
***Emerita analoga* (Stimpson) as an Indicator Species
for Paralytic Shellfish Poisoning Toxicity
Along the California Coast**

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ABSTRACT

Paralytic shellfish poisoning toxins (PSPT) pose a serious threat to public health worldwide. Along the west coast of North America, *Mytilus californianus* (California mussel) has long been used as the primary indicator species for monitoring levels of PSPT in the environment. However, because the distribution of this species is limited to rocky shores, vast stretches of sandy beaches are not monitored for PSPT. This lack of information greatly reduces our ability to track and predict harmful algal bloom development and movement along the west coast of much of North and South America. Early studies on *Emerita analoga* (sand crab), a common sandy shore invertebrate of the eastern Pacific, showed that this species can sequester saxitoxin (STX, the primary neurotoxin produced by PSPT blooms) in its tissues. The purpose of this study was to develop a PSPT extraction protocol for *E. analoga*, and to compare the utility of this species as a PSPT indicator with that of *M. californianus*. Samples of both species were spiked with known amounts of saxitoxin and processed (*M. californianus* with the standard acid extraction procedure and *E. analoga* with the new adapted acid extraction process). Spike and recovery results show that *E. analoga* recovery rates were very similar to those of *M. californianus*.

To compare the accumulation and depuration rates of PSPT for the two species under nearly identical field conditions, samples of each were collected at six pairs of adjacent rocky and sandy beaches along the central coast of California. *M. californianus* and *E. analoga* samples collected in 1998 and 1999 from April through November, the season of historically high paralytic shellfish poisoning toxicity, indicate that *E. analoga* would make an excellent complement to the current PSPT monitoring program. Results from the 2-year study reveal that any PSPT event detected by *M. californianus*, was also detected by *E. analoga*. In fact, in one instance, *E. analoga* detected a spike in HAB activity, that was not reflected by *M. californianus*. The initial costs to include *E. analoga* with the regular monitoring regime are minimal, making the use of *E. analoga* as an bioindicator for PSP toxicity, not only cost efficient, but effective.

I. INTRODUCTION

The incidence of harmful algal blooms (HAB's) in California has increased over the past two decades (Laycock et al., 1994). HAB's, although somewhat seasonally predictable, often vary in toxicity from year to year (Laycock et al., 1994). Toxins produced by these blooms are commonly known as Paralytic Shellfish Poisoning Toxins (PSPT's), a group of saxitoxin (STX) derivatives produced by dinoflagellates mainly belonging to the genus *Alexandrium* spp. (Van Egmond et al., 1994). Ingestion of STX blocks sodium channels in the body, which can result in respiratory paralysis, and possibly death (Laycock et al. 1994, Price et al. 1991). Since PSP toxins have been shown to enter the food web via filter-feeding invertebrates, particularly molluscs and crustaceans, they can present a serious health hazard to higher predators such as fish, birds, marine mammals and humans (Cembella et al., 1994).

In California, the Department of Health (CDH) is responsible for notifying the public about the occurrences of harmful algal blooms. The PSPT monitoring program relies on the collection and testing of mussels (*Mytilus californianus*) from natural populations, and outplants (Price et al., 1991). Each county along the coast is required to submit samples to the CDH at bi-weekly intervals during the mussel quarantine season (normally May 1 through October 31), and monthly, during the off season. Testing of these samples is based on the mouse bioassay, where saxitoxin concentration is correlated with the time elapsed between injection and mouse death (Laycock et al., 1994). Toxicity levels above the FDA quarantine limit of 80 µg STX/ 100g tissue signal the onset of a PSPT producing bloom. This program is a part of a nation-wide effort to: 1) prevent the human consumption of contaminated marine organisms, 2) track the blooms as they migrate along the coast, and 3) increase understanding of the causes of harmful algal blooms.

Mussels are an ideal monitoring species for HAB's because they are abundant, widely distributed, easily obtainable from rocky intertidal habitats, and are known to rapidly concentrate and release the toxins as HAB's appear and disappear (Price et al., 1991).

However, in California sandy beaches constitute 64% of the coastline and are unsuitable for mussel colonization (. Consequently, much of the California shore is left unmonitored making it difficult or even impossible to track HAB's as they spread along the coast. Without advanced warning of impending harmful algal blooms, there is an increased potential for serious damage to marine fisheries and bivalve industries, and a greater risk to public health.

There is clearly a pressing need for an indicator species suitable for monitoring PSP toxins along sandy beach environments. The variety of organisms commonly found on California's sandy shores offers a diverse range of candidates for use in toxicity testing. The goal of this study was to identify, develop and test one of these as an appropriate bioindicator for PSPT monitoring.

Based on Wenner's (1988) guidelines for selecting a bio-indicator for sandy beach environments, the following set of criteria was adopted for identifying the species to be evaluated in this study as a PSPT-indicator:

1. The organism should accumulate the levels of pollutant encountered in the environment without substantial mortality.
2. The organism should be abundant throughout the study area.
3. The organism should be sufficiently long-lived to allow the sampling of more than one-year class, if desired.
4. The organism should be of reasonable size, giving adequate tissue for analysis.
5. The organism should be easy to sample.
6. The organism (or its population) should reveal gradations relative to the amount of pollutants in the environment.

Several species, such as the bean clam (*Donax spp.*), purple olive snail (*Olivella sp.*), annelid worm (*Euzonius spp.*) and the mole or sand crab (*Emerita analoga*), were considered for use as an indicator for PSP toxicity. The organism that best fulfills all the criteria for an indicator species is the sand crab, *Emerita analoga*. *E. analoga* occurs intertidally on sandy beaches along the western coast of North America from Kodiak Island, Alaska to Bahia de San Francisco, Baja California and along the west coast of South America from San Lorenzo, Ecuador to False Bay, Argentina (Efford, 1976). *E. analoga* tends to aggregate in dense patches, filter feeding with their long secondary antennae. These feeding appendages appear as characteristically "V-shaped" ripple marks in the swash zone as waves recede (Ricketts, 1985). As a common inhabitant of California beaches, *E. analoga* could make an excellent complement to mussels in PSPT testing.

Sand crabs have previously been used in testing for environmental pollutants such as DDT and heavy metals (Wenner, 1988). Sommer (1932) was the first to conduct a study on the occurrence of PSP toxins in *E. analoga*. Through a series of tests, he was able to document the concurrent rise and fall of PSPT concentrations within sand crabs and mussels. Sommer's extraction method, however, involved the extremely tedious dissection and removal of the crab's liver, a technique not amenable to development as a routine monitoring lab procedure. In 1936, Sommer et al. published an additional study comparing the toxicity levels of several marine organisms including *E. analoga* and *M. californianus*. *E. analoga* and *M. californianus* PSPT levels were measured at 6 different sites over a 3-year period. Results from this study show that retention and release rates for both species are very similar and generally parallel one another. Curiously, despite Sommer's provocative results, there has apparently been no further research published on the use of sand crabs as indicators for PSP toxicity.

The purpose of this study was to assess the utility of *E. analoga* as a monitoring tool for PSPT along sandy beaches, and to develop an effective procedure for routine PSPT extraction from this species. To this end the project objectives were to:

- Develop a protocol for an extraction of PSPT from *E. analoga*, for use in a mouse bioassay.
- Determine the rate of toxin uptake and depuration for *E. analoga* as compared to *M. californianus*.
- Determine the concentration of PSPT retained by *E. analoga* as compared to *M. californianus*.

The general approach taken was twofold. In the laboratory, the *M. californianus* extraction protocol was used as a baseline, and refined for *E. analoga* using spike and recovery testing. Simultaneous field sampling of mussels and sand crabs at pairs of rocky and sandy shore sites exposed to the same HAB conditions were used to compare PSPT concentration time series for both species. The results from these paired samples were then used to test the following hypotheses:

H₀ : *Emerita analoga* does not retain PSPT in its body or tissues when exposed to a PSPT producing HAB.

H₁ : *Emerita analoga* does retain PSPT .

H₂ : *Emerita analoga* does retain PSPT at concentrations similar to *Mytilus californianus*.

H₃ : *Emerita analoga* accumulates PSPT at the same rate as *Mytilus californianus*.

H₄ : *Emerita analoga* depurates PSPT at the same rate as *Mytilus californianus*.

I. METHODS

A. Adaptation of Saxitoxin Extraction Procedure for *Emerita analoga*

A new extraction procedure was needed in order to process *E. analoga* samples collected to determine PSP toxicity. The standard acid extraction method for *M. californianus* was used as a basis for the adaptation process for use on *E. analoga*.

Tissue Collection and Preparation

Emerita analoga and *Mytilus californianus* samples were collected from a paired rocky and sandy site at Pebble Beach in early October of 1998.

E. analoga were haphazardly collected in 100g increments, along the beach, within the swash zone, using a shovel and a 1/4 inch mesh net. The tissues collected were rinsed of noticeable particulates (such as sand or algae) and stored in labeled ziploc bags that were subsequently frozen at -70°C.

M. californianus tissue was collected in 100g sample sizes, and prepared according to the protocol set forth by the California Department of Health (Appendix A). *M. californianus* samples were rinsed before shucking to remove any foreign material. The soft tissue was separated from the shell by severing the abductor muscle from the inside of the shell on either side. The labeled tissues were frozen at -70°C.

PSPT Extraction Process for *Mytilus californianus*

The acid extraction method currently used on *M. californianus* species, by the California Department of Health, is listed in Appendix B.

Adaptations to *M. californianus* Extraction Procedure for *E. analoga*

Several modifications to the original extraction procedure used by the California Department of Health were tested for use with *E. analoga*. Initially we included both the exoskeleton and the soft tissues of *E. analoga*. However there was difficulty controlling the pH of the extract due to the reaction between the calcium carbonate in the exoskeleton and the hydrochloric acid added during processing. Modifications made to the preparation of the crab tissue prevented the pH variation by removing the calcium carbonate from the sample.

To prepare the *E. analoga* tissue, it was necessary to separate the exoskeleton from the soft tissue with a potato ricer. The ricer compressed the whole bodies of the crabs, and extruded only *E. analoga*'s viscera and ovum.

The tissue was homogenized, and a sub-sample of 50g was transferred to a glass beaker for acidification with an equal mass of 0.18 M hydrochloric acid (HCl). The pH of the acidified homogenate mixture was determined, and if necessary, adjusted with 5 M HCl to a pH between 2 to 4. If the sample reached a pH of less than 2 or more than 4, any toxins present would either become more toxic, or be deactivated, respectively. The acidified *E. analoga* tissue was boiled for 5 minutes to ensure the proper breakdown of cells in order to release the saxitoxin.

There were difficulties encountered during the boiling of the acid/ *E. analoga* mixture. *E. analoga* tissue tends to boil very quickly, and at a lower temperature than *M. californianus*. Samples had to be watched diligently in order to keep the mixture from erupting out of the beaker. After the sample began to boil, constant stirring by hand was often required until the 5 minute boiling time had elapsed.

After cooling, the sample was again adjusted for pH and brought back to its' original weight with 3 mM HCl. *E. analoga* samples also tended to have a higher lipid content than

M. californianus, which made separating the solids from the supernatant difficult. Centrifuge times for *E. analoga* were increased to 10 minutes, which was twice as much as the time used for *M. californianus*. The sample was centrifuged, and the supernatant was separated and stored at -70°C until shipped to the California Department of Health for testing in a mouse bioassay.

At the CDH, the extracts were injected intraperitoneally into 3 different mice. The behavior of the mice was documented, and the time of death was used to correspond to a known amount of saxitoxin. Results were reported in µg STX/100g of tissue.

B. Spike and Recovery

The goal of the spike and recovery was to determine what, if any, matrix effects the *E. analoga* tissue would have on saxitoxin. In *M. californianus*, the epimerization of saxitoxin can occur where the molecular composition of the STX molecule is altered when the toxin is transferred from the algae to the shellfish tissues (Ralonde, 1996). This molecular transformation can decrease the original toxicity encountered by *M. californianus* by 11 times. With such a difference in toxicity, it is important to determine if such a reaction also occurs in *E. analoga*.

Tissue Collection and Preparation

Both *M. californianus* and *E. analoga* were collected haphazardly on June 21, 1999 from Natural Bridges State Park, Santa Cruz.

Fifty gram samples of *E. analoga* were collected and prepared according to the instructions listed under section A. The smaller sample size of 50 g (as opposed to 100g) was chosen in order to create the least amount of impact to the natural populations of both *M.*

californianus and *E. analoga* at Natural Bridges State Park. Approximately 50 grams per sample of *M. californianus* tissue were also collected from each site and prepared according to the instructions listed in Appendix A.

Processing

For the spike and recovery, a deionized (DI) water sample was added to act as a control, or baseline, to determine if the procedure itself was producing any by-products that might interfere with the mouse bioassay. *M. californianus* samples were processed according to the procedure used by the California Department of Health (Appendix B). *E. analoga* tissues were extracted with the newly adapted procedure, and the DI water samples were treated as *M. californianus* tissue, and also processed with the CDH's original procedure.

Triplicate 50g samples, each of *E. analoga*, *M. californianus* tissue and deionized (DI) water, were spiked at each of the 5 different toxicity levels with 50, 80, 200, 500 and 1000 µg of purified saxitoxin. The stock STX was obtained from Sherwood Hall of the Food and Drug Administration, and had a concentration of 1.89mg STX/ mL. Samples were spiked by pipette into the samples before homogenization, and then processed according to each of samples' individual extraction procedures. The resulting extracts were kept at -70°C before being sent to the CDH for mouse bioassay.

C. Field Comparison of *M. californianus* and *E. analoga* as Bioindicators

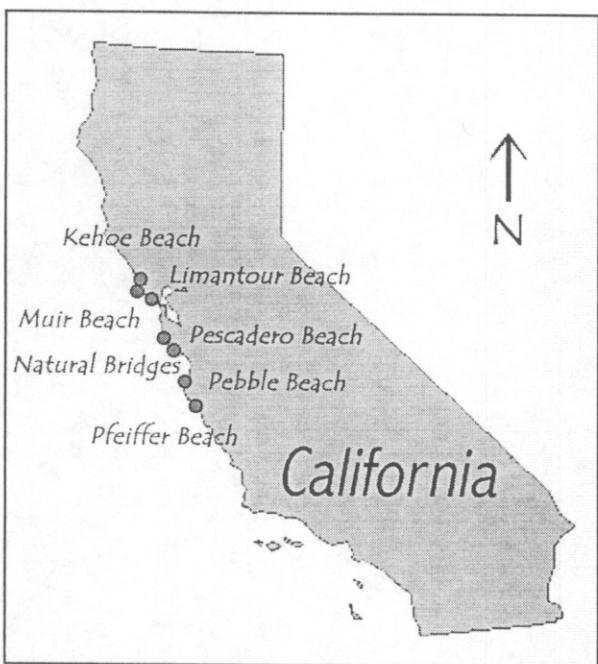


Figure 1. Map of sample collection sites in California.

Tissue Collection and Extraction

Between the months of April and September in 1998, and April to November in 1999, samples of *Emerita analoga* and *Mytilus californianus* were collected from six pairs of adjacent rocky and sandy beaches along the California coast. The 6 sites: Kehoe Beach, Limantour Beach, Muir Beach, Pescadero State Beach, Pebble Beach and Pfeiffer State Beach, have historically exhibited a downward gradation in toxicity from the North to the South (Cembella 1998, Price et al. 1991). During the 1998 and 1999 sampling period, both species were collected bi-weekly and within 24 hours of each other. When possible, collection was done on days with particularly low tides.

Approximately 100 grams of *M. californianus* tissue were collected from each site and prepared according to the protocol set forth by the California Department of Health as

described above in Section A. All the samples were stored at -70°C, where they were kept until shipped to the California Department of Health's labs at UC Berkeley. From the *M. californianus* tissue, the CDH produced extracts using the standard saxitoxin extraction method described in Appendix B. The resulting extract was used in a mouse bioassay to determine what levels of saxitoxin were present, if any.

Fifty-gram samples of *E. analoga* were haphazardly collected along the beach for each of the 6 sites during the 2-year study. *E. analoga* samples were collected and prepared using the methods described in section A.

Not all of the *E. analoga* samples collected during 1998 and 1999 were processed. Results from the *M. californianus* tissue for all of the sites during both years were used to determine which subsets of the *E. analoga* samples would be used. Samples that were processed for this study remained frozen for up to two years until the new PSPT extraction procedure was developed for *E. analoga* (see section A).

The supernatant from the extraction of *E. analoga* samples (see section A) were kept frozen at -70°C, and later shipped to the California Department of Health for toxicity analysis with the mouse bioassay. Results were reported in µg STX/ 100g tissue.

II. RESULTS

Results from the spike and recovery experiment (figure 2) with *Mytilus*, *Emerita* and DI water samples spiked at the 5 different toxicity levels (50, 80, 200, 500 and 1000µg), demonstrated that saxitoxin recovery for *E. analoga* closely parallels that of *M. californianus*. There was no significant difference between recovery levels for the 3 sample types. *E. analoga* retained a slightly higher level of saxitoxin than *M. californianus* at the

lower toxicity levels (50, 80, 200 and 500 µg), and at the 1000 µg level, *M. californianus* had a higher recovery rate than *E. analoga*. Even though there was a small difference in recovery levels, there was no significant difference between the two species. Also, as expected, DI water samples showed the highest recovery rates of any of the three sample types, with the exception of the 50 µg level.,

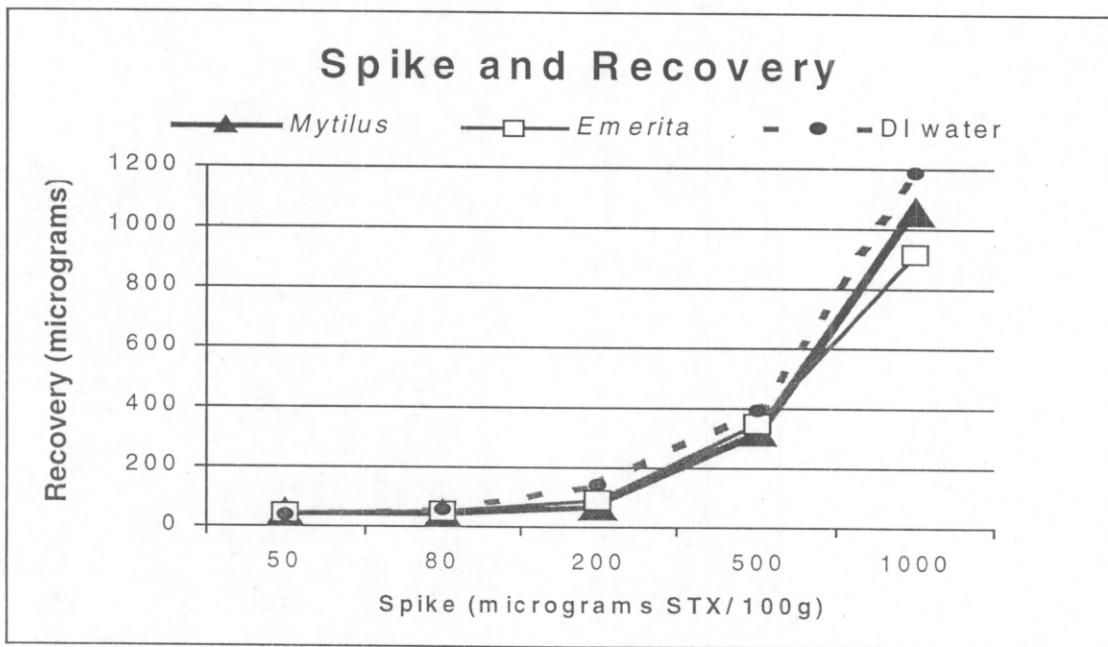
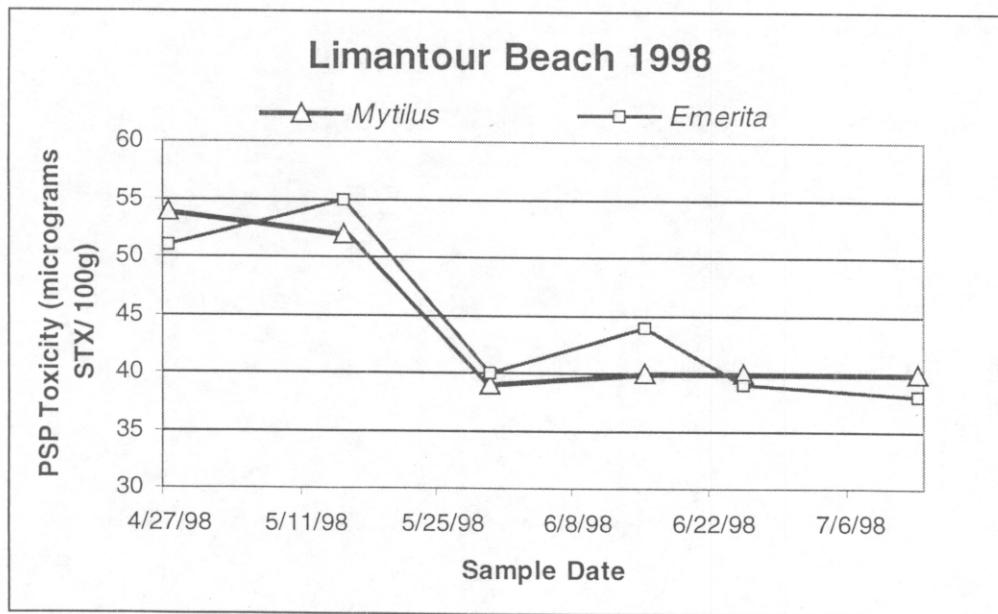
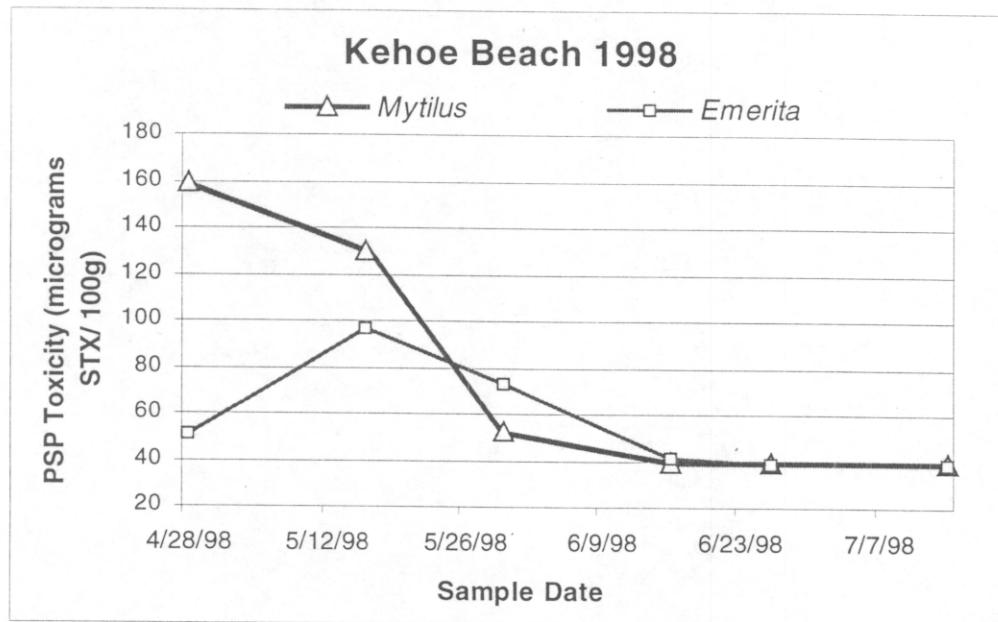
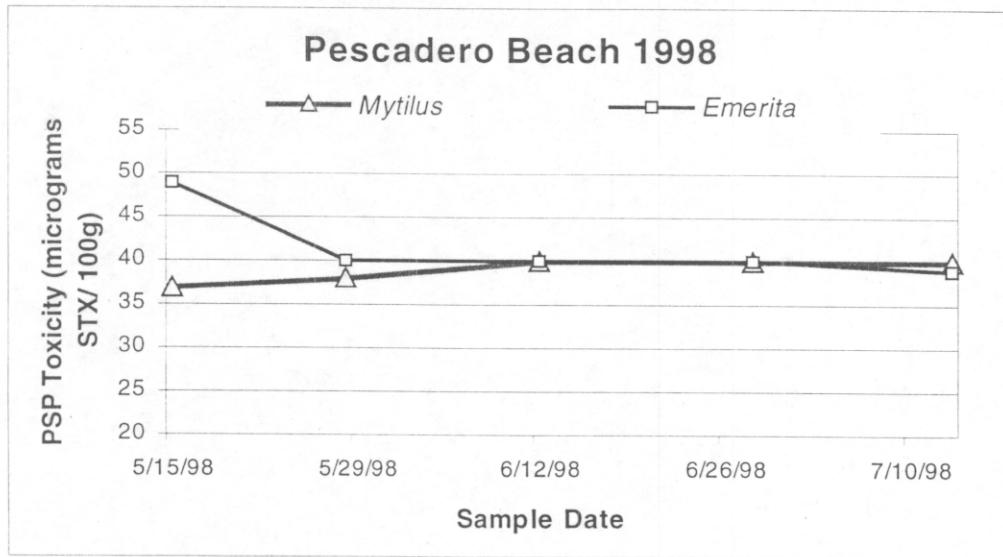
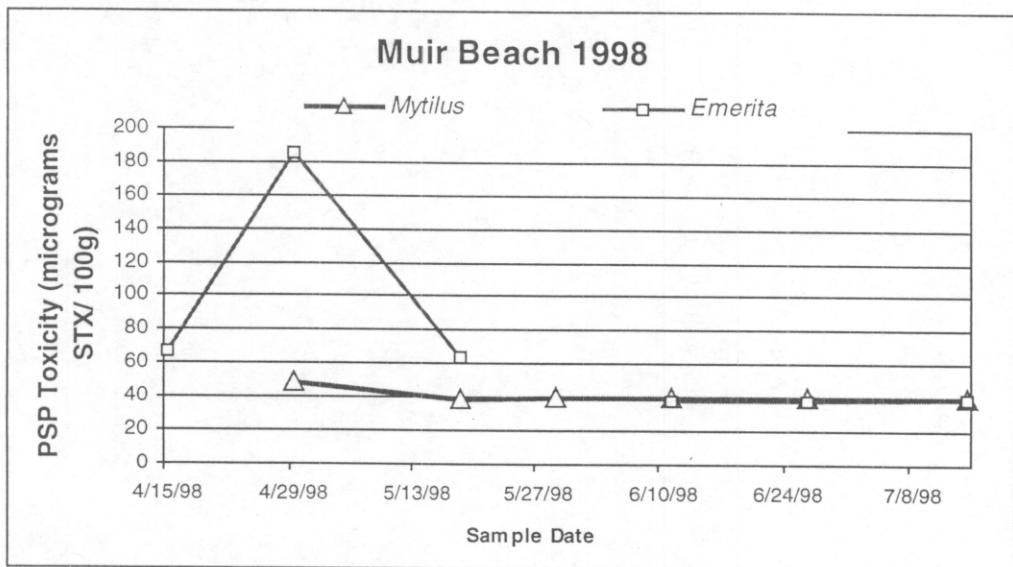


Figure 2. Graph of the average spike and recovery levels for comparable samples of *M. californianus*, *E. analoga*, and DI water

Throughout the sampling period for the 2-year field comparison, PSPT levels for either *M. californianus* or *E. analoga* only rose above the 80 µg STX/ 100g quarantine level at 6 sites: Kehoe, Muir and Pebble Beach in 1998 (figure 3a, b, e), Limantour, Muir and Pescadero in 1999 (figure 4a, b, c). Toxicity results for the other 5 sites in 1998 and 1999 (figure 3c, d, f and 4d, e) remained below 80µg / 100g, and were generally close to the detection limits (38 µg / 100g) of the mouse bioassay test.





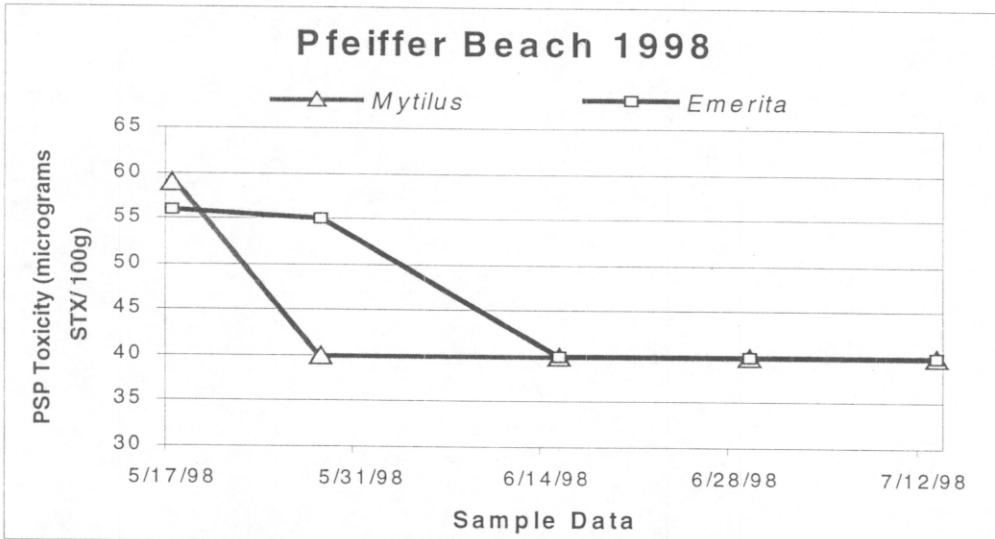
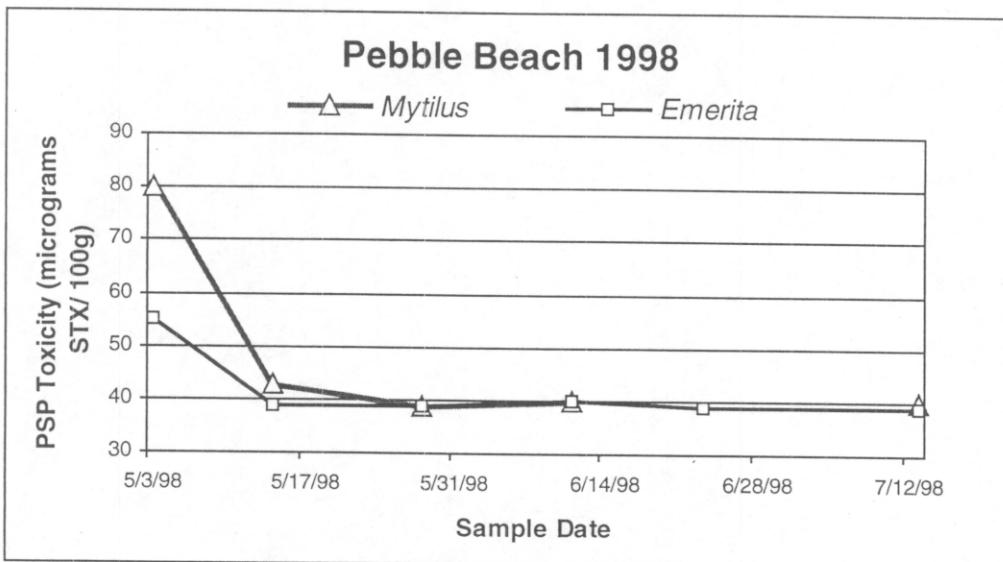


Figure 3. Graphs of Kehoe (a), Limantour (b), Muir (c), Pescadero (d), Pebble Beach (e) and Pfeiffer (f) sites in 1998.

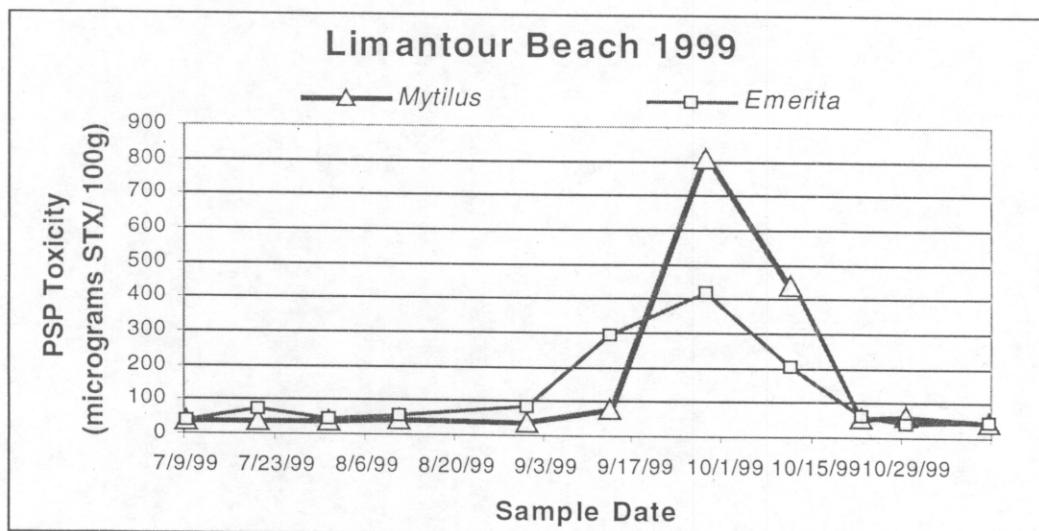
When sampling began at Kehoe beach in late April, *M. californianus* toxicity was already at 160 μ g (figure 3a), and then declining. *E. analoga* samples did not show elevated toxicity levels until later in mid-May, and then they only peaked at 97 μ g.

At Limantour beach (figure 3b), *E. analoga* and *M. californianus* toxicity levels rose and fell in synchrony showing how well the two species paralleled each other at these low levels.

Results from Muir beach (figure 3c) show that only *E. analoga* detected levels of PSPT above 80 μ g STX, while *M. californianus* toxicity stayed near the detection limit. Even though peak toxicity for *M. californianus* was greater than that for *E. analoga* at Limantour and Kehoe, the 186 μ g peak detected in *E. analoga* at Muir (figure 3c) was not even observed in *M. californianus*, which peaked only at 49 μ g.

PSPT activity for Pescadero beach (figure 3d) never reached above the 80 μ g quarantine limit. Peak PSP toxicity during this period was detected by *E. analoga* with 49 μ g, which is slightly higher than that detected at the same time interval by *M. californianus*.

At Pebble beach, the highest PSPT level was detected by *M. californianus* which peaked at 80 μ g. *E. analoga* PSPT levels also increased during the same time period as *M. californianus*, but never rose above 55 μ g. Both *M. californianus* and *E. analoga* PSPT values leveled off after mid-May with an average of 40 μ g.



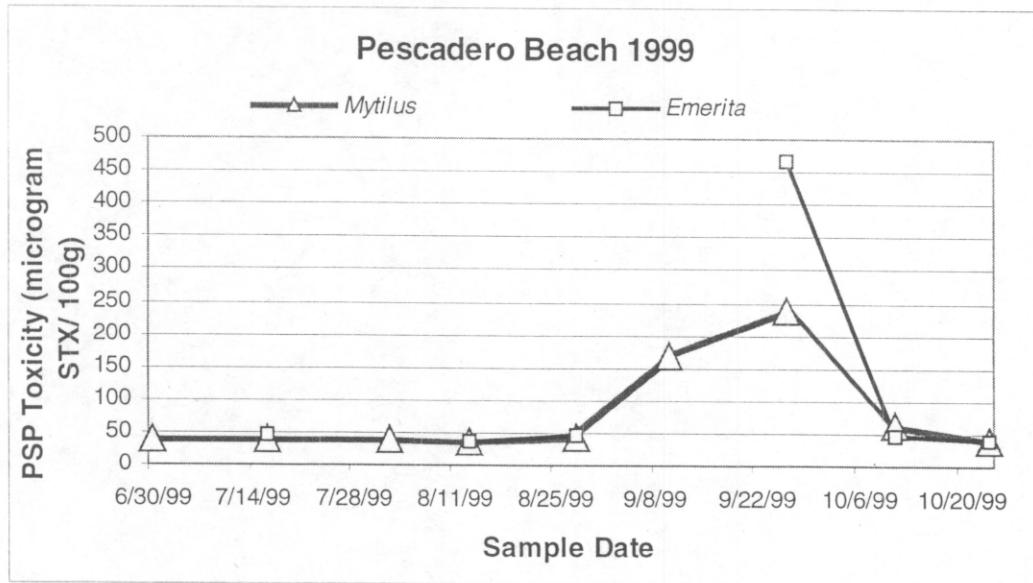
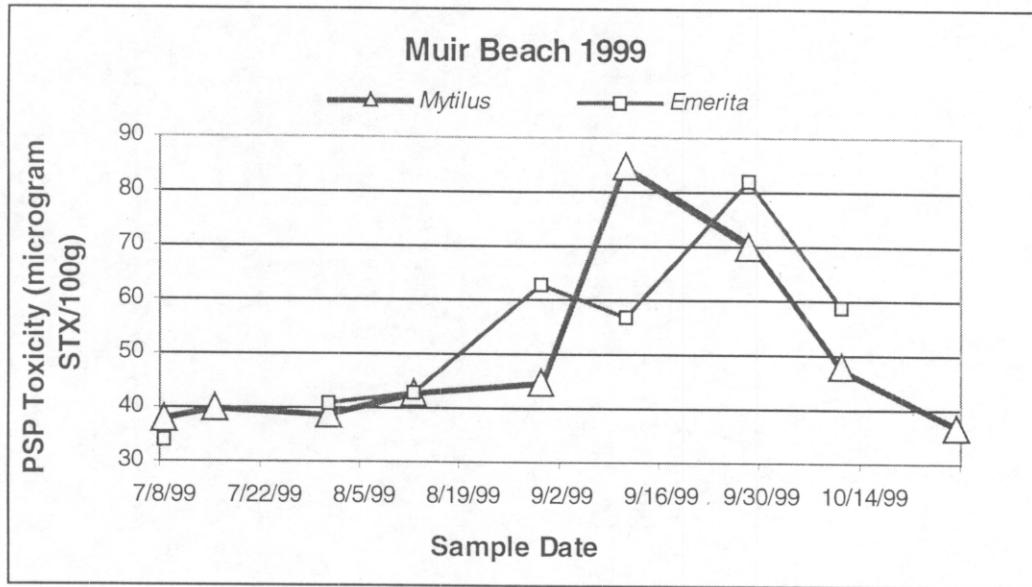


Figure 4. Graphs of PSP toxicity for Limantour (a), Muir (b) and Pescadero (c) sites in 1999.

Limantour beach samples in 1999 (figure 4a) show an obvious spike in toxicity starting in early September, and ending in late October. *E. analoga* was the first to detect PSPT activity (83 µg) in late August. Two weeks later *M. californianus* PSP toxicity was still under the quarantine limit 79 µg, while *E. analoga*'s levels had reached 293 µg. Levels of *M. californianus* peaked 2 weeks later in late September at 810 µg. *E. analoga* samples also peaked in late September, at 419 µg. PSP toxicity in both species dropped down below 80 µg in mid to late October.

In figure 4b, *E. analoga* PSPT levels at Muir beach rose slightly in late August , followed by a small decline in toxicity. *M. californianus* toxicity levels rose quickly in early September and peaked at 85 µg. *E. analoga* peaked in late September (82 µg) and then followed *M. californianus* in a declining trend.

PSP toxicity levels at Pescadero Beach (figure 4c) remained near the detection limit until late August, when they begin to rise. The data set for of *E. analoga* was incomplete, and it is hard to determine if the elevated toxicity for *E. analoga* at 468 µg, is really the peak of PSPT for that time period, or not. After *E. analoga* peaked in late September, the levels of PSPT decreased rapidly and by the following sampling interval, the PSPT level was below the quarantine limit. *M. californianus* toxicity levels peaked at the same time as *E. analoga*, and then decreased during subsequent sampling intervals to below 80 µg.

III. DISCUSSION

The importance of determining the role of *E. analoga* as a bioindicator for PSP toxicity, stems from a need for a more extensive monitoring program for harmful algal blooms. HAB distribution is patchy due to environmental conditions such as winds, tides and currents that can either concentrate, or disperse the cells of a toxic algal bloom along our coast. Vast stretches of beach in California are not being monitored, and the uneven toxicity that results from the effects of oceanographic conditions on a HAB, make it difficult to monitor for these variances. With the addition of *E. analoga* to the current monitoring regime, these gaps in information can be bridged. Results from the 2-year study reveal that any PSPT event detected by *M. californianus*, was also detected by *E. analoga*. In fact, at the Muir beach site (figure 4b), *E. analoga* detected a spike in HAB activity (186 µg) that was not reflected by *M. californianus* (49 µg).

H₁ : *Emerita analoga* does retain PSPT in its body or tissues when exposed to a PSPT producing HAB.

The results indicate that *E. analoga* does indeed sequester the Paralytic Shellfish Poisoning toxin, saxitoxin, within their tissues during periods of a harmful algal bloom. In figure 4a, which shows the STX levels of paired *M. californianus* and *E. analoga* samples from Limantour Beach in 1999, there was a PSPT producing bloom that occurred between late August and October. The graph clearly shows a definite increase in toxicity levels for *M. californianus* and *E. analoga*, (810 and 419 µg respectively), thus demonstrating *E. analoga*'s ability to sequester saxitoxin when exposed to a harmful algal bloom. In the work that Sommer published in 1932, he was also able to show that *E. analoga* retained PSP toxins in their tissues, and in most cases at the same toxicity as *M. californianus*.

H₂ : *Emerita analoga* does retain PSPT at concentrations similar to *Mytilus californianus*.

The ability of *E. analoga* to retain levels of PSPT comparable to *M. californianus* is apparent in figure 3b, where the graph shows *E. analoga*'s ability to detect PSPT levels comparable to that of *M. californianus*. For figure 3b, statistical analyses showed no significance for a difference in toxicity between *E. analoga* and *M. californianus*. There is an indication that at elevated toxicity levels (above 200 µg), *M. californianus* detected higher levels of PSPT than *E. analoga* (figure 4a), but that was only observed at Limantour in 1999. When there were significant differences between toxicity levels between species at a specific time interval (810 µg for *M. californianus*, and 419 µg for *E. analoga*), levels were above the FDA quarantine limit of 80 µg.

Sommer et al. (1936) compared the PSP toxicity of *M. californianus*, *E. analoga* and *Saxidomus nutallii* (Washington clam) over a 6-year period at six different sites along the central coast of California. A graph of the toxicity shows that *E. analoga* generally parallels PSPT levels in *M. californianus*.

H₃ : *Emerita analoga* accumulates PSPT at the same rate as *Mytilus californianus*.

In figure 3b, the increases in PSP toxicity for both species are very similar, and occur at the same time interval during that sampling period. In other cases (figures 3d, e), results approaching the non-detect level for PSPT (~ 40 µg), show that both species retain nearly identical amounts of saxitoxin at the same rate. Of course because these levels are so near the non-detect level, it's hard to say with any certainty if this is the case.

There are several examples where *E. analoga*'s rate of uptake does not correspond to the rate of uptake for *M. californianus*. Both species at Kehoe beach in 1998 (figure 3a) indicated increased HAB activity in late spring, though *M. californianus* picked up the toxicity levels sooner than *E. analoga*. The rate of uptake for *E. analoga* did not follow the rate of uptake for *M. californianus* in this case.

At Limantour beach in 1999 (figure 4a), *E. analoga* and *M. californianus* PSPT levels paralleled one another. Though *E. analoga* detected the increased levels of PSPT 2 weeks before *M. californianus*, both species retained their high levels of toxicity for the same length of time. Toxicity levels for both species dropped to below the quarantine limit at the same time in mid-October.

H₄ : *Emerita analoga* depurates PSPT at the same rate as *Mytilus californianus*.

During the 1998 sampling period, *M. californianus* and *E. analoga* samples from Limantour Beach (figure 3b), showed that both species retained and released similar amounts of PSP toxins at the same time. In figure 3e, the results from Pebble beach show that there was a slight increase in PSP toxicity for both species at the beginning of May, but by the following sampling interval, both species PSPT levels dropped on an average of 33% to the mouse bioassay non-detect levels.

In Sommer et al. (1936), *M. californianus* and *E. analoga*'s PSPT levels declined at approximately the same rate. Sommer et al. (1936) also stated that higher levels of PSPT in *E. analoga* are sometimes still detectable, even when they can no longer be detected by *M. californianus*.

From the results, it appears that *E. analoga* is an excellent indicator for the presence of PSPT bloom. There were a few instances where *E. analoga* detected a rise in toxicity where it had not been reflected by *M. californianus* samples.

In order to determine if the extraction procedure adapted for use with *E. analoga* is suitable for monitoring at higher levels of PSPT (>1000), further studies involving feeding experiments with PSPT producing algae and *E. analoga* and *M. californianus* should be done.

Cost/ Benefit Analysis

If there were sufficient funds, a large-scale study could be conducted along the entire coast of California to assess the utility of the newly adapted *E. analoga* extraction procedure in comparison to current monitoring tactics. However this type of study would require an enormous amount of collection and processing, which can be costly and time consuming.

The overall costs of adding *E. analoga* to the current monitoring program at this time are minimal. Field collection costs lie in the initial purchase of nets and shovels to collect samples of *E. analoga*. These are more costly tools than those used for the collection of *M. californianus*, but once purchased, they can be used repeatedly over many years. The financial cost to set up sampling for *E. analoga* is offset by the lowered risk that is involved in its collection. *M. californianus* collection requires periods of low tides in order to collect safely and effectively, whereas *E. analoga* can be collected at virtually any tidal height with very little risk. Time and information are lost while waiting for a window to collect *M. californianus*. The time commitment to train personnel to collect *E. analoga* samples is also basically the same as that for *M. californianus*.

Laboratory costs for including *E. analoga* in California's monitoring program are also quite small. Since the extraction procedure for *E. analoga* was adapted from the procedure used currently for *M. californianus*, all of the reagents are the same. The equipment used to process *E. analoga* samples is also identical, except during tissue preparation. *M. californianus* tissue is removed from its shell with a shucking knife. *E. analoga* tissue is removed from its carapace with a stainless steel potato ricer, which can be purchased from any kitchenware store for around \$10. The costs to introduce *E. analoga* into the monitoring program are small compared to the knowledge that added monitoring stations would bring.

IV. CONCLUSION

The procedure adapted for the extraction and concentration of saxitoxin from *E. analoga*, is as efficient as the procedure for *M. californianus*. Preparation of both species involves the same amount of time, however the collection of *E. analoga* may take slightly longer, depending on the seasonal changes in beach morphology and how that may affect their distribution. Additional attention during the boiling stage of the extraction process for *E. analoga* is necessary in order to avoid changing the properties of the sample. If this added time does affect the cost efficiency of running *E. analoga* extractions, it is only by a small percentage, if at all. Overall, the processing of *E. analoga* is comparable to that of *M. californianus* in relation to time and materials.

E. analoga's ability to detect levels of PSP toxicity compares well with that of *M. californianus*. In the presence of an algal bloom that had been detected by *M. californianus*, *E. analoga* also reflected elevated levels of PSPT's at nearly the same time. In some cases, *E. analoga* detected the PSPT bloom sooner than *M. californianus*. In one case, *E. analoga* detected a bloom when *M. californianus* did not. The depuration rates of PSP toxins for *E. analoga* and *M. californianus* appear to be very similar. In most instances, the toxicity for both species after a algal bloom, return to the minimum detection level at the same time.

The results from this study show considerable promise, and since the mussel quarantine levels for the FDA are only 80 µg STX/ 100g, *E. analoga* is ready to be used as a bioindicator for PSPT blooms in California.

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VII. APPENDIX

Appendix A

Field Sampling Protocol: Shellfish Marine Biotoxin Monitoring and Control Program

Prepared by

California Department of Health Services
Environmental Management Branch
And
Environmental Microbial Diseases Laboratory

INTRODUCTION

The following field sampling protocol will accommodate analysis for both paralytic shellfish poisoning (PSP) toxins and domoic acid (DA). Because preservative cannot be used at the time of sample collection, it is imperative that the field collectors take care to ensure the integrity of each sample.

1. SAMPLING: A sample should consist of a single species of bivalve shellfish collected randomly from the sampling site. Each sample should include a minimum of 20 individuals and at least 200 grams of drained shellfish meat; this provides adequate material for the analysis with a reserve as insurance against a possible lab accident. This is equivalent to a volume of about one (1) cup of shucked meats. It takes up to 40 small sea mussels (about 2 inches shell length) to produce 250 grams of meat. Avoid collecting only a few very large specimens: this may provide misleading information on the presence or absence of toxin.

2. FIELD PREPARATION OF SAMPLES:

Shucked samples.

- (1) Thoroughly clean the outside of shellfish with water.
- (2) Open shell by cutting the adductor muscle(s). DO not use heat or anesthetic before opening shell. Cut carefully to avoid damage to body of mollusk.
- (3) Rinse the opened shellfish to remove sand or other foreign materials.
- (4) For mussels, cut off byssal threads (attachment hairs) with scissors and discard, saving all meat.
- (5) Remove meat from shell by scraping off all attachments to shell.
- (6) Drain meat (preferably with a #10 sieve).
- (7) Place drained meat into a wide-mouthed, 16-ounce sample bottle. About 1/2 to 2/3 of a sample bottle of shellfish meat provides the desired amount. NOTE: Do not overfill; be sure to leave an air space to accommodate expansion upon freezing.
- (8) Tighten cap securely. Refrigerate immediately in the field.
- (9) **Freeze sample** as soon as possible; ensure that sample is frozen prior to shipment.

Appendix B

Extraction Process for Shellfish Toxins

1.0 APPARATUS & SUPPLIES

- 1.1 Electrical Blender - Oster
- 1.2 Analytical Balance - for shellfish puree: Mettler Toledo PB 3002 (0.5-3100 g)
- 1.3 Centrifuge - Fisher Centrifuge Model 225 with polypropylene housing.
- 1.4 Hot Plate - Stirring, Thermolyne 13,000 Multistir or Thermolyne Cimarec 2
- 1.5 1" magnetic stirring bar - teflon covered
- 1.6 Chemical fume hood
- 1.7 600 mL beakers - Pyrex or Kimax
- 1.8 Blender jar, blades, rubber seal and ring - Osterizer Mini-Blender
- 1.9 Sieves, No. 10 stainless steel - VWR Scientific with stacking collection pans.
- 1.10 8-inch diameter, Cat. No. 10SS8H071646
- 1.11 Two - tongue depressors, 6" X 7/8"
- 1.12 Nylon Brush - National Cat. No. 0103
- 1.13 Shucking knife

2.0 REAGENTS

- 2.1 Hydrochloric Acid, (conc.) HCl (12M), (5M), (0.18M) and (3.0 mM)
- 2.2 Sodium Hydroxide, NaOH 50% and 1M

3.0 PREPARATION OF SHELLFISH FOR PSP ANALYSIS - Cleaning and Shucking (Clams, Oysters and Mussels)

Note: Safety glasses or goggles must be worn throughout the following procedures.

- 3.1 Twelve or more animals with the net weight of the meat weighing 100-150 g is considered an adequate sample.
- 3.2 Place unshucked shellfish sample in plastic #10 sieve in sink. Clean outside of shellfish thoroughly with water; use nylon brush for removing dirt from shells. When shellstock have been cleaned, place in a plastic pan with label.
- 3.3 Open shells by inserting knife between shells and cutting the adductor muscles. Avoid damaging visceral body of shellfish, which would cause a loss of PSP toxic material. Rinse inside the shell with fresh H₂O to remove sand or other foreign material after opening.
- 3.4 Remove animal body (meat) from shells by scraping abductor muscles and tissue from shells with knife and dropping intact animal body onto #10 wire mesh sieve.
- 3.5 Drain shellfish in a single layer on a #10 wire mesh sieve for five minutes, discarding fluids.

4.0 HOMOGENIZATION, ACIDIFICATION AND EXTRACTION OF TOXIN FROM SHELLFISH MEAT:

Note: At this point turn hotplates on to preheat.

- 4.1 Use two tongue depressors to remove meat from sieve to Oster blender jar. Fill blender jar with meat to "maximum fill line".

- 4.2 When processing sample (without shells) submitted in a mailing jar which has been previously drained in the field on a #10 wire mesh sieve as described above, whether previously frozen or not, should be placed in blender in its entirety including all fluids.
- 4.3 Place rubber gasket, blades and ring on opening of jar and secure. Grind tissue in blender until homogenous (60-120 seconds). Remove blade unit assembly.
- 4.4 Place 600 mL beaker on zeroed digital balance and record tare weight on worksheet. Add approximately 100 grams blended meat to beaker using the tongue depressor. Record net weight of blended shellfish sample. Record all weights to the nearest 0.1 gram. Add to the beaker an amount of 0.18M HCl by weight equal to that of the blended shellfish (and fluid) in beaker. Record total weight. Mix meat and liquid with tongue of blade until blended.
- 4.5 Determine pH of blended shellfish-acid mixture using pH paper with a range of 0-6, and a sensitivity of 0.5 pH units. Adjust pH to 3.3 (range 2.0 - 4.0). To lower pH, add 5M HCl drop-wise with 5 mL pipette and mix well; to raise pH, add 0.1M NaOH drop-wise with constant stirring to prevent local alkalization and consequent destruction of shellfish toxin (PSP).
- 4.6 Carefully place stir bar into shellfish-acid mixture in beaker. Cover with watch glass to avoid evaporation of water from sample. Using stirring hot plate, heat mixture with gentle stirring to boil gently for 5 minutes. Thermometer must be placed in beaker to determine when 100C boiling point has been reached and the 5 minute boiling time is to begin.
- 4.7 When the 5 minute boiling time has elapsed, let mixture cool to room temperature. Beakers can be placed in an ice water bath (cold packs) to accelerate cooling.
- 4.8 Remove stir bar. Recheck pH, and if necessary, adjust to between pH 2.0 and 4.0 (ideally, pH 3) as in step 4.5.
- 4.9 Bring weight of the total amount of sample/ HCl mixture back up to the beginning weight with 3 mM HCl (pH approx. 3.3) on digital balance.
- 4.10 Pour blended tissue into 5 inch sterile test tubes and label. Balance opposing centrifuge cups with tubes containing tissue and place in centrifuge at the maximum speed setting for 6 minutes.
- 4.11 Separate centrifuged shellfish supernatant extract from solid tissue.