FINAL WORKSHOP REPORT
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Denman Island Disease Risk Assessment and Risk Management Workshop Tacoma, Washington

October 11-12, 2004

Workshop Report

Prepared by the
Pacific Shellfish Institute
Olympia, Washington

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Project Overview

A project to assess risk and formulate risk management strategies in regard to the hazard presented by Denman Island disease (causative agent *Mikrocytos mackini*) of Pacific oysters (*Crassostrea gigas*) was conducted by the Pacific Shellfish Institute, Olympia, Washington, with funding from a Saltonstall-Kennedy Program grant administered by the National Marine Fisheries Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce (grant number NA03NMF4270186).

Prior to the project workshop a detailed project report was drafted and provided to workshop participants. This report consisted of conducting a risk assessment procedure and surveying various interest groups including regulatory authorities in potential importing countries (of live oyster products), regulatory authorities in west coast states of the United States, shellfish disease experts and shellfish producers. This final report entitled Risk Assessment and Risk Management for *Mikrocytos mackini*, Causative Agent of Denman Island Disease of Oysters, is provided in addition to this workshop report.

The workshop was held in Tacoma, Washington, USA on October 11 and 12, 2004.

Key Project Personnel

Project Manager: Ralph Elston, Pacific Shellfish Institute (email: elston@pacshell.org)
Project Report Writer and Researcher: Karen Humphrey, Pacific Shellfish Institute
Overall Workshop Facilitator: Kevin Amos, National Marine Fisheries Service
Workshop Assistant Facilitators: Theodore Meyers, Alaska Department of Fish and Game and Tom Kasari, Animal and Plant Health Inspection Service, U.S. Department of Agriculture
Workshop Rapporteur: Dan Cheney, Pacific Shellfish Institute.
Workshop Logistics: Andy Suhrbier, Pacific Shellfish Institute

Workshop Participants, Affiliations and Expertise

Denman Island Disease Risk Assessment and Risk Management Workshop - PARTICIPANTS	Shellfish Disease or Risk Assessment Expert	State or Federal Regulatory Official	Shellfish Industry Producer	Sponsoring Organization - Pacific Shellfish Institute	Affiliation
Amos, Kevin		●			National Marine Fisheries Service
Becker, Peter			●		Little Skookum Shellfish Growers
Bower, Susan	●	●			Department of Fisheries and Oceans, Canada
Bloomfield, Steve			●		Seattle Shellfish
Burnett, Nicole			●		Lummi Nation Hatchery
Cheney, Dan				●	Pacific Shellfish Institute
Cudd, Sue			●		Whiskey Creek Shellfish Hatchery
Dewey, Bill			●		Taylor Shellfish
Dolphin, Craig			●		Lummi Nation Hatchery
Edwards, Judy			●		Coast Seafoods Company
Elston, Ralph	●			●	Pacific Shellfish Institute
Friedman, Carolyn	●				University of Washington
Gibbons, Jim			●		Seattle Shellfish
House, Marcia	●				Northwest Indian Fisheries Commission
Humphrey, Karen				●	Pacific Shellfish Institute
Kasari, Tom	●				USDA, APHIS (Risk Analysis Specialist)
Kuiper, Linda			●		Kuiper Mariculture
Meyer, Gary	●				Department of Fisheries and Oceans, Canada
Meyers, Ted	●	●			Alaska Department of Fish and Game
Moore, Jim	●	●			California Department of Fish and Game
Rogers, Russell		●			Washington Deparment of Fish and Wildlife
Suhrbier, Andy				●	Pacific Shellfish Institute
Whitney, Jennifer		●			Washington Department of Fish and Wildlife
Attendees by group:	**8**	**6**	**9**	**4**	
Total attendees:	**23**				

Summary of Workshop Narrative, Discussion, Conclusions and Recommendations

The workshop considered risk assessment and risk management for the hazard identified as *Mikrocytos mackini*, causative agent of Denman Island disease of Pacific oysters (*Crassostrea gigas*) and other species. Workshop participants were provided with the pre-workshop report about two weeks prior to the workshop. The workshop first considered the subjects of the report including hazard identification, release assessment, exposure assessment, and consequence assessment. Subsequently, using an approach codified in federal regulations of the United States, the workshop estimated risk of exporting Denman Island disease from selected export farms and areas that produce live molluscan shellfish products. In addition, the workshop identified key research needs that need to be fulfilled in order to more effectively manage the identified hazard.

The workshop utilized 10 factors in considering risk of exporting *Mikrocytos mackini*. These factors are authority, organization and infrastructure; negative disease status of region; disease status of adjacent regions; disease control programs; separation from adjacent regions; movement control from higher risk regions and biosecurity; livestock demographics and marketing practices; disease surveillance; diagnostic laboratory capacity and emergency response capability. Considering these factors in detail, the workshop found that there was negligible risk of exporting *Mikrocytos mackini* with live shellfish products from seven production areas that are recognized USDA shellfish export farm areas in the states of Washington, Oregon and California.

The following research needs were identified by the workshop (listed in order of priority): Perform PCR validation and optimization, determine alternative hosts or vectors – e.g. Manila clams and geoduck clams, and other oyster species; determine how long pathogen can survive outside of host; determine temperature regime of disease; develop more detailed information on the pathogenesis and etiology of the disease to include the following information: susceptibility to DID vs age of host animal, appropriate testing of DID susceptibility in larvae, development of more detailed *M. mackini* life-cycle information; validation of *in situ* hybridization (ISH) method for *M. mackini*; evaluation of effects of other environmental variables such as salinity on disease; review European test results and/or test susceptible oysters.

With regard to risk management, the workshop made the following recommendations: Surveillance on old, at risk, or relic oysters be continued and preferably expanded; shellfish handlers should be trained to identify presence or absence of suspected DID in shellfish; adoption of a best management practices program such as the PCSGA High Health Shellfish Risk Management Plan (this has been done by requirement of USDA-APHIS for authorized export hatcheries and nurseries); consistency should be promoted in shellfish health management between states, tribal governments, and federal governments; and the workshop recommended that relic and at-risk oysters be removed, if possible, during normal culture practices and management of public oyster beds.

Workshop Narrative, Discussion, Conclusions and Recommendations

The workshop agenda is attached to this summary. This document is a summary of the workshop discussion, conclusions and recommendations. Workshop participants were given an opportunity to review this summary.

Hazard Release and Exposure Assessment Discussion

What is the definition of “release”? When a hazard is identified, how is it transmitted? How is the disease released to the general population of shellfish and the environment? Release is equivalent in meaning to “pathway.”

The hazard (*Mikrocytos mackini*) must be present in the exported product for release to occur in the waters of the importing country. The accuracy of the determination of the presence of this hazard in oysters depends on the detection method used. Misidentification/misdiagnosis can occur at the gross inspection level as abscesses from *Mikrocytos mackini* infection can be mistaken for abscesses resulting from *Nocardia crassostreae* infection due to the similarity in appearance at the gross inspection level. Thus it is important not to assume that a lesion is due to *M. mackini* on gross appearance only. Differentiation of nocardiosis from mikrocytosis is relatively easy with histology and cytology.

The question was raised as to whether there are different strains of *M. mackini* or different conditions which cause *M. mackini* to manifest with different virulence levels. There is inadequate information available to answer this question.

As noted in the Risk Assessment and Risk Management Report, Pacific oysters are identified as a primary carrier but the level of susceptibility of other species is less well documented. The Bower et al. (1997) study, conducted at the Pacific Biological Station (PBS) in Nanaimo, British Columbia, used available oysters for challenge including *Ostrea edulis* as a host. There were differences between *Crassostrea gigas* and *C. virginica* and *O. edulis* susceptibility. *Crassostrea gigas* appears to contain the pathogen more effectively than the other species, although DID development among individual oysters of all tested species is not consistent even with high dose inoculation in the laboratory. The reasons for this are not understood. Differences in susceptibility are an important issue in assessing the disease by officials of the European Commission and fisheries agencies in European countries. They are concerned about susceptibility of both *O. edulis* and *C. gigas*.

A few responses to the pre-workshop survey were received from European regulators. According to one response, the perception was that infection with *M. mackini* was responsible for the decline in native Olympia oysters (*Ostrea conchaphila*). There is no scientific evidence documenting any association between DID and the decline of the Olympia oyster

populations. The disease has never been detected in native oysters in Washington State despite surveillance testing by both PCR and histology. Although the Bower et al. (1997) experiments showed that native oysters are infected in nature, it is not known if Denman Island disease is or was responsible for the decline of that oyster in either British Columbia or Washington State. The workshop participants agreed that a greater degree of surveillance for DID in Washington State native oysters would be useful. It is difficult to detect suspected DID grossly in the small Olympia oyster, so PCR and/or histological testing would be beneficial. Due to the current interest in restoration of native oyster populations in Washington State, it is important to assess prevalence and intensity of infection in this species and the biology of the parasite in the oyster. Presently, there is insufficient knowledge to know if DID is endemic in species along the west coast of North America. Its origin (imported or native parasite) is unknown. While the disease has not been detected in Asia, there has apparently been limited or no testing on that continent.

M. mackini has not been detected in crabs or fish, but such testing may have had limited sensitivity. Tests for presence of the parasite, using the PCR method, in geoduck clam seed were negative when the clams were exposed experimentally by Bower and colleagues at PBS. If other bivalves are not susceptible, they may be able to destroy the parasite and prevent the initiation of or sustaining of an infection.

Is there resistance to DID occurring within oyster populations? This seems possible due to the low prevalence found in some populations, although this could also be due to a low transmissibility of the parasite. Individuals or populations of *C. gigas* may be resistant to infection, resulting in the low observed prevalence in Washington State. Resistance might also be a factor in the laboratory oysters that did not manifest DID despite heavy inoculation with the parasite.

Other poorly understood biological factors include the apparent tendency for only older oysters to become infected and the inconsistent ability to transmit the disease in laboratory challenges, the loss of infectivity of the parasite in experimental challenges and the apparent resolution of the infection in infected oysters. However, experimental work by scientists at the Pacific Biological Station, Nanaimo, British Columbia, has shown that juvenile oysters can become infected. However the relative susceptibility by age of oysters has not been experimentally evaluated.

Regarding geographic extent, it was noted that the parasite has not been detected in Alaska, although the cold seawater temperatures would be permissive for infection. The surveillance data for the State of Alaska is contained in an appendix of the risk assessment and risk management report.

Regarding host susceptibility, if it is possible to infect a species experimentally, then field trials are more important and should form a basis for risk assessment. Infection in laboratory trials with a heavy pathogen burden may not accurately reflect degree of host susceptibility in the field. Species for which susceptibility is unknown include *C. ariakensis* and other *Crassostrea* spp., but field experiments have shown that *Crassostrea virginica* is susceptible to infection.

The question was raised as to whether Denman Island disease might already exist in Europe and whether this has been examined by surveillance. The disease does not spread easily and can be very unapparent unless a surveillance program is specifically looking for it. DID was not apparent in Washington State until specifically looked for in older "feral" oysters. This subject was held for discussion later in the workshop.

Another concern raised was whether or not Manila clams and mussels can be vectors of *M. mackini*. The parasite has never been detected in Manila clams in British Columbia by histopathology and has not been detected by PCR in Manila clams held adjacent to parasite positive oysters in a test site in Washington. The Pacific Biological Station (Department of Fisheries and Oceans, Canada; Dr. Susan Bower and Gary Meyer) are planning to repeat Manila clam challenge trials, which were not finished in a first attempt due to technical problems. In this first trial these researchers planned to expose Manila clams to high levels of DID, then hold clams in parasite-free water to assess reduction in parasite load but they were unable to infect the clams or create a vector condition. They plan to attempt a repeat of this experiment as soon as parasites are available – possibly in Spring 2005 or earlier. At present, there are no plans to test mussels or other potential vector species, but such tests will probably be carried out in future trials. The primary issue that has currently been posed is "can Manila clams act as vectors or carriers of *M. mackini*?" and this question will be tested first.

It was noted by workshop participants that laboratory challenges with massive concentrations of parasite exposure do not replicate field conditions. A compromise would be to place Manila clams and other test species in locations where parasites are present in field conditions. According to the PBS researchers, such field trials are planned for the future.

Experimental work is limited by the fact that *M. mackini* cannot be cultivated or cultured *in vitro*, and therefore, researchers must rely on parasites that are available in living oysters. The parasite requires cooler temperatures (less than 15°C) or oysters apparently do not become infected. Exposed oysters require several months at reduced temperatures (10°C) before the disease will develop. There may also be a seasonal component or cycle of infection. It is also difficult to maintain infected animals in the lab. Cryopreservation is possible, but some mortality of the parasite occurs. To obtain heavily infected oysters, the PBS researchers first isolate *M. mackini* from naturally infected oysters in the spring and then transmit the parasite in the laboratory via syringe inoculation to increase infection intensity. Inoculated oysters are maintained at less than 10°C for 8-12 weeks. However, less than 50% of the oysters develop heavy infections. This procedure required 6 months to get infected oysters from which parasites could be harvested - a 3 month field exposure followed by 3 months at cold temperature in the laboratory. An additional limitation, even in British Columbia, is that the distribution of the parasite is patchy and it is usually difficult to find areas with high prevalence and intensity of infection.

Recent research by PBS investigators found infected juvenile oysters from a known Denman Island disease enzootic intertidal zone, using *in situ* hybridization (ISH). These

oysters were not expressing signs of disease and there was no mortality in juveniles. In another location where 42% of the 4 year old *C. gigas* in suspended culture had macroscopic signs of DID and an estimated 7% mortality, uninfected juvenile oysters were suspended in close proximity to the infected oysters and placed on the nearby intertidal beach. After 10 weeks of exposure, *M. mackini* was detected in the suspended group but not in the intertidal group. We have no information on occurrence of *M. mackini* in the water column, but it is assumed that larval oysters would ingest such parasites, if present. It is unknown as to whether or not *M. mackini* can be carried with gametes or discharged during spawning. The disease can be transmitted in water via cohabitation experiments, but there has been no sampling and testing of water over infected animals in the field due to technical difficulties of sampling and detection in the water column. Due to dependence of the parasite on host cell mitochondria, its survival period outside the host is expected to be short. If the disease is easily transmitted there should be many more infected oysters. There may also be some host factors causing variations in rates or level of infection.

Age of oyster and susceptibility. How is the age of the oyster related to susceptibility to infection by *M. mackini*? Commercial oysters are moved at various ages. Seed is shipped with the intention of placing it in the water. Older animals may also be placed in the water. In Washington State, positive oysters are generally those considered "feral" (i.e. old oysters outside of or on the periphery of managed growing areas). Over 10 years of histopathological surveillance and 1 ½ years of PCR testing in hatcheries, there have been no positives in seed, larvae or broodstock. A more precise definition of wild, feral and cultured oysters would facilitate the discussion. Cultured oyster seed is produced in a hatchery, planted to grow-out beds and generally harvested in 2 ½ years. However, it is possible that some older oysters from prior plantings could be harvested. Such larger oysters may be processed by shucking, or could be sold as single oysters but according to producers, most such oysters which they consider an incidental or specialty market, are sold frozen into Asian markets.

Broodstock management. Broodstock from exporting hatcheries are maintained in areas that have a long disease-negative surveillance history. To increase biosecurity, it would be additionally advisable to remove any oysters over three years old from such areas. With regard to OIE recommendations, disease-free zoning can be done by farm areas but directives of the European Commission state that the water supply must be certified disease free; or shown to be unexposed to disease-carrying animals. The distance that broodstock or harvest animal populations must be separated from the site of an infected population to be considered "unexposed" to disease-carrying animals has not been determined.

Testing in relation to control of disease. What are appropriate tests? In Washington State and California, there is as much as 20 years of surveillance data, based on histology. What is the statistical value of these repetitive, but less sensitive tests? How does this relate to PCR testing? In regard to testing, the policy of the State of Alaska is that the results of the three types of testing used must be considered together. For example, Lummi Hatchery (a hatchery not approved for export by the U.S.D.A.) has negative histology on seed product but a positive PCR reaction. The follow-up ISH test, specific for the parasite, was negative. As a

result, seed produced in this facility were allowed to be shipped into the State of Alaska. A researcher from British Columbia (G. Meyer) concurred with this interpretation of results.

Discussion of presence of parasite vs presence of disease. If the disease was present it would have been detected with histology. But the possibility was raised that the parasite was more widely distributed or present for a longer period of time but did not cause disease (from Washington State southward). There has been ample opportunity for transmission to and from Washington State associated with movement of broodstock and live oysters back and forth. Environmental conditions may have differed historically between Washington and British Columbia thereby imposing a barrier to the transmission of *M. mackini*. Water temperature may be an important factor regulating presence or absence of the disease.

The historical record in older animals (2 to 5 yrs old) and broodstock, has shown no DID positive oysters in Washington State and areas to the south. However, British Columbia and recent Washington State surveillance, where large older oysters were specifically targeted for sampling, did detect DID by PCR at a low prevalence (maximum population prevalence about 6% in Washington State). The large volume of historical surveillance data is mainly from locations with broodstock or seed production, whereas recent DID-positive collections are from one non-exporting hatchery in a disease enzootic zone, from public harvest sites that contain old oysters or from privately managed tidelands within Denman Island disease enzootic areas.

The historical record of histological samples could be examined by ISH because the DNA sequences in sections are very stable. However, this would be very expensive and probably not cost effective. The historical record of histology is substantial and should be confirmed going forward with supplementary molecular methods, primarily PCR. It was further noted that sampling should take place prior to mid-June because the disease appears to go into remission with warming water temperatures, or susceptible oysters may have died by this time of the season. Sampling of Pacific oyster broodstocks by commercial hatcheries in Washington has intentionally been conducted in April of each year in order to allow detection of DID if present.

Discussion of testing methods and their interpretation. A recent case involved positive results by PCR but negative results by histology and ISH. The positive PCR test could be a DNA fragment or a laboratory technical error. In such a case it was considered by some to be advisable to conduct additional PCR testing. In addition, tests at the positive non-exporting hatchery showed that the positive PCR signal could be depurated by holding the oyster seed in a different location for three days. This test should also be repeated to increase confidence in the results.

The PCR assay for *M. mackini* needs to be validated. The existing PCR method is still at an early stage of development and needs further validation. The existing *M. mackini* PCR is a good screening tool as it has been shown to be more sensitive than histology. However, positive PCR results should be confirmed by other testing methods (histology, ISH). Further validation of the PCR test is needed to build confidence that there is no cross reaction with any organism with a similar DNA sequence and, until further testing is done, it is not possible

to be completely sure that the primers amplify a sequence from *M. mackini* exclusively. Also, there may be more appropriate primers that could be selected for the PCR test, as indicated by a primer selection computer program recently run at the University of Washington. In addition, the PCR test should be fully optimized as well. The need to validate and optimize the PCR test for *M. mackini*, including the development of alternative primer sequences, was agreed to be a high priority by the workshop.

PCR positive results should always be confirmed, as every laboratory test has false-positive results. As pointed out by the APHIS representative, the rate of false-positive results for any test can be statistically predicted based on the sensitivity/specificity of the test. Even the most highly refined test, like the test for BSE, will have a false-positive rate. It may be as low as one in every 10,000 tests for a test with a 99.99% specificity—but if you never have a false-positive, there is something wrong with the test. Because a certain rate of false-positives is to be expected, positive results should be confirmed by re-running the PCR, and, if still positive, by other testing as appropriate.

The molecular testing protocol is not currently an OIE issue since there are no molecular methods specified for *M. mackini* in the OIE Aquatic Animal Diagnostic Manual. However, for exports to the European Union, it may be necessary to have a validated PCR and ISH method. From a risk management standpoint, there is a need to establish detection limits as well as sensitivity and specificity information for the test. The problem with ISH is that it is expensive and time-consuming. ISH was agreed best for confirmation testing.

The current OIE Diagnostic Manual recommends a number of diagnostic methods for some molluscan diseases. However, the general feeling of the workshop is that molecular tools are good research tools, but not appropriate for normal diagnostic testing until fully validated. There is still great value in histology in detection of disease – although it may not detect presence of organisms in subclinically-infected oysters.

Based on the historic record (10 or more years) using clinical and histological examinations, there is no evidence that the disease occurs in cultured broodstock populations in Washington State. Because these samples were collected under conditions that would optimize detection of both the disease and the parasite, these data strongly support a conclusion that the parasite is absent from the tested populations. Again, if there was sufficient reason, ISH used on archived samples could confirm this supposition, but this would be a considerable expense and probably not cost effective, but the workshop group agreed that this was a technically sound approach.

For the future, it was agreed by the workshop participants that an optimized and validated PCR test for *M. mackini* would provide a useful detection and screening tool. Thus the workshop considered what would be needed to optimize and validate the test and concluded the following:

- Design and test new primer sets
- Optimize primer and DNA concentration in test
- Sequence the amplicons from these primer sets

- Establish the detection limit of the test
- Compare samples blindly against samples of known status from other labs and conduct a cross-laboratory validation involving at least three laboratories
- Establish the numerical sensitivity and specificity of the test
- Compare PCR against histology

These objectives were given a high priority for future research and funding by workshop participants.

Discussion of surveillance for M. mackini and Denman Island disease.

Shellfish producers: Production managers should maintain a log of the health status of cultured shellfish that can be assessed at harvest or, as needed, prior to shipment. This is already a requirement for those farms participating in the Pacific Coast Shellfish Growers Association Shellfish High Health Program. However, detection sensitivity by gross diagnosis by shellfish farmers will be difficult when there is a low prevalence, as occurs in Washington State. It will require some training or educational material for shellfish plant managers and oyster shuckers to recognize DID. End users such as consumers and restaurants are likely to notice gross lesions of the disease unless the disease is in an early stage of development.

In California, there is a regular history of individual farm surveillance and seed surveillance for certain larger producers in Humboldt Bay (where 70% of California oysters are produced) but the health inspection history may be sporadic for smaller farms not located in Humboldt Bay. The California Department of Fish and Game recently completed a state-wide disease survey using histology and sampling for PCR. There have been no positives for *M. mackini* but the PCR tests have been completed only for Humboldt Bay.

In Oregon there is an approximate 10 year history of larval certifications and some broodstock surveillance in Netarts Bay, the site of an export hatchery. There has been no detection of *M. mackini* from this location.

The presence or absence of the disease in Europe is unknown, but until tested or found positive, European shellfish are considered negative for the disease.

In Alaska, certification to import Pacific oyster seed requires considerable information from hatcheries: (1) animals must be evaluated by histology and PCR (broodstock and larvae); no oysters larger than 20 mm can be imported into the state; no oyster seed is exported from Alaska; a site producing Pacific oyster seed for sale into Alaska must be certified by surveillance examination for one year, followed by review of status once every 3 years. In Alaska, instate transport is also regulated. If the distance of proposed transport of shellfish is greater than the natural planktonic drift zone, the shellfish must be examined. In addition, sampling by growers is required and the State has up to 20 years of disease surveillance records. There is one in-state shellfish hatchery that cannot supply sufficient Pacific oyster seed for the State's needs. In Alaska, water temperatures rarely exceed 10°C.

Most surveillance inspections are done in the spring. In the future, histological examinations will be supplemented by PCR testing.

In Washington State, health certifications are required for transfer of shellfish from inside a Denman Island disease surveillance area to outside of the area and may be required for shellfish movements within a surveillance area. It is optional for shellfish producers to determine whether histology or PCR is to be used. If a population is found positive for *M. mackini*, it is declared a "prohibited area" and a larger area surrounding the prohibited area is the designated "surveillance area." Within Washington State, Denman Island disease has only been found in Puget Sound and not in the major oyster growing areas of Willapa Bay and Gray's Harbor. The regulatory authority in Washington State, the Washington Department of Fish and Wildlife, conducts annual surveillance of 10 to 14 sample areas in areas of likely high risk. High-risk animals such as larger oysters from lower tidal elevations (- 2 feet MLLW) are targeted, although oysters found in other conditions may also be sampled. It was noted that in British Columbia, researchers are finding infections at tidal elevations as high as +3 feet and +4 ft MLLW and also in suspended systems.

The Lummi Hatchery (a facility not approved for export by the U.S.D.A) is in a Denman Island disease enzootic zone, but the hatchery is testing all batches of Pacific oysters produced using the PCR test of *M. mackini*.

The workshop agreed that it could be stated with a high degree of confidence that there is no evidence of Denman Island disease in the states of Oregon, California, Alaska and Hawaii and that the low prevalence of Denman Island disease found in Washington State occurred in both public and private oyster beds with variable intensities of management.

Future surveillance could consider routine sampling (150 once a year, or alternatively, 60 twice a year, although the latter may be inconsistent with OIE guidelines) coupled with field and in-plant observations by the industry. While there may be added cost, this step could improve credibility to foreign importers. However, the possible need to screen all processed oysters would greatly increase the cost, and the risk reduction benefit of doing so would have to be considered in view of the low prevalence of the disease. While most recorded surveillance testing has been in broodstock, surveillance on private tidelands has found many areas to be disease free but several tests of oysters on private tidelands were positive at low prevalence for the disease (e.g. P.S.I. 2000 and WDFW surveillance data).

Control program for Denman Island disease. Eradication seems untenable in British Columbia as it would be extremely difficult and expensive, if not impossible, to remove all oysters because infected animals are widely distributed and there may be unknown host species. In Washington State, there are limited and defined infected populations. Thus it could be possible to remove all infected animals but more surveillance data on the distribution of DID in Puget Sound should be obtained in order to better evaluate the probability of success of an eradication program. Such surveillance should focus on oyster growing areas important for export. We presently have data on presence of DID in the areas already sampled but more comprehensive geographic sampling is needed. Alternatively, greater effort should be expended on improved understanding of the biological responses of the

animals to associated field conditions. It appears that some of the infected oysters are found in public shellfish beds that have not been harvested for many years. Therefore, it was suggested that the Washington Department of Fish and Wildlife could reduce the prevalence and concentration of infective units in the environment by allowing or facilitating the harvest of such old oysters to an end use. Similarly, the removal of old oysters from privately owned tidelands could have a similar beneficial effect, if such a method was tested and found effective, regardless of management of the tideland areas.

Is eradication technically feasible? It would be useful to evaluate the results of removal of diseased oysters from a prohibited area (including adjacent animals and empty shells). It was mentioned that such a program would not likely be compromised by the illegal movement of wild or cultured adult oysters. However, evaluating the success of an eradication program is partially constrained by our limited understanding of the disease – i.e. what level of eradication would reduce the risk of transmission to the population?

In conclusion of the subject, should eradication be considered? Generally, it was concluded that we need more information prior to attempting an eradication program. There is a need to survey oyster beds adjacent to known infected foci to determine the extent of the disease. Without more comprehensive information on the distribution of the disease, an eradication effort would probably not be sufficient to resolve the perception problem in Europe. The real problem with DID is that it may be viewed as a bigger problem than actually exists, and if an eradication program were begun today, many uninfected sites might be eradicated as well as known foci of infection in Washington. Setting up study areas to determine the effectiveness of systematic removal of relic and at-risk oysters in eliminating DID is warranted. If this is effective for eradicating DID, it could be considered as a management tool, but the effort and cost would need to be justified by showing that this method is effective.

Surveillance Zones for Denman Island Disease in Washington State

Surveillance zones for DID are now erected in Washington State. If these surveillance zones were eliminated in Washington, individual transfer permits for each and every shellfish movement would be required. With or without a surveillance zone, importers of Washington-produced shellfish would likely require suitable documentation from hatcheries or growers to ensure products are DID-free. For live shellfish exports to the European Union, batch-testing has been an acceptable method of determining disease-free status. Based on OIE recommendations there is growing interest in developing a zoning approach to infectious disease control for live mollusks. Also, the expectation is that interstate trade practices will be the same as international practices. However, it is recognized that the erection of specific pathogen zones requires a substantial investment in surveillance and organization, and resources would need to be identified to support the development of such a system. Under the current system of private producer and state surveillance, data for disease enzootic and disease-free zones has been accumulated in some areas and is being accumulated in other areas.

The workshop discussed and noted that it is the basic understanding that the parasite is very dependent on the host cell and host cell mitochondria. Once they are separated from host cells, it is likely they have a very short survival time. This fact suggests a possible rationale for the observed extremely patchy distribution and low prevalence of DID in Washington State.

Consequence Assessment

The workshop considered the question: "What does the disease mean to you?" in regard to shellfish producers in British Columbia and Washington State. The following comments are paraphrased from workshop participants:

British Columbia. Not much effect occurs in B.C. from DID. Until last summer, BC growers managed around the disease. Typically there have been sporadic events, and processors have noted a few diseased animals. The worst case to date, which stands out from the norm, is that in 2004, one processor rejected product with many diseased oysters from a suspension system farm which held the oysters in culture one year longer (to 4 years of age) than is typical. Rejected oysters went to a landfill. Beaches in the vicinity of the diseased oysters were known to have infected oysters in the past but it is not known where or when the infected hanging stock oysters acquired the parasite.

Washington. The disease is of no consequence to oyster producers because it has not caused any observable losses and has never been found in any stocks from APHIS registered export farms. The only consequence has been regulatory and financial since the finding of the disease at a uniformly low prevalence in Washington oysters, particularly older oysters, may have caused reluctance to accept Manila clam shellstock in European countries.

Consequence of DID for native oysters, Ostrea conchaphila.

In regard to infection of native oysters, the Pacific Biological Station Scientists have conducted one laboratory and one field exposure study in British Columbia, both of which confirmed that *O. conchaphila* was susceptible to infection. They were also able to demonstrate that native oysters collected in the field can be infected with the parasite. In Washington, there are serious efforts to restore native oysters to the northern reaches of Puget Sound where populations are minimal, in contrast to the southern reaches of Puget Sound where the oyster is still relatively abundant. In contrast to the situation in British Columbia, all native oyster populations tested for *Mikrocytos mackini* (using PCR or histology) in Washington State have tested negative. A morphologically similar microcell has been observed in Olympia oysters from San Francisco Bay but appears to be genetically distinct from *Mikrocytos mackini* (Friedman et al. 2005).

Evaluation of water treatment strategies

Scientists at the workshop postulated that it is likely that treatment with UV irradiation can kill the parasite. This is an issue in regard to shipment of live shellstock product into Europe because some rehydrating facilities in Europe don't have depuration equipment. It would also be useful to do an untreated seawater depuration test.

Because DID is not viewed as an important issue in the United States because the disease is so limited in prevalence and has no biological impact on oyster farming, it is difficult to justify requests for external funding (such as from state or national Sea Grant Institutes or the U.S. Department of Agriculture). However, the disease has a potentially great impact on the ability of U.S. shellfish producers to export product due to misperceptions of importing countries, lack of scientific documentation of possible vector status of non-oyster shellfish and insufficient surveillance data on the distribution of wild product as well as the need for a pro-active management program of the disease in Washington State. It was therefore deemed important to educate such funding agencies that there is a very significant economic impact and to provide substantiated cost estimates of that impact.

Cost of testing

The cost of testing is an issue for hatcheries and production facilities, particularly if they are located in a disease enzootic area. Only one hatchery (non-exporting) in Washington State is located in a disease enzootic area but this hatchery has had additional testing costs in 2003 and 2004 to identify sources of *Mikrocytos mackini*-free broodstock and to verify that their seed stock production of both Pacific and native oysters and Manila clams are Denman Island disease-free, which they have been to date.

Risk Estimation

Risk Assessment Scenarios. The workshop considered the risk of exporting shellfish product from different embayments in the four states of the United States that export live molluscan shellfish (Washington, Oregon, California and Hawaii).

The question of whether or not sufficient similarities exist between different production sites to allow grouping of sites was first considered. The consensus was that there are not sufficient similarities to allow grouping of sites. Therefore, the workshop considered sites separately in terms of assessing risk. The following information, based on farm and agency authority monitoring was stated and agreed upon by workshop participants:

1. No Denman Island disease has ever been reported in Alaska, Hawaii or California.

2. In Oregon, there is a published report of microcells similar in morphology to Denman Island disease in Yaquina Bay but there are no reports of microcells in other Oregon bays which have a history of surveillance, including Netarts Bay and Coos Bay. It

was stated that 99%+ of shellfish growers in Oregon receive seed produced within Oregon but the actual percentage value was not documented.

3. In Washington State, the major oyster producing embayment, Willapa Bay, is free of Denman Island disease, based on an extensive surveillance history and recent and ongoing confirmation by the use of the PCR technique for detection of *Mikrocytos mackini*.

The risk assessment was conducted on the basis of the following factors (Risk Factors Considered in the Animal and Plant Health Inspection Service [APHIS, U.S. Department of Agriculture] Regionalization Rule), agreed upon by the workshop, with the discussion facilitated by U.S. Department of Agriculture, Animal and Plant Health Inspection Service risk analyst Dr. Tom Kasari:

1. Authority, organization and infrastructure
2. Negative disease status of region
3. Disease status of adjacent regions
4. Disease control program
5. Vaccination status [*not applicable to mollusks*]
6. Separation from adjacent regions
7. Movement control from higher risk regions and biosecurity
8. Livestock demographics and marketing practices
9. Disease surveillance
10. Diagnostic laboratory capacity
11. Emergency response capability
12. Risk assessment based on consideration of factors 1 to 11.

In regard to risk classification the workshop agreed that the history of surveillance and volume of product shown to be disease-free are also important criteria. For purposes of this evaluation, the risk estimation is based on historical data, existing research data, and understanding and documentation of biological and environmental influences. This approach is based on the US Code of Federal Regulations (9CFR92.2) that provides guidance for required or likely requested documentation.

Based on this approach, major oyster producing embayments in Washington, Oregon, California and Hawaii were considered in terms of risk assessment in regard to the risk of spreading Denman Island disease. The results of this assessment are presented in Workshop Table 1, supplemented by the following discussion notes for each location.

The workshop agreed not to consider the risk in terms of any particular importing regulatory framework, but from a purely biological risk assessment standpoint, as noted in the 12 features of risk assessment used by USDA - APHIS (less item number 5 which is not relevant to mollusks).

While species other than oysters are considered for export, there has never been a detection of *M. mackini* in non-oyster species. The workshop agreed that when Denman Island disease

was considered for a given area, other species would be included in the consideration but that it was important to consider the volume of the other species product that was under consideration.

The following table summarizes the conclusions of the workshop on site and product specific risk assessment. Narrative commentary relevant to the sites considered and represented in Workshop Table 1, (constructed during workshop deliberations), is included in the text following Workshop Table 1.

Humboldt Bay, California

Humboldt Bay, California produces Manila clam seed, Pacific oyster and Kumamoto oyster seed for export, as well as shell stock products for sale domestically. There are two APHIS recognized export farms in Humboldt Bay, Coast Seafoods Company and Kuiper Mariculture. Shellfish larvae and seed, subject to examination and permitting of the larvae, seed, and the broodstock, are produced in Quilcene Bay, Washington or Netarts Bay, Oregon, both from APHIS recognized export facilities.

A negligible (= acceptable) risk determination was unanimously made by the workshop for all oyster species, in regard to Denman Island disease, based on testing in Washington, Oregon and California and on the approximate 20 year history of examination of oysters in Humboldt Bay with all negative results for Denman Island disease. Water temperatures in Humboldt Bay may be too high to support the maintenance of *M. mackini*. While the parasite or the disease has never been observed by gross and histologic examination, the workshop agreed that it would be useful and further build confidence if additional tests were done to confirm that oysters and clams produced in Humboldt Bay were not carriers of the parasite.

It was also noted that Kumamoto oysters have been tested in California and elsewhere and there has never been a positive finding for DID.

Netarts Bay, Oregon

Whiskey Creek Shellfish Hatchery has produced a variety of larvae, including Manila clam larvae and Pacific oyster larvae for export for approximately 20 years. The facility is an APHIS-recognized export facility. There have been no detections of Denman Island disease from any location in Oregon and there is a long surveillance history for product from the facility and a recently established (in 2002) surveillance history for broodstock used at the facility. The facility may receive broodstock from Tillamook Bay, Oregon, Willapa Bay or Totten Inlet, Washington but all broodstocks are certified to be free of Denman Island disease and other Notifiable shellfish diseases. In consideration of the negative DID history and the APHIS export status of the facility (requiring a Shellfish High Health Program), a negligible risk determination was agreed upon by the workshop.

Willapa Bay, Washington

In reference to #8, livestock movement and demographics, some products for processing are transported to processing plants on Willapa Bay and these products may come from Denman Island disease enzootic areas. However, such movements of shell stock from DID enzootic areas must be done by permit from the Washington Department of Fish and Wildlife and safeguards are incorporated into the permit, such as processing is only allowed in areas of the plant that are isolated from discharges to Willapa Bay, process water must be disinfected and processed shell must be placed at uplands areas away from the Bay. There is little transport of seed stocks out of Willapa Bay, if any. Cultch shell may be transported out of the region. Selected areas of Willapa Bay are used for broodstock maintenance. Two years of sampling selected sites by histology and PCR for *M. mackini* has failed to produce any positive findings for the parasite.

Workshop Table 1. Summary of workshop *Mikrocytos mackini* risk consideration and assignments for production sites.

Location	Humboldt Bay, California	Netarts Bay, Oregon	Willapa Bay, Washington	Totten Inlet, Washington	Oakland Bay, Washington	NEHLA Aquaculture Facility, Kailua-Kona, Hawaii	Quilcene and Dabob Bays, Washington	All *Mikrocytos mackini* negative sites	All *Mikrocytos mackini* positive sites
Species:	All	All	All	All	All	All	All	Geoduck	Pacific Oysters
1 - Authority	Y	Y	Y	Y	Y	Y	Y	Y	Y
2 - Status	NEG	NEG	NEG	NEG	NEG[2]	NEG	NEG	NEG	POS
3 - Distance	600 MI	350 MI	140 MI	12 mi	14 MI	1000'S MI	60 mi		0 MI
4 - Control	Y	Y	Y	Y	Y	Y	Y	Y	Y
5 - Vaccination									
6 - Separation	Y	Y	Y	Y	Y	Y	Y	Y	Y
7 - Biosecurity	Y	Y	Y	Y	Y	Y	Y	Y	Y
8 - Marketing	Y	Y	Y	Y	Y[3]	Y[4]	Y	Y	Y
9 - Surveillance	Y	Y	Y	Y	Y	Y	Y	Y	Y
10 - Diagnostics	Y	Y	Y	Y	Y	Y	Y	Y	Y
11 - Emergency response	Y	Y	Y	Y	Y	Y	Y	Y	Y
12 - Risk	Negligible	Negligible	Negligible	Negligible[1]	Negligible[1]	Negligible	Negligible	Negligible	

[1]Proximity to DID source possible concern.
[2]See discussion in narrative
[3]Flupsy -- High volume of product, mostly seed, moving interstate and international (= "feedlot")
[4]High volumes of product moving through facility; adjacent to other operators producing other product with good separation and Shellfish High Health Program in place.

Totten Inlet, Washington

Oakland Bay was previously thought to be positive for *M. mackini*, based on early PCR results. However, this area, which was the closest DID positive to Totten Inlet, was later repeatedly tested for *M. mackini* and found negative while reevaluation of the original results strongly indicated that the positive was a false positive. Therefore, the nearest positive area for *M. mackini* to Totten Inlet is McMiken Island, Puget Sound, Washington.

Totten Inlet has valuable broodstock, is an extensively studied area and biosecurity measures, as outlined in the Shellfish High Health Program guideline, are in place. Pedigreed families from the Molluscan Broodstock Program are highly controlled. The issue of risk of sabotage by rogue farmers who might have an interest in damaging a competitor was raised. There is no indication that this has ever occurred. A bigger risk factor may be the presence of older oysters which could be harboring DID in Totten Inlet. Surveillance in Totten has been directed to cultured animals. The workshop agreed that there was a negligible risk of exporting Denman Island disease using Totten Inlet broodstock, based on a nine year surveillance history, and the management of broodstocks using the Shellfish High Health Program.

Oakland Bay, Washington

Denman Island disease status in Oakland Bay: Pacific oyster adults sampled in July 2002 tested PCR positive and histologically negative in initial testing; 1 ½ months later native oysters also tested PCR positive. Representative samples of the same Pacific and native oyster samples were ISH negative. An opinion from the testing scientist at the Pacific Biological Station (Gary Meyer) was that the results, taken together, should be interpreted as a negative. Oysters from the same area, if not precisely the same site, were re-sampled in February 2003 and tested by *M. mackini* PCR and histology and all samples were negative. Sampling was at the optimal time for manifestation of Denman Island disease.

In summary, the initial positive was probably a false positive through some lab error or other problem (such as an unknown, but related species). The DID-PCR test is only 2 years old and needs better primers, optimization, validation, and other measures to improve performance. The Oakland Bay episode was the first time the PCR test was performed and the outcome of the test suggested a false positive (i.e. too high a proportion of positives by PCR that could not be confirmed by other methods or resampling).

Nonetheless, the Washington Department of Fish and Wildlife remains concerned that there is still a risk of unreported disease – and that this information should be reported to importers. However, the workshop pointed out that PCR is not an accepted method in the OIE manual and that it is the federal authority (APHIS) that has the responsibility and expertise to make a determination of whether the finding rises to the level of OIE reportability. The initial positive result was, in greatest likelihood, a laboratory technology problem. For the Alaska Department of Fish and Game, which employs an expert with training and experience in shellfish disease management, the fact that further testing was negative and infectious agents were not present resulted in a determination that there would

be no restrictions on import. The opinion was expressed that it may be reasonable to inform importers that we have a false positive test that was not confirmed. However, USDA policy is to not announce false positives unless they can be confirmed with follow-up testing. Multiple tests are needed to fully confirm the results (e.g. histology and *in situ* hybridization). It was noted that transparency in representing results is desirable but a threshold of technical credibility and confirmation of the result must be achieved. In addition, economic and other issues may alter the way results are presented. A problem with low-prevalence diseases is that false positives increase – which is a particular problem when the diagnostic procedure is at an early or undeveloped stage of development. In summary, based strictly on scientific grounds, all positives need to be confirmed. OIE guidance on this matter should be followed.

The establishment by the Washington Department of Fish and Wildlife of the DID disease prohibited area in Oakland Bay, in view of the uncertainty of the results from Oakland Bay, provided assurance to neighboring states that action had been taken to contain the disease, if it did indeed occur in Oakland Bay.

In addition to the follow-up testing in Oakland Bay, extensive testing of old oysters and other invertebrates in the vicinity of the floating upweller by PCR and histology produced only negative results.

A high volume of product moves in and out of the Oakland Bay floating upweller. This consists of relatively small seed coming from one hatchery source. Mixed species are held in very high densities, somewhat analogous to a "feed lot."

After discussion, the workshop unanimously agreed that the risk of transferring Denman Island disease from this Oakland Bay seed production site was negligible.

Natural Energy Laboratory of Hawaii Authority (NELHA) and HOST (Hawaii Ocean Science and Technology) Park.

This is an aquaculture zone that is highly regulated by the Hawaii Department of Agriculture and the Natural Energy Laboratory of Hawaii Authority (NELHA). In addition to producer Shellfish High Health Programs that may be in place for export farms approved by APHIS, NELHA has an Aquatic Animal High Health Program by which all tenants must abide. In addition, producer records and surveillance records indicate no findings of Denman Island disease. Therefore, the workshop agreed that a negligible risk assignment was justified.

Quilcene and Dabob Bays, Washington

These two oyster producing embayments were combined for consideration by the workshop, due to their geographic proximity. The workshop agreed that a negligible risk

determination was justified based on surveillance history. The following issues were raised in regard to future management of facilities in these locations:

Geoduck seed from Puget Sound locations produced at the Dabob Bay hatchery:

(1) How old are the animals and what are the DID exposure conditions?
(2) Genetic origin of the broodstock must be known
(3) Other micro-organisms may be present, and a precautionary approach is recommended. Related to DID, geoducks are unlikely to be infected but could be carriers.

Native oyster (Ostrea conchaphila) production at Dabob hatchery:

(1) There are very few animals in northern Puget Sound while the species is common in southern Puget Sound. The lower numbers in some areas pose a problem for sampling for DID. There has been no DID found in native oysters in Washington to date, and there has been sampling by PCR and histology of at least six populations (n=60). There is a plan to produce these oysters in the Dabob hatchery and Oakland Bay floating upweller. This could be a future problem if DID prohibited zones are extended to locations where native oyster broodstock are available. For example, Samish Bay is positive for DID, and is a possible source of native oyster broodstock. However, broodstock are now obtained from the San Juan Islands, Washington.

Feral Oysters

There is a need to consider specific locations. Relic feral oysters found around production facilities (Dabob hatchery) and all of Hood Canal are negative. Other risk factors must be incorporated in the analysis, including mitigation (farm management, treatment, etc.). A Puget Sound-wide approach is needed to manage the disease, particularly if it becomes known to be more widespread, although there is no indication at present that this is the case.

Risk Management Recommendations

The Workshop considered risk management recommendations. The following risk management recommendations were made by the Workshop.

1. It is important that surveillance on old, at risk, or relic oysters be continued and preferably expanded

2. Shellfish shuckers and shellfish handlers should be trained to identify presence or absence of suspected DID in shellfish

3. Adopt as a BMP the PCSGA High Health Shellfish Risk Management Plan (this has been done by requirement of USDA-APHIS for authorized export hatcheries and nurseries)

4. Promote consistency in shellfish health management between states, tribal governments, and federal governments

5. The workshop recommended that whenever practical, relic and at-risk oysters be removed during normal culture practices and management of public oyster beds.

Needed Research in Regard to *Mikrocytos mackini* and Denman Island Disease

The following subjects were agreed upon by the workshop to need additional research. These are arranged in approximate order of priority with a priority reading taken from workshop participants. The validation and optimization of the PCR method received the highest priority rating.

1. Perform PCR validation and optimization (Rated #1 priority by 20+ workshop participants)
2. Determine alternative hosts or vectors – e.g. Manila clams and geoduck, and other oyster species (Rated #1 priority by 3 participants, rated #2 priority by 20+ participants).
3. Determine how long pathogen can survive outside of host (Rated #3 priority by 20+ participants)
4. Determine temperature regime of disease (Rated #2 priority by 3 participants, rated #4 priority by 20 participants)
5. Develop more detailed information on the pathogenesis and etiology of the disease (rate #5 priority by 20+ workshop participants), to include the following information:
    a. Susceptibility to DID vs age of host animal
    b. Appropriate testing of DID susceptibility in larvae (are larvae potential carriers?)
    c. Develop more detailed *M. mackini* life-cycle information
6. Validate *in situ* hybridization (ISH) method for *M. mackini* (rated # 6 priority by 20+ workshop participants)
7. Evaluate effects of other environmental variables on disease, such as salinity (rated #7 priority by 20+ workshop participants)
8. Review European test results and/or test susceptible oysters (rated #8 priority by 20+ workshop participants)

Additional discussion about research priorities included the following. These points are included in the prioritization of research needs.

- What about depuration to clear the cells from mantle cavity and shells? Chlorine emersion is typically used to treat seed. It would be relatively easy to design an experiment to assess potential for such depuration from non-infectable species.

- How long can *M. mackini* survive outside the host and over what geographic distance can infective forms travel?

- Can species such as Manila clams and other species act as disease vectors?

- There should be more sampling and applied research directed toward old or "feral" oysters in Washington State to more fully define any possible risk from such oysters.

- Workshop participants noted that there is some ongoing or planned research including the assessment of carrier status of Manila clams, and research that addresses other recommended priorities, by scientists at the Pacific Biological Station in Nanaimo, British Columbia and the evaluation of new PCR primers at the University of Washington. For the latter study it is important to select more appropriate PCR primers and to establish numerical sensitivity with paired tests in separate labs.

Literature Citations

Bower SM, Hervio D, Meyer GR (1997) Infectivity of *Mikrocytos mackini*, the causative agent of Denman Island disease in Pacific oysters *Crassostrea gigas*, to various species of oysters. Dis Aquat Org 29:111–116.

Friedman CS, Brown HM, Ewing TW, Griffin FJ, Cherr GN. 2005. Pilot study of the Olympia oyster *Ostrea conchaphila* in the San Francisco Bay Estuary: description and distribution of diseases. Diseases of Aquatic Organisms. *In Press*.

P.S.I. 2000. High Health Management of Pacific Oysters, Final report to Saltonstall-Kennedy Program, National Marine Fisheries Service, U.S. Department of Commerce for grant number NA86FD0262 to Pacific Shellfish Institute, Olympia, Washington.