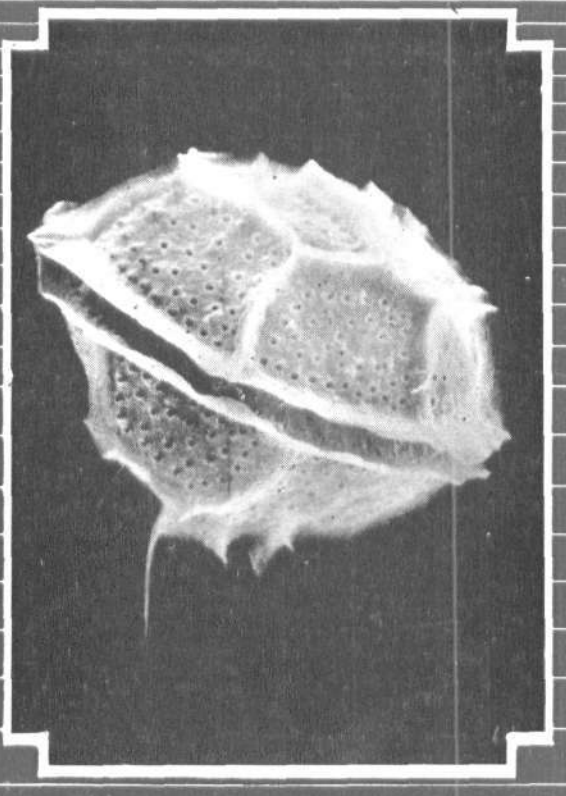
CMFRI SPECIAL PUBLICATION
Number 16

MANUAL ON MARINE TOXINS IN BIVALVE MOLLUSCS AND GENERAL CONSIDERATION OF SHELLFISH SANITATION



ISSUED ON THE OCCASION OF THE WORKSHOP ON

MARINE TOXINS IN BIVALVE MOLLUSCS AND GENERAL CONSIDERATION OF SHELLFISH SANITATION

ORGANISED BY

THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
HELD AT TUTICORIN FROM 3RD TO 5TH MAY 1984

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Front cover : *Pyrodinium bahamense* var. *compressa.*

Back cover : A chain of *Pyrodinium bahamense* var. *compressa.*

MANUAL ON MARINE TOXINS IN BIVALVE MOLLUSCS AND GENERAL CONSIDERATION OF SHELLFISH SANITATION

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(LIMITED DISTRIBUTION)

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PRINTED IN INDIA
AT PAICO PRINTING PRESS, ERNAKULAM, COCHIN-682 031

PREFACE

A major programme of the Centre of Advanced Studies in Mariculture of the Central Marine Fisheries Research Institute is conducting of researches in mariculture to evolve appropriate methods of culturing molluscan shellfish of economic value. Progress is being made in several areas such as hatchery systems, seed production, bottom and off-bottom culture, predator and disease control and processing and marketing of molluscan shellfish. In addition to increasing shellfish production through modern mariculture techniques, it is necessary to ensure that the shellfish harvested are of good quality and be safe for human consumption. Without assurance of high quality and purity of shellfish, India's potential for increased production of shellfish for domestic consumption and exports will not be fully realised.

Several serious illnesses may sometimes result from the consumption of shellfish. The causative agents are natural marine toxins and enteric pathogens. The purity of shellfish for human consumption can be assured only by monitoring the shellfish for toxins, sanitary quality and the cleanliness of waters inwhich shellfish is grown. The quality of the shellfish must be guarded from harvest upto delivery to the consumer. The problem of maintenance of sanitary quality of shellfish can be expected to increase as a result of growth of human population and the accompanying rise in sewage pollution.

In India little work has been done on the toxicity of molluscan shellfish and methods of evaluating shellfish quality. Therefore, Dr. Sammy M. Ray, Coordinator of Graduate Programme and Dean of Moody College of Marine Technology, Texas A & M University at Galveston, Galveston, Texas, U.S.A. who has done considerable work on parasites of oysters, marine toxins and shellfish sanitation, has been invited as FAO/UNDP Expert Consultant to give consultancy in oyster biology and oyster culture with particular reference to shellfish poisoning and assessment of sanitary quality of shellfish. During the consultancy Dr. Sammy M. Ray has prepared this **Manual on Marine Toxins in Bivalve Molluscs and General Consideration of Shellfish Sanitation.** This manual which was issued at the time of the Workshop conducted by

Dr. Sammy M. Ray on the subject at Tuticorin, deals with various aspects including the nature, symptoms, causative factors, treatment and prevention of molluscan shellfish poisonings, and the procedures for assessment of sanitary quality of the shellfish growing waters and shellfish meat. It is hoped that this manual will be useful to Scientists interested in the subject and will lead to taking up of studies in this important field. With the informations presented in this manual and references provided, fisheries and public health agencies could initiate programmes to ensure the safety and high quality of shellfish produced in India.

I express my sincere thanks to Dr. Sammy M. Ray for writing this manual in which he has treated most lucidly the various aspects of shellfish poisonings and procedures for assessment of shellfish quality. I thank Shri K. Nagappan Nayar, Scientist of the Institute for his support and valuable discussions and Dr. K. Satyanarayana Rao, Scientist for assistance given in the preparation of the manual. I am thankful to Shri K. Rengarajan, Scientist for editing the manual.

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Cochin - 682 018.
August 1984.

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INTRODUCTION

Although only a small percentage of marine and estuarine organisms are toxic or harmful to man and other animals, there is an increasing interest in understanding the harmful effects, source, nature as well as the potential for useful application of marine toxins. This manual deals only with those toxins that are transmitted to human and other warm-blooded vertebrates through intake of bivalve molluscs.

Another aspect of public health significance *i.e.*, the transmission of human enteric diseases such as typhoid fever, paratyphoid fever, cholera, infectious hepatitis, etc. by consumption of bivalve molluscs contaminated by human waste materials will receive brief attention. Although very important human illnesses caused by the consumption of bivalves that have accumulated anthropogenic substances such as heavy metals, pesticides, PCBs and other man-made materials will not be considered in this manual. Minamata disease which occurred in Japan as a result of human consumption of shellfish harvested from sea water heavily contaminated with mercury present in industrial effluents, is a well known example of this type of shellfish poisoning.

At this point the definition of a few terms may be useful. *Toxin:* when used in the broadest sense, refers to a substance which adversely affects the health or well being of the victim. Thus, a toxin may either be a *venom* or a *poison*. Venoms, usually proteinaceous substances, are secreted by venom glands or specialised secretory cells in the causative organisms and injected into the victim by venom apparatuses such as fangs, spines, nematocysts, etc. On the other hand, poisons are substances which produce harmful effects in the victim as a result of the ingestion of organisms

containing such substances. Generally, the words toxin and poison are used interchangeably. The values are given in both mouse units (MU) and amount of purified toxin (μg). A mouse unit is the minimum amount of poison that will kill a 20 gram mouse in 15 minutes when one ml of a solution of extract at about pH 4 is injected intraperitoneally.

As pointed out by Hashimoto (1979), marine toxins are frequently named according to the organisms carrying or transmitting the toxin. For example, paralytic shellfish poison has been referred to as *mussel poison* or *mytilotoxin* if derived from mussels of the genus *Mytilus* whereas the same toxin is known as *saxitoxin* or *clam poison* when derived from butter clams of the genus *Saxidomus*. Moreover, a thorough search of the literature will reveal that several other names have been applied to the marine toxin(s) at present commonly known as paralytic shellfish poison(s).

We agree with Hashimoto's view that naming toxins or poisons for the marine organisms bearing or containing them is confusing. For example, oysters may accumulate more than one type of marine toxin. Furthemore, it has been demonstrated that toxins isolated from mussels (*Mytilus*), clams (*Saxidomus*) and culture of a dinoflagellate (*Gonyaulax catenella*) may have the same chemical and toxicological characteristics. Therefore, naming systems that utilize the characteristics of the toxin rather than the name of the toxic organisms is more appropriate. Thus, more uniformity and less confusion prevail with a name that depicts the clinical response of the victim to a particular marine toxin.

An excellent example of this approach to naming marine toxins is *paralytic shellfish poison*. The term paralytic shelfish poison is commonly and uniformly applied for toxins obtained from several species of bivalve molluscs distributed in widely separated areas of the world. Wherever possible we shall follow such a naming procedure in this manual.

It should be pointed out, however, that the naming of toxins has been complicated even more by the recent discoveries that poisonous organisms may contain or produce multiple toxins that are associated with a particular set of clinical symptoms. This factor will receive further mention in the chapter dealing with the source and nature of shellfish toxins.

The more common shellfish poisonings are transmitted by filter feeding bivalve molluscs that have ingested toxin-bea.ing

phytoplankters. In most shellfish poisonings dinoflagellates have been incriminated as the source of the toxin. The clinical symptoms as well as the severity of the toxicity are often related to the species of dinoflagellates and bivalve molluscs involved in the incidence.

Paralytic shellfish poisoning referred to as PSP, is by far the most commonly known and most widely distributed form of shellfish poisoning. In this manual we shall use PSP with reference to either the toxin(s) or the poisoning. Until recently most reports of PSP were from the temperate regions of the world and the majority occurred in the northern hemisphere. However, during the past ten or fifteen years PSP has been reported from semitropical and tropical areas of both the hemispheres. Thus, PSP is not only the most common, but probably the most widely occurring type of shellfish poisoning known at the present time.

Although several species of bivalve molluscs serve as transvectors of PSP, most toxicities result from the consumption of mussels and clams. Dinoflagellates belonging to two genera *Gonyaulax* in temperate waters and *Pyrodinium* in semitropical and tropical regions are the most common known sources of PSP. This form of shellfish poisoning is often fatal, but survivors usually recover rapidly without noticeable after-effects.

Neurotoxic shellfish poisoning (NSP) is known only from the west coast of Florida following the consumption of oysters and hard clams harvested during red tides caused by the dinoflagellate *Ptychodiscus brevis* (= *Gymnodinium breve*). So far NSP is known only from the west coast of Florida in the Gulf of Mexico. Several cases of toxicity were reported, but no deaths have been attributed to NSP.

A kind of food poisoning recently discovered following the consumption of scallops and mussels has been named diarrhetic shellfish poisoning (DSP). As the name implies, diarrhoea among other gastro-intestinal disturbances is the primary symptom in human victims of the toxicity. Thus, DSP differs from PSP and NSP in that paralysis and/or neurological symptoms are lacking. Also, as in the case of NSP, no deaths have been reported for DSP. Dinoflagellates of the genus *Dinophysis* and possibly some species of *Prorocentrum* have been implicated as the cause of DSP. Although initially discovered in the coastal waters of Japan, DSP is possibly world-wide in its distribution. Moreover, shellfish poisonings that have been called gastro-intestinal or choleratic actually may be DSP.

Venerupin shellfish poisoning, generally referred to as VSP is also called oyster or *asari* poisoning. Sporadic and sometimes massive outbreaks of highly lethal food poisonings have followed consumption of oysters and the short-neck clams *Tapes japonica* (= *Venerupis semidecussata*) harvested from certain coastal areas of Japan. Unlike PSP and NSP, paralytic or neurological symptoms are absent in VSP. Initially gastro-intestinal symptoms prevail, followed by damage to liver and kidney. The source of the poison has not been definitely established. A dinoflagellate belonging to the genus *Prorocentrum* has been suggested to be responsible for this type of poisoning.

Other types of shellfish poisonings, which will not be dealt with in this manual, have been reported under a variety of names as follows: gastro-intestinal or choleratic, erythematous or allergenic and Minamata disease. Except for Minamata disease, none of the known shellfish poisonings cause paralytic and neurotoxic symptoms that characterise PSP while NSP shows neurological symptoms alone.

The interested reader should consult Halstead (1965) for general accounts of various shellfish poisonings and Halstead (1967) for Minamata disease. Hashimoto (1979) provides an excellent account of various marine toxins and other biologically active marine substances. Some other general publications dealing with marine toxins include those of Baslow (1969), Martin and Padilla (1973), Scheuer (1973) and Taylor and Seliger (1979).

As previously mentioned human enteric diseases may result from the consumption of bivalve molluscs contaminated by domestic sewage or improper handling of harvested shellfish. If consumer safety, confidence and satisfaction are to be ensured, the sanitary and taste qualities of molluscan shellfish meat must be protected at all steps from production upto delivery to the consumer. Without such protection to increase internal and foreign acceptance of shellfish as food, the enormous potential for expanding the bivalve shellfish resources of semitropical and tropical regions through aquaculture will not be achieved. We shall present general methods and practices for maintaining and monitoring shellfish sanitary quality as well as procedures by which contaminated shellfish could be purified.

2

SYMPTOMS AND TREATMENT OF SHELLFISH POISONINGS

2.1 Paralytic Shellfish Poisoning

Since symptoms of paralytic shellfish poisoning are very distinctive, the illness may be readily separated from other kinds of shellfish poisonings. The principal symptoms of PSP are summarized from Halstead (1965), Quayle (1969) and Prakash *et al.* (1971). Symptoms usually develop within 30 minutes of intake of toxic shellfish. Early effects, such as a tingling or burning sensation of lips, gums and tongue, may occur within a few minutes, and paresthesia generally progresses to the neck, arms and legs, causing a feeling of numbness. This is followed in severe cases by ataxia (inability to co-ordinate voluntary movements) and general motor inco-ordination. Victims often report a 'feeling of lightness' as though floating in air. Other symptoms may include weakness, dizziness, drowsiness, incoherence, headache, rapid pulse, slightly subnormal temperature, slight respiratory distress and impairment of vision. Less common are gastro-intestinal symptoms, nausea, vomiting, diarrhoea and abdominal pain. Mental processes do not appear to be affected and most victims remain calm and conscious during the illness. Characteristically, in the advanced stages of this illness, muscular paralysis becomes progressively more severe. In fatal cases, death results from respiratory paralysis and cardiovascular collapse 3 to 13 hours after consuming shellfish containing PSP. Patients surviving the first 12 hours generally recover rapidly without permanent after effects.

The following tabulation of symptoms according to the level of toxicity (mild, severe or extreme) is taken from Prakash *et al.* (1971):

Tingling sensation or numbness around lips, gradually spreading to face and neck. Prickly sensation in fingertips and toes. Headache, dizziness, nausea.	MILD	
Incoherent speech, progression of prickly sensation to arms and legs, stiffness and non co-ordination of limbs. General weakness and feeling of lightness, slight respiratory difficulty, rapid pulse.	SEVERE	
Muscular paralysis, pronounced respiratory difficulty, choking sensation.		EXTREME

Treatment of PSP is primarily symptomatic. No specific antidote is known for this poison. Digitalis and alcohol are not recommended. The stomach should be evacuated as soon as possible, with an emetic followed by a rapidly acting laxative. In cases of respiratory difficulty, artificial respiration should be given promptly and continued as long as necessary. Treatment for primary shock may be required. Evans (1969) has suggested that increase urine production should hasten recovery.

Although the source of the toxin responsible for two outbreaks of shellfish poisonings which occurred in India in 1981 (Silas *et al.*, 1982) and 1983 (Karunasagar *et al.*, 1984) is unknown, the symptoms in both cases leave no doubt that the shellfish poisonings were of the paralytic type. In the incidence of shellfish poisoning at Vayalur in Chingleput District on the east coast in 1981, the symptoms reported were tingling sensation of the lips, tongue and fingertips, numbness of limbs, back pain, sensation of flying in the air, blurred vision and gastro-intestinal disturbances. Similar symptoms *viz.*, numbness of lips within 30 minutes of consumption of clams followed by vomiting and numbness of arms and legs progressing to paralysis were reported by Karunasagar *et al.* (1948) for the outbreak at Arikadu. All the victims recovered within 48 hours except for a 14 year old boy who died of respiratory paralysis.

2.2 Neurotoxic Shellfish Poisoning

This type of shellfish poisoning is possibly the least common of the shellfish poisonings recorded in humans. It produces some of the symptoms, such as tingling or numbness around the mouth, face and extremities, suggestive of a mild case of PSP (McFarren *et al.*, 1965). Hughes (1979) reported on the symptoms noted in two outbreaks of NSP involving five individuals. The incubation

period among the five patients ranged from 30 minutes to 3 hours and the duration of the illness varied from 40 to 48 hours. The symptoms were characterised by sensory abnormalities consisting mainly of numbness or tingling around the mouth. Also, gastro-intestinal symptoms, nausea, vomiting, abdominal cramps or diarrhoea were experienced by some individuals in each outbreak. Generally, symptoms disappear after 3 days. One individual showed signs of cranial nerve disfunction, typically consisting of weakness of eye muscles, speech and swallowing difficulties. Although the patient was not paralyzed, ventilatory support was required when the patient became comatose after a generalised seizure. No deaths have been attributed to NSP.

Moreover, during *Ptychodiscus brevis* red tides, humans venturing on to beaches and nearby surf zones may suffer pronounced respiratory and eye irritations. Individuals suffering from asthma or other respiratory ailments are especially vulnerable to this effect. Generally, relief of the respiratory difficulty occurs immediately after one leaves the beach area. The treatment for NSP is primarily symptomatic as indicated for PSP and as mentioned previously, artificial respiration may be required in severe cases.

2.3 Diarrhetic Shellfish Poisoning

This most recently discovered type of shellfish poisoning was first reported and named by Yasumoto *et al.* (1978) following outbreaks of food poisonings in Japan in 1976. Among several gastro-intestinal symptoms, nausea, vomiting, abdominal pain and diarrhoea, the last mentioned is the most prominent. Although numerous cases have been reported generally following the consumption of mussels and scallops, none have been fatal as far as we could ascertain. The incubation period required for the onset is 30 minutes in severe cases to a few hours in most cases, but seldom more than 12 hours. Victims of DSP recover after 3 days regardless of treatment and there appears to be no after-effects (Yasumoto *et al.*, 1978). In severe cases vomiting and diarrhoea may occur 10 to 20 times a day; usually these symptoms occur about four times a day. Since we have no information regarding treatment of DSP, we presume that it is primarily symptomatic.

2.4 Venerupin Shellfish Poisoning

The symptoms of venerupin shellfish poisoning, are categorised by Halstead (1965), Okaichi and Imatomi (1979) and Hashimoto (1979). The incubation period is usually 24 to 48 hours after consuming toxic shellfish. However, it is thought by some

that the incubation may extend upto 7 days. The early symptoms include anorexia, nausea, gastric pain, vomiting, constipation, headache and malaise. Within 2 to 3 days there is nervousness and bleeding from the mucous membranes of the nose, mouth and gums accompanied by heavy liver and kidney damage. Halitosis (bad breath) and petechial haemorrhage of the skin about the chest, neck and upper arms and legs are characteristic of VSP. Blood analysis shows lycocytosis and reduced clotting time. There is no evidence of paralysis and/or neurotoxic symptoms in VSP such as those noted in PSP and NSP. In fatal cases death occurs within a week after the onset of symptoms; and it may sometimes occur within 24 to 48 hours. According to Halstead (1965), treatment is symptomatic with bed rest, intravenous feeding of glucose and administration of vitamins B, C and D as well as insulin.

3

PUBLIC HEALTH AND ECONOMIC SIGNIFICANCE OF SHELLFISH QUALITY

In view of the relatively low incidence in comparison with other health problems, bivalve molluscan shellfish poisonings do not constitute a major public health concern. The sporadic and unpredictable nature of outbreaks, however, creates difficult and expensive problems for public health and fishery agencies having the responsibility of the safety of vast quantities of shellfish consumed throughout the world. It is difficult to estimate the economic losses that may result from temporary cessation of shellfish harvest during toxic periods and reduced consumption of bivalve shellfish due to apprehension regarding possible toxicity following outbreaks.

In addition to economic losses attributed to shellfish poisonings, public health procedures, including depuration where required for protecting consumers from human enteric diseases are costly. Although we have no estimates of the cost for programmes required to assure good sanitary quality of molluscan shellfish, they will be considerable. In the United States of America as well as some other countries of the world no molluscan shellfish may be marketed unless they have been certified as safe for human consumption by State or Municipal public health agencies and also Federal public health agencies if interstate shipments are involved. Generally, the State and Federal Agencies work co-operatively on setting and monitoring shellfish sanitary standards.

In some countries such as U.S.A., shellfish safety is achieved through monitoring of the sanitary quality of waters in which it is grown, processing facilities and shellfish meats prior to delivery to the consumer. In other countries such as Australia for

example, an additional step-depuration to be carried out prior to marketing is required by law. During a recent visit to Australia two of the largest oyster culturists in New South Wales informed Sammy M. Ray that it costs about 15–20 Australian dollars to depurate one bag of (about 1,000) oysters for which they received about $ 200. Thus, depuration costs the oyster farmer about 10% of the price he receives for the product. Just as in the case of incidence of shellfish poisonings, economic effects of the loss of consumer confidence following outbreaks of human enteric diseases attributed to shellfish consumption are difficult to determine.

In several instances, bivalve molluscan shellfish that have been consumed for many years from a particular area, have become toxic suddenly without any warning whatsoever. On the other hand, in some areas certain species such as the butter clam (*Saxidomus giganteus*) may maintain a high level of toxicity throughout the year and from year to year (Chamber and Magnusson, 1950; Quayle, 1969; Schantz, 1969). In southeastern Alaska great quantities (conservatively estimated at approximately 2½ million pounds) of butter clams are not utilized because of persistent toxicity (Lehman, 1966). The toxicity of meat of clams of the area cannot be reduced economically by processing or cooking.

According to Halstead (1965) more than 957 cases of paralytic shellfish poisoning which resulted in more than 222 deaths, have been reported to have occurred between 1793 and 1962 (a single case was reported from France in 1689). Atleast three outbreaks not listed by Halstead occurred (Table 1) prior to 1962 (Ray, 1971). These outbreaks were from Norway in 1939 (6 cases, no deaths); from Toyakosi city, Japan (12 cases, 1 death); and a third from the west coast of Portugal (several cases, 1 death). A brief account of the outbreaks that occurred from 1962 to 1970 have been summarised (Ray, 1971). Ray (1971) did not include the occurrence of PSP in Papua, New Guinea. Worth *et al.* (1975) reported that seven known deaths and 125 hospitalised cases had been recorded upto 1971. Furthermore, Prakash *et al.* (1971) estimated that about 1,600 cases of PSP with about 300 deaths have been recorded upto 1971 (Table 1).

More recently in the period 1971–1979, additional outbreaks of PSP have been recorded from the South Pacific in New Guinea, New Britain and Borneo, northeast coast and Pacific Coast of U.S.A., Canada (British Coloumbia, Quebec and Maritime Provinces), Venezuela, Chile, the Pacific Coast of Mexico and the Atlantic Coast of Spain. During this 9 year period, 760 cases resulting in about

30 deaths have been reported. Thus, the total number of cases and deaths recorded from 1689 to 1979 over a span of nearly 300 years are approximately 2,300 and 330 respectively (Table 1).

TABLE 1. *Summary of estimated number of reported paralytic shellfish poisoning cases and deaths during* 1689–1979*

Period	No. of cases	No. of deaths	References
1689–1793	?	1 death in France	Halstead, 1965
1794–1962	957**	222**	–do–
1963–1970	*ca.* 1,600	*ca.* 300	Prakash *et al.*, 1971
1971–1979	*ca.* 2,300	*ca.* 330	Ray, unpublished

* The number of cases and deaths for each period are cumulative; deaths are included with the number of cases.

** Does not include four outbreaks of PSP involving more than 38 cases with 3 deaths (Ray, 1971).

Despite the considerable increase in the number of PSP cases reported after 1962, the fatality rate has shown a notable decrease (Table 1). From 1793 to 1962 (about 200 years) the fatality was about 23% (957 cases with 222 deaths). Although during the succeeding eight years (1963–1970) there were another 650 additional reported cases, the fatality rate (about 78 deaths) was about one-half (about 12%) of that in the previous period of about 200 years. During the next nine years (1971–1979) the number of additional PSP cases reported was similar, about 700 to that reported in the previous eight years. However, the fatality rate (about 30 deaths) was only one-third (about 4%) of that noted for the 1963–1970 period. Since 1970 deaths have occurred most often among children. In some outbreaks children are the only fatal victims.

Two very recent outbreaks of PSP in India follow the pattern of rather low fatalities, which have been restricted to children. India's first documented case of PSP occurred at Vayalur in Chingleput District, Tamil Nadu on the east coast on August 12, 1981 (Silas *et al.*, 1982). In this outbreak 85 individuals were affected after consuming the backwater clam *Meretrix casta* taken from Buckingham Canal. Three of the victims, children less than 15 years old, died. The second outbreak occurred at Arikadu near Kasaragod in northern Kerala on April 3, 1983 (Karunasagar

et al., 1984). In this incidence 15 persons became ill after eating *M. casta* from Kumble Estuary and a boy less than 15 years old died of respiratory paralysis.

The causes for the apparent increase in PSP cases and decrease in fatalities in recent years are not readily explained. The increase in cases may be due to overall improvement in the diagnostic and reporting systems worldwide. Perhaps the reduction in fatalities may be due to better diagnosis and the availability of improved medical care throughout the world. Also, both the lay population and medical personnel in endemic areas may be better informed concerning the cause, symptoms and treatment of PSP.

Although illnesses due to consumption of bivalve molluscan shellfish obtained during outbreaks of *Ptychodiscus brevis* red tides on the west coast of Florida have been reported, McFarren *et al.* (1965) were the first to demonstrate that *P. brevis* caused shellfish poisoning. In late 1962 and early 1963 several mild cases of neurotoxic shellfish poisoning were experienced by individuals who had eaten oysters and clams from areas where red tides prevailed. Since 1970 two outbreaks of NSP involving five individuals have been reported to the National Communicable Disease Centre, Atlanta, Georgia, U.S.A. (Hughes, 1979). Neurotoxic shellfish poisoning is generally thought to be of minor public health importance since no deaths have occurred and illness is of short duration. However, the need to give one patient artificial respiration has caused more concern regarding the alarming seriousness of NSP than previously noted. Florida public health officials ban the harvest of bivalve molluscs in affected areas during *P. brebis* red tides.

Diarrhetic shellfish poisoning was not recognised as a clinical complaint until 1976 at which time 42 persons became ill following consumption of mussels and scallops (Yasumoto *et al.*, 1978). Since that time about 800 cases have been recorded in Japan (Murata *et al.*, 1982). Although no fatalities have been reported for DSP, a monitoring programme was initiated in 1978 in the Tohoku and Hokkaido areas in northern Japan (Yasumoto *et al.*, 1980). The harvesting and marketing of shellfish, usually scallops and mussels is not permitted if the toxin content exceeds 0.05 MU/g (Takagi *et al.*, 1982). In 1978 the scallop aquaculture industry in northern Japan suffered an estimated loss of over ten million dollars due to DSP (Shimizu, 1983). It has been reported by Shimizu (1983) that recently the Japanese Government has rejected fresh-frozen, green-lipped mussels from New Zealand because diarrhetic shellfish poison had exceeded the allowed limit.

Shellfish poisonings exhibiting the characteristic gastro-intestinal disorders of DSP have been reported from Chile and the North Sea Coast of Holland (Yasumoto *et al.*, 1980). These authors did not indicate the number of cases of illness involved.

The fourth type of shellfish poisoning, generally referred to as venerupin or *asari* poisoning, is a serious illness that occurs sporadically in certain areas of Japan. According to Halstead (1965) and Hashimoto (1979) seven outbreaks of VSP have occurred between 1889 and 1950. The first occurrence was in 1889 in which 81 individuals became ill and 54 died. More than 50 years elapsed before the next occurrence of VSP in 1941. After such a long hiatus between the first and second outbreaks, it is puzzling that six more outbreaks should occur within a decade (1941–1950). Details of the last six outbreaks as given by Halstead and Hashimoto are as follows: 1941 – 6 cases, 5 deaths; 1942 – 334 cases, 114 deaths; 1943 – 16 cases, 6 deaths; 1949 – 67 cases, 3 deaths; 1949 – 26 cases, 3 deaths; 1950 – 12 cases, no death. Both Halstead and Hashimoto state that no incidence of VSP has been reported since 1950.

Although occurring infrequently and very limited in its distribution, venerupin shellfish poisoning appears to be more lethal than the paralytic type. For example, the fatality rate for PSP upto 1979 is about 15% (*ca.* 2,300 cases with *ca.* 330 deaths) whereas fatality rate for VSP upto 1950 is 34% (542 cases with 182 deaths). However, both VSP and PSP show a great variability in fatality rate among outbreaks. It is notable, however, that as mentioned previously for PSP, the fatality rate during earlier outbreaks of VSP was greater than in later ones. The four outbreaks occurring upto 1943 showed fatality rates that ranged from 34% to 83%. On the other hand, during the last three outbreaks, 1949 (2) and 1950, fatality ranged from 0% (no death in 12 cases) to 12% (3 deaths in 26 cases). Perhaps the same reasons suggested to account for the decline of PSP fatality rates, namely, a greater awareness of the local population and medical personnel in endemic areas as to the causes, symptoms and treatment of VSP are responsible for the fall in fatality rates.

4

GEOGRAPHICAL AND SEASONAL DISTRIBUTION OF SHELLFISH POISONINGS

Prior to 1962 paralytic shellfish poisonings was considered primarily as a problem of the north temperate regions of the world. Halstead (1965) records only two outbreaks (in Vera Cruz, Mexico in 1797 and Manus Island, New Guinea in 1962) in subtropical and tropical regions (between 30° N and 30° S). Since the Vera Cruz outbreak was attributed to the eating of fish, its classification is open to question. Perhaps the poisonings resulted from ciguatera. Until the 1927 outbreak near San Francisco, California, most cases were reported from northern Europe. Subsequent to 1968 most incidences have occurred in North America. In North America the Pacific Coast between central California and the Aleutian Islands has suffered from this affliction at various times, whereas on the Atlantic Coast the occurrence of PSP has been limited to the St. Lawrence Estuary in Canada and the Bay of Fundy in Canada and the U.S.A. A small outbreak occurred in the Gulf of California in 1939 (Halstead, 1965). Until 1972, documented cases of PSP along the Atlantic Coast of U.S.A. were questionable. From 1972 to 1975 about forty cases, but no death have been reported from the New England Coast, primarily Massachusetts.

Furthermore, prior to 1970 reports of PSP from South America were notably absent. The lack of reports of PSP from South America was surprising at the time, in view of the fact that water temperature, especially along the west coast of Chile and Peru, appear to be suitable that is, sufficiently low for the growth of toxic dinoflagellates such as *Gonyaulax catenella*. Ray (1971) received an oral report of a PSP outbreak (several illnesses with 2 deaths)

that occurred off the coast of Chile in 1972. Since 1970, however, additional illnesses have been reported from Chile and a large number of cases occurred in Venezuela in 1976. Also, the first incidence of PSP on the Pacific Coast of Mexico, near Mazatlan occurred in 1979 (Casper, V. 1980, personal communication, Dallas, Texas).

Moreover, the large number of cases reported from the South Pacific (New Guinea, New Britain and Borneo) are noteworthy in view of the scarcity of reports of PSP in the tropics prior to 1970. Recently in 1980 and 1981 high levels of paralytic shellfish poisoning were demonstrated in bivalve molluscs in Palau (Harada *et al.*, 1982). An incidence of PSP has been reported from New Zealand also.

A number of European countries including England, Wales, France, Scotland, Germany, Norway, Ireland, Belgium, Denmark, Portugal and Spain have had PSP outbreaks. On the continent of Africa, only South Africa principally the west coast has experienced outbreaks. Until recently the coastal areas of Japan appear to be the only areas in the Far East from where PSP has been reported. During a science exchange visit to the People's Republic of China in the spring of 1982, Ray (personal communication) sought information on the occurrence of paralytic shellfish poisoning. Discussions with several fishery scientists and marine biologists in several cities (Peking, Dalian, Nanking, Hangchou and Canton) brought the same response: there was no problem of PSP in China.

Only very recently India became the first country of mainland Asia to experience outbreaks of paralytic shellfish poisoning. The first record outbreak occurred on the east coast in 1981 (Silas *et al.*, 1982) and a second one on the west coast of India in 1983 (Karunasagar *et al.*, 1984).

In summary there appears to be a definite increase in the incidence of PSP in subtropical and tropical regions and in the southern hemisphere as well. The world distribution of PSP from 1869 (inclusive) is presented in Fig. 1.

In the northern hemisphere PSP is most prevalent during the warm months May to October (Meyer *et al.*, 1928; Sommer and Meyer, 1937; Medcof *et al.*, 1947). Quayle (1969) noted that PSP may develop along the coast of British Columbia any time between April and November, inclusive. Except for the butter clams which are capable of storing poison in the siphon throughout the year,

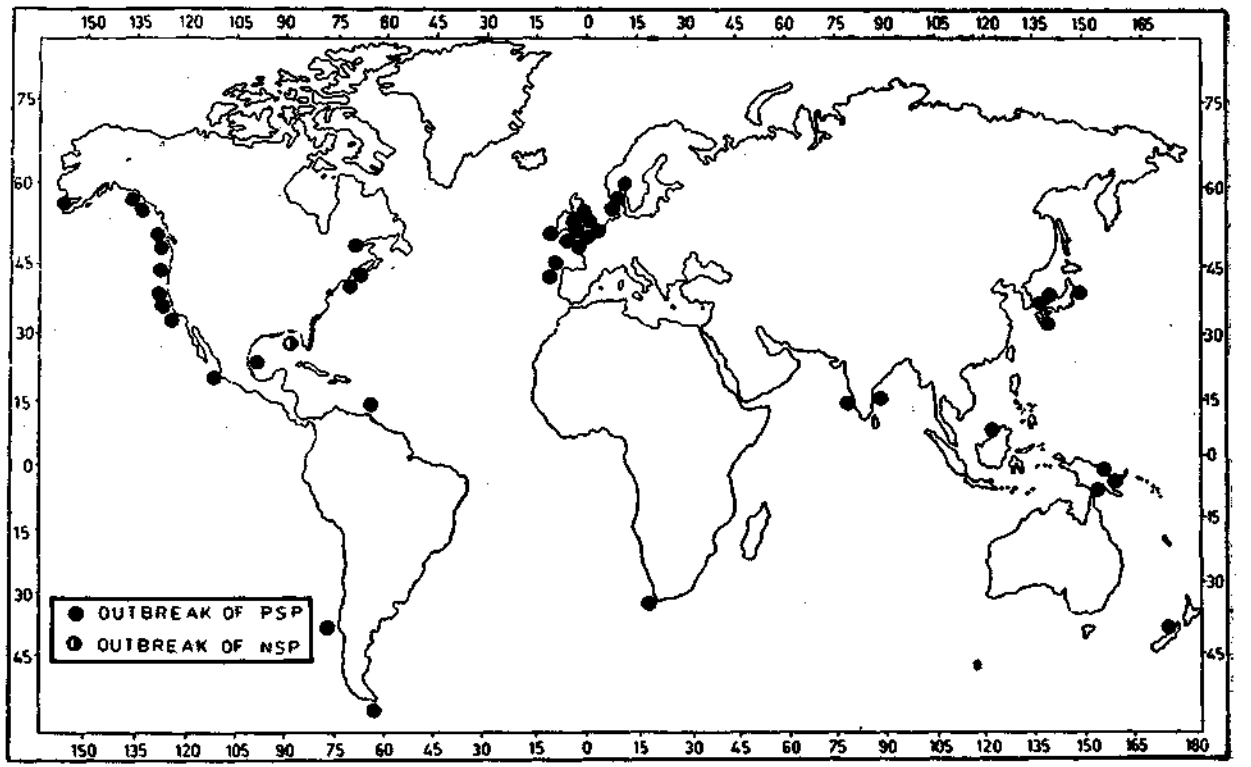

Fig. 1. World distribution of paralytic shellfish poisoning (PSP) 1689–1979 and neurotoxic shellfish poisoning (NSP) 1964–1979 (modified after Halstead, 1965 and Prakash *et al.*, 1971).

the molluscs of British Columbia contain virtually no toxin during the winter. In eastern Canada, Prakash *et al.* (1971) observed that inshore species such as soft-shell clams and mussels show little or no toxin during winter and spring. However, the toxin levels rise rapidly to a peak in summer and then decline in autumn. A major outbreak that involved more than eighty victims in England in 1968 occurred during the summer (Ingham *et al.*, 1968). A major outbreak on the Pacific Coast of Mexico near Mazatlan occurred in late April, 1979.

The more recent outbreaks in Japan have occurred in May, July and February (Hashimoto *et al.*, 1950; Kawabata *et al.*, 1962; Akiba, 1970, personal communication, Tokyo). The incidences in South Africa have been observed in April, May and December (Grindley and Sapeika, 1969). In the South Pacific, PSP outbreaks occurred during the March – July period in Port Moresby, New Guinea area (Worth *et al.*, 1975). Also cases were recorded during the March – May period in Sabah (North Borneo), Malaysia (Maclean, 1979). In Palau the toxic dinoflagellates as well as several species of toxic bivalves were detected in December of 1980 and May of 1981 (Harada *et al.*, 1982). Although the bivalves were more toxic in May than in December, these authors indicated that toxic dinoflagellates and associated shellfish presented a danger throughout the year.

The results obtained in Palau support the view that PSP outbreaks in semitropical and tropical regions are less likely to show the seasonal characteristics noted in temperate regions of the world.

Neurotoxic shellfish poisoning is known only from the west coast of Florida during *Ptychodiscus brevis* red tides (Fig. 1). Although other areas in the Gulf of Mexico, east coast of Mexico and coast of South Texas have experienced *P. brevis* fish kills, no cases of NSP have been reported from these areas. On rare occasions the red tides have extended tc the east coast of Florida.

No particular seasonal pattern has been ascribed for NSP. Generally *P. brevis* red tides begin between August and November and they may last only a few days or may have an extended duration of several months. Frequently outbreaks of red tide follow shortly after periods of high rainfall such as that produced during a tropical storm in the Gulf of Mexico. There is always a possibility for bivalve molluscs to become toxic during a *P. brevis* red tide.

Diarrhetic shellfish poisoning was first discovered in Japan in 1976 (Yasumoto *et al.*, 1978). In recent years DSP has been prevalent in Tohoku (northern Honshu) and Hokkaido areas (Fig. 2) in Northern Japan (Yasumoto *et al.*, 1980). These authors and Murata *et al.* (1982) have stated that PSP is a potential threat for the entire coast of Japan from Kyushu to Hokkaido since the causative dinoflagellate *Dinophysis fortii* occurs in many areas along the coasts of Japan. Shellfish poisonings with the characteristics of DSP have been reported from North Sea Coast of Holland and Chile (Yasumoto *et al.*, 1980) and Spain (Tanger, 1982). Recently the Japanese Government rejected a shipment of green-lipped mussels (*Perna canaliculus*) from New Zealand which contained more than the allowable level of diarrhetic shellfish poisons (Shimizu, 1983). It is likely that DSP will prove to be worldwide in distribution (Yasumoto *et al.*, 1980, Shimizu, 1983). Several species of the genus *Dinophysis* occur and two of them have been implicated as causes of this type of shellfish poisoning.

DSP outbreaks of 1976 and 1977 in Japan occurred in late June (Yasumoto *et al.*, 1978). Takagi *et al.* (1982) reported that an outbreak occurred in the fall of 1980 on the Okhotsh Coast in Hokkaido Island. In another reference to Japanese outbreaks of DSP, Murata *et al.* (1982) indicate that shellfish may be toxic for several months in some areas. Tanger (1982) cites an outbreak of DSP in the Netherlands that occurred in September–October 1981.

Among the four major types of bivalve shellfish poisoning, Venerupin shellfish poisoning probably has the most restricted geographical (Fig. 2) and seasonal characteristics. Two of the outbreaks in 1889 and 1941 occurred in Kanagawa Prefecture and the other five outbreaks between 1941 and 1950 occurred in Shizuoka Prefecture (Halstead, 1965; Hashimoto, 1979; Okaichi and Imatomi, 1979). Lake Hamana in Shizuoka Prefecture was the area of massive outbreaks in 1942 and these poisonings occurred during the period of February to April (Hashimoto, 1979). Also Hashimoto did not indicate whether any of the other outbreaks extended beyond the month of the initial appearance of the outbreak except for the one which occurred in 1942. Hashimoto indicated that all the other outbreaks occurred in March, except the 1941 incidence which occurred in February.

A 1979 outbreak of food poisoning in Norway that followed the consumption of mussels contaminated with toxins attributed to *Prorocentrum minimum* has been related to venerupin poisoning

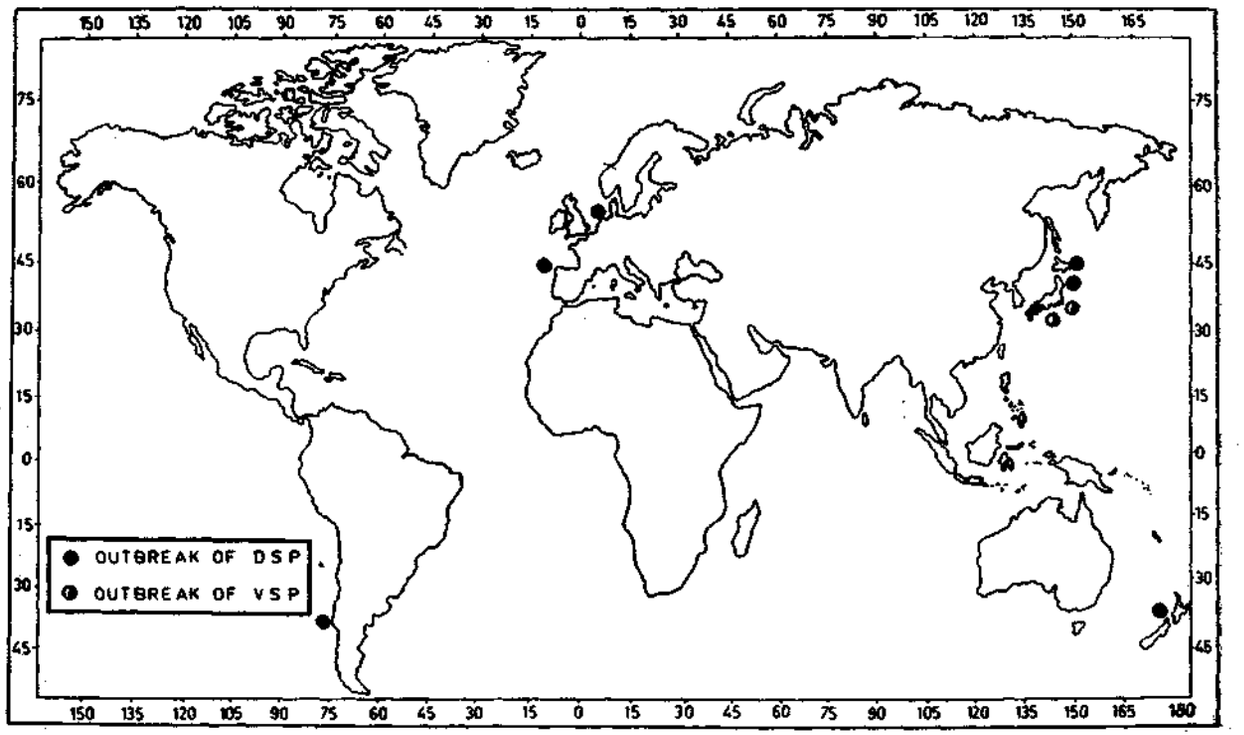

Fig. 2. World distribution of diarrhetic shellfish poisoning (DSP) 1976–1981 and venerupin shellfish poisoning (VSP) 1889–1950.

by Tanger (1982). If this outbreak involving 41 individuals with no death is verified to be VSP, it will be the first incidence to be reported outside of Japan. Tanger (1982) states that gastro-intestinal symptoms noted in the Norway outbreak were more similar to those attributed to VSP in Japan than gastro-intestinal disorders accompanying DSP reported from the Netherlands and Spain.

Although many of the 41 victims showed symptoms similar to the initial ones for VSP, none of them exhibited the more serious ones such as bleeding from mouth, gums and nose as well as petechial haemorrhage of the skin of the chest, neck and upper arms and legs. Without further evidence, we do not believe that the symptoms reported and the association of toxic shellfish with blooms of the dinoflagellate *Prorocentrum minimum* justify attributing the outbreaks in Norway to VSP. We believe that the illness as described is more similar to DSP.

5

TRANSVECTORS OF SHELLFISH POISONS

The consumption of bivalve molluscs, especially mussels and clams, is the most common cause of recorded incidences of paralytic shellfish poisoning (Halstead, 1965). Of these two groups of bivalves, by far the greatest number of human poisonings have been traced to various species of mussels. Among the mussels, the blue or bay mussel (*Mytilus edulis*) and the California or sea mussel (*Mytilus californianus*) are the most important.

A number of other molluscan species, oysters, scallops, predatory and filter-feeding gastropods and some non-molluscan invertebrates, filter-feeding crustaceans (barnacles and sand crabs), and a starfish are known to accumulate PSP (Sommer and Meyer, 1937; Goggins, 1961; Halstead, 1965; Quayle, 1969; Prakash *et al.*, 1971; Tufts, 1979). Because of their great filter feeding capacity, bivalve molluscs are unquestionably the most serious cause of poisonings. Nonetheless, the potential danger of consuming any organismst hat accumulate PSP should not be disregarded.

Fish have been implicated in atleast three outbreaks of PSP. Two of these incidences, in Vera Cruz, Mexico in 1797 and Table Bay, South Africa in 1837, are listed by Halstead (1965). The listing of the Vera Cruz outbreak as PSP is open to question since the poisonings were attributed to eating fish. Perhaps, the poisoning resulted from ciguatera. Grindley and Sapeika (1969) suggested that the South African epidemic may have been gastro-enteritis resulting from the eating of decaying fish rather than PSP. More recently outbreaks of human illnesses involving both bivalve molluscs and fish have been reported (Beales, 1976) from the coastal area of Brunei (northern Borneo) in March, 1976. The fish involved in the Brunei incidence were chub mackerel *Rastrelliger*

species. (Scombridae) and scad *Selar* sp. (Carangidae). It is most unusual for fish to serve as transvectors of PSP. Beales, however, presents a plausible explanation to account for poisoning by consumption of chub mackerel which is a plankton feeder. In Brunei, it is common practice to consume the gut and internal organs with the fish flesh or as a separate dish with rice.

As noted by Steidinger (1973), the dinoflagellates that produce shellfish poisonings in humans and other animals are rarely directly responsible for extensive mortality of marine organisms with two exceptions, *P. bahamense* var. *compressa* (PSP) and *Ptychodiscus brevis* (NSP). There have been no reports of illnesses due to the consumption of fish killed by a *P. brevis* red tide. Moreover, Maclean (1973) reports that no ill effects occurred following the consumption of fish and sea turtles killed during a *P. bahamense* var. *compressa* red tides in New Guinea.

Fish kills were reported by the villagers around Kumle Estuary to have occurred the day before the 1983 outbreak (Karunasagar *et al.*, 1984).

White (1981 a, b) has demonstrated in both laboratory and field studies that toxins of *Gonyaulax excavata* (=*G. tamarensis*) may be accumulated and retained by numerous herbivorous marine zooplankters such as barnacle nauplii, copepods, pteropods, etc. Thus, planktonic herbivores provide a mechanisms for transmission of PSP and other marine toxins. Fish kills of herring have been noted during *G. excavata* red tides and PSP has been detected in stomach contents of dead fish. In acute poisonings death of fish occurs so rapidly that toxins are not accumulated in muscle tissues. Nevertheless, based on the Brunei fish poisonings, the consumption of visceral organs of plankton feeding fish (dead or alive) should be avoided during red tides caused by PSP producing dinoflagellates.

Predatory gastropods deserve some mention in this respect since they may accumulate PSP by feeding on toxic bivalves (Goggins, 1961; Medcof *et al.*, 1966; Ingham *et al.*, 1968; Quayle, 1969). The rough whelk (*Buccinum undatum*) from the north shore of the St. Lawrence Estuary has been confirmed as causing 12 mild cases of PSP in 1937 and four persons were poisoned in 1970; and there was one fatality after eating whelks from the south shore of the St. Lawrence Estuary (Prakash *et al.*, 1971). In 1972 there was a report of two unconfirmed cases of PSP in British Columbia due to consumption of whelks (Prakash, 1980, personal communication, Ottawa, Canada) and two cases in 1975 in Massachusetts

attributed to eating predatory snails *Lunatia* (=*Polinicies*) were reported by Tufts (1979). In Palau, a gastropod *Tectus* sp. has been reported as being mildly toxic at a time when several bivalves were highly toxic (Harada *et al.*, 1982). Beales (1976) found toxicity in 4 out of 20 gastropod samples collected during the PSP outbreak in Brunei. Although predatory and filter-feeding snails are less important than bivalve molluscs as transvectors of PSP, one should be cautious and avoid eating gastropods during periods of shellfish toxicity.

Despite the scarcity of reports of PSP in the South Pacific prior to 1970 a large number of species of bivalves either have been involved in poisonings, or have been shown to be carriers of poisons (Maclean, 1975; Worth *et al.*, 1975; Harada *et al.*, 1982). Transvectors from New Guinea include cockle (*Anadara maculosa*), spiny oyster (*Spondylus* sp.), mussel (*Modiolus auriculatus*), oysters (*Crassostrea echinata*, *Ostrea trapezina* and *Pycnodonta hyotis*), pearl oyster (*Pinctada maxima*), pen shell (*Pinna* sp.), *Barbatia parvivillosa*, *Pterocarpo* sp. and *Chama* sp. In Palau several bivalve molluscs, the spiny oyster *Spondylus butleri*, giant clam *Tridacna crocera*, *Septifer bilocularia*, *Lopha cristagalli*, oyster *Saccostrea mordax*, mussel *Modiolus* sp. and *Barbatia* sp. have been established as transvectors of PSP. *Spondylus butleri* was found to be the most toxic of the above listed bivalves. Harada *et al.* (1982) commented that the toxicity of *S. butleri* with a highest level of 1,100 mouse units/g was exceptionally high, even when compared with PSP contaminated molluscs in U.S.A., Canada and Japan. The appearance of *T. crocera* among the list of PSP bearers is somewhat surprising, since tridacnid clams are thought to be weak filter feeders. The giant clams are generally believed to receive most of their nutrition from zooxanthellae species cultured in their mantle tissues. In addition to several unidentified species of bivalves, Beales (1976) reported the presence of PSP in two species of fish (*Rastrelliger* sp. and *Sardinella* sp.) and two unidentified crustacean species (Penaeid prawn and crab).

The PSP outbreaks in India involved the consumption of the backwater clam *Meretrix casta*. Originally the 1981 incidence at Vayalur on the east coast was attributed to eating mussels. However, follow up investigations made by the Madras Research Centre of Central Marine Fisheries Research Institute revealed that the mollusc responsible for the shellfish poisoning was not mussel, but the clam *M. casta*. Although not involved in the 1983 PSP outbreak, oysters (*Crassostrea cucullata*) collected from the same area (Kumble *Estuary*) as the toxic clams were poisonous (Karunasagar *et al.*,

1983). Clams and oysters collected on April 4, 1983 showed PSP levels of 18,793 MU/100 g and 6,680 MU/100 g respectively. Despite the initially greater toxic levels of the clams, they lost toxicity more rapidly than the oysters. The clams were free of detectable PSP after about 5 weeks whereas the oysters retained small amounts of toxin 7 weeks after initial evaluation.

The transvectors for poisonings in South America and the epidemic outbreaks of western Europe in 1976 were various species of mussels. Oysters (37%) and clams (63%) were implicated in the large outbreak (43 cases) on the Pacific Coast of Mexico (Casper, 1980, personal communication, Dallas, Texas).

Prakash *et al.* (1971) provide an excellent account of the relative hazards of the PSP transvectors encountered in eastern Canada. The principal shellfish transvectors in decreasing order of hazard are: soft-shell clams, mussels and rough whelks. Clams account for 65% of the cases compared with 9% for whelks. Also clams account for 65% of the deaths, whereas whelks have been incriminated in 4% of the deaths.

Rates of accumulation and loss of poisons as well as their anatomical distributions vary from species to species (Sommer *et al.*, 1937; Sommer and Meyer, 1937; Medcof *et al.*, 1947; Quayle, 1969; Prakash *et al.*, 1971; Maclean, 1975; Harada *et al.*, 1982). Nontoxic organisms have been known to become toxic within a day or two under both natural and experimental conditions (Meyer *et al.*, 1928; Prakash, 1963). Generally the digestive gland variously known as the digestive diverticula, hepatopancreas, liver, midgut gland or dark gland accumulates the greatest concentration of the poisons and the latter appear to be eliminated without appreciable accumulation in other organs. In some species, however, organs such as gills and siphons become the principal storage sites of PSP following the initial accumulation in the digestive gland (Medcof *et al.*, 1947; Quayle, 1969; Prakash *et al.*, 1971).

Although the siphon of the Pacific Coast butter clam (*Saxidomus giganteus*) is the primary site of toxin accumulation, the siphon of the soft-shell clam has a low capacity for toxin accumulation (Prakash *et al.*, 1971). As previously mentioned, the stability and marked accumulation of PSP in the siphons of butter clams have serious economic and health consequences. In the butter clam most of the toxins become localised primarily in the gills and siphon, as much as $\frac{1}{2}$ to $\frac{3}{4}$ of it occurring in the latter organ, which contitutes about 20% of the total meat weight. The distal third of

the siphon, which is black due to the presence of large amounts of melanin, accumulates the greater portion of the toxins found in siphons (Quayle, 1969).

With regard to melanin, studies by Price and Lee (1972 a) indicate that melanin serves as a binding agent for PSP *in vivo*. Furthermore, the same workers (Price and Lee, 1972 b) showed that interaction of PSP and melanin of butter clams is strongly influenced by the presence of cations. Also they found that the PSP - melanin interaction is reversible and electrostatic in nature. This finding offers an approach for possible reduction of the toxicity of poisonous butter clams.

The muscular tissues (white meat) of molluscs, *i.e.* the mantle muscles, adductor muscles, foot and body exclusive of digestive glands tend to store relatively small amounts of poison (Medcof *et al.*, 1947; Bourne, 1965; Quayle, 1969). Similarly limited studies carried out on bivalve molluscs of the South Pacific area show that the digestive glands and gonads of bivalve molluscs such as the oyster *Crassostrea echinata* and the pearl oyster *Pinctada maxima* contained most of the shellfish toxins (Maclean, 1975). Since the digestive glands and gonads are difficult to separate, Maclean has assayed both organs together. Except for palps of the edible oyster and the heart (mainly ventricle) of the pearl oyster, all other tissues, *viz.* mantle, gills, adductor muscle and foot of the pearl oyster were only mildly toxic. Likewise, the studies on the anatomical distribution of toxins in spiny oyster *Spondylus butleri* from Palau showed that 98% of the poisons were in the viscera, whereas the other organs gills, mantle and adductor muscle each contained less than 1% of the total toxin detected (Harada *et al.*, 1982).

The limited accumulation of PSP in adductor muscles of bivalve molluscs is a fortunate circumstance. It can be and is used to reduce the danger of ingesting toxic levels of shellfish poisons. Scallops may become toxic for long periods (Medcof *et al.*, 1947; Bourne, 1965). However, since only the adductor muscle is marketed, human poisoning is unlikely unless the entire animal is eaten. Maclean (1975) has pointed out another fortunate circumstance regarding the low levels of toxins in the adductor muscle of the pearl oyster *Pinctada maxima* in New Guinea. At harvest time, which may occur during toxic red tides in Port Moresby, the pearl farm workers eat the adductor muscles of harvested pearl oysters. Thus, as in the case of scallops, the consumers escape poisoning since they eat only the adductor muscle.

Fortunately in North America, except for butter clams and scallops most molluscs including both the siphonated (clams) and the non-siphonated (mussels and oysters) species lose the poison rather rapidly. Thus, the poisonous condition is transitory in most cases (Medcof *et al.*, 1947; Schantz and Magnusson, 1964; Halstead, 1965; Quayle, 1969) lasting from a few days to a few weeks and with a maximum of two months. Quayle (1969) studied the rate of toxin loss in eight species of bivalves and found that most species including both the siphonated and non-siphonated lost most of the toxins in a few weeks, whereas butter clams required approximately two years to be free from accumulated toxins. The non-siphonated forms the blue or bay mussels (*Mytilus edulis*) and the Pacific oyster *Crassostrea gigas* lost toxins very rapidly. Moreover, oysters and cockles *Clinocardium nuttallii* accumulated far less toxin than the other species examined.

In New Guinea the oyster *Crassostrea echinata* remained mildly toxic for at least four months after the subsidence of the toxic red tide (Worth *et al.*, 1975). However, oysters of the same species from the same red tide became nontoxic after three weeks in a closed sea water system (Maclean, 1975). Two other toxic bivalves *Anadara maculosa* and *Modiolus auriculatus* were free from toxin within six weeks after the toxic dinoflagellate bloom disappeared. On the contrary, the spiny oyster *Spondylus* sp. was still highly toxic after the same period.

Although oysters are consumed in large quantities throughout the world, the relatively low capacity for storing PSP probably accounts for the low frequency with which oysters are incriminated in PSP outbreaks.

Two bivalves, the American oyster *Crassostrea virginica* and the southern hard clam *Mercenaria* (=*Venus*) *campechiensis* have been incriminated in cases of neurotoxic shellfish poisoning on the west coast of Florida. Morton and Burklew (1969) found that *C. virginica* loses its toxicity in two to six weeks after the disappearance of *Ptychodiscus brevis* red tides. Therefore the consumption of any filter feeding bivalves during or immediately after a *P. brevis* red tide may result in neurotoxic shellfish poisoning.

Diarrhetic shellfish poisonings commonly follow the consumption of scallops and mussels in certain areas of northern Honshu and northern Hokkaido Islands in Japan (Takagi *et al.*, 1982; Yasumoto *et al.*, 1978). Mussels (*Mytilus edulis*) and scallops (*Patinopecten yessoensis*) from these areas are also highly toxic (Yasumoto *et al.*, 1980; Murata *et al.*, 1982). Based on

weight, mussels appeared to be more toxic than scallops while oysters (*C. gigas*) showed low levels of toxin (Yasumoto *et al.*, 1978). In both of the bivalves the major portion of the toxins were contained in the hepatopancreas or digestive glands. Another scallop (*Chlamys nipponensis akazara*) along with mussels, has been implicated in one outbreak; and an edible sea squirt *Halocynthia roretzi* taken from the area of toxic shellfish proved to be non-toxic (Yasumoto *et al.*, 1978). It has been commented by Yasumoto *et al.* (1978) that DSP is not likely to occur from eating oysters. Consumption of blue mussels (*M. edulis*) was blamed for a DSP outbreak in the Netherlands (Tanger, 1982) and green-lipped mussels (*Perna canaliculus*) from New Zealand were cited by Shimizu (1983) as bearers of DSP.

According to Yasumoto *et al.* (1978), in the 1976 outbreak the maximum toxicity occurred in the shellfish on June 9 and gradually decreased and finally disappeared in August. In 1977 maximum toxicity was recorded on June 28, and the toxicity decreased more rapidly than in 1976. These workers also monitored the loss of toxin in mussels maintained in the laboratory tanks; about half of the toxin disappeared in one week and it was barely detectable after one month. Murata *et al.* (1982) noted that shellfish (species not mentioned) toxicity may last for several months. We could not ascertain whether this was due to either a long retention time of the toxins or continuous exposure to toxic dinoflagellates. The results obtained by Yasumoto *et al.* (1978) indicates that the retention time for the toxin in mussels is no more than one to two months.

Venerupin, the most lethal of the shellfish poisonings, has resulted from the consumption of either short-necked clams *Tapes japonica* (=*Venerupis semidecussata* or *Tapes semidecussata*) or the oyster *Crassostrea gigas*. According to Halstead (1965), Hashimoto (1979) and Okaichi and Imatomi (1979), no other transvectors are known for confirmed cases of VSP. Four of the seven outbreaks (1889, 1941, 1943 and 1949) involved the consumption of oysters and three outbreaks including the massive one of 1942 as well as the 1944 and 1950 incidences were attributed to the consumption of short-necked clams (Hashimoto, 1979). The two earliest outbreaks (1889, 1941) which occurred in Kanagawa Prefecture, involved the consumption of oysters. On the other hand, of the last five outbreaks all which occurred in Shizuoka Prefecture, resulted from consumption of oysters in two incidences (1943 and 1949) and short-necked clams in three of them (1942, 1949 and 1950). It is of further interest to note that the outbreak (1942) with the greatest number of deaths (114) and the only one

(1950) with no death (12 cases) both resulted from the consumption of short-necked clams.

Hashimoto (1979) has referred to studies by Akiba (1943) in which various species of molluscs from Lake Hamana were examined for toxicity by injection of methanolic extracts into mice. Toxicity was detected in three bivalves, short-necked clams, oysters and *Dosinia japonica;* two other bivalves *Meretrix lusoria* and *Mactra veneriformis* and a gastropod *Batillaria multiformis* were nontoxic. The digestive gland was the primary site of toxin concentration. Toxicity was detected from February to May. Hashimoto has also noted that nontoxic bivalves became toxic within a short period when transplanted to a toxic area and toxic bivalves lost their toxicity when maintained in a nontoxic area.

6

SOURCE AND NATURE OF SHELLFISH POISONS

Sommer and his colleagues (1937) solved the mystery of the cause of paralytic shellfish poisoning through outstanding researches. While investigating various aspects of a series of outbreaks that began on the west coast of U.S.A. in 1937, these workers proved that the dinoflagellate *Gonyaulax catenella* was the primary source of PSP in that region. Since that time, additional species belonging to the genus *Gonyaulax* and other genera as well, have been shown either to be the source or have been implicated as such in various areas of the world. *Gonyaulax catenella* and *Pyrodinium bahamense* var. *compressa* chain forming species and *G. tamarensis* a non-chain forming species, are the most commonly known causes of PSP and probably represent the most poisonous of the dinoflagellates. Another non-chain forming dinoflagellate *G. acatenella,* is known to cause PSP on the Pacific Coast of British Columbia, Canada (Prakash and Taylor, 1966).

The source of the shellfish poison in the two outbreaks in India is not known. The toxicity of the shellfish at Vayalur in Chingle put District was attributed to dinoflagellates consumed by them (Dr. Ramesh Bhatt, Food and Drug Toxicology Research Centre, National Institute of Nutrition, Hyderabad, Andhra Pradesh). However, the identity of the microalgae had not been established. A comment was made that toxins injected in mice produced toxic manifestations. It is also not clear from this report whether the toxins referred to were from a sample of the shellfish consumed by the victims or from the shellfish harvested from the same area from where the toxic shellfish were originally collected. *Gonyaulax polygramma* which is considered by Maclean (1937) to be a toxic dinoflagellate is known to occur in Buckingham Canal (Gopinathan, C. P., Personal communication, Tuticorin). In view of the

high water temperature in the area of both incidences in India, we speculate that the toxic dinoflagellates may have been *Pyrodinium behamense* var. *compressa* or some other unknown warm water producer of paralytic shellfish poison.

Perhaps a brief description of dinoflagellates will be useful at this point. The following identifying characters have been taken from Steidinger's excellent review (1983) of the biology and ecology of toxic dinoflagellates. Under the light microscope these microscopic algae (Pyrrophyta) show two identifying characters: a prominent, large nucleus with continually condensed choromosomes and a motile, bi-flagellated stage at sometime in the life-cycle. Dinoflagellates are characterised at the electron microscope level by: a vesicular cell covering a nucleus with a persistent nuclear membrane, a 2–3 nuclear chloroplast envelope and chromosomes are attached to the nuclear envelope. Although most dinoflagellates are free-living and photosynthetic, some are parasitic and heterotrophic. Some free-living species may be both photosynthetic and heterotrophic. Dinoflagellates have distinct photosynthetic pigments *e.g.* xanthophylls such as peridinin. Furthermore, Steidinger (1983) notes that less than 2% of the known dinoflagellate species (1,000–1,500) have been confirmed as toxic or poisonous to other organisms, including humans.

Currently there is considerable debate concerning the taxonomical relationships of species of *Gonyaulax* that have been implicated as the source of paralytic shellfish poisons. Since most of the dinoflagellates were originally described and classified by light microscope characters, it is understandable that new information gained through electron microscopy will lead to changes in the systematics of dinoflagellates and other organisms as well. We shall attempt to point out some of the recent taxonomical changes so that the general reader will not be confused by constant reclassification of species of economic significance such as toxic dinoflagellates. Steidinger (1983) has indicated that a recommendation has been made to the Botanical Nomenclature Committee for the conservation of specific names of economically important species.

Intensive laboratory and field studies by a number of scientists in various parts of the world indicate that large complexes of *catenella* and *tamarensis* types of organisms may exist rather than distinct single species entities as *G. catenella* and *G. tamarensis*. Isolates of morphologically indistinguishable forms may differ in such attributes as toxicity and bioluminescence. Because of such differences, the current tendency is to consider such forms as different varieties or strains of the same species. For example, it was recently

thought that three characteristics ventral pore, bioluminescence and toxicity could be useful for differentiating *G. excavata* (New England and eastern Canada species) from *G. tamarensis* (English and European species). However, Schmidt and Loeblich (1979 a) have found that these characteristics occur in all but one combination among various Pacific, Atlantic and English Coast isolates. Since all isolates have identical thecal plate tabulation, these authors recognize them as different varieties; *G. tamarensis* var. *tamarensis* (with ventral pore) and *G. tamarensis* var. *excavata* (without ventral pore, with a luminescent/toxic form of a single species. Also they have observed that some isolates and natural populations identified as *G. acatenella, G. catenella, G. excavata* and *G. tamarensis* are more closely related than previously thought. They consider that these four species very likely belong to one species *G. tamarensis.*

Schmidt and Loeblich (1979 b) found that some strains of both *tamarensis* varieties are toxic to mice. Moreover, these authors tested 23 dinoflagellates using the mouse bioassay and found that only those species belonging to the *Catenella* Section produce PSP. To date, all dinoflagellates known to produce PSP except *Gymnodinium catenatum* (cause of PSP on the Pacific Coast of Mexico) are armoured (with thecal plates) and belong to the *Catenella* Section. This naked (without thecal plates), chain-forming species is probably a derivative of the armoured chain-forming species *Gonyaulax catenella* (Steidinger, 1983).

Another chain-forming species *Pyrodinium bahamense* var. *compressa* is the cause of PSP in the south Pacific area. The other variety of this species *P. bahamense* var. *bahamense* is associated with nontoxic red tide blooms and bioluminescence in subtropical and tropical waters. Although these two varieties may be distinguished by expert algologists based on morphological features, the primary difference is physiological. Var. *compressa* produces PSP and var. *behamense* is nontoxic (Steidinger *et al.*, 1980).

Recently some species belonging to the genus *Gonyaulax* including known PSP producers *viz., G. catenella, G. acatenella* and *G. tamarensis* have been assigned to the genus *Protogonyaulax* (Steidinger, 1983). In this manual, we chose to retain the generic name *Gonyaulax*, since this name appears most often in the extant literature. Those interested in the taxonomic aspects of *Gonyaulax* may consult Schmidt and Loeblich (1979 a, b), Loeblich and Loeblich (1975, 1979), Taylor (1979), Wall (1975) and Steidinger (1983).

Researches on the chemical characterisation and neurophysiology of paralytic shellfish toxins are being conducted in numerous institutions throughout the world, especially in those areas where poisonings are known to occur. To date at least, 12 paralytic shellfish toxins isolated from various species of toxic dinoflagellates and bivalves have been named and partially characterised. The literature dealing with the subject is too voluminous to summarise. For a general summary on the subject see Steidinger (1983). The PSP's are water soluble, non-proteinaceous, have low molecular weight and are heat stable. Saxitoxin is a white solid, that is water soluable, only slightly soluble in ethanol and methanol and isoluble in most organic solvents such as chloroform, diethyl ether and petroleum ether. The molecular formula is $C_{10}H_{17}N_7O_4$. 2 HCl, and molecular weight 372. This poison is inactivated when reduced with hydrogen (Schantz, 1979). Saxitoxin, the major toxin produced by *Gonyaulax catenella,* has been completely characterised after many years of research by several investigators. Schantz *et al.* (1975) determined the structure of saxitoxin.

Twelve of the toxins, including saxitoxin, neosaxitoxin and gonyautoxins I–VIII, have similar chemical structures (Steidinger, 1983). Harada *et al.* (1982) isolated saxitoxin, neosaxitoxin, gonyautoxin V and two unidentified toxins from *Pyrodinium bahamense* var. *compressa* in Palau. The two unidentified toxins were labelled PBT–1 and PBT–2. They believe that PBT–1 is a new compound and that PBT–2 may be similar to gonyautoxin VI. According to Schantz (1979), saxitoxin from California sea mussels (*Mytilus californianus*) and Alaska butter clams (*Saxidomus giganteus*), which is the major toxin in these bivalves, is identical to saxitoxin (STX) from *G. catenella* from central California. Schantz notes further that a weakly basic poison found in cultured *G. tamarensis* and scallops from the Bay of Fundy accounted for 80 to 90% of toxicity. Chemical analyses and properties as well as NMR spectra showed that the structure was the sulphate ester of 11-hydroxysaxitoxin. This poison had normal basic properties like saxitoxin when the sulphate group was hydrolised from it to form 11-hydroxysaxitoxin. Gonyautoxin III is α 11- (OSO_3) STX.

The ratio of saxitoxin and the other known PSP's are variable among bivalve species and in tissue distribution within species. Bioconversion or transformation of various paralytic shellfish toxins in the scallop *Placopecten magellanicus* from the Bay of Fundy, to saxitoxin as the final product has been demonstrated by Shimizu and Yashioka (1981). Also these workers found that the locomotory organs, foot and adductor muscle, caused marked

changes in the toxin profile. Furthermore, they found that the adductor muscle of scallops can inactivate toxins. This finding may explain the lack of PSP in the adductor muscle of highly toxic scallops and other bivalves as well. As mentioned previously, this is a fortunate circumstance since the adductor muscle is the only portion of scallops which is usually consumed.

Paralytic shellfish poisons are among the most potent nonproteinaceous natural human poisons. Schantz (1973, 1979) has procided some general information of the toxicity of these poisons to humans. The values are given in both mouse units (MU) and amount of purified toxin (saxitoxin). One MU is equivalent to 0.18 μ gm of saxitoxin. The amount of toxins required to produce sickness or death in humans may vary considerably. Meyer (1953) has estimated that illness may result from the ingestion of 1,000 to 20,000 MU and estimated that at least 20,000 MU or more was required to cause death. Workers in Maritime Provinces of Canada (Bond and Medcof, 1958) estimated that much smaller amounts (about 600 MU) were required to produce sickness and that death could occur with 3,000 to 5,000 MU. In terms of purified toxin, Schantz (1966) considered 100 μg by oral ingestion to be a lethal dose for humans.

The availability of saxitoxin (purified butter clam poison) has prompted numerous studies of the toxicological and pharmacological properties if this toxin (Murtha, 1960; Kao, 1966, 1967; Evans, 1967, 1969 a, b; Bull and Pringle, 1968). Kao (1966) presents an excellent review of this subject. Likewise, Baslow (1969) has reviewed this topic in his book on marine pharmacology. Saxitoxin and tetrodotoxin (puffer poison) have provided extremely valuable tools in the study of neuromuscular physiology (Kao, 1966; Evans, 1969 a).

Although very different in chemical structure and biological origin, both saxitoxin and tetrodotoxin have a direct paralysing action on striated (skeletal) muscles and nerve fibres, reducing membrane permeability to sodium ions (or ions that may be substituted for sodium) by blockage of sodium channels (Kao, 1966; Evans, 1969 a). The blockage of sodium channels by paralytic shellfish poisons inhibits the passage of an impulse along a nerve axon or muscle fibre. The peripheral nervous system along with associated muscles is the primary site of action and subsequently the central nervous system if affected. Steidinger (1983) cited the work of Kobayashi and Shimizu (1981) who speculated that marked difference in toxicity between gonyautoxins III and VIII may be due to the differences in the binding action between the

toxin molecules and the sodium channels. She also noted that saxitoxin (2,045 MU/μmol) and gonyautoxin III (2,234 MU/μmol) are the most potent of the paralytic shellfish poisons.

Poisons, one of which is similar to saxitoxin, are produced by the freshwater blue-green alga *Aphanizomenon flos-aquae* (Jackim, and Gentile, 1968). According to Schantz (1973) several species of poisonous crabs in Japanese waters have been shown to contain a poison (saxitoxin) identical to that produced by *Gonyaulax catenella*. Surprisingly, Kodama *et al.* (1983) isolated a nontoxic high molecular fraction from highly toxic pufferfish liver that released tetrodotoxin (about 70% of total toxicity) along with a small amount of saxitoxin and other unidentified toxins on RNase digestion.

The association of *Ptychodiscus brevis* red tides with a mild form of shellfish poisoning on the west coast of Florida was first made by Eldred *et al.* (1964) and McFarren *et al.* (1965). The naked dinoflagellate *P. brevis* has been confirmed as the source of poisons known as neurotoxic shellfish poisons (Ray and Aldrich, 1965, 1967; Morton and Burklew, 1969; Cummins *et al.*, 1968; Cummins and Stevens, 1970). McFarren *et al.* (1965) extracted similar toxins with lipid (organic) solvents from toxic clams, oysters and *P. brevis* cultures. Based on the lipid solubility of toxins and absence of paralytic symptoms in the poisonings, these workers indicated that NSP was more like ciguatera poisoning caused by toxic fish in semitropical and tropical areas rather than PSP.

Red tides caused by *P. brevis* are better known for extensive fish kills along the west coast of Florida than for shellfish poisonings. In addition to humans and fish, *P. brevis* poisons are toxic to mice, chicken and some invertebrates but not bivalve molluscs (Gunter *et al.*, 1948; Ray and Wilson, 1957; McFarren *et al.*, 1965; Ray and Aldrich, 1965; 1967; Sievers, 1969). This dinoflagellate produces a haemolytic fraction in addition to neurotoxic substance (Steidinger, 1983).

In recent years several groups of investigators have worked on characterisation, toxicology and pharmacology of toxins produced by unialgal cultures of *P. brevis*. Most of these workers have used a strain of *P. brevis* isolated by the Late W.B. Wilson (Marine Biology Dept., Texas A & M University at Galveston). Steidinger (1983) pointed out that Wilson's 1953 strain had lost its ability to reproduce sexually. Thus, she suggests that this strain may differ from more recent isolates, which do reproduce sexually, with regard to the nature and levels of metabolic products.

Several different poisons have been isolated and these poisons have been given different names as identification toxin numbers (T-numbers). The use of different extraction and purification procedures by various research groups has led to complications in efforts to determine the chemical and physical nature of *P. brevis* poisons. Schantz (1979) remarked that either several kinds of poisons are produced by *P. brevis* or that the isolation and purification procedures alter the molecular structure. The publications concerning the nature of poisons produced by *P. brevis* are too numerous to be treated in this manual. Schantz (1979) and Steidinger (1983) have briefly summarised the work done so far on this subject. According to Schantz (1979) all of the toxins isolated from cultured *P. brevis* cells are lipid soluble, nonaromatic and nonproteinaceous with molecular weight of approximately 800. The structure of the major toxin, brevetoxin B (also known as GB-2, T 47 or T 34) has been determined by Lin *et al.* (1981). According to these workers brevetoxin B (BTX-B) is the major toxin of a unique, new series of marine toxins isolated from *P. brevis* that are characterised by strong ichthyotoxicity and neurotoxicity. The planar structure and relative configuration of BTX-B, $C_{50}H_{70}O_{14}$ was determined by X-ray analysis (direct methods) and the absolute configuration by converting BTX-B, by a 5-step process, into a di-p-bromobenzoate and then applying the dibenzoate chirality method. The structure of BTX-B containing eleven contiguous ether rings represents an unprecedented structural class. Another neurotoxin referred to as brevetoxin A also known as GB-3, T 46 or T 17 (Lin *et al.*, 1981) has been isolated.

According to Steidinger (1983) the oral lethal dose for NSP (500 µg/kg) is much greater than that for PSP (10 µg/kg). She notes that relative toxicity of brevetoxin B (T 34) and brevetoxin A (T 17) to mice depends on the manner of administration (Baden, 1983). Brevetoxin A is more toxic orally than brevetoxin B, whereas the reverse is true in the case of intraperitoneal or intravenous injection. She further notes that the speculations of Baden *et al.* (1982) that brevetoxin A (T 17), a reduced form of brevetoxin B (T 34) is the cause of NSP illnesses and "red tide aerosol" effects that produce coughing and sneezing among people in contact with surf sprays during *P. brevis* red tides.

Steidinger (1983) cites the mode of action of NSPs according to Baden *et al.* (1982); these toxins depolarise the resting potential of nerve membranes by increasing the sodium ion influx, thus increasing the frequency of end plate potentials ultimately resulting in the depletion of the neurotransmitter, acetycholine, at synapses.

The poisons do not exert anticholinesterase activity. Steidinger notes that brevetoxins A and B may act synergistically.

Although unknown until 1976 the source and nature of diarrhetic shellfish poisons were determined much more rapidly than for other shellfish poisonings. This is undoubtedly due to advances made through investigations on PSP and NSP. Many of the techniques and procedures developed in the studies of these poisonings and poisons are applicable to new poisonings.

Moreover, the demonstration that many marine poisons are derived from planktonic or benthic algae has contributed significantly to identifying the source of such poisons. Yasumoto *et al.* (1978) determined that DSP was a lipid soluble toxin found principally in hepatopancreas of mussels and scallops. Quite early the source of DSP in Japan was traced to the dinoflagellate *Dinophysis fortii.* No red tides or fish kills have been associated with DSP outbreaks in Japan. Another member of the *Dinophysis* viz., *D. acuminata* has been associated with DSP on the Dutch coast of Holland (Kat *et al.*, 1982). Various species of *Dinophysis* occur throughout the world. Thus, it is likely that many cases of mild gastro-enteritis of unknown etiology will be proved to be DSP.

Since the toxic substances produced by some species of *Prorocentrum* are lipid soluble and similar in structure to DSP, it is possible that species of this genus may prove to be producers of diarrhetic shellfish poisons. Although Kat (1979) observed an association of gastro-intestinal illness of mussel consumers and red tides caused by *Prorocentrum redfieldi* and *P. micans*, she was unable to establish in laboratory studies that either of the dinoflagellates produced toxins.

Yasumoto *et al.* (1980) isolated toxins from mussels and *D. fortii* that behaved similarly in both permeation and partition chromatography and these toxins produced similar responses in mice that received intraperitoneal (I.P) injections of the toxins. Both the mussel and dinoflagellate toxins produced prostration, laboured breathing and weak paralysis in hind limbs following I.P. injections. A mouse assay technique has been developed for monitoring the toxicity of shellfish (Yasumoto *et al.*, 1978; 1980). These workers defined one mouse unit (MU) as the minimum amount of toxin required to kill a 20 g mouse within 24 hours. The toxicity of shellfish is expressed as mouse units contained in 1 g of the hepatopancreas (MU/g). Approximately 13,000 cells of *D. fortii* are stated to be required to produce 1 MU and about 12 MU of toxin from

the hepatopancreas will produce a mild case of DSP (Yasumoto *et al.*, 1978).

The major toxin in diarrhetic shellfish poisoning was isolated from hepatopancreas of the mussel *Mytilus edulis* and was named dinophysistoxin-1 (DTX–1) by Murata *et al.* (1982). These workers also determined the structure and physical properties of DTX–1. This toxin was also detected in the dinoflagellate *Dinophysis fortii*, providing further confirmation that this organism is a source of DSP. Dinophysistoxin-1 is a colourless solid: mp. 134°C, $(\alpha)_D^{20}$ 28° (0.57 mM, chloroform). The minimum lethal dose for mice is 160 μg/kg by I.P. injection. Comparison of DTX–1 with okadiac acid isolated from the marine dinoflagellate *Prorocentrum lima* (Murakami *et al.*, 1982) indicates that DTX–1 is 35^S–Methyl okadaic $C_{45}H_{70}O_{13}$. Dinophysistoxin-1 accounts for about 88% of the initial toxicity observed in toxic mussels.

Murata *et al.* (1982) isolated two other toxins from mussels in addition to DTX–1. One was an unstable toxin which comprised 4% of the original toxicity, that was extracted with the hexane layer. A second toxin comprising about 8% of the original toxicity was contained along with DTX–1 in the aqueous ethanol layer. The two toxins were separated from silicic acid column by different organic solvents. No further work was done on the two minor toxins since one was unstable and the other was not available in sufficient amount.

Another group of Japanese workers (Takagi *et al.*, 1982) isolated a fat soluble toxin from scallops (species not mentioned) from the Okhotsk Coast of northern Hokkaido Island. These workers found that the major toxic components of this toxin were polyenoic acids in the form of free fatty acids. A series of pure unsaturated fatty acids were assayed for toxicity with the killifish *Oryzias laptipes* and by I.P. injection of mice. Linoleic (18:3) and arachidonic (20:4) and 5, 8, 11, 14, 17 – eicosapentaenoic (20:5) acids were more highly toxic to fish and mice than linoleic (18:2) acid (slightly toxic) and 4, 7, 10, 13, 16, 19 – docosahexaenoic (22:6) acids (mildly toxic). They found that toxicity of the unsaturated fatty acids was nearly comparable for fish and mice. Thus, they suggested that fish may be used for perliminary tests in assaying shellfish for DSP. They also noted that conversion of free fatty acids to methyl esters eliminated their toxicity.

Yasumoto *et al.* (1978) carried out studies on DSP isolated from mussels and found that I.P. injections in mice produced initial symptoms of inactivation and general weakness within 30 minutes to several hours and death may take place within 100 minutes

to 47 hours after injection. The oral lethal dose for mice was about 16 times greater than by I.P. injection. Chicks are less sensitive than mice; about 13 MU are required to kill 100 g chick by I.P. injection. Chicks showed no ill effects when fed with mussel hepatopancreas containing 10 MU of toxin. Cats showed vomiting but no diarrhoea when voluntarily fed steamed and homogenised toxic hepatopancreas of mussels and scallops. Mussels appeared to be more toxic to cats than scallops.

The source of venerupin shellfish poisoning has not been established with certainty. Nakazima (1965 a, b, c) reported that a dinoflagellate *Prorocentrum* sp. which was later identified (Nakazima, 1968) as *Exuviaella mariae-lebouriae* was the source of VSP. Subsequent to 1968, this dinoflagellate has been named *Prorocentrum minimum* var. *mariae-lebouriae*. Nakazima reported that cells of *P. minimum* var. *mariae-lebouriae*, extracts of such cells and extracts of the digestive glands of shellfish that had fed on cultured cells of this species induce the same type of pathological damage when administered to mice as noted in human poisonings. Nakazima's conclusion regarding the source of VSP is not fully convincing to Hashimoto (1979). He agrees, however, that it is reasonable to conclude that bivalves become toxic by feeding on plankton.

However, recent studies by Okaichi and Imatomi (1979) lend support to Nakazima's contention that *P. minimum* var. *mariae-labouriae* is the cause of VSP. These workers extracted two toxic substances from cultures of the dinoflagellate implicated by Nakazima. A methanolic extract (75%) partially purified by XAD–7 column chromatography had a lethality of 500 mg/kg mouse by I.P. injection. In response to a dose of 15 mg of this toxin, the mice showed prostration and diarrhoea. Also, the mice lost 10 – 20% of their weight when they died within 24 – 48 hours. Paralysis and tonic spasm were not observed, but occasionally lifting of the tail was noted. Moreover, centrilobular necrosis of the liver was noted on autopsy. Two toxic fractions were separated from the methanolic extract by chromatic methods and identified as TF–2 and TF–3. Each of the toxic fractions had lethality of 250 mg/kg mouse. Toxic fraction F–3 was negative to the Dragendorff reagent; no further studies have been conducted on this fraction. Toxic fraction F–2 has been purified and it was found to be positive to the Dragendorff reagent, but negative to ninhydrin, Sakaguchi and Libermann reagents. No appreciable absorption was noted in the ultraviolet region from 200 to 350 μm. With infrared spectroscopy, the presence of OH radical, -C-O-C- and aliphatic tertiary amino radical were determined. The fraction TF–2 had a lethality of less than 125 mg/kg mouse.

7

PREVENTION AND CONTROL OF SHELLFISH POISONINGS

The surveillance of edible molluscs during potential danger periods constitutes the most effective means of preventing shellfish poisonings. Many of the procedures and steps that we shall present for preventing and controlling shellfish poisonings have been applied previously to paralytic shellfish poisoning. Nevertheless, most of the measures or the same with some modifications thereof are applicable to the other forms of shellfish poisonings. Of these the mouse bioassay is the standard method for monitoring the safety of shellfish. The most widely used and perhaps best known mouse bioassay for shellfish poisoning was first developed by Sommer and Meyer (1937) and later modified by Medcof *et al.* (1947) for detecting PSP. The details of the procedures employed for assaying the various shellfish poisons *i.e.* PSP, NSP, DSP and VSP are dealt with in this Chapter.

Although the mouse bioassay for PSP has been used successfully for many years, it is costly, time consuming and difficult to standardise. White and Maranda (1978) pointed out that the mouse assay is not very sensitive or accurate. When carried out under very carefully controlled conditions, the mouse bioassay has a precision of $\pm 20\%$ (Horwitz, 1980). In view of the inherent difficulties with the mouse bioassay there has been considerable search for alternate means of detecting and quantitating shellfish poisons, especially PSP. A chemical method for quantitating PSP, based on the Jaffe test, has been developed by McFarren *et al.* (1958), and a serological test has been developed by Johnson and Mulberry (1966). As of 1966, Pringle (1966) concluded that mouse bioassay was still the most suitable method to quantify PSP.

According to White and Maranda (1978), several chemical assays have been developed recently for saxitoxin (Bates and Rapoport, 1975; Buckley *et al.*, 1976; Gershey *et al.*, 1977; Proctor *et al.*, 1975). They considered the alkaline peroxide oxidation-fluorometric method (APOF) of Bates and Rapoport (1975) to be the most promising for routine monitoring programmes. White and Maranda (1978) used the APOF method and the standard mouse bioassay to monitor PSP in *Gonyaulax excavata* (= *G. tamarensis*) cultures and three species of toxic bivalves from the east coast of Canada. They concluded from these studies that APOF method which measures saxitoxin (STX) was less reliable than the standard mouse bioassay for measuring paralytic *G. excavata* toxin in shellfish. These workers stated that the APOF method does not indicate total toxin content. As previously mentioned *G. tamarensis* of east coast of North America produces several toxins in addition to STX whereas *G. catenella* (west coast of North America) appears to produce only STX. Thus White and Maranda indicated that APOF method for STX, which is 100 times more sensitive than the bioassay method and which requires only small samples (2 g) has great potential for use in monitoring PSP on the west coast of North America. However, the recent detection of neosaxitoxin in some samples of Alaskan butter clams raises doubt that saxitoxin is the only paralytic shellfish poison produced by *G. catenella* or that *G. catenella* is not the sole source of paralytic shellfish poison accumulated in Alaskan butter clams (Oshima *et al.*, 1977; Reichardt *et al.*, 1978).

An improved modification of the fluorometric method was later developed by Bates and Rapoport (1978). The fluorometric method has been criticised on grounds that it does not detect total toxicity and needs further refinements (Steidinger, 1983).

Various research groups are working on the development of rapid immunoassays for PSP, NSP and ciguateria poisoning (Steidinger, 1983). According to Steidinger, immunoassays require that a purified nonproteinaceous toxin be conjugated to a protein in order that antisera can be produced in a host animal such as the rabbit. She states that investigators have produced antigen complexes for some toxins and that work is being done on NSP. Furthermore, she speculates that field immunoassay kits that require little incubation time will be available to detect the various shellfish poisons as well as other marine poisons.

Moreover, we learned recently that John Sullivan and Wayne Iwaoka, researchers at the University of Washington* have

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developed a PSP detection technique that uses high pressure liquid chromatography. Comparison of this technique with the mouse bioassay showed high correlation in shellfish samples containing about 60 μg of toxin per 100 g of meat. In shellfish containing higher amount of toxin, the average variation was 25%. As noted previously the precision of mouse bioassay is ±20%.

The public health and fisheries agencies of U.S.A. and Canada have co-operated extensively in developing a certification programme to ensure the safety of interstate shipments of fresh or canned shellfish. In U.S.A. and Canada growing areas are quarantined when PSP is known to reach 400 mouse units (80 μg)/100 g of shellfish or more (McFarren *et al.*, 1960; Quayle, 1969). According to McFarren *et al.* (1960) the U.S. Food and Drug Administration does not permit the marketing of fresh frozen or canned shellfish when the average toxicity exceeds 400 MU/100 g and no individual package may exceed 2,000 MU. Minced or chopped shellfish meats must have an average toxicity of less than 2,000 MU/100 g and individual packages may not exceed 2,000 MU. While adherence to the above standards may cause appreciable economic losses to the shellfish industry, their strict enforcement by U.S.A. and Canadian authorities may be credited with the notable lack of PSP cases traceable to commercially processed shellfish. Except for Canada and U.S.A. the establishment of toxic limits of PSP for restricting the harvest and marketing of shellfish has not been noted. However, we have seen an editorial note (Hashimoto, 1979 to this effect "In 1978, the Fisheries Agency of Japan promulgated a tentative regulation that scallops with toxin level higher than 4 MU/g of the edible part shall not be marketed." We have no further information regarding this tentative regulation.

Along the west coast of Florida, the harvest of bivalve shellfish is prohibited during a *Ptychodiscus brevis* red tide in shellfish growing areas. The area normally remains closed to shellfish harvesting until water samples are negative for *P. brevis* cells and two consecutive shellfish meat samples are negative for NSP (Keys, 1975).

Furthermore, we are aware of only one other official regulation which establishes the allowable limits of shellfish poisons permitted for harvest and sale of shellfish. This regulation involves shellfish contaminated with diarrhetic shellfish poison in Japan. Since 1978 a surveillance system has been established for DSP in the northern parts of Honshu and Hokkaido Islands.

Harvesting and marketing of shellfish inwhich the toxicity exceeds 0.05 MU per g of hepatopancreas are prohibited by

regulation unless the hepatopancreas is properly eliminated (Yasumoto *et al.*, 1980; Takagi *et al.*, 1982).

Aside from strict quarantine of commercial harvesting and marketing of poisonous shellfish, a number of other precautionary measures are employed to control PSP. Signs are posted in shellfish growing areas and on beaches to warn the general public of the potential dangers in consuming toxic shellfish. The news media newspapers, television and radio are used extensively to warn picnickers and tourists as well as local residents of the hazards of PSP and NSP during the danger season. Such use of the news media to issue prompt warnings of PSP outbreaks is credited by McCollum *et al.* (1968) with preventing an even greater epidemic than was actually experienced in England in 1968. Similar means of warning the public were used in the Brunei shellfish poisoning incidence in 1976 (Beales, 1976). Also other measures were employed in the Brunei incidence, including general ban on seafood sales for several days, ban on harvest of chub mackerel and scad until the dinoflagellate blooms disappeared. Harvesting of molluscan shellfish was banned for nearly four months even after the toxic blooms disappeared. Measures similar to those mentioned above are applicable to other forms of shellfish poisonings.

Following the 1981 outbreak of paralytic shellfish poisoning at Vayalur on the east coast of India, the National Institute of Nutrition, Hyderabad, Andhra Pradesh made the following recommendations: that sewage should not be discharged untreated into the canal, but should be let out into the sea to prevent occurrence of dinoflagellate blooms in the canal due increased nutrients, that signs should be put up along the canal warning people against possibilities of shellfish in the canal being toxic, that the public health authorities should educate the populations in affected and neighbouring villages on the hazards of consuming toxic shellfish, that the public health authorities should educate the public in vulnerable areas to contact the Primary Health Centre in times of emergency and that a warning should be made through media like radio, television and newspapers when there are outbreaks of shellfish poisoning.

The eating of whole uncooked shellfish from endemic areas may be extremely dangerous. Cooking does not completely destroy PSP and NSP since they are rather heat stable. Nevertheless, cooking in any form is especially effective in reducing the toxicity of PSP, reduction being more than 70% (Medcof *et al.*, 1947). Because of their water solubility, much of the PSPs are concentrated in the nectors and liquors produced by cooking. The toxic liquors

or 'soups' of cooked shellfish were responsible for several deaths in New Guinea (Maclean, 1973). The villagers, also, cooked other foods in the 'soups' and became ill and some died without eating the shellfish meats. Moreover, Maclean reported that one woman in Talasea (New Britain) died within two hours after eating food cooked in water contaminated with red tide. All fluids produced by cooking should be discarded. Despite the high level of toxicity among mussels in the 1968 PSP outbreaks in England, no deaths resulted from more than 80 poisonings. The common practice of discarding the liquor from cooked mussels is credited by McCollum *et al.* (1968) with reducing the severity of the English epidemic. Moreover, the discarding of certain portions of the shellfish, such as siphons (butter clams), digestive glands and gills prior to cooking further reduces the hazards of paralytic shellfish poisonings.

We have found no data regarding the extent to which cooking and discarding of resultant liquors decreases the toxicity of NSP, DSP and VSP. Unless the toxins involved in these forms of shellfish poisonings are more heat labile than PSPs, cooking may not be as effective in reducing toxicity as is the case with PSP. The main toxins of NSP and DSP are lipid soluble and as such probably would not be concentrated in liquor produced by cooking. The toxins causing VSP have not been identified with certainty. Hashimoto (1979) has indicated that a suspected toxin is soluble in water, methanol, acetone and acetic acid, but insoluble in other organic solvents. Thus, it is possible that cooking may be effective in reducing the total toxicity of shellfish containing VSP.

Since most of the diarrhetic shellfish poisons are concentrated in the hepatopancreas, the removal of this organ is recommended by Japanese authorities to make scallops safe for human consumption (Yasumoto *et al.*, 1978). However, they pointed out that this procedure was impracticable for mussels since the hepatopancreas is difficult to remove without sacrificing much of the edible portion of mussels. Also the mussels accumulate a moderate amount of the toxins in the gills. Most, if not all of the toxins are accumulated in hepatopancreas of scallops. Therefore, Yasumoto *et al.* (1978) recommended a ban on the harvesting and marketing of mussels during the toxic season.

Commercial canning of shellfish is more effective than cooking in reducing PSP toxicity. Medcof *et al.* (1947) showed that precooking fairly toxic clams (*Mya arenaria*) with steam for 10 minutes reduces the PSP content by about 90%. Considerable effort has been made to develop processes to render highly toxic butter clams in southeastern Alaska safe for human consumption. Dassow

(1966) reports that trimming the siphons from shucked meats and steaming the meats for 10 minutes followed by retorting at 250°F for 75 minutes reduces the poison content of butter clams by as much as 93% without affecting the quality of the product. The Alaskan shellfish industry, however, has not used this process since it is considered unprofitable. The apparent ease with which PSP can be extracted from molluscan tissues prompted the U.S. Bureau of Commercial Fisheries to consider pretreating butter clams in a mild acid solution to reduce toxicity (Anonymous, 1976). No reduction in PSP was noted in butter clams (live and chopped meats) held in acid solutions of pH 5.0 - 6.0 for periods upto 17 hours.

Other means of inactivating shellfish poisons have been attempted. Chin (1970) tested 7 chemical disinfectants (β-propiolactone, Lysol, formaldehyde, iodine, peracetic acid, sodium hypochlorite and alkyl dimethyl benzyl ammonium chloride) for efficiency in neutralizing saxitoxin. Sodium hypochlorite (NaOCl) was the most effective; concentrations of 3 ppm NaOCl completely neutralized 1 μg of saxitoxin at room temperature (25°C) in 15 minutes. Likewise ozone treatment appears to be effective in reducing the toxicity of PSPs and NSPs (Thurberg, 1975) as well as detoxifying live surf clams (*Spisula solidissima*) in two weeks (Blogoslawski and Stewart, 1978).

The monitoring of vast areas of shellfish growing waters in British Columbia and southeastern Alaska presents an expensive and formidable task (Quayle, 1969). As pointed out by Schantz (1969) and others (Felsing, 1966), one of the most important problems facing the shellfish industry is the need for an economic commercial process that can reliably reduce the toxicity of butter clams from Alaska and British Columbia to safe levels. Numerous efforts to develop commercially feasible means of detoxifying raw butter clams, including transplanting live animals to nonendemic waters and storage in air, or ice or in a frozen state as well as irradiation, have been unsuccessful (McFarren *et al.*, 1960). Since outbreaks of PSP (Sommer *et al.*, 1937; Needler, 1949; Prakash, 1963; Wood, 1968; Quayle, 1969; Prakash *et al.*, 1971; Maclean, 1975; Worth *et al.*, 1975; Harada *et al.*, 1982) and NSP (Eldred *et al.*, 1964; McFarren *et al.*, 1965) are definitely associated with dinoflagellates, the systematic search for dinoflagellate blooms as indicated by discoloured water (red tides or red water) and plankton sampling for toxic dinoflagellates may be used to study where and when poisonous shellfish are likely to occur. Clark (1968) has suggested that biological features such as presence of sick and dying birds and sand eels, and luminescence at night which preceded

the 1968 English outbreak, might serve as useful warnings of impending PSP toxicity of shellfish. Blooms of dinoflagellates are often accompanied by spectacular displays of luminescence or phosphorescence at night. This feature is reported by Quayle (1969) to have been used by the Indians along the Pacific Coast of North America as a warning of shellfish toxicity. Similarly the phosphorescene of discoloured water at night signals the villagers of New Guinea to stop eating shellfish (Maclean, 1973).

Although direct fish kills and occasional mass mortality of marine invertebrates are well known for *Ptychodiscus brevis* red tides on the west coast of Florida, such events are rarely noted with red tide caused by PSP producing dinoflagellates belonging to the genus *Gonyaulax*. However, White (1977, 1980, 1981) has reported several kills of the Atlantic herring *(Clupea harengus harengus)* in the Bay of Fundy, Canada as a result of PSPs transmitted by zooplankton that had fed on *Gonyaulax excavata* (=*G. tamarensis*). Fish kill associated with a PSP producing dinoflagellate *(Pyrodinium bahamense* var. *compressa* occurred in Brunei (northwestern Borneo) in 1976. A scattering of several hundred small fish (namely *Lethrinus* sp. and *Stolephorus* sp.) was floating in the harbour and also stranded on the beaches during early stages of this incidence (Beales, 1976). In New Guinea and New Britain about half of the toxic *Pryrodinium* red tides cause mass mortality of marine organisms (Maclean, 1973). Various species of fish and air-breathing sea turtles and dolphins are victims of some of the red tide outbreaks.

The monitoring of shellfish growing waters for toxic dinoflagellates as well as assaying the dinoflagellates for toxicity are means for preventing shellfish poisonings. However, as pointed out by Hashimoto (1979) this method is unreliable unless it is carried out in conjunction with the mouse bioassay. The reasons put forward by Hashimoto include:

i. Sometimes as few as 200 *Gonyaulax* sp. per ml may result in toxic shellfish; such a low level of dinoflagellates may be overlooked.

ii. Shellfish may become toxic during short outbreaks of toxic dinoflagellates and retain toxin for a time after the bloom subsides.

iii. Toxic species of dinoflagellates are known to produce non-toxic strains.

Nevertheless, the presence of toxic or suspected toxic dinoflagellates should provide a signal to refrain from consuming shellfish unless their safety has been verified by mouse bioassay. The monitoring of the shellfish growing waters for the dinoflagellate *Dinophysis fortii* has been suggested as an aid for predicting diarrhetic shellfish poison (Yasumoto *et al.*, 1980). This dinoflagellate, which is large (average 70 μm) and peculiarly shaped is easy to recognise. These authors caution that population densities as low as 200 cells per litre may cause sufficient levels of DSP in shellfish to produce illness in humans. In examing sea water for low densities of toxic dinoflagellates, one should keep in mind that most, if not all dinoflagellates are positively phototrophic. This characteristic may be used to concentrate low populations of these phytoplankters. The best procedure is to keep the whole sea water sample in a vessel such as a volumetric flask that has a narrow neck. The sample should extend into the narrow portion of the vessel. Then the vessel containing the sea water sample should be placed directly under a flourescent lamp (about 0.3 m above sample) for two to four hours. After this period a sample should be taken from the meniscus and examined microscopically for dinoflagellates. Subsequent to microscopic examination the sample may be shaken to redistribute the phytoplankters for population density determination. Another procedure for monitoring dinoflagellate population is to make plankton tows with nets of proper size to retain dinoflagellates. This procedure permits the concentration of phytoplankters from greater volumes of water than is possible through the examination of whole samples of sea water.

The mouse bioassay, first developed by Sommer and Meyer (1937) and later modified by Medcof *et al.* (1947) is the standard method for monitoring for PSPs in shellfish. Details of the procedure for quantifying PSP by the mouse bioassay are available from a number of sources (Medcof *et al.*, 1947; McFarren, 1959; McFarren *et al.*, 1960; Halstead, 1965; Anonymous, 1970 b; Horwitz, 1970, 1975, 1980). Although the MU as originally defined by Sommer and Meyer (1937), is the average minimum amount of toxin that will kill a 20 g white mouse in 15 minutes, more consistent results are obtained when the death time is between 4 and 8 minutes (Schantz, 1973). According to Schantz, there is a direct relationship between dose and time from injection to death, death times of 3, 4, 5, 6, 7, 8 and 15 minutes are equivalent to 3.7, 2.5, 1.9, 1.6, 1.3 and 1.0 MU respectively. The MU is a measure that may vary with the species and condition of the mice and various other factors.

With purification of clam and mussel toxins (Mold *et al.*, 1957; Schantz *et al.*, 1957, 1958) recommended the use of purified

clam toxin as a standard in mouse bioassays. The purified clam toxin is known as saxitoxin (STX) since it is extracted from the siphons of butter clams (*S. giganteus*). Thus, the amount of toxin may be reported by weight (micrograms) rather than mouse units. According to McFarren *et al.* (1960), the average conversion factor (CF) obtained by several laboratories gave an average of 0.191 μg of poison per mouse unit. Using this average CF, the previously accepted tolerance level of 400 MU/100 g was converted to 80 μg/100 g (Jensen, 1959). Despite the recommendation that toxicity be reported by weight, most workers continue to report in mouse units. The use of pure STX for standardising the mouse bioassay is probably most useful along the Pacific Coast of North America since the major PSP toxin accumulated by shellfish in the general area appears to be STX. Steidinger (1983) suggests that there is a weakness in the mouse bioassay for PSP since it is standardised with STX. It is known that STX may not be the major toxin fraction in shellfish on the east coast of North America and European Coasts and she notes that STX may not be the predominant orally toxic fraction.

Paralytic shellfish poison standard solution (100 μg/ml) as an acidified 20% alcoholic solution (standard is stable indefinitely when maintained in cool place) is available from Division of Microbiology, Food and Drug Administration, 1090 Tusculum Ave; Cincinnati, OH 45226.

The procedure for performing the mouse bioassay for paralytic shellfish poisons with slight modifications is from Anonymous (1970 b).

7.1 Method of Mouse Bioassay

7.1.1 Materials

PSP reference solution (1 μg/ml): Dilute 1 ml standard solution to 100 ml with distilled water. Final pH should be between 2.0 and 4.0. Reference solution is stable for several weeks at 3–4° C.

Healthy white mice, 19–21 g in weight from a stock colony are used for routine assays. If the weight of mice is < 19 or >21g, apply correction factor to obtain true death time (Table 2). Do not use mice weighing >23 g; do not reuse mice.

7.1.2 Standardisation of bioassay

Dilute 10 ml aliquots of 1 μg/ml PSP reference with 10, 15, 20, 25 and 30 ml distilled water respectively, until an intraperitoneal

injection of 1ml dose into a few mice causes a median death time of 5 – 7 minutes; pH of dilutions should be 2 – 4 but not >4.5. Test additional dilutions in 1 ml increments of distilled water, *e.g.* if 10 ml diluted with 25 ml distilled water kills mice in 5 – 7 minutes, test dilutions diluted 10 + 24 ml and 10 + 26 ml. The pH of these dilutions must be between 2.0 and 4.0.

TABLE 2. *Death time: mouse unit relations for paralytic shellfish poison (acid)*

Death* time	Mouse units	Death* time	Mouse units	Death* time	Mouse units	Death* time	Mouse units
1:00	100.0	3:00	3.70	5:00	1.92	10.00	1.11
10	66.2	05	3.57	05	1.89	30	1.09
15	38.3	10	3.43	10	1.86	11.00	1.075
20	26.4	15	3.31	15	1.83	30	1.06
25	20.7	20	3.19	20	1.80	12.00	1.05
30	16.5	25	3.08	30	1.74	13	1.03
35	13.9	30	2.98	40	1.69	14	1.015
40	11.9	35	2.89	45	1.67	15	1.00
45	10.4	40	2.71	50	1.64	16	0.99
50	9.33	45	2.73	6:00	1.60	17	0.98
55	8.42	50	2.66	15	1.54	18	0.972
2:00	7.67	55	2.50	30	1.48	19	0.965
05	7.04	4:00	2.54	45	1.43	20	0.96
10	6.52	05	2.48	7:00	1.39	21	0.954
15	6.06	10	2.32	15	1.35	22	0.948
20	5.66	15	2.36	30	1.31	23	0.942
25	5.32	20	2.21	45	1.28	24	0.937
30	5.00	25	2.26	8:00	1.25	25	0.934
35	4.73	30	2.12	15	1.22	30	0.917
40	4.48	35	2.18	30	1.20	40	0.898
45	4.26	40	2.04	45	1.18	60	0.875
50	4.06	45	2.00	9:00	1.16		
55	3.88	50	2.00	30	1.13		
		55	1.96				

* Minutes and seconds.
From Sommer and Meyer (1937).

Inject a group of 10 mice with each of 2 or preferably 3 dilutions that fall within median death time of 5–7 minutes. Inject a 1 ml dose intraperitoneally into each mouse and determine death time as elapsed between injection and the last gasping breath of the mouse.

Repeat assay one or two days later using dilutions prepared above which differ by 1 ml increments of distilled water. Then repeat entire test starting with testing dilutions prepared from a freshly prepared PSP reference solution.

Calculate median death time for each group of 10 mice used for each dilution. If all groups of 10 mice injected with any one

dilution give median death times of <5 or >7 minutes, disregard results from this dilution in subsequent calculations. On the other hand, if any of the groups of 10 mice injected with one dilution gave a median death time falling between 5 and 7 minutes, include all groups of 10 mice used for that dilution, even though some of the median death times may be <5 or >7 minutes. From median death time for each group of 10 mice in each of the selected dilutions, determine the number of mouse units per ml from Table 2. Divide the calculated µg poison per 1 ml by the MU/ml to obtain conversion factor (CF value) expressing µg poison equivalent to 1 MU. Calculate the average of the individual CF values and use this average as a reference point to check routine assays. Individual CF values may vary significantly within a single laboratory if the techniques and mice are not rigidly controlled. This situation will require continued use of reference standard or secondary standard, depending on the volume of assay work performed.

7.1.3 Use of standard with routine assay of shellfish

Check CF value periodically. If shellfish products are assayed less than once a week, determine CF value on each day assays are performed by injecting 5 mice with an appropriate dilution of the reference standard. If assays are made on several days during each week, only one need be made per week on a dilution of the standard such that median death time falls within 5–7 minutes. The CF value thus determined should check with the average CF value within $\pm 20\%$. If it does not check within this range, complete group of 10mice by adding 5 mice to the 5 mice already injected, and inject a second group of 10 mice with the same dilution of standard. Average the CF value determined by the second group with that of the first group. Take the resulting value as the new CF value. A variation of $>20\%$ represents a significant change in the response of mice to poison or in the assay technique. Changes of this type require a change in the CF value.

Repeated checks of CF value ordinarily produce consistent results within $\pm$ 20%. If wider variations are found frequently, the possibility of uncontrolled or unrecognised variables in the method should be investigated before proceeding with routine assays.

7.1.4 Preparation of sample

Thoroughly clean outside of shellfish with fresh water. Open and rinse inside with fresh water to remove sand or other foreign

material. Remove meat from shell by severing adductor muscle(s) taking care not to cut or damage the mollusc's body. Do not use heat or anaesthetics in opening shellfish. Collect about 150 g of meats in a glazed dish. Immediately transfer meat to a No. 10 sieve in a single layer and let drain for 5 minutes. Pick out pieces of shell and discard drainings. Grind meat in a blender until homogeneous.

For shellfish such as scallops separate edible portion (adductor muscle) and test this portion alone. Drain and grind as indicated above for other shellfish.

For canned shellfish place entire contents of can (meat and liquid) in a blender and blend until homogeneous. For large cans drain in a large sieve and collect all liquid. Determine weight of meat and volume of liquid. Recombine portions of each in proportionate quantities. Blend recombined portions in blender until homogeneous.

7.1.5 Extractions

Weigh 100 g of well-mixed material into a tared beaker. Add 100 ml 0.1 N HCl, stir thoroughly and check pH (should be less than 4.0, preferably about 3.0). If necessary, adjust pH as indicated below. Heat the mixture, boil gently for 5 minutes and let cool to room temperature. Adjust cooled mixture to pH 2.0 – 4.0 (never >4.5). To lower pH, add 5 N HCl dropwise while stirring. To raise pH, add 0.1 N NaOH dropwise with constant stirring to prevent local alkalinisation and consequent destruction of poison. Transfer mixture to graduated cylinder and dilute to 200 ml. (note: some laboratories recommend using varying normalities of HCl, depending on species of shellfish being extracted, *e.g.* extracts of soft-shell clam (*Mya arenaria*) prepared with 0.18 N HCl seldom require pH adjustment).

Return mixture to beaker, stir to homogeneity and let settle until portion of supernatant is clear and can be decanted free of solid particles large enough to block a 26-gauge hypodermic needle. If necessary, centrifuge mixture or supernatant for 5 minutes at 3000 RPM or filter through filter paper. Only enough liquid to perform bioassay is necessary.

7.1.6 Mouse test

Inject each test mouse intraperitoneally with 1 ml of the acid extract. Note time of injection and observe mice carefully for

death time as indicated by last gasping breath. Record death time with stop watch or clock with sweep second hand. One mouse may be used for initial determination, but 2 or 3 are preferred. If the death time or the median death time of several mice is <5 minutes, make a dilution to obtain death times of 5–7 minutes. If the death time of 1 or 2 mice injected with undiluted sample is >7 minutes, a total of 3 or more mice for each dilution must be injected toes tablish the toxicity of the sample. If large dilutions are necessary, adjust the pH of the solution by dropwise addition of dilute HCl (0.1N) to 2.0–4.0, never >4.5. Inoculate 3 mice with a dilution that gives death times of 5–7 minutes.

7.1.7 Calculation of toxicity

Determine median death times of mice, including survivors and from Table 2 determine corresponding number of MU. If test animals weigh <19 g or >21 g, make correction for each mouse by multiplying MU corresponding to death time for that mouse by the weight correction factor (WCF) for that mouse (from Table 3) and determine the median MU for the group. Consider the death time of survivors as >60 minutes or equivalent to <0.875 MU in calculating the median MU. Convert MU to μg poison per ml by multiplying by the CF value.

TABLE 3. *Correction Table for weight of mice*

Weight of mice (g)	Mouse units	Weight of mice (g)	Mouse units
10	0.50	17	0.88
10.5	0.53	17.5	0.905
11	0.56	18	0.93
11.5	0.59	18.5	0.95
12	0.62	19	0.97
12.5	0.65	19.5	0.985
13	0.675	20	1.000
13.5	0.70	20.5	1.015
14	0.73	21	1.03
14.5	0.76	21.5	1.04
15	0.785	22	1.05
16	0.84	22.5	1.06
16.5	0.86	23	1.07

From Sommer and Meyer (1937).

7.1.8 Quantity of poison

μg poison/100 g meat =(μg/ml x dilution factor) x 200. Consider any value >80 μg or >400 MU/100 g of shellfish meat as hazardous and unsafe for human consumption.

The standard mouse bioassay for detecting shellfish toxins produced by the dinoflagellate *Ptychodiscus brevis* (=*Gymnodinium breve*) was developed by McFarren *et al.* (1965). Detailed procedures for NSP in shellfish may be obtained by consulting McFarren *et al.* (1965), Cummins and Hill (1969) and Anonymous (1970 b). This bioassay is based on the relationship of dose to death time of mice injected intraperitoneally with crude toxic residues extracted from shellfish with diethyl ether. One mouse unit (MU) is the amount of crude toxic residue that, on the average will kill 50% of the test animals (20 g mice) in 930 minutes. The procedures to follow are from Anonymous (1970) with slight modifications.

7.1.9 Reagents

Hydrochloric acid, conc. (12 N); sodium chloride (reagent grade); cottonseed oil *e.g.* Wesson oil and diethyl ether (purified for fat extraction).

7.1.10 Test animals

Healthy mice weighing 20 g ± 1 g from stock colony used for routine assay. Mice of different weights can be used, but do not use mice weighing < 10 g or >25 g. Do not reuse mice.

7.1.11 Preparation of sample

Thoroughly clean outside of shellfish and then shuck; drain shell liquor and homogenise shellfish meats in a blender.

7.1.12 Extraction

Place 100 g of shellfish homogenate into a tared 400 ml beaker. Add 1 ml conc. HCl and 5 g of NaCl to the homogenate. Heat to boiling with frequent stirring and cook for 5 minutes, then cool.

Transfer the cooled homogenate to a 250 ml centrifuge vessel; add 100 ml of diethyl ether, stopper and shake vigorously for 5 minutes (open frequently). Centrifuge for 10 to 15 minutes at 2000 RPM. Transfer the ether extract to a 1000 ml separatory funnel. Extract the shellfish homogenate 3 more times with 100 ml of diethyl ether, transfer each ether extract to the 1000 ml separatory funnel. Drain off any emulsion from the combined ether extracts. Transfer the ether extract to a 400 ml beaker.

Evaporate most of the ether on a steam bath under a chemical hood. Quantitatively transfer the remaining extract to a tared

100 ml beaker. Evaporate ether; be sure that no ether remains after the last evaporation step.

7.1.13 Mouse assay

Accurately weigh the remaining viscous oil residue. On the assumption that the density of the oil residue is the same as cottonseed oil, *e.g.* Wesson oil (0.917 g $\simeq$ 1 ml), gravimetrically bring the residue upto 9.17 g (10 ml). Since the resulting solution of the toxin in cottonseed can be readily handled, any subsequent dilution can be made volumetrically. The toxin solution must be well mixed before injection into the mice. Some investigators have substituted Tween 80 (polysorbate 80) for cottonseed oil (Spikes *et al.*, 1968). In this modification of the technique the crude NSP extract is mixed with 0.5% Tween 80 in isotonic saline and 0.5 to 1.0 ml of toxin solution is injected intraperitoneally into mice.

Inject 1 ml of the residue-cottonseed oil mixture intraperitoneally into each of two mice (about 20 g weight). Record the time of injection and observe the mice for 24 hours. The death time is the elapsed time between injection and the last gasping breath of the mouse. Record the death time of each mouse surviving a given period of continuous observation as greater than the duration of that *continuous* observation period (in minutes). For example, assign a death time of $>$ 930 minutes to those mice that survive a 930 minutes (15.5 hours) continuous observation period, but die (unobserved) sometime thereafter during the required 24 hours observation period.

We suggest that one or two mice be injected with 1 ml of cotton-seed oil or Tween 80 as controls to determine the effects of injection technique.

If the first two mice do not die within 360 minutes (6 hours), inject three more mice and observe all five mice for 24 hours. If the first two mice die in less than 110 minutes, a dilution of the toxin solution is made with cottonseed oil and each of two mice injected with one ml of the diluted toxin. Recommended dilution 1:1.25, *e.g.* add 2 ml of cottonseed oil to the remaining 8 ml of residue cottonseed oil mixture. If required, repeat the identical dilution-injection sequence. When a dilution is found that causes the mice to die between 110 and 360 minutes after injection, three more mice are each injected as previously described and observed for 24 hours. The duration of the continuous observation period will determine the lower limits of the assay's sensitivity. For example, 10 MU/100 g shellfish meat is the lower amount of toxin that can usually

be quantified with 20 g mice. This level of sensitivity can be achieved only by continuously observing the mice for 930 minutes (15.5 hours). In case the mice are not observed continuously for longer than 360 minutes, 20 MU/100 g shellfish meat will be the lower limit of sensitivity with 20 g mice.

7.1.14 Calculation of toxicity

Determine the number of MU/ml corresponding to the death time of each of the five mice injected (Table 3). Assign indeterminate less than (<) to those MU corresponding to indeterminate death times.

TABLE 4. *Relationship of dose to death time and weight of mice injected with* Gymnodinium breve *toxins(s) extracted from shellfish*

Death time in minutes (20 g mice)	Mouse units per 1 ml	Mouse weight correction	
		Weight of mice (g)	Correction factor
8	10.0	10	0.39
10	9.0	11	0.45
12	8.0	12	0.51
14	7.0	13	0.57
16	6.0	14	0.63
18	5.0	15	0.69
20	4.5	16	0.75
30	4.0	17	0.81
38	3.8	18	0.87
45	3.6	19	0.94
60	3.4	20	1.00
83	3.2	21	1.06
105	3.0	22	1.12
140	2.8	23	1.18
180	2.6	24	1.24
234	2.4	25	1.30
300	2.2	26	1.36
360	2.0	27	1.39
435	1.8	28	1.41
540	1.6	29	1.42
645	1.4	30	1.43
780	1.2		
930	1.0		

From McFarren *et al.* (1965).

Adjust the MU for differences in weight of each mouse in the following manner: multiply the number of MU per ml by the weight correction factor (WCF) for that mouse (Table 4) *e.g.* the number of MU per ml corresponding yo a > 930 min. death time of a 22 g mouse (1.0 MU/ml) x 1.12 WCF for 22 g mouse) = 1.12 MU per ml.

Determine the mean number of MU per ml if 100% mortality is observed and the death times are determinate. Determine the median number of MU per ml if 100% mortality is observed and the death times are indeterminate. Determine the median number of MU per ml if *less than* 100% mortality is observed (include survivors).

Calculate the number of MU/100 g shellfish meat in the following manner: multiply the *mean* or *median* number of MU per ml injected by the dilution factor (if applicable) and by 10 (residue orginally made up to 10 ml with cottonseed oil), *i.e.* MU/100 g shellfish meat = (MU/ml) x (dilution factor) x 10.

7.1.15 Reporting procedure

When relative toxicity is determinate, report number of MU/100 g shellfish meat. When relative toxicity is indeterminate *(e.g.* 10 MU/100 g shellfish meat, report: (i) number of mice which died in 24 hours; toxin detectable < 100 MU/100 g shellfish meat; or (ii) no mice died in 24 hours; toxin not detectable <10 MU/100 g shellfish meat. Consider any detectable level of toxin/100 g shellfish meat potentially unsafe for human consumption.

In case mice are unavailable for assaying for NSP, we suggest that one or two day old chicks be injected intraperitoneally with shellfish extract as recommended for the mouse bioassay. Although chick have not been standardised with regard to units of toxicity an indication of shellfish toxicity may be obtained using chicks as the bioassay organism. Chicks may be used in the forced feeding of raw shellfish homogenate (1 g/chick to detect the presence of NSP or any other shellfish poisons to which chicks are sensitive. Ray and Aldrich (1964) demonstrated that chicks receiving 1 g of a fresh homogenate of oysters that had fed on cultures of *Ptychodiscus brevis* died within 24 hours. Since the adductor muscle of bivalves does not appear to accumulate significant amounts of shellfish toxins, we recommend that adductor muscle(s) be removed from shellfish to be assayed by force feeding fresh homogenates thereof to chicks.

Yasumoto *et al.* (1978) developed a fairly simple mouse bioassay for the routine analysis for diarrhetic poisons in shellfish. Their procedures with only slight changes are presented as follows:

1. Clean outside of shellfish with fresh water. Carefully flush and remove shellfish meat. Remove hepatopancreas from

shellfish; obtain about 10 g of hepatopancreas for each shellfish sample.

2. Extract hepatopancreas sample in a blender with three 50 ml portions of acetone. Extract for 2 minutes at room temperature and combine the three extractions.

3. Free the acetone from combined extracts under reduced pressure.

4. Suspend residue in 1% Tween 60 (polysorbate 60) solution to make up 2 ml of original shellfish test solution.

5. Inject aliquots of 0.5 to 1.0 ml of this solution or dilutions thereof, if required, intraperitoneally into female white mice (weight 15 – 20 g).

6. Observe mice for 48 hours. The minimum of toxins required to kill a mouse within 48 hours is designated as one mouse unit (MU).

7. Toxicity of shellfish is expressed in terms of MU/g of hepatopancreas.

8. Toxicity of shellfish in excess of 0.5 MU/g of hepatopancreas has been established by Japanese health officials us unsafe for human consumption unless the hepatopancreas is properly removed. The harvesting and marketing of shellfish is legally forbidden when the established safe limit of 0.5 MU/g is exceeded.

Following intraperitoneal injection of toxic extracts, mice become inactive and generally weakened. Depending on the dose, symptoms may appear as early as 30 minutes or be delayed for several hours; death occurs between 100 minutes and 47 hours after injection of toxic extract. The lethal dose for mice forced fed with toxin (in cottonseed oil) by stomach tube is 16 times greater than by intraperitoneal injection.

The effects of DSP on test animals other than mice were investigated by Yasumoto *et al.* (1978). White leghorn chick (male) weighing 100 g required 13 MUs to produce death when injected intraperitoneally. Chicks forced fed mussel hepatopancreas containing 10 MU of DSP showed no ill effects. Cats that were voluntarily fed steamed and homogenised hepatopancreas of toxic mussel

and scallops containing about 33 MU/g exhibited vomiting, but no diarrhoea during the 48 hour observation period.

According to Hashimoto (1979), the toxic factor(s) for venerupin shellfish poison remain(s) unknown. It does not appear that a standard bioassay technique for detecting VSP is available at present. However, Hashimoto refers to studies by Akiba (1943) in which toxicity was shown in mice injected with 75% methanolic extract of shellfish from Lake Hamana. Schantz (1973) briefly mentions that a hepatotoxic shellfish poison (VSP) from oysters in Japan can be detected by feeding or injecting water extracts of oysters into mice and then observing them for pathological changes in the liver and other organs. In order to detect small quantities of toxin the mice must be observed for several days.

Okaichi and Imatomi (1979) isolated two toxic fractions from 75% methanol extracts of cultured *Prorocentrum minimum* var. *mariae-lebouriae*, which is the suspected cause of VSP. One of the fractions was positive to the Dagendorff reagent and the other was negative to it.

8

MISCELLANEOUS SHELLFISH POISONINGS

Two other forms of shellfish poisonings, Japanese callista and giant clam poisonings have occurred rarely and only in certain localities. Accounts regarding these two forms of shellfish poisonings have been given by Hashimoto (1979).

According to Hashimoto, many people have been poisoned after eating the Japanese callista *Callista brevisiphonata*, in the vicinity of Mori-machi, Hokkaido Island. This bivalve mollusc, locally known as *oasari*, is a common Hokkaido species that was not eaten for a long time because it was considered to be poisonous. Twenty six individuals developed callistin poisoning following the consumption of this bivalve in Mori-machi between mid-June and mid-July in 1950. Following this incidence, the Mori Health Center prohibited the harvesting and marketing of Japanese callista. Despite this ban, four individuals were poisoned after eating these clams in May, 1952.

The principal symptoms are urticaria, asthma and gastrointestinal disorders. About fifty per cent of individuals involved in the 1950 incidence showed all the three above mentioned symptoms and about one fourth of the victims exhibited gastrointestinal disorders alone. Although some patients complained of hoarseness for four days, most of them recovered in one or two days. No death has been reported from callistin poisoning.

In *Callista brevisiphonata* the ovaries are thought to be the site of toxin concentration. In Hokkaido the gonads of this clam mature in late August and spawning occurs by the end of September. Fortunately mature ovaries have a white, granular appearance that permits easy differentiation from the 'milky' testes.

Little is known of the toxic substance found in *C. brevisiphonata* and its mode of action. A large amount of choline, but no histamine has been detected in the ovaries of this shellfish. The symptoms suggest that the toxin is allergenic and cholinergic. Calcium and antihistamines have been effectively used in treating callistin poisoning. Test animals such as mice, rabbits, cats and dogs appear to be insensitive to callistin poison. It appears that only humans are sensitive to this shellfish poison. Three human volunteers who ate ovaries of *C. brevisiphonata* developed urticaria whereas those who ate testes and digestive glands showed no symptoms.

Hashimoto (1979) states that investigators had reported the presence of several compounds in the ovary of *C. brevisiphonata.* These compounds included various betaines, such as glycine betaine, homoserine betaine, valine betaine, γ-butyrobetaine, δ-valerobetaine and stachydrine and the choline content was low.

Hashimoto (1979) reported that humans and domestic animals on Bora Bora Island in French Polynesia were poisoned after eating the giant clam *Tridacna maxima* during the months of April to July, 1964. Thirty three individuals were poisoned and two of them died. Eleven of the severely poisoned persons required several weeks of hospitalisation. Also many domestic animals were found dead during this incidence. Symptoms of poisonings appeared within fifteen to thirty minutes after eating the shellfish. Initially the victims developed irritation of the nostrils, pain in nose and lips, hyperemia of the face, lacrimation and perspiration. These symptoms were followed by gastro-intestinal disorders such as stomach ache, diarrhoea, nausea and vomiting. Within six to twenty four hours after eating the toxic shellfish, a variety of neurological symptoms developed in the victims. Both water and fat soluble toxins have been isolated from toxic giant clams but nothing is known of their properties.

Furthermore, Hashimoto (1979) reported on giant clam (*Tridacna* sp.) poisoning that occurred at Ie in Kunigami, Okinawa. The local residents believed that toxicity of the clams was due to toxic algae that grew in a particular area. Hashimoto and his coworkers found that the 'toxic seaweeds' were actually a sea anemone that had adhered to the mantle of the clam. When the clam was eaten, the undischarged nematocysts of the anemones caused itching and swelling in the throat.

9

GENERAL CONSIDERATION OF SHELLFISH SANITATION: TECHNIQUES FOR MONITORING QUALITY AND PURIFICATION OF SHELLFISH

9.1 Shellfish Sanitation

In addition to contamination with marine toxins, shellfish may also become unsafe for human consumption through contamination with enteric pathogens. The latter form of contamination is probably a more serious public health and economic problem than the former. Sewage pollution of shellfish growing waters is a world wide problem which is continuously exacerbated by ever increasing human populations. The lack of adequate sewage treatment as well as shellfish processing, storage and marketing facilities in many subtropical and tropical areas appear to offer major impediments to expanding bivalve molluscan production for domestic consumption and foreign export. Although shellfish contaminated with enteric pathogens may be rendered safe for human consumption by adequate cooking, generally higher payments are got for shellfish that are safe and palatable for raw consumption. Thus, in order to meet safety standards internally and abroad, fisheries and public health officials should institute measures to ensure adequate sanitary quality of shellfish. Such action is paramount to the development of viable and profitable shellfish industry.

Velankar (1955) conducted a survey for marine bacteria about 3 km from shore in the Palk Bay and Gulf of Mannar near Mandapam in 1950–51 and determined the incidence of coliforms by the Most Probable Number technique. Coliforms were found in fairly large numbers (50 to 100/100 ml of sea water) at distances upto about 360 m from shore, but they were generally absent in

sea water samples taken about 3 km from the shore. Coliforms were not noticed in any of the deep water samples. When coliforms were found in surface water samples, the total bacterial count of the water was high, which indicated chance pollution probably of a local nature.

With regard to sanitary quality of shellfish growing waters in India, we have found that published reports are very few. Venkataraman and Sreenivasan (1955) have dealt with mussel (*Perna viridis*) pollution in Korapuzha Estuary, near Calicut. The study showed the presence of *Escherichia coli* type I in the estuary throughout the year which is an indicator organism of human faecal contamination. The peak of pollution immediately follows the beginning of the southwest monsoon and until summer the coliform numbers as well as total bacterial counts were low. *Salmonella-Shigella* group and cholera vibrios were found to be absent These findings indicate faecal contamination of Korapuzha Estuary.

Earlier people of Calicut Coast believed that green mussels (*Perna viridis*) of the area were poisonous during the monsoon months (Jones and Algarswami, 1973) and ascribed it to factors like turbidity, presence of sand and mud particles in the mantle cavity, lowered salinity and occurrence of the pea crab *Pinnotheres* sp.

Stephen *et al.* (1975) have reported the presence of enteropathic *Escherichia coli* from the freshwater mussel *Lamellidens marginalis* from southwest coast of India. Enteripathic *E. Coli* has been associated with gastro-enteritis in children. Seven out of twenty specimens of *L. marginalis* studied were positive for enteropathis *E. coli* of serotypes 0111 and 0127, which are identical with some of those isolated from acute cases of gastro-enteritis in children.

Bacteriological and toxicological analysis carried out at the Inspection Laboratory of the Marine Products Export Development Authority, Cochin indicated that the meat of oysters from the oyster farm of C.M.F.R. Institute at Tuticorin did not contain *E. coli*, *Staphylococcus* and *Salmonella*. The contents of heavy metals (mercury, copper and cadmium) were also found to be below permissible limits (Silas *et al.*, 1982).

The occurrence of the enteric pathogen *Vibrio parahaemolyticus* in India was reported by Bose and Chandrasekharan (1976) from the slime and guts of prawns and sea water off the southeast coast of India at Nagapattinam, Tamil Nadu. Although various strains of *V. parahaemolyticus* have been isolated from several areas

in the world, it appears to be of greatest public health significance in Japan. Acute gastro-enteritis associated with the Japanese custom of eating raw fish and shellfish is caused by *V. parahaemolyticus* (Le Clair *et al.*, 1970). These authors reported that the organism has been isolated from fish, shellfish, sea water, sea sediments and from the faeces and vomitus of victims as well as from the intestinal contents of fatal victims.

The occurrence of marine vibrios including *Vibrio parahaemolyticus* in Vellar Estuary at Porto Novo on the southeast coast of India have been reported by Nair *et al.* (1980). During the six months period February-July 1978, 27 strains of the enteropathogen were isolated from water and sediment samples. Moreover, 9 strains with intermediate properties between *V. parahaemolyticus* and *V. alginolyticus* were isolated from water samples.

Pillai (1980) observed that bacterial concentration of the cultured brown mussel *Perna indica* at Vizhinjam was higher than that of mussels in natural beds and the levels of coliforms *E. coli*, faecal streptococci and coagulase-positive staphylococci were steady in the mussel meat and sea water samples analysed. The bacteria *Pseudomonas*, *Vibrio* and *Micrococcus* were considered to be normal flora of mussels and coastal waters in the area.

Another published report dealing with enteric pathogens in shellfish in India is that of Karunasagar (1982) on the survival of *Vibrio parahaemolyticus* in an estuarine clam. Karunasagar (1982) studied the survival of *V. parahaemolyticus* in association with clams maintained in a closed system of estuarine mud and water. Six strains, three Kanagawa positive and three Kanagawa negative were employed in this study. It was found that *V. parahaemolyticus* survived in association with the clams for two months. Since the clams died before the third month's sample could be taken the survival time represents only a minimum time. There was no significant difference in the survival time of the six strains. Also there was no indication that the Kanagawa reactivity was altered by association with clams.

In India there are frequent large out-breaks of gastro-enteritis of cholera in various States which may be due to contamination of drinking water or food by vibrios. The incidences in the maritime States may be due in part to the consumption of sea foods *viz.* fish or shellfish contaminated by *Vibrio parahaemolyticus*. Outbreaks of gastro-enteritis or cholera occur especially when there are very high catches of fish like oilsardine and shad which may be contaminated by vibrios and are consumed.

The Central Marine Fisheries Research Institute has constructed molluscan shellfish depuration tanks at Tuticorin (Nayar *et al.*, 1980), where bivalve shellfish are purified by cleaning the exterior of the shellfish and keeping them in flowing filtered sea water for 24 hours. Balachandran and Nair (1975) have reported that mussels kept in sea water for 24 hours and then in chlorinated water (5 ppm) for 2 hours had reduced sand content and no faecal or pathogenic bacteria.

In this manual we shall outline the general procedures employed in various areas of the world to provide raw shellfish that are safe for human consumption. We shall not deal with the details of such procedures since these are readily available from several sources.

The best way for producing shellfish of satisfactory sanitary quality is to grow them in waters that receive little or no untreated human waste material. However, large numbers of shellfish grow naturally in waters polluted with sewage effluents. In U.S.A. the oysters are relaid on privately owned growing grounds in sanitarily approved waters for a minimum of 2–3 weeks to allow the shellfish to free themselves of human enteric pathogens. This procedure varies in slight detail from State to State, but is always carried out under the surveillance of State public health and fisheries agencies. Unless the oyster farmer is receiving a very good price for shellfish, relaying of oysters is generally unprofitable for him. This technique is practised on a small scale in Galveston Bay, Texas. During the two months May and August of the closed season May to October, the oyster farmers are allowed to remove a prescribed quantity of oysters from polluted public reefs and relay them on their oyster beds. If the oysters come from areas with high heavy metal pollution, they must remain in unpolluted water for a minimum of 90 days instead of the usual minimum 2 weeks.

In addition to relaying contaminated shellfish in clean waters shellfish may be purified (depurated) by allowing them to feed on filtered, clean or purified sea water in tanks for 36 to 48 hours. Several techniques have been used for purifying sea water for use in depuration systems. These techniques include chlorination, U.V. treatment and ozonation of sea water. The general procedure for depuration of shellfish will be presented later in this Chapter.

Standard procedures have been developed in U.S.A. and other countries for the bacterial examination of sea water and shellfish. Although these procedures are similar in all countries for which such standards have been established, we shall present those

employed in U.S.A. Details of the procedures for examination of sea water and shellfish are presented by Anonymous (1970 a). Also, standard procedures for microbiological examination of water are available (Anonymous, 1976). These procedures provide a count of indicator organisms of faecal pollution such as *Escherichia coli* rather than enteric pathogens *per se*.

9.2 Examination of Sea Water

9.2.1 Collection and transportation of sample

Sea water samples for bacteriological examination should be collected in clean sterile bottles. During sampling stopper and neck should be protected from contamination. To facilitate mixing do not fill bottle completely. When possible bacteriological analysis should be initiated promptly after collection, preferably within 1 hour after collection. In case there is going to be delay in initiating bacteriological analysis the samples should be stored at 10°C or less until they are examined. Samples should not be tested after keeping them for more than 30 hours.

9.2.2 Tests for members of coliform group

The *Coliform* group of organisms as determined by the multi-tube test includes all aerobic and fecultative anaerobic gram-negative nonspore-forming rods that ferment lactose with gas formation within 48 hours at 35° C.

A. Presumptive test

Lactose broth or lauryl sulphate lactose broth in fermentation tubes, shall be used in the Presumptive test medium. Inoculate each of 5 tubes of double strength presumptive test broth with 10 ml of sample. Inoculate each of 5 tubes of single strength presumptive test broth with 1 ml and 0.1 ml of sample respectively. In order to avoid indeterminate results extension of dilutions suggested above may be required. It is usually necessary to inoculate at least 4 decimal dilution.

Incubate the fermentation tubes at 35°C ± 0.5°C. Examine each tube at the end of 24 ± 2 hour and if no gas is formed, return tubes to incubator and examine again at the end of 48 ± 3 hour. Record presence or absence of gas in the fermentation tube at each examination of tubes. Absence of gas formation at the end of 48 hr of incubation constitutes a negative test for coliform group organisms.

B. Confirmed test

All presumptive test fermentation tubes showing any gas at the end of 24 or 48 hr of incubation should be subjected to Confirmation test. If there is active fermentation prior to expiration of 24 hour incubation period, it is advisable to transfer cultures without waiting for completion of full incubation period. Conditions under which all positive presumptive tubes need not be confirmed are presented in the detailed procedures.

Gently mix the positive Presumptive test fermentation tube and transfer one loopful of the medium to a fermentation tube containing brilliant green lactose bile broth (BGL BB). Incubate the inoculated tubes for 48±3 hr at 35°C±0.5° C. The formation and presence of gas in the fermentation vial within 48 ± 3 hour constitutes a Confirmed test for coliform group organisms.

C. Completed test for coliform organisms

Streak one or more plates of endo or Levine's eosin methylene blue agar from each primary fermentation tube showing gas formation or from each BGL BB fermentation tube showing gas formation. Incubate the streak plates in an inverted position for24±2 hour at 35° C±0.5° C. Three types of colonies will develop on the endo or Levin's eosin methylene blue agar on incubation within 24 hours:

i. Typical coliform: nucleated with or without metallic sheen.
ii. Atypical coliform: opaque unnucleated and pink.
iii. Noncoliform: all others.

Pick one or more typical coliform colonies from each plate. If no typical coliform colonies are present, pick two or more atypical colonies considered most likely to be coliform organisms from each plate. If possible choose well-isolated colonies.

Inoculate separate lactose broth tubes and nutrient agar slants. Incubate agar slants and lactose broth tubes at 35°C ± 0.5°C for 24 ± 2 hour. Record gas formation and if no gas is formed, incubate for another 24 ± 2 hr. Examine microscopically gram-stained preparations from the agar slants corresponding to gas positive lactose broth tubes and record gas production.

The formation of gas in lactose broth tubes and demonstration of gram-negative nonspore-forming rods in agar slant constitute a

Positive Completed test for coliform organisms. On the other hand, absence of gas formation in lactose broth or failure to demonstrate gram-negative nonspore-forming rods in the agar culture constitutes a Negative Completed test.

9.3 Computing and Recording Result

Results of tests for coliform organisms or for various members of the coliform group of organisms by the multi-tube dilution method are to be reported in terms of the 'Most Probable Number' (MPN) index. One must realise that the MPN is not a precise enumeration of the number of bacteria in a given volume of sample. The precision and confidence limits of the test using any given number of tubes and of the MPN method of estimating densities have been determined and are available from reports listed in the bibliography of Anonymous (1970 a).

The accuracy of the results is dependent upon the number of portions of each dilution planted in the multi-tube fermentation procedure. For example, the approximate lower and upper 95% confidence limits when 3 tubes each of 3 dilutions are planted, may be estimated as 21% of the MPN for the lower end and 395% for the upper. By comparison, the approximate 95% confidence limit for 5 tubes in each of 3 decimal dilutions has been stated to be 29% for the lower and 290% for the upper.

The MPN results for a variety of planting series and positive results are shown in the tabulation given. The quantities shown at the heads of the columns merely indicate decimal dilutions and need not be considered as indicative of the volumes planted.

The figures may be used in computing the MPN in larger or smaller portion planting in the following manner. If instead of portions of 10, 1.0 and 0.1 ml, a combination of portions of 100, 10 and 1 ml is used, the MPN may be recorded as 0.1 times the figure in the tabulation (Table 5). On the other hand, if a combination of corresponding portions of 1.0, 0.1 and 0.01 ml is used, record 10 times the figure in the tabulation. Should a combination of portions of 0.1, 0.01 and 0.001 be planted, record 100 times the figures in the tabulation.

When more than 3 dilutions are employed in a decimal series, the results from only 3 of them are significant. Consult Anonymous (1970 a) for procedure for selecting the 3 significant dilutions when 4 or more dilutions are employed in a decimal series of dilutions.

Although the methodology to differentiate rapidly and absolutely coliforms of faecal origin from those of nonfaecal source is currently unavailable, a practical method has been developed that favours the selection and growth of faecal coliform organisms to

TABLE 5. *Most Probable Numbers (MPN) per* 100 *ml of sample, planting 5 portions in each of* 3 *dilutions in geometric series*

Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes			
10 ml	1 ml	0.1 ml	MPN	10 ml	1 ml	0.1 ml	MPN	10 ml	1 ml	0.1 ml	MPN
0	0	0		1	0	0	2	2	0	0	4.5
0	0	1	1.8	1	0	1	4	2	0	1	6.8
0	0	2	3.6	1	0	2	6	2	0	2	9.1
0	0	3	5.4	1	0	3	8	2	0	3	12
0	0	4	7.2	1	0	4	10	2	0	4	14
0	0	5	9.0	1	0	5	12	2	0	5	16
0	1	0	1.8	1	1	0	4	2	1	0	6.8
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2
0	1	2	5.5	1	1	2	8.1	2	1	2	12
0	1	3	7.3	1	1	3	10	2	1	3	14
0	1	4	9.1	1	1	4	12	2	1	4	17
0	1	5	11	1	1	5	14	2	1	5	19
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3
0	2	1	5.5	1	2	1	8.2	2	2	1	12
0	2	2	7.4	1	2	2	10	2	2	2	14
0	2	3	9.2	1	2	3	12	2	2	3	17
0	2	4	11	1	2	4	15	2	2	4	19
0	2	5	13	1	2	5	17	2	2	5	22
0	3	0	5.6	1	3	0	8.3	2	3	0	12
0	3	1	7.4	1	3	1	10	2	3	1	14
0	3	2	9.3	1	3	2	13	2	3	2	17
0	3	3	11	1	3	3	15	2	3	3	20
0	3	4	13	1	3	4	17	2	3	4	22
0	3	5	15	1	3	5	19	2	3	5	25
0	4	0	7.5	1	4	0	11	2	4	0	15
0	4	1	9.4	1	4	1	13	2	4	1	17
0	4	2	11	1	4	2	15	2	4	2	20
0	4	3	13	1	4	3	17	2	4	3	23
0	4	4	15	1	4	4	19	2	4	4	25
0	4	5	17	1	4	5	22	2	4	5	28
0	5	0	9.4	1	5	0	13	2	5	0	17
0	5	1	11	1	5	1	15	2	5	1	20
0	5	2	13	1	5	2	17	2	5	2	23
0	5	3	15	1	5	3	19	2	5	3	26
0	5	4	17	1	5	4	22	2	5	4	29
0	5	5	19	1	5	5	24	2	5	5	32

TABLE 5 (Contd.)

Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes			
10 ml	1 ml	0.1 ml	MPN	10 ml	1 ml	0.1 ml	MPN	10 ml	1 ml	0.1 ml	MPN
3	0	0	7.8	4	0	0	13	5	0	0	23
3	0	1	11	4	0	1	17	5	0	1	31
3	0	2	13	4	0	2	21	5	0	2	43
3	0	3	18	4	0	3	25	5	0	3	58
3	0	4	20	4	0	4	30	5	0	4	76
3	0	5	23	4	0	5	36	5	0	5	95
3	1	0	11	4	1	0	17	5	1	0	33
3	1	1	13	4	1	1	21	5	1	1	46
3	1	2	18	4	1	2	26	5	1	2	64
3	1	3	20	4	1	3	31	5	1	3	84
3	1	4	23	4	1	4	36	5	1	4	110
3	1	5	27	4	1	5	42	5	1	5	130
3	2	0	14	4	2	0	22	5	2	0	49
3	2	1	17	4	2	1	26	5	2	1	70
3	2	2	20	4	2	2	32	5	2	2	95
3	2	3	24	4	2	3	38	5	2	3	120
3	2	4	27	4	2	4	44	5	2	4	150
3	2	5	31	4	2	5	50	5	2	5	180
3	3	0	17	4	3	0	27	5	3	0	79
3	3	1	21	4	3	1	33	5	3	1	110
3	3	2	24	4	3	2	39	5	3	2	140
3	3	3	28	4	3	3	45	5	3	3	180
3	3	4	31	4	3	4	52	5	3	4	210
3	3	5	35	4	3	5	59	5	3	5	250
3	4	0	21	4	4	0	34	5	4	0	130
3	4	1	24	4	4	1	40	5	4	1	170
3	4	2	28	4	4	2	47	5	4	2	220
3	4	3	32	4	4	3	54	5	4	3	280
3	4	4	36	4	4	4	62	5	4	4	350
3	4	5	40	4	4	5	69	5	4	5	430
3	5	0	25	4	5	0	41	5	5	0	240
3	5	1	29	4	5	1	48	5	5	1	350
3	5	2	32	4	5	2	56	5	5	2	540
3	5	3	37	4	5	3	64	5	5	3	920
3	5	4	41	4	5	4	72	5	5	4	1,600
3	5	5	45	4	5	5	81				

From Anonymous (1970 b).

the exclusion of many, but not all of the types considered to have little or no public health significance. The elevated temperature test using a modified Eijkman reaction has the necessary sensitivity and specificity required for a reliable confirmatory test. From a practical standpoint, gas production under the conditions of the test is an indication of the presence of recent faecal contamination. Absence of gas production is indicative of a source other than the

intestinal tract of warm-blooded vertebrates and is considered non-faecal in origin. Normally routine tests associated with sanitary surveys of shellfish growing waters of commercial processing of shellfish do not require these differential tests.

However, if there is a need to differentiate faecal coliforms from nonfaecal coliforms, the method of choice is the multiple tube enrichment procedure with confirmation of gas positive tubes in EC medium for 24 ± 2 hour in a circulating covered water bath at 44.5°C±0.2°C to be used for sea water and shellfish. This method is presented in Anonymous (1970 a). The faecal coliform MPN is computed in the same manner as the coliform MPN. Moreover, if there is a need to identify faecal coliforms upto species, consult Anonymous (1970 c, Appendix, pp. 69–70).

Another bacteriological test required for evaluating the sanitary quality of sea water is the standard plate count. Since the details of this procedure are too numerous to summarise in a useful manner, the reader should consult Anonymous (1970 a).

In recent years the membrane filter method has been found useful and applicable for the enumeration of coliform organisms in some sea waters. The method's limitations are chiefly in waters that have a high turbidity, large numbers of noncoliforms as related to low coliform counts, or that contain toxic substances, as indicated above for the standard plate count. The procedures for membrane filter technique for evaluating the sanitary quality are too detailed to summarise in a brief manner. Thus, the reader should consult Anonymous (1970 a) for membrane filter procedures for enumeration of coliform group organisms, faecal coliforms and faecal streptococcal group organisms in sea water.

9.4 Examination of Shellfish

Samples of shellfish in the shell and shucked unfrozen shellfish should be examined within 6 hr after collection and in no case shall they be examined if there is more than a 24 hour delay. The report shall include the elapsed time between collection and examination. Individual containers of shellfish samples shall be marked for identification and the same shall be put on the descriptive form that accompanies the sample. A history and description of the shellfish sample shall accompany the sample to the laboratory and it should include: date, time and place of collection, area of harvest, date and time of harvest, and storage conditions between harvesting and collection. All these informations may not be obtainable for shellfish samples collected in market areas. In such cases the name of the

shipper, date of shipment and the harvesting area as well as the date, time and place of collection should be determined.

Samples of shellfish shall be collected in clean containers, which shall be waterproof and abrasion resistant. Shellfish samples shall be kept in dry storage at a temperature above freezing point but lower than 10°C (35-50°F) until they are examined. Shellfish shall not be allowed to come in contact with ice.

Generally, a minimum of 12 shellfish shall be taken in order to obtain a representative sample and to allow for sound animals suitable for shucking. With most species, allowing for the necessary culls, approximately 200 g of shell liquor and meat will be obtained. Shellfish of large size such as the Pacific oyster *Crassostrea gigas*, the surf clam *Mactra solidissima* and the hard clam *Mercenaria mercenaria* may give more than 200 g of shell liquor and meat when 10 or 12 individuals are used. It is desirable to use at least 10 animals for a sample. Thus, the use of large blender jars may be indicated. When sufficiently large blender jars are not available, the 10–12 shellfish meat and shell liquor should be ground for 30 seconds. Then 200 g of this meat homogenate should be blended with 200 g of sterile buffered phosphate water or 0.5% sterile peptone water for 60 seconds. On the other hand, 10 or 12 individuals of small species such as Olympia oyster *Ostrea lurida* and small sized clam *Protothaca staminea* and *Tapes japonica*, may produce much less than 100 g of shell liquor and meat. Thus, blender containers of small size should be used. However, even the smallest blender containers may require 20 to 30 of the small bivalves to produce an adequate volume for proper blending.

Shucked shellfish meat should be placed in sterile, watertight containers of adequate capacity. Sterile technique should be used in handling shellfish meat. Samples of the final product of shucking plants and repacking establishments may be in the final packing containers. Consumers size packages are acceptable for examination, provided they contain an adequate number of animals. Samples of shucked shellfish shall be packed in crushed ice immediately after collection and retained under such refrigeration until they are examined.

One or two packages of frozen shucked shellfish may be used provided a package contains at least 10 or 12 animals. Using sterile technique, samples from large blocks may be taken by coring with a suitable instrument or by quartering. Such samples shall be transferred to sterile containers for transportation to the laboratory.

If possible the frozen shucked shellfish sample should be maintained in the frozen state at temperatures close to those at which the commercial stock was maintained. When such treatment is not possible, samples of frozen shucked shellfish shall be maintained in crushed ice till they are examined.

9.5 Preparation of Shellfish

In preparing shell stock (shellfish in shell) for shucking, certain precautions to prevent contamination should be taken. The hands of the examiner should be thoroughly scrubbed with soap and water. Scrape off all growth, loose material from the shell and scrub the shell stock with a sterile stiff brush under running fresh water of drinking quality. Particular attention should be paid to the crevices at the shell junctions. Place cleaned shell stock in clean containers or on clean towels and allow to dry in the air.

Prior to removing shell contents, the examiner's hands should be thoroughly scrubbed with soap and water and rinsed with 70% alcohol. Using sterile instruments and techniques, open the shellfish and collect the appropriate amount of shell liquor and meat as indicated above. Place shellfish content in sterile blender containers or other suitable sterile containers. Transfer a suitable quantity of meat and liquor to a sterile tared blender jar or other container with sterile technique. Weigh the sample to the nearest gram. Transfer the weighed sample to a sterile blender jar and add an equal amount, by weight of sterile phosphate buffered dilution water or 0.5% sterile peptone water. (A dilution of equal amounts by weight of shucked pack of certain shellfish results, after grinding in a blender, in a mixture that has too heavy a consistency for pipetting. The meats of hard clam, surf clam and butter clam often are of this nature. Transfer such species undiluted to the culture tubes. In these cases the use of greater proportions of dilution water is permissible. The addition of 3 parts by weight of dilution water is suggested. With such dilution 4 ml of ground sample is equal to 1 g of shellfish and this should be used in the inoculation procedure. Adjustment of the presumptive broth concentration should be made).

Grind shellfish meat and shell liquor for 80–120 seconds in a blender operating at approximately 14,000 RPM. In general, a grinding time of 60–90 seconds will be sufficient for all species. Excessive grinding in small blender containers should be avoided to prevent overheating.

9.6 Presumptive Test

The ground sample should be cultured within 2 minutes after completion of grinding. On standing separation occurs in the sample. Thus, to avoid errors in volumetric measurements of the suspension, the sample should be thoroughly mixed immediately before transfer to the presumptive medium if there has been any delay between grinding and culturing of the sample.

Lactose broth or lauryl broth, single strength in fermentation tubes, shall be used as the presumptive test medium. Sterile phosphate buffered dilution water or 0.5% sterile peptone water shall be used for dilutions. Just before pipetting, all dilutions shall be shaken 25 times through an arc of 30 cmi n a time interval not exceeding 7 seconds. Inoculate:

i. Each of 5 tubes 2 ml of ground sample (=1 g of shellfish).

ii. Each of 5 tubes with 1 ml of a 1:10 of ground sample (=0.1g of shellfish).

Note : The 1:10 dilution may be made by adding 20 ml of ground sample to 80 ml of sterile dilution water.

iii. Each of 5 tubes with 1 ml of a 1:100 dilution of shellfish sample (=0.01 of shellfish).

Note : The 1:100 dilution may be made by adding 11 ml of the 1:10 dilution prepared in (ii) above to 99 ml of sterile dilution water.

iv. Each of 5 tubes with 1 ml of a 1:1000 dilution of shellfish sample (=0.001 g of shellfish).

Note : The 1:1000 dilution may be made by adding 1 ml of the 1:10 dilution prepared in (ii) above to 99 ml of sterile dilution water.

In order to avoid indeterminate results, extensions of the dilutions suggested above may be required. The amounts of sample selected for inoculation shall be such that the largest portions will give positive results in all or the majority of tubes and the smallest portions will give negative results in all or the majority of tubes. To attain this with any degree of assurance with samples of doubtful quality, it will be necessary to inoculate additional decimal dilutions.

Inoculate the fermentation tubes at 35°C ± 0.5°C. Proceed with (i) Presumptive test (ii) Confirmed test and (iii) Completed test as prescribed under section 9.2 Examination of sea water.

The coliform density shall be expressed as 'Most Probable Number' (MPN) per 100 g of sample. Compute the MPN as directed in 9.2 Examination of sea water or Standard Methods for the Examination of water and waste water (Greenberg *et al.*, 1981).

$$\text{MPN/100 ml} = \frac{\text{No. of positive tubes x 100}}{\sqrt{\text{ml sample in negative tubes}} \times \text{ml sample in all tubes}}$$

9.7 Test for Faecal Coliforms in Shellfish

The Presumptive test for faecal coliforms is conducted in the same manner as the Presumptive test for coliform organisms.

Proceed with Confirmed test as prescribed under 9.2 Examination of sea water.

When individual faecal coliforms are to be identified, consult Anonymous (1970 c).

The faecal coliform density in shellfish shall be expressed MPN per 100 g of shellfish. Compute the MPN as prescribed in 9.2 Examination of sea water.

9.8 Standard Plate Count

The standard plate count of samples of shellfish shall be conducted as prescribed under 9.2 Examination of sea water except that the plates shall be incubated at 48±3 hr rather than at 24±2 hr. In the examination of shellfish 0.1 ml inocula of the original shellfish sample shall not be used. One ml of an appropriate dilution shall be used. Express the results as Standard Plate Count per g of shellfish at 35°C, 48 hr. Ordinarily it is not desirable to plant more than 0.1 g of shellfish sample in a plate because of excess turbidity produced by larger quantities of sample. Therefore when the total number of colonies developing from 0.1 g is less than 30, it will be necessary to record the results as observed.

According to Mr. Richard Thompson, Director of Shellfish Sanitation, Austin, Texas (personal communication, 1984), the

baoteriological standards currently in use in U.S.A. are as presented below:

i. Minimum acceptable standards for shellfish growing waters:
Total coliforms : A median of all samples not to exceed 70 MPN/100 ml water. Not more than 100% of sample to exceed 230 MPN/100 ml of water.

Faecal Coliforms : A median not to exceed 14 MPN/100 ml water. Not more than 10% of samples to exceed 43 MPN/100 ml water.

Work under the assumption that the number of total coliforms and faecal coliforms are statistically equivalent.

ii. Recommended guidelines for shellfish meat :
Faecal coliforms : Not to exceed 230 MPN/100 g meat.
Standard Plate Count: Not to exceed 500,000/g meat.

Note : This may not be an accurate standard because consideration is under way to evolve a more specific *Escherichia coli* standard).

9.9 Purification of Shellfish

The basic principal for controlled purification or depuration of contaminated shellfish involves providing clean or purified sea water in ponds or tanks whereby the shellfish filter and pump such water for a period of 36 to 48 hours or more if required. The untreated shellfish are culled removing damaged or moribund ones and cleaned externally of debris and silt by spraying and washing with sea water. Then the shellfish are placed in trays or baskets at a density of one or two layers deep, but never more than 15 cm in depth. For ease of handling, one-half to one bushel of oysters could be placed in each tray. Some operators use a single tier of trays while others may use multi-tiers of trays depending on the design of the purification tanks. In the case of multi-layers of trays there should be some means of separating the tiers of trays. The trays are placed in concrete or wooden tanks preferably with slight gradient (*ca.* 3%) and a drain plug at the bottom to facilitate cleaning and flushing silt, faeces, pseudofaeces and other debris from the tank. Trays should be raised from the tank bottom by runners or 2.5 cm PVC pipes arranged longitudinally to the water flow. This arrangement allows accumulated debris to be easily flushed from the tank. A stand pipe is used at the drain end of the tank to regulate the water level, which should be at least 10 to 15 cm above the top layer of shellfish.

Ideally a shellfish purification or depuration plant should be located near the least polluted source of sea water in the vicinity of shellfish growing and marketing areas. Also the physical characteristics (salinity, temperature, turbidity, dissolved oxygen, etc.) of the sea water used in the depuration plant should not be radically different from that of the shellfish growing waters. The level of dissolved oxygen (D.O.) in purification tanks should not be allowed to drop below 2 mg/1. Aeration with compressed air should be employed when D. O. levels fall below 2 mg / 1. According to Neilson *et al.* (1978), *Crassostrea virginica* may be successfully depurated with an ultraviolet (UV) light system in sea water with turbidities as high as 77 mg/1. In the case of excessive turbidities, however, it may be necessary to filter the ambient sea water before purifying it. Sand filters are quite suitable for such purposes.

Most shellfish purification plants are operated as 'flow through' or 'once-through' systems, *i.e.* treated sea water is passed through the purification tanks at a fixed rate and then returned to its source. The flow rate of purified sea sea water required to depurate shellfish depends upon the contamination load of the shellfish and the method of sea water purification employed. Presnell and Cummins (1967) found that *C. virginica* could be satisfactorily purified with UV treated sea water at low rates of 0.5 to 5 litres per hour per oyster in a 24 hour period. Neilson *et al.* (1978) working with *C. virginica* in Chesapeake Bay reported that the flow of UV treated sea water should be at least 1 gallon per minute per bushel of oysters to obtain satisfactory depuration in 48 hours. A general diagram showing the flow of sea water and shellfish through depuration is presented in Fig. 3. Neilson *et al.* (1978) stated that a plant should be able to process 15 to 20 bushels of shellfish at a time in order to be profitable. Also these workers commented that depuration periods of more than 48 hours usually were not cost effective.

Although seldom used, most depuration plants are equipped for operating on a 'closed' or 'recycling' system during periods of abnormal ambient sea water quality (low salinity, low temperature, etc.). Under such conditions, the addition of artificial salts or heating the ambient sea water may be required to provide suitable sea water for shellfish filtration. Such measures greatly increase the cost of depuration. Thus, it is usually not feasible to operate depuration plants during periods of abnormal ambient sea water.

Regardless of the method used for sea water purification, the efficiency may be increased by draining the tank and washing the tank and oysters with a strong spray of clean or treated sea water to flush silt, faeces, pseudofaeces and other debris from the tank

about midway during the depuration process. Thus, if a 36 hours or 48 hours purification period is used, it is more effective to drain, clean and refill the system at 18 and 24 hours respectively.

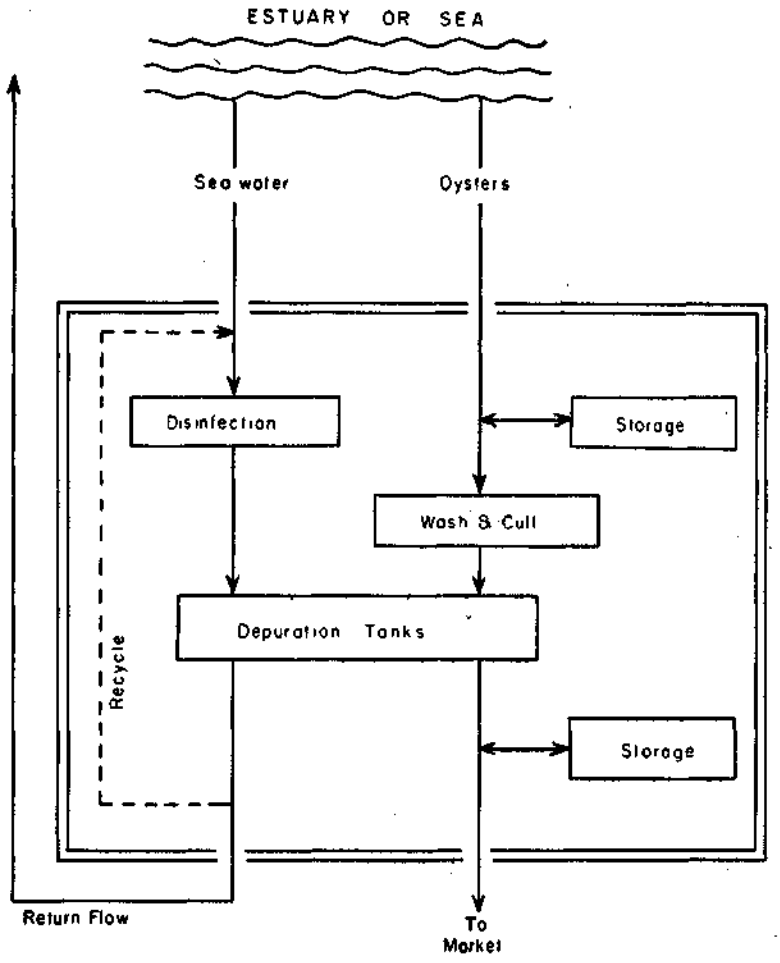

Fig. 3. Flow of water and oysters through a depuration plant (Adapted from Neilson, 1978).

This procedure prevents the resuspension of micro organisms removed by way of entrapment in faeces and pseudofaeces. Moreover,

the organic matter which may reduce the D.O. content of the sea water and thereby reduce the filtering efficiency of the shellfish, is eliminated from the system. The shellfish and the purification tank should be similarly cleaned at the end of the depuration period. The shellfish should be culled for damaged or moribund individuals and then packed for immediate delivery to market or stored in cool dry storage area until shipped to market for further processing.

The efficiency of the depuration procedure employed should be evaluated by bacteriological examination of the treated sea water treated shellfish by means of techniques presented earlier in this Chapter. Should the treated shellfish fail to meet the required bacteriological standards, the plant design and sea water purification system should be examined for deficiencies and modified as needed.

Four processes or some combinations thereof have been used to clean or purify sea water for purging shellfish contaminated with human pathogens. They are: i. Filtration, ii. Chlorination, iii. Ultraviolet light irradiation and iv. Ozonation.

The simplest procedure for purifying shellfish, provided they are not heavily contaminated is placing them in a flow-through purification system receiving clean or filtered sea water for 48 hours.

Although chlorinated sea water has a long history of use in depurating shellfish there is concern about the use of this strong oxidant for food processing (Blogoslawski, 1980). Chlorine or its oxidative by-products are known to reduce the pumping action of bivalves. Galtsoff (1946) noted that 0.2 ppm chlorine caused a 46% decrease in the pumping action of *C. virginica.* Other studies have shown that oysters subjected to levels as low as 0.01 ppm may reduce pumping rate (Blogoslawski, 1980). Blogoslawski has pointed out further that this sensitivity to chlorine may have an advantage in that the inner tissues of shellfish generally do not come in contact with chlorine since most animals cease pumping when chlorine level in ambient sea water is as low as 0.2 ppm. Thus, chlorine is effective in disinfecting the exterior of shells of bivalves at levels generally employed *viz.*, 2 to 3 ppm. In systems employing chlorine the shellfish are usually cleansed by chlorinated sea water that has been dechlorinated. In addition to the problems indicated above, Blogoslawski states that the expenses of chlorine depuration in terms of equipment and manpower makes it less attractive when compared with most other forms of sea water purification, *i.e.* UV treatment, ozonation and filtration.

General procedures used with the chlorine-sea water depuration method are briefly presented by Blogoslawski (1980). Some examples of the procedure described by Blogoslawski are given below. In the Dodgson procedure used in England shellfish are exposed to a 3 ppm chlorine in sea water for 3 hours, then in dechlorinated sea water for 20 hours. The process is repeated and then the shellfish are dipped in a solution of calcium hypochlorite and packed for marketing or shipment The Chabal procedure used in France involves the sterilisation of the exterior of shellfish with active chlorine. This process is followed by 3 days of depuration in filtered sea water. Another variation of the chlorine-sea water method, the Tarbett procedure is used in U.S.A. In this procedure the shellfish are washed with chlorinated (2 ppm) sea water, the shellfish are depurated for 20 hrs in sea water chlorinated at 2 ppm and then dechlorinated. The process is repeated.

According to Blogoslawski (1980), a depuration plant for cleansing mussels (*Mytilus edulis*) has been operated in Conway, Wales for nearly 60 years with a faultless record. In this plant sea water is pumped in and sterilized for at least 8 hours with chlorine at a concentration of 3 ppm and dechlorinated with sodium thiosulphate. Contaminated mussels are treated for 24 hours in concrete tanks. The water is drained and the shellfish are hosed to remove faeces and debris from mussels and the tank. Subsequently the mussels are held in dechlorinated sea water for an additional 24 hours. After hosing and flushing again the outside of the shells are sterilized by immersing the mussels for one hour in sea water containing 2–3 ppm of active chlorine. The mussels are then ready for sale to the public. The three concrete purification tanks at Conway have a total capacity of 50,000 imperial gallons of sea water and 13.5 tonnes of mussels. During the season which extends from October to March, the Conway plant processes 350–450 tonnes of mussels from the local fisheries.

For many years chlorine purification was the method of choice in France (Blogoslawski, 1980). Until early 1960s six of the seven depuration plants used chlorine as hypochlorite. The seventh station, at Sete, used chlorine gas for purifying sea water. Comparative bacteriological experiments conducted at Sete demonstrated that ozone cleansed shellfish more rapidly and completely than gaseous chlorine. The chlorine treatment reduced the initial coliform content of 120,000/litre to 1,200/litre after 5 days, whereas the ozone treatment reduced the coliform content to 0 after 5 days. Subsequent to this experiment, all French depuration stations changed over to ozone depuration. Moreover, the use

of ozone eliminated two problems attributed to the use of chlorine - a 'chemical' taste and 'chewy' texture of shellfish meat.

Only a few depuration plants throughout the world continue to use the chlorine method for depuration shellfish. The greatest number of depuration plants appear to be located in France and Australia. As previously mentioned the French plants have converted to ozone treatment. In Australia where depuration of shellfish is mandatory by law, the UV method is used by all depuration plants except one. Recently one of us (S. Ray) visited two of the largest depuration plants in Australia located at Port Stephens.

The Australian plant in which UV treatment is used for purification of sea water has two concrete purification tanks each with a capacity of 20 bags of oysters (*ca* 1,000 oysters per bag). The oysters are placed in plastic covered trays two layers thick. The sea water is purified by passage through two quartz UV units at the rate of 4 to 4½ thousand gallons per hour. The oysters in the purification tanks are allowed to feed or pump untreated water for 12 hours. Then the tank and oysters are hosed down to remove the accumulated silt and faeces. Later UV treated sea water is supplied to the purification tank for 18 hours after which the hosing and flushing process is repeated and the shellfish are treated for another 18 hours. The oysters are hosed down again and then culled, sized and packed for shipment to market. The cost of depurating a sack of oysters for the farmer who receives about $ 200, is $ 15 or 20.

The depuration plant equipped for ozonation of sea water mentioned above is the largest in Australia. The plant has four concrete purification tanks. These tanks have a 3% gradient to facilitate cleaning and flushing them. The sea water is pumped into a large settling reservoir to allow removal of silt before passage through the two ozonator vessels which have the capacity of treating 15–20 litres of water per second. The shellfish. to be treated are placed in trays, two layers thick and two trays high. The oysters are treated for 18 hours, the tanks are drained and the oysters are sprayed with treated sea water under high pressure by a mobile sprayer that travels the entire length of the tank. The tanks are refilled and the shellfish are treated for another 18 hours. The treated shellfish are sprayed once again with treated sea water, culled and packed for shipment to market.

Neilson *et al.* (1978) have presented plans and details for the construction and operation of UV facilities for depuration of shellfish. We have no reference to offer for a source of similar information for an ozonation depuration facility.

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DISCUSSION

Extensive blooms of dinoflagellates, commonly referred to as red tides or red water since the water may develop a brownish to reddish discolouration because of great densities of organisms, occur from time to time in various areas of the world, especially, in marine and estuarine waters. The literature on this phenomenon is voluminous. For a general reference we refer the reader to the work of Brongersma-Sanders (1948). For a review of modern concepts of the ecology of dinoflagellate blooms see Steidinger (1983). Blooms of dinoflagellates are often accompanied by spectacular displays of luminescence or phosphorescence at night. This feature has been used by Indians along the Pacific Coast of North America Quayle, 1969) and villagers of Papua, New Guinea (Maclean, 1973) as a warning of shellfish toxicity.

Although ecological factors responsible for such blooms are not well known, considerable progress has been made in the last ten or fifteen years towards a better understanding of the factors involved in the initiation, support, maintenance and dispersion of dinoflagellate blooms. A number of factors such as upwelling, tides and currents, salinity and fresh-water influx, amount of sunshine and light penetration, wind intensity and direction, water temperature and turbulence (degree of calmness), introduction of nutrients and growth factors (including pollutants) from land drainage, abundance of dinoflagellate predators (ciliates and other zooplankters) and competitors (diatoms), naturally produced cytolytic substances (produced by blue-green algae) and so on, have been considered by various observers. The role and interaction of various factors are not sufficiently understood, however, to permit prediction of dinoflagellate blooms with any degree of accuracy. Nevertheless, the general areas of occurrence and approximate seasonality of some toxic dinoflagellates are known well enough

to justify the establishment of stations in potential danger areas to routinely monitor for toxic dinoflagellates and toxic shellfish.

Until recently there was a tendency to attribute dinoflagellate blooms to factors that caused sudden population explosions due to increased cell division rate. Steidinger (1983) has pointed out that this is not the case She states that in red tides, population increases proceed at normal rates (usually upto 1 division per day). Blooms are the result of factors that concentrate or confine the planktonic organisms. Moreover, she has summarised the information regarding the four sequential stages in dinoflagellate bloom development, which are: initiation, growth, concentration and dispersion (including termination by dilution).

The recent concept of the role of benthic seed populations, as espoused by several authors, has provided a major advance in understanding the occurrence as well as the seasonality of dinoflagellates. According to Steidinger (1983), both armoured and unarmoured dinoflagellates form benthic resting stages (hypnozygotes), which may excyst to produce motile cells. These benthic resting stages accumulate to form *seed beds*; a concept introduced by Steidinger. The motile cells derived from dormant hypnozygotes may inoculate the over-lying waters. And with suitable physical and nutrient conditions dinoflagellates increase through normal cell division. Subsequently, favourable physical conditions such as winds, tides, currents, convergence, divergence and pynoclines serve as concentrating agents. On the other hand, these same agents can cause dispersal of high concentrations of dinoflagellate cells thereby either reducing or terminating the outbreak.

Furthermore, Steidinger (1983) has suggested that the mapping of localised seed beds, which has been done in New England waters, has value in forecasting potential areas of red tide blooms. She also states that mapping of benthic resting stages has advantage over the mouse bioassay or chemical assay of shellfish poisons. Depending upon the availability of personnel and facilities, we believe that, mapping of seed populations will provide an additional means for monitoring toxic dinoflagellates.

Blooms are caused by a variety of dinoflagellate species; mortality of aquatic organisms occurs in some incidences but not always. Such mortality may result either from indirect efforts such as oxygen deficiency and attendant deleterious substances resulting from decomposing masses of dinoflagellates and/or other dead aquatic life, or from toxins.

Mass mortality of aquatic organisms characteristically accompanies blooms of *Ptychodiscus brevis* and *Gonyaulax monilata*, which occur in the Gulf of Mexico. *Ptychodiscus brevis* (Gunter *et al.*, 1948; Ray and Wilson, 1957; Ray and Aldrich, 1967; Sievers, 1969) and *G. monilata* (Connell and Cross, 1950; Gates and Wilson, 1960; Aldrich *et al.*, 1967; Ray and Aldrich, 1967; Sievers, 1969) have been shown to produce substances that are toxic to fish and some invertebrates. Two other dinoflagellates *Gymnodinium veneficum* and *Gonyaulax grindleyi* appear to produce substances that are toxic to marine organisms. Those produced by *G. veneficum* are toxic to fish and some invertebrates whereas those produced by *G. grindleyi* cause considerable mortality of numerous invertebrates including molluscs, with little evidence of fish kills along the west coast of South Africa (Grindley and Nel, 1968; Grindley and Sapeika, 1969.

The toxins produced by the above organisms appear to have different specificities. Both *Ptychodiscus brevis* and *Gymnodinium veneficum* are toxic to fish and mice; and generally these species show little or no toxicity to invertebrates. *Gonyaulax monilata* is toxic to fish and a number of invertebrates including molluscs, but shows little evidence of toxicity to mice and chicks. In this respect *Gonyaulax grindleyi* appears to be similar to *G. monilata* except for the apparent lack of toxicity of the former organism to fish. Extracts of *Gonyaulax grindleyi* proved nontoxic to mice (Grindley and Nel, 1968; Grindley and Sapeika, 1969).

As a general rule, the mortality of aquatic organisms have been notably absent during blooms of dinoflagellates associated with paralytic shellfish poisoning outbreaks. Mortality of marine animals (fish, turtles and dolphins) was observed during 6 of the 13 *Pyrodinium bahamense* var. *compressa* PSP outbreaks that occurred between 1956 and 1973 in Papua, New Guinea (Maclean, 1973). It is not known whether *P. bahamense* var. *compressa* produces toxins that may kill fish or the toxin is obtained through the food chain as has been demonstrated for Atlantic herring in the Bay of Fundy in Canada during *Gonyaulax tamarensis* red tides (White, 1977, 1980, 1981). Maclean (1973) noted that the mortality of marine animals in New Guinea was not a simple case of suffocation since deaths of air-breathing turtles and dolphins occurred. It is most likely that these air-breathing aquatic animals obtained the toxins by way of the food chain.

Another incidence of mass mortality of marine animals occurred along the north-east English Coast during the 1968 PSP outbreak which was caused by *Gonyaulax tamarensis*. Extensive mortality

of sand eels (*Ammodytes*), birds especially shags *Phalacorax aristotelis* which had fed on the eels, some bivalve molluscs (primarily non-commercial species) and some animals that feed upon mussels occurred shortly before and during the outbreak (Adams *et al.*, 1968; Clark, 1968; Coulson *et al.*, 1969; Ingham *et al.*, 1968). Most of the bivalves harvested for human consumption such as mussels (*Mytilus*), scallops (*Pecten*) and queens (*Chamys*) accumulated large quantities of toxins without apparent harmful effects (Adams *et al.*, 1968; Clark, 1968). It has been noted in these investigations that other molluscs such as cockles (*Cardium*), clams (*Venus*), *Tellina*, *Macoma*) and starfish (*Asterias*) suffered considerable mortality in some areas. The restriction of fish mortality to sand eels suggests that ingestion of toxins through the food chain rather than an indirect effect such as oxygen deficiency was the cause of death among the eels. Assuming that the noncommercial bivalves were not killed by some indirect cause, it appears that some molluscs are more sensitive to *Gonyaulax tamarensis* toxins than others.

There remain numerous problems related to the causative organisms of shellfish poisons and other marine poisons such as puffer and ciguatera poisons. Steidinger (1983) points out that a new dimension has been added to the study of toxic dinoflagellates. Historically ecological studies of toxic dinoflagellates have dealt with only water column bloom dynamics. However, with the discovery that dinoflagellates such *as Gambierdiscus toxicus* and *Prorocentrum* spp. produce ciguatera poisons, more attention must be directed to benthic habitats. These and probably other toxic dinoflagellates exist as epiphytes on tropical and subtropical macroalgae. Thus the population dynamics of these benthic dwellers will differ greatly from those of planktonic red tides.

The ecological conditions responsible for the development of massive populations of toxic dinoflagellates require further study. The solution of some problems requires integrated field and laboratory investigations concerning taxonomic status, toxin-producing capability and the specificity of such toxins, physical and chemical nature of the toxins and toxicological and pharmocological relationships between causative and transvector organisms. Most of the studies mentioned above require the development of pure cultures of known and suspected causative organisms. The various studies may be patterned after those conducted for *Gonyaulax catenella* and *G. tamarensis* beginning with the pioneering work of Sommer and his co-workers. With proper identification and isolation of known causative organisms, knowledge of their nutritional and physical requirements as well as ecological interaction of such

factors, may be used in developing means for predicting the timing and location of toxic shellfish. The current progress in isolating and characterising various shellfish poisons hold promise that a technique to replace the cumbersome mouse bioassay for detecting shellfish poisons will be forthcoming soon.

Moreover, new information regarding the toxicological, pharmacological and chemical nature of various toxins is being rapidly acquired. We can be hopeful that such knowledge will lead to the development of satisfactory antidotes for human shellfish poisonings.

The current bacteriological procedures employed for monitoring the sanitary safety of shellfish growing waters and shellfish meats, although cumbersome and time consuming, appear to afford adequate protection to shellfish consumers. In U.S.A. for example, cases of food poisoning resulting from consumption of raw commercially produced shellfish are rare since they are normally taken from sanitarily approved growing waters and processing plants. There is increasing concern, however, that the bacterial indicators currently in use, are not reflective of the concentration of enteric viruses in sea water (Gerba *et al.*, 1980; LaBelle *et al.*, 1980). Another problem is the occurrence of false-positive reactions in the Presumptive Coliform test (Austrin *et al.*, 1981; Hussong *et al.*, 1981). The occurrence of false-positive reactions require that the Completed Coliform test we carried out to confirm the presence of faecal coliforms. For reasons indicated above as well as other reasons, the currently used bacteriological procedures for evaluating the sanitary quality of shellfish growing waters and shellfish meat require improvement and refinement. However, until improved techniques become available, the procedures presented in this manual represent the best available ones for monitoring the safety of shellfish for human consumption.

In India bivalve molluscan shellfish especially clams and mussels are utilised as food regularly in good quantities in many parts of the west coast and to a small extent in other areas (Rao, 1958; Jones, 1968; Alagarswami and Narasimham, 1973; Nayar, 1980; Silas *et al.*, 1982). In view of the hazards of consuming molluscan shellfish contaminated by enteric pathogens and toxins, we recommend that monitoring for sanitary quality of shellfish growing waters and shellfish meats be undertaken immediately in areas of shellfish production and utilisation. Such studies are particularly needed near large population centres where sewage pollution is likely to be very high. Monitoring of the shellfish toxicity and toxic dinoflagellates in the areas where there have

been outbreaks of shellfish poisoning *i.e.*, Buckingham Canal and Kumble Estuary is also suggested. The surveillance programmes for shellfish quality and shellfish poisons should be extended to other production areas especially on the west coast when facilities and manpower are available.

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* Out of print.