	$W=-0.266689\ L^{2.640613}$	0.54
5	May	$W=0.362199\ L^{1.67414}$	2.31
6	June	$W=-0.02574\ L^{2.323303}$	0.94
7	July	$W=-0.414356\ L^{2.861426}$	0.39
8	August	$W=0.285425\ L^{1.825578}$	1.93
9	September	$W=-0.240778\ L^{2.659828}$	0.58
10	October	$W=0.346444\ L^{1.717092}$	2.23
11	November	$W=0.037453\ L^{2.204396}$	1.09
12	December	$W=-0.00022\ L^{2.229469}$	1.00
13	January 2000	$W=-0.316751\ L^{2.734452}$	0.48

TABLE 13

Length-weight relationship and conditon factor for *Perna viridis*

SNo	Month	Length weight relationship $W=aL^b$	Condition factor
1	January 1999	$W=-0.882363\ L^{2.559256}$	0.13
2	February	$W=-0.683346\ L^{2.262458}$	0.20
3	March	$W=-0.62487\ L^{2.27125}$	0.24
4	April	$W=-0.496051\ L^{2.124854}$	0.32
5	May	$W=-0.066309\ L^{1.650396}$	0.87
6	June	$W=-0.303623\ L^{1.87784}$	0.51
7	July	$W=-0.352244\ L^{1.999414}$	0.45
8	August	$W=-0.799345\ L^{2.54123}$	0.16
9	September	No sample	---
10	October	No sample	---
11	November	$W=0.582042\ L^{1.05468}$	3.90
12	December	$W=-0.679055\ L^{2.350123}$	0.21
13	January 2000	$W=-0.874824\ L^{2.566617}$	0.13

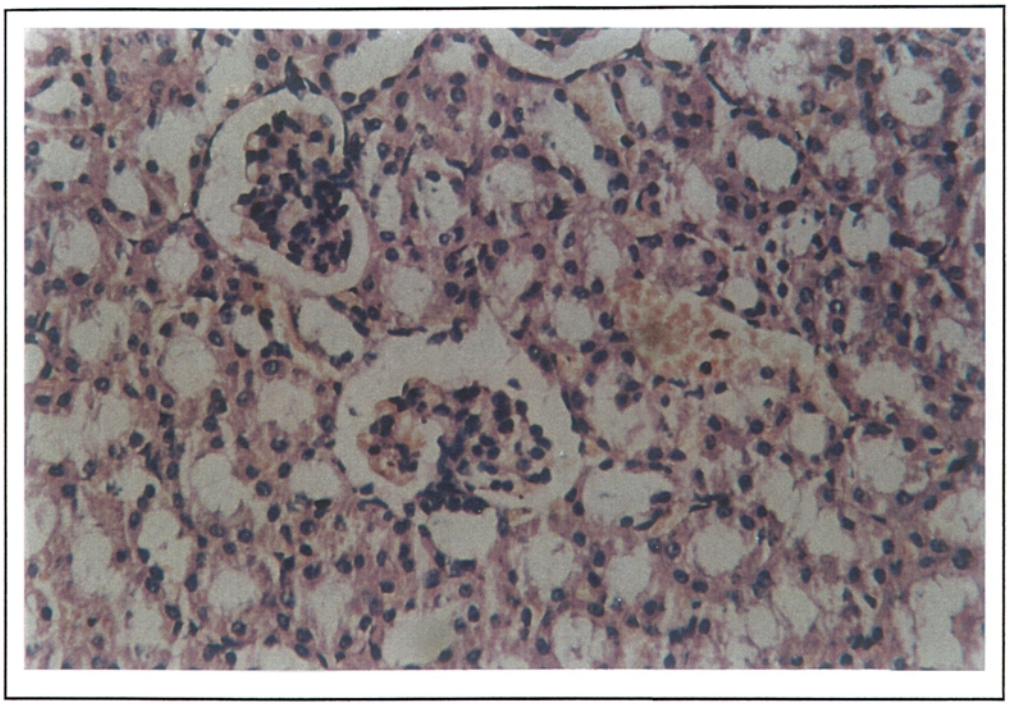

Plate 1: C.S. of kidney of mice injected with 1.0 ml of normal saline

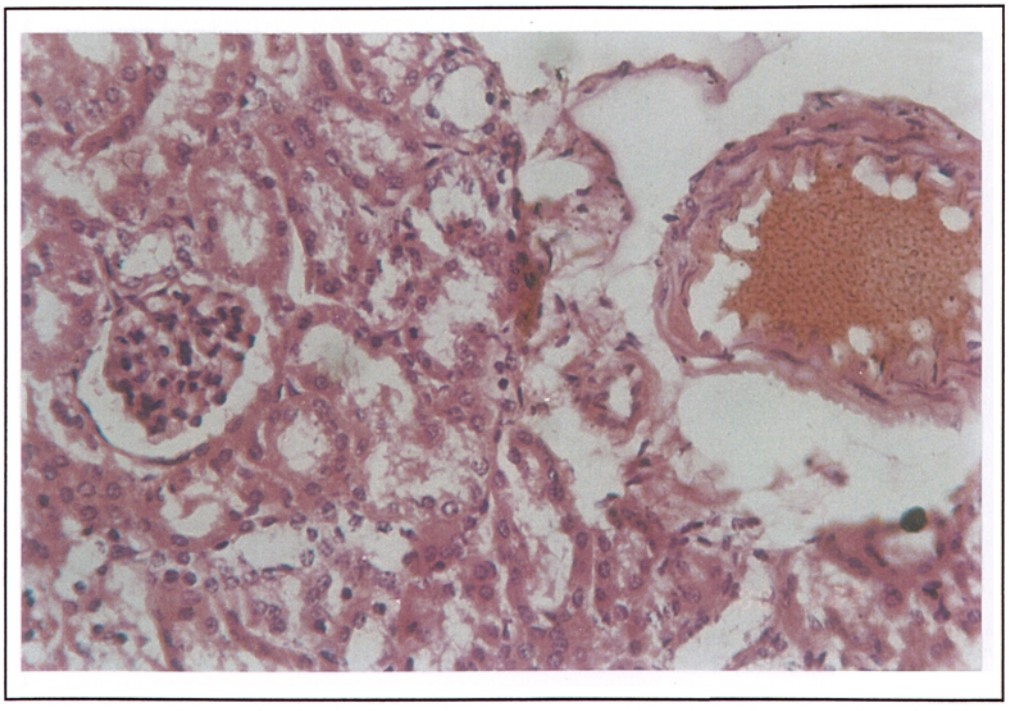

Plate 2: C.S. of kidney of mice injected with 1.0 ml of crude extract of mussels

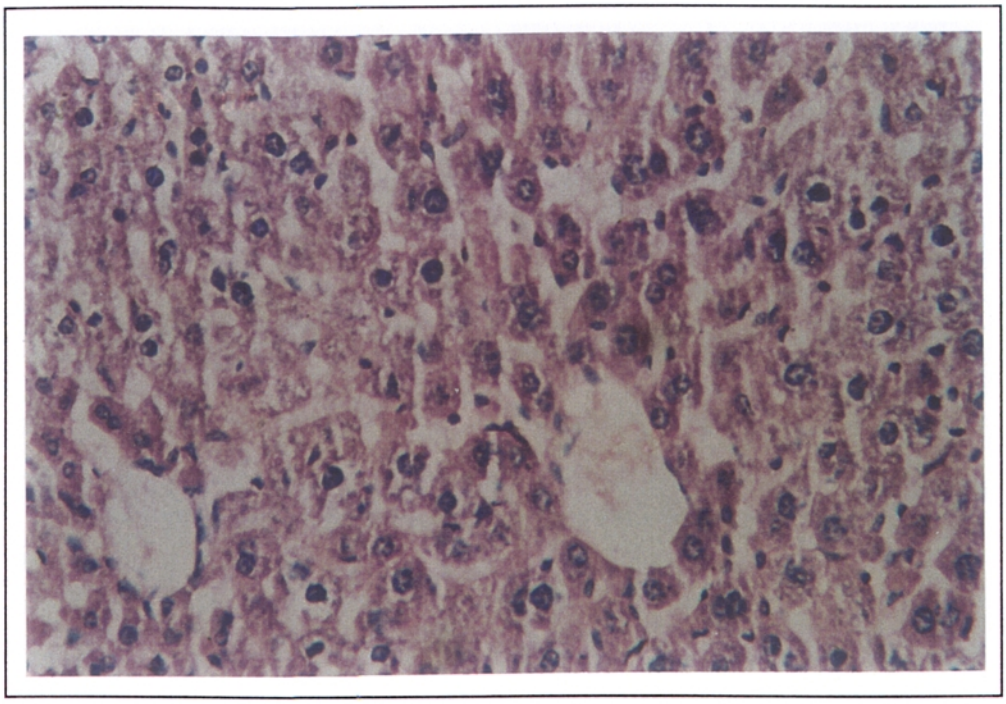

Plate 3: C.S. of liver of mice injected with 1.0 ml of normal saline

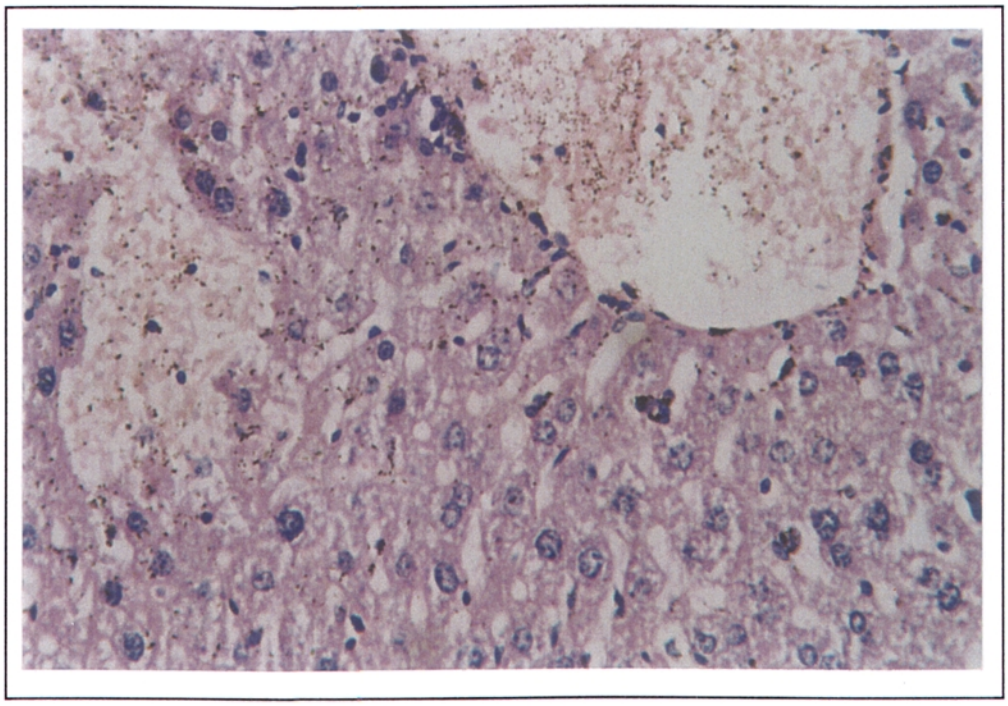

Plate 4: C. S. of liver of mice injected with 1.0 ml of crude extract of mussels

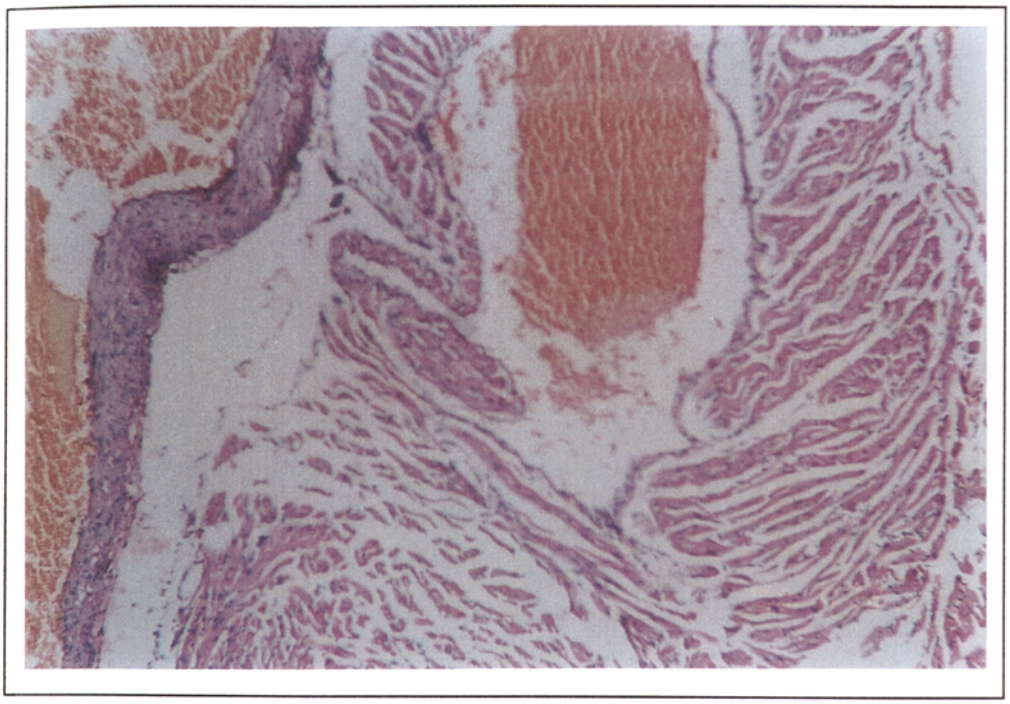

Plate 5 C.S. of heart of mice injected with 1.0 ml of normal saline

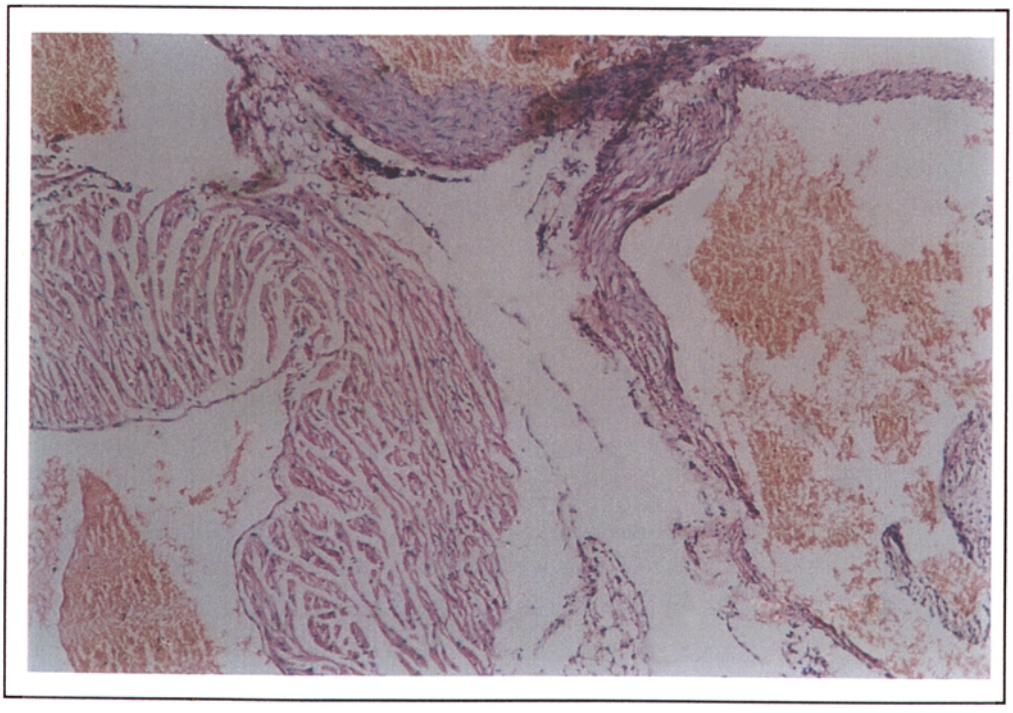

Plate 6 C.S. of heart of mice injected with 1.0 ml of crude extract of mussels

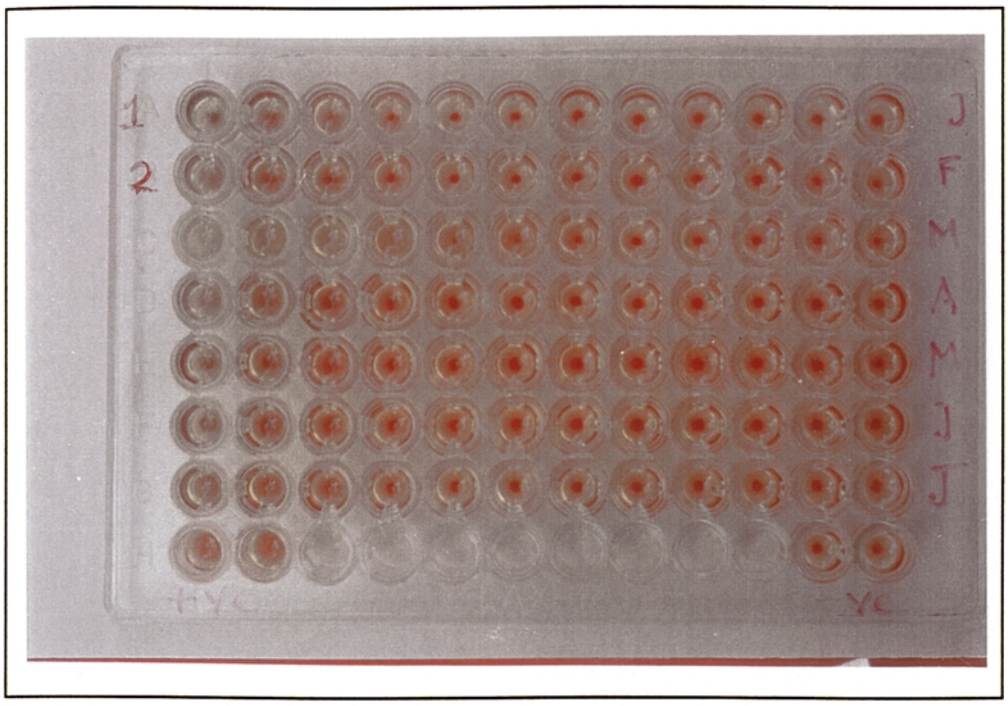

Plate 7: Showing the hemolytic activity of crude toxin extracted from Clams (Row 1-7: January 1999 to July 1999)

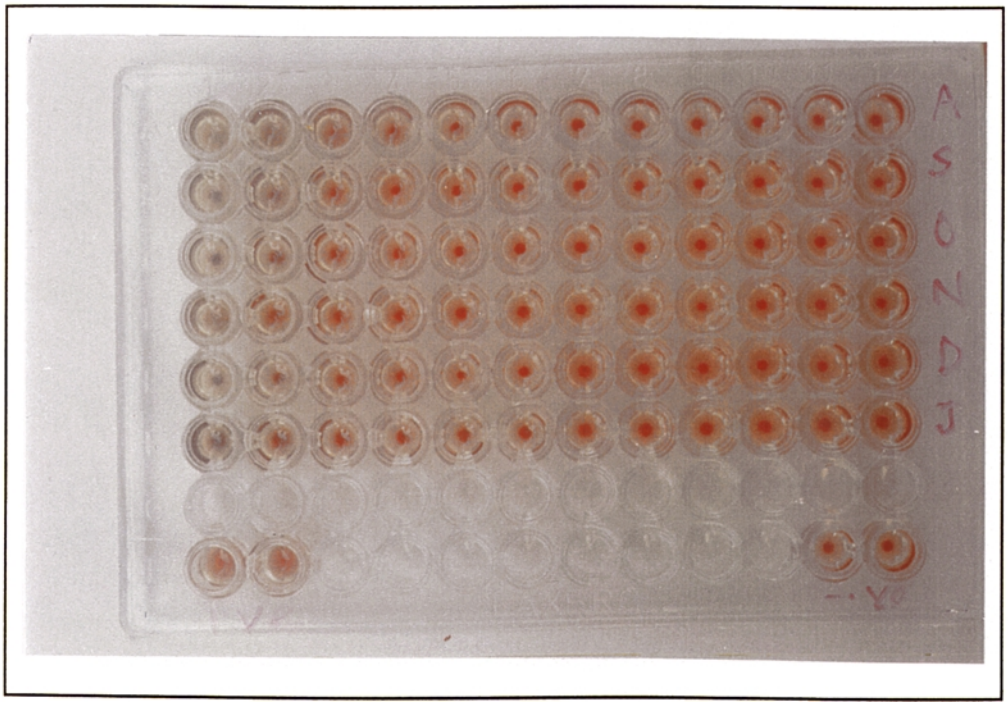

Plate 8: Showing the hemolytic activity of crude toxin extracted from Clams (Row 1-6: August 1999 to January 2000)

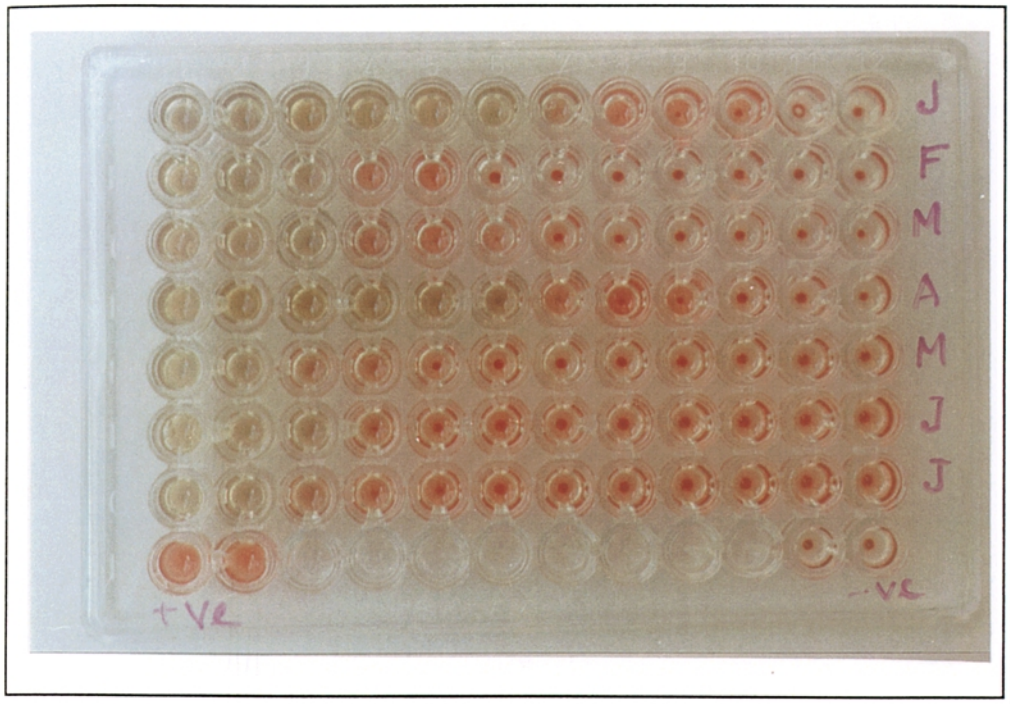

Plate 9: Showing the hemolytic activity of crude toxin extracted from Mussels (Row 1-7: January 1999 to July 1999)

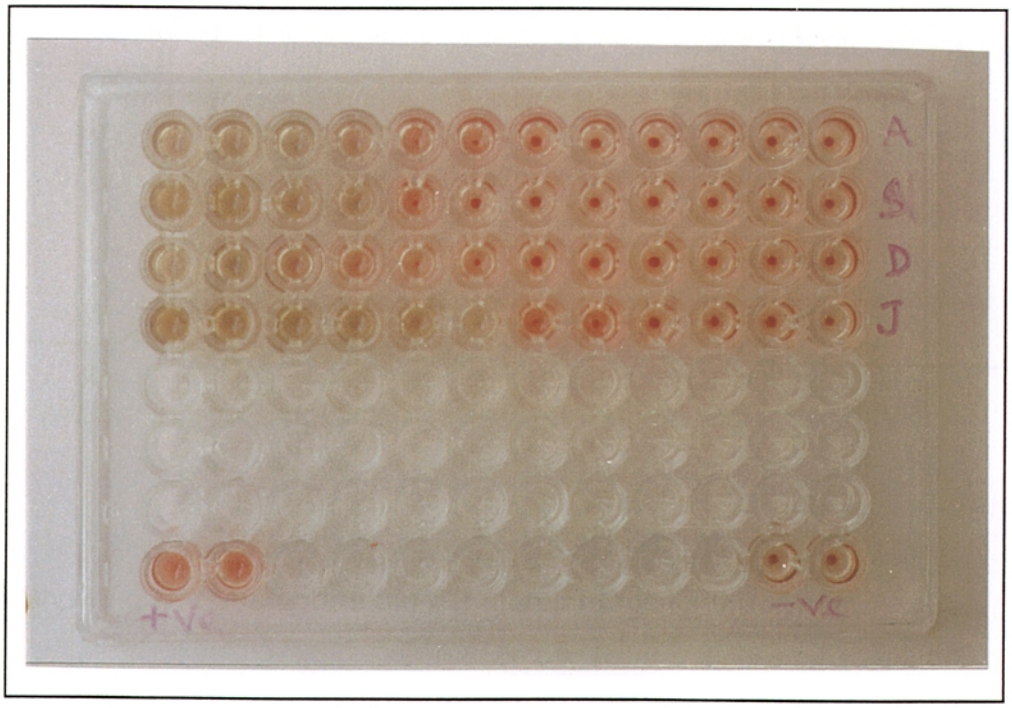

Plate 10: Showing the hemolytic activity of crude toxin extracted from Mussels Row 1-4: August 1999 to January 2000 (except September and October)

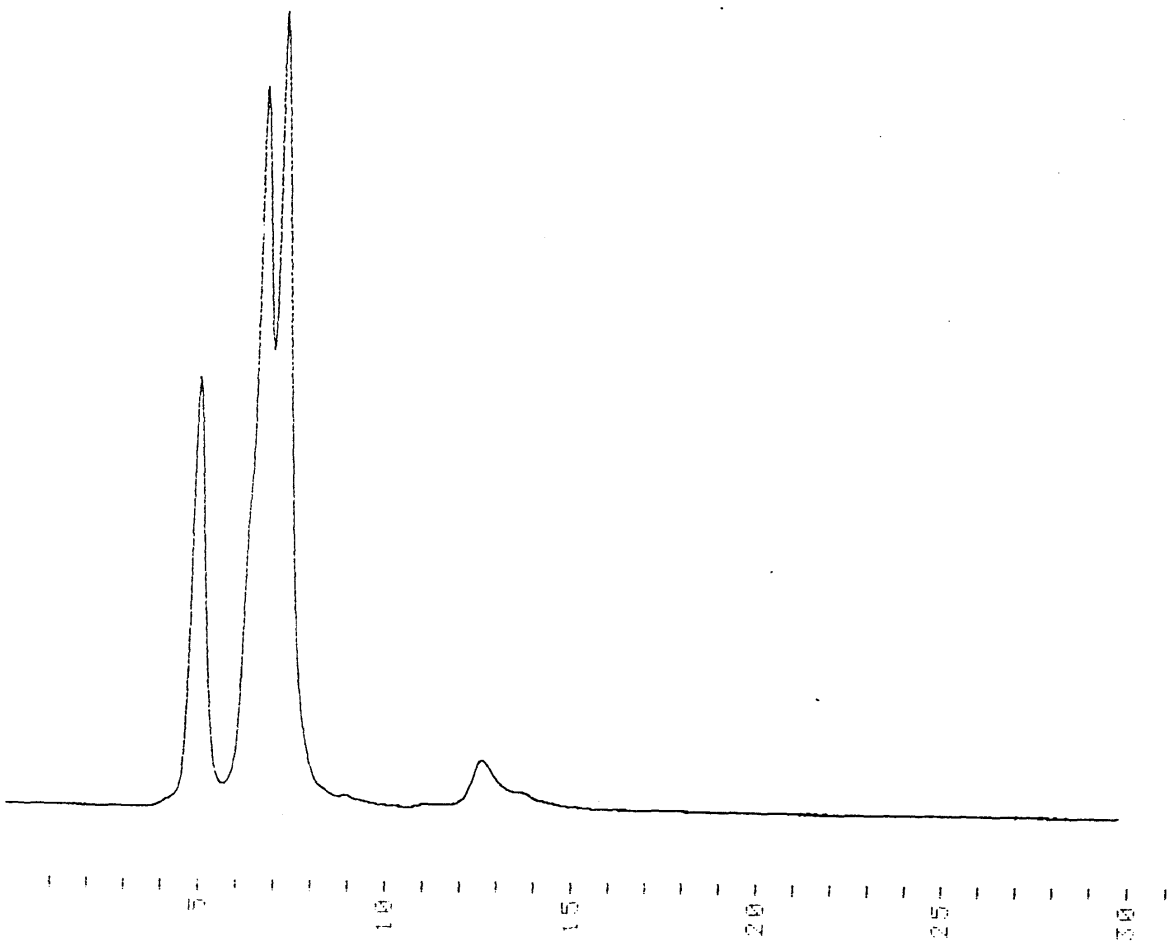

Fig. 1 Showing Chromatogram of PSP standard run @ 0.6 ml/min.

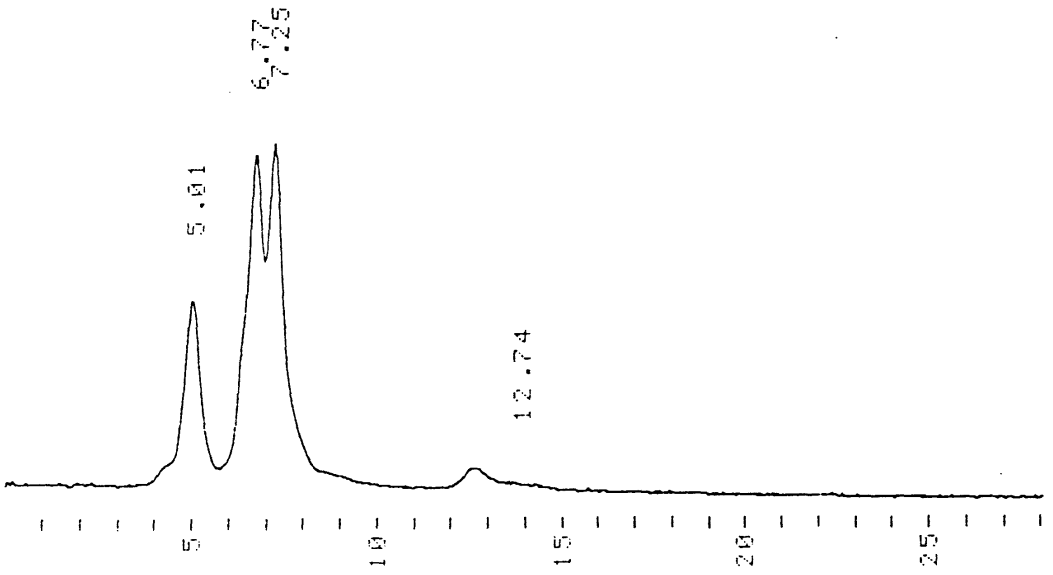

Fig. 2 Showing Chromatogram of PSP standard run @ 1.0 ml/min.

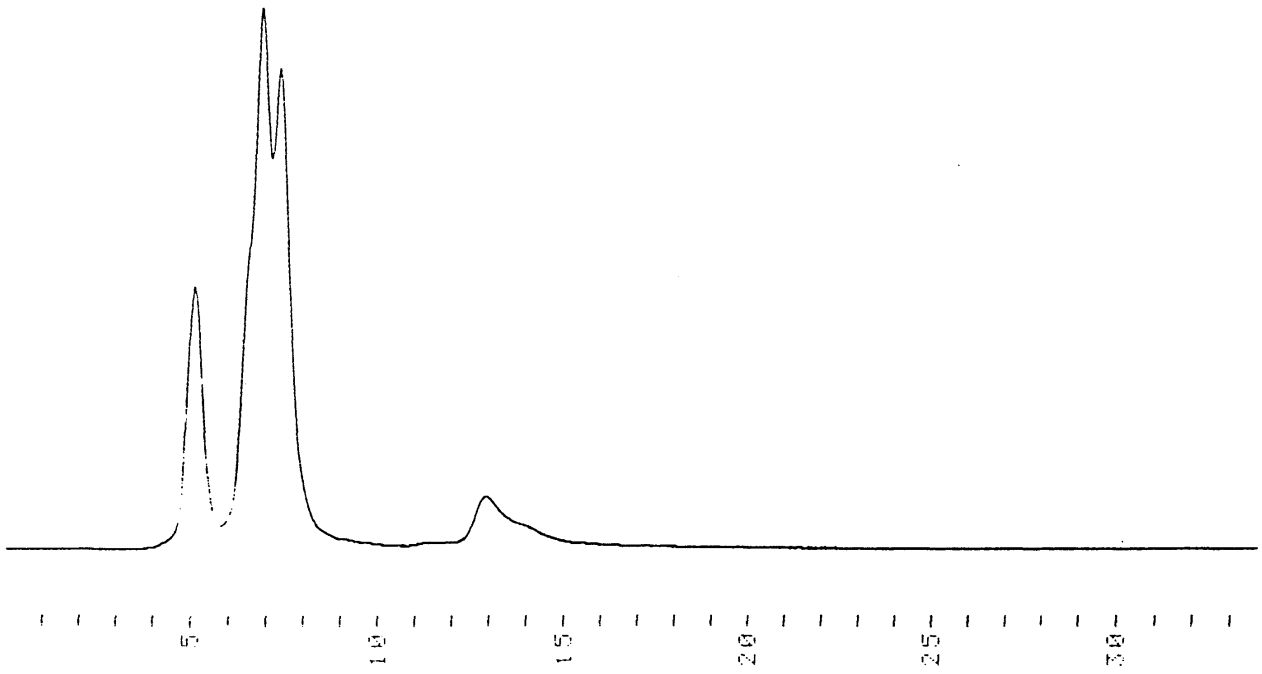

Fig. 3 Showing Chromatogram of PSP standard run @ 0.8 ml/min.

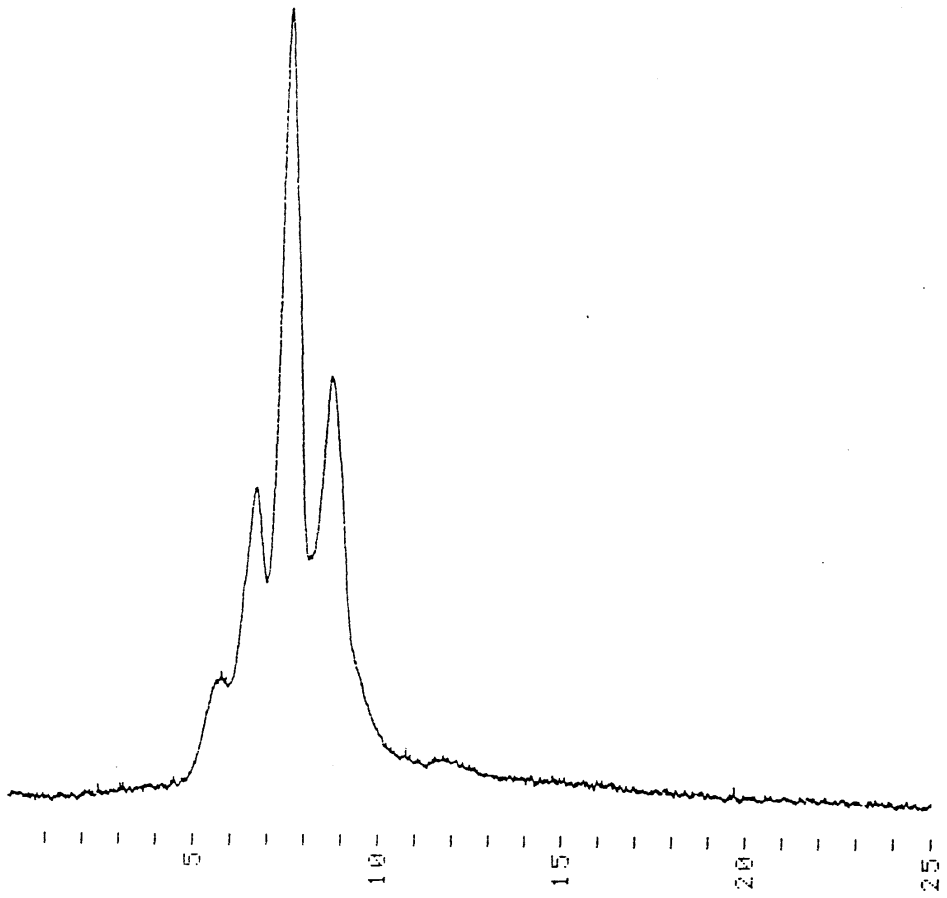

Fig. 4 Showing Chromatogram of crude extract from Clam run @ 0.8 ml/min.

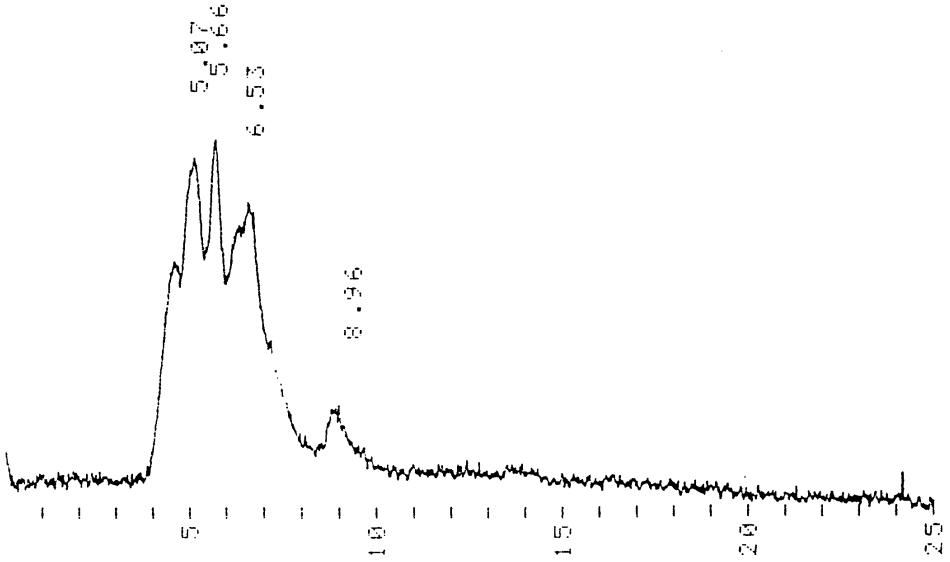

Fig. 5 Showing Chromatogram of crude extract from Mussels run @ 0.8 ml/min

DISCUSSION

5. DISCUSSION

5.1 TOXICITY

The bioaccumulation of the paralytic shellfish toxins in the tissues of both *Meretrix meretrix* and *Perna viridis* has been clearly indicated in the present study. The results of the mouse bioassays reveal the lethality of the accumulated toxins. Considerable seasonal variation was discernible in the observed levels of toxicity; the crude extracts from *M. meretrix* were not lethal during January, February and September and those from *P. viridis* were lethal only during March, April, December and January. It is common knowledge that considerable variation occurs in the biotoxicity of all poisonous and venomous aquatic organisms based on geographic location, season, sex, life-history stage and even among different specimens of the same sex and size from the same locality at the same time (Halstead and Corville, 1965; Hashimoto, 1979). Red tides themselves being a seasonal phenomenon, restricted to the warmer months, it is but natural that the expression of the bioaccumulated toxins would also be seasonal in nature being pronounced during months immediately succeeding the red tides. It is said in USA, that bivalves are not to be eaten during months not having a "R" in its name (*viz.* May, June, July and August). However, the variations found in the toxicity of the clam and mussel extracts during the present study do not show any fixed pattern to draw clear-cut conclusions. The problem is further complicated by the absence of information on the seasonality or otherwise of red tide phenomenon in Mumbai waters.

The poison absorbed from the toxic dinoflagellates are stored in the organs of the shellfish in variable amounts of toxin depending on the species. In the mussel *Mytilus*, the greatest amount is in the digestive organs or liver (Meyer, 1953) while in the soft-shell clams (*Mya* sp.) and in bar clams (*Mactra* sp.), the gills are more laden with poison (Medcof *et al.*, 1947). In case of Alaskan butter clams (*Sarcidomus* sp.), siphons are the primary binding site for the toxin (Chambers and Magnusson, 1950). The amount and concentration of the toxin in the various organs varies seasonally. More poison is found in the digestive organs during the summer, and in the gills during the fall and water. The present study, however, did not address this sort of differential storage in various tissues since toxin was extracted from whole tissue

Moreover, the quantity of the PSP toxins recorded in terms of mouse units following AOAC methods was always less than 0.875 indicating the low levels of toxin accumulation in the bivalves under study. The toxin levels were found to exceed 18,000 MU, containing mainly of GTX 1-8 and epiGTX 1-8 during the PSP outbreak at Karnataka in 1983 (Karunasagar, 1984). Even the PSP levels observed in clams and oysters along the Karnataka Coast during April 1985 (Segar *et al.*, 1988) were much higher than those in the present study, but within the permissible limits (<400 MU/100 mg). The observed PSP concentrations having a potency less than 0.875 MU over an entire year are, therefore, indicating the lack of accumulation of the toxins to dangerous levels in this area. However, it may be erroneous to draw concrete conclusions in the absence of supporting data on diatom and dinoflagellate concentrations in

the open waters, feeding rates of the bivalves studied, and the oceanographic conditions favouring the algae to bloom.

However, the study clearly shows that bioaccumulation does indeed take place albeit in sublethal concentrations. Prolonged exposure to sublethal concentrations can have deleterious impacts on human health. It is in this context that the present study assumes importance since the histopathological observations during the present study indicate the effect of these toxins on the kidney and liver of the test animals. Although no human fatalities can occur under the present situation, the possibilities of impairment of human health in terms of impairment of gastrointestinal and urinary functions in and around Mumbai, and may be in the coastal areas of Maharashtra due to long-term exposure cannot be ruled out.

In India DSP has been detected by mouse bioassay in several shellfish samples along the coast of Karnataka (Karunasagar *et al.*, 1989). DSP producing dinoflagillates *Dirophysis fortii* and *D. norvegiea* have been detected in these waters. These are non bloom-forming dinaflagellates and *D. fortii* could render shellfish toxin even when the all numbers are 200/l (Yasumoto *et al.*, 1990). The symptoms of DSP include stomach pain, nausea, vomiting and diarrhoea (WHO, 1984). Lack of chemical reports of DSP in India could due to the mild nature of the illness that may be confused with other causes of diarrhoea. More studies are required on DSP and its public health implications in India.

5.2 HEMOLYTIC AND HEMAGGLUTINATING ACTIVITY

Potent hemolytic activity was discernible in the crude PSP extract from both *P. viridis* and *M. meretrix*. Extracts from the mussel *P. viridis*, however,

showed a much higher activity than those from the clam *M. meritrix*. Neither extract showed any hemagglutinating property. Results of the hemolytic property of the toxins studied conform with those reported in earlier studies such as Targett and Mitsui (1979) Bass *et al.* (1983), Yasumoto *et al.* (1990), and Rao *et al.* (1991). The present study is, however, at variance from certain earlier ones such as those of Onoue and Nozawa (1989) wherein both hemolytic and hemagglutinating properties were contained within the same toxin. The highest hemolytic units (HU) recorded in the clam (64) and mussel (512) are much higher than those reported earlier. Hemolytic activity is indicative of cytolytic activity and most cytotoxins have considerable potential as anticancer and antiviral agents.

Most of the published research on cytotoxic exotoxins from marine algae have been devoted to a single toxin, prymnesin, produced by *P. parvium* (Shier, 1988). Prymnesin appears to act by causing a change in the permeability of cell membranes. Since this change permits calcium ions to enter the cytoplasm of the cell from extracellular fluids (Shier, 1985), it is capable of stimulating a variety of responses in cells, including activation of endogenous phospholipase activity and concomitant prostaglandin synthesis (Shier and Du Bourdieau, 1982).

Bass *et al.* (1983) reported a hemolysin from *G. monilata* that caused fragmentation of the human erythrocyte membrane. Present HPLC results indicated the presence of GTX in both the species studied and the results of hemolytic activity thus confirm the presence of GTX.

The presence of lectins or agglutinins in the present samples can, however, be ruled out since crude extracts from both the bivalves failed to elicit any hemagglutination.

5.3 ANALGESIC ACTIVITY

Analgesic activity of any PSP toxin has so far been not reported. Crude extracts of both bivalves in the present study showed a certain amount of analgesic activity as compared to the controls, up to about 150 times, but not better than paracetamol. Cai *et al.* (1997) reported that extracts from river clams possess a marked analgesic effect on mice, but there is no evidence as to the algal origin of the active components. The present experiments on analgesic activity were restricted only to crude extracts of both the bivalves studied. It is quite possible that better levels of analgesic activity would be discernible if the active component is separated and then tested for its analgesic activity. Nonetheless, the present finding is the first of its kind in as much as PSP toxins are concerned and, thus, opens up new vistas for future research for analgesic compounds of harmful algal bloom origin.

5.4 TOXIN IDENTIFICATION

The toxins extracted from clams contained *N*-Sulfocarbamoyl toxins C1 and C2, and decarbamoyl toxins and carbomate toxins, *i.e.* dcGTX/STX. In case of mussels, carbomate toxins GTX-2 and GTX-3 and *N*-sulfocarbamoyl toxins C1 and C2 were present.

So far, many different kinds have been identified (Gennah and Shimizu, 1981; Shimizu, 1978; Shimizu *et al.*. 1975; Shimizu, 1988) and characterized as Saxitoxin (STX), Neosaxitoxin (neoSTX), Gonyautoxins 1-8 (GTX 1-8), C 1-4, B 1-2, dcGTX and dcSTX. All these toxins with known structure are

alkaloids. Shimizu (1978) found that all these toxins are very hygroscopic and thus, very water-soluble. STX, GTX-2 and GTX-3are more stable in acid than in alkaline medium, even at low temperatures (Shimizu, 1978).

5.5 STABILITY

From the present experiment, it was found that lyophilization, which is supposed to be the best method for long-term storage of most of the biotoxins, has a destructive effect on the PSP toxin. The present results are in agreement with the report of Louzao *et al.* (1994) that the PSP toxins were rendered ineffective by lyophilisation.

It has already been reported that the PSP toxin is stable in acidic solution at pH range of 2.0-4.5. Beyond this range of pH, the toxin was not at all stable (Sommer *et al.*, 1937; Chang *et al.*, 1988). The present study is in conformity with these studies. It has also been found that a relationship exists between the temperature and the pH of the toxin; change of any one of these two parameters makes the toxin unstable (Chang *et al.,* 1988).

The present study revealed that storage of the PSP toxin in a deep freezer at a temperature level of –80°C was the safest way to store this toxin if they were to be used for biological experimentation or for development of pharmaceutical and other beneficial compounds. There is not much work on this particular aspect, but the present study reveals that even after six months or more of storage at –80°C the toxin remains stable and retains its activity. Since the toxin is pH sensitive, the toxin should be stored at acidic pH.

Heating or boiling the PSP toxin also causes its destruction as reported by Medcof *et al.* (1947) and Sommer *et al.* (1948). The heating of the toxin destroys the toxicity even when the solution is acidic, but the percentage of

destruction will be less in acidic medium (Sommer *et al.*, 1948). But Medcof *et al.* (1947) reported that pan-frying is much more destructive for the toxin than boiling and, thus, a better way of cooking to prevent human health hazards associated with the consumption of bivalves that might be contaminated.

Since the toxin is not stable at higher temperatures, there is a very large decrease in toxicity in cooked molluscs, especially when higher temperatures are employed in cooking, as in pan frying (Concon, 1988). Sommer and Meyer (1941) recommended adding sodium bicarbonate to the cooking medium and heating for 20-30 minutes. Since the toxin is water soluble, the broth in which the mollusc is cooked should contain a significant amount of the toxins and should be discarded (Cocon, 1988).

Canned products are probably generally safe to eat, since the condition used in canning generally reduces the toxicity, if any (Concon, 1988). For instance, the pH during steaming may be as high as 8.2 and decreases to 6.5 after addition of vinegar. This is still high enough to further decrease the toxicity while retorting the product at 121°C for 45 minutes (Concon, 1988).

5.6 DEPURATION

Result of the present study indicate that ozone treatment was more effective compared to chlorine treatment for the depuration of the bivalves. A similar observation was made by Gacutan *et al.* (1984). The result showed that there is a sharp decline in the toxicity level between the 5^{th} and 10^{th} days of depuration. There have been a handful of similar studies on the effective inactivation by ozone of PSP toxins in mussels, oysters and other shellfish that were observed from *G. tamarensis, G. catenella and Gymnodinium breve*

blooms (Thurberg, 1975; Dawson *et al.*, 1976; Blogoslawski and Stewart, 1977; Blogoslawski *et al.*, 1979) and also from *Pyrodinium* spp. (Gacutan *et al.*, 1984). Detoxification process takes shorter time if we use flow through system for depuration rather than static condition. *Mya arenaria* lost a considerable amount of toxin within 24 hours when it was subjected to flowing water (Blogoslawski *et al.*, 1979).

A number of works have been carried out on depuration using ozone. *G. breve* toxins were progressively inactivated using ozone, while the untreated extracts brought total mortality to mice in 7-10 minutes (Blogoslawki *et al.*, 1975, 1979). Depuration of shellfish using ozone is one of the most popular methods used world over. The major advantage of ozone-assisted depuration over chlorination is that it does not change the taste or appearance of the shellfish. Ozone may react in seawater with bromide that can be oxidized and become a disinfecting agent. It is also important to consider oxidant contact time, concentration, temperature and pH of the disinfection system (Pichet and Hurtubise, 1976). In addition to ozone's ability to inactivate bacteria and viruses, it is capable of oxidizing marine dinoflagellate toxins (Thurberg, 1975). Blogoslawski and Stewart (1977) reported ozone's ability to degrade tetrodotoxin (TTX).

Chlorine had been found to be less effective than ozone for depuration purpose. Gacutan *et al.* (1984) also suggest that chlorine is not suitable for detoxification; chlorine treatment took 14 days to bring the toxicity down to slightly lower than the safe level, whereas ozone treatment took only 7-8 days. In studies with *G. catenella* toxins, chlorine neutralized the toxin over a long

period (Chin, 1970); it also lowered flavour quality and decreased acceptability despite the fact that it was cost effective (Gacutan *et al.*, 1984).

There are some other ways of depuration apart from usage of ozone and chlorine. PVP-iodide-iodine can be used for this purpose that proved to be effective than chlorine (Gacutan *et al.*, 1984). But its use is still not widespread although there are many studies showing its ability to act against a wide variety of microorganisms (Casagrande, 1978). At SEAFDEC, PVP iodide–iodine has been used exitensively to depurate heavily contaminated oyster, *Crassostrea iredalei* (Gacutan *et al.* 1984).

The use of filtered seawater is another method of depuration. Waiwood *et al.* (1995) used this method to depurate giant scallop from the Bay of Fundy, but found that the depuration time of one year was not sufficient to reach total animal toxicity below the legislated level.

Work on ozone and other depuration methods should be intensified with the purpose of finding the effective time and dosage for total inactivation of the toxin due to public health and safety considerations. To obtain definite results, more replications must be set up. The complexity of ozone's chemistry has led many to investigate its effects. When added to seawater, it reacts with free bromide ions to form hypobromite ions and hypobromous acid. Blogoslawski *et al.* (1979) believed that hypobromous acid, in addition to dissolved ozone itself, was responsible for inactivating PSP toxins.

5.7 LENGTH-WEIGHT RELATIONSHIP AND CONDITION FACTOR

The present results are inconclusive as to the general well being of the bivalves studied, especially since the clam showed a condition factor less than 1.0 for most part of the year, while the mussel exhibited alternating patterns

with K values less than 1.0 for most part of the year while the mussel exhibited alternating patterns with K values less than 1.0 and as high as 3.0 to 4.0. The present results indicate, however, allometric growth in both the bivalves studied, conforming to most studies reported earlier but at a variance from Mohan (1980) who reported that, in case of *P. viridis,* growth was not allometric.

Condition factor is an indication of the overall well-being or otherwise of the animals; values of 1.0 and above indicate that all is good, whereas values less than 1.0 indicate a stress condition. Condition factor has rarely been used to correlate with toxicity of any organism, although stress has been implicated in cases of increased toxin production. Deo (2000) showed the toxicity of the proteins in the mucus of certain Ariid catfishes to be correlated to stress conditions indicated by a condition factor less than 1.0. However, no such conclusions could be drawn from the present study.

5.8 SCOPE FOR FURTHER STUDIES

1. Through the application of computerised molecular modeling and synthetic organic chemistry, marine toxins responsible for PSP, ASP, DSP, NSP, and ciguatera poisoning may provide some of the most interesting new specific tools for the study of essential ligand receptor interactions in living system.

2. The toxin responsible for PSP, DSP, ASP and NSP are reported to enhance or inhibit nerve conduction through the voltage-sensitive sodium channel. So detailed pathways of their action may be studied.

3. Apart from the use as pharmacological tools, each toxin may be derivatized to specific forms which provide affinity columns for receptor

purification, assay kits and standards for the public health and mariculture industries, radioactive and enzyme-linked forms for receptor localization and metabolism studies and as complete immunogens for development of toxin-specific antibodies.

4. Based on the knowledge of the molecular mechanism of action, synthetic "toxins" may be designed to modulate living systems in therapeutically important ways.

5. Latex antibody test (LAT) has been used for detection of marine toxins in ciguateric fish and it would be worthwhile to find out the usefulness of LAT in studies of shellfish poisons.

6. PCR based method for detection of dinoflagellate toxins is another emerging field of interest.

7. Studies of involvement of bacteria in production of paralytic shellfish toxin and its conversion from one form of toxin to another is another major field for future study.

8. Use of tissue culture for the purpose of bioassaying PSP also a promising field for study, especially in view of the ethical issues associated with animal bioassays.

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