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Denman Island disease in Washington State, USA: distribution and prevalence in Pacific and Olympia oysters

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ABSTRACT: We sampled over 2400 wild, feral, and cultured Pacific oysters *Crassostrea gigas* and Olympia oysters *Ostrea lurida* in Washington State, USA, from 2002 to 2006 to estimate the prevalence of infection with *Mikrocytos mackini*, the causative agent of Denman Island disease. Both histology and qualitative PCR methods were used. Estimates of true prevalence of *M. mackini* infection in *C. gigas*, after accounting for imperfect test sensitivity, ranged from mean values of 0 to 10.0% by histology and 0 to 8.4% based on pooled PCR samples. *M. mackini* was not detected in any of the *O. lurida* samples. Results suggest a lower prevalence of the pathogen and severity of this oyster disease in Washington than that indicated in previous reports from British Columbia, Canada, potentially attributable to higher seawater temperatures in the Washington sample locations.

KEY WORDS: Denman Island disease · *Mikrocytos mackini* · *Crassostrea gigas* · *Ostrea lurida*

INTRODUCTION

An infectious disease causing morbidity and mortality of Pacific oysters *Crassostrea gigas* was first described in 1960 by Quayle (1961, 1982, 1988). The disease occurred in the vicinity of Henry Bay of Denman Island, which lies in the Strait of Georgia on the eastern aspect of Vancouver Island, British Columbia, Canada. Upon histological evaluation, the lesions were found associated with 'microcells' infecting vesicular connective tissue cells of the oysters and, in active advanced cases, causing grossly visible raised yellow to green pustules on the body surface or within the adductor muscle. These microcells were designated *Mikrocytos mackini* by Farley et al. (1988). *M. mackini* is a small (2–3 µm diameter) intracellular protistan parasite that lacks mitochondria. Although it has some superficial similarities to another oyster pathogen, *Bonamia* spp., its taxonomic affinities remained undetermined (Carnegie et al. 2003, Carnegie & Cochennec-Laureau 2004, Abbott et al. 2011) until its recent classification within a newly described parasite order, the Mikrocytida (Burki et al. 2013, Hartikainen et al. 2014).

Mikrocytosis is commonly referred to as Denman Island disease (DID). DID has a similar clinical appearance to nocardiosis of Pacific oysters (Elston et al. 1987, Friedman & Hedrick 1991, Friedman et al. 1998), but the latter disease is expressed in the Pacific oyster host in direct relationship to rising temperatures and usually occurs in mid- to late summer, in contrast to DID which requires temperatures below 10°C to cause significant disease in Pacific oysters (Hervio et al. 1996).

Between 1985 and 2002, over 22 000 oysters from brood stock or intensive culture areas in Washington, Oregon, and California, USA, were examined grossly and by histology in commercial oyster production

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surveillance programs, and no instances of the pathogen or DID were detected. The absence of findings in cultured Pacific oysters suggested at first that the distribution of DID did not extend to Washington or other Pacific states. These surveillance efforts, however, focused on managed shell stock or brood stock populations and thus typically involved relatively young oysters 2 to 3 yr of age.

In spring of 2002, Fisheries and Oceans Canada and AquaTechnics conducted a preliminary survey on a population of feral Pacific oysters in Dungeness Bay, Clallam County, Washington, located on the southern aspect of the Strait of Juan de Fuca and less than 25 km south of British Columbia. We sampled a low tidal level habitat containing a typical assemblage of 4 to 5 yr and older oysters in an unmanaged and unharvested oyster bed, where infected oysters were most likely to be found, if present (Quayle 1961, Bower 1988). This detailed investigation of 50 such individual oysters revealed 3 oysters infected by *M. mackini*, based on subtle resolving gross lesions and confirmed by histology and a polymerase chain reaction (PCR) method developed at the Fisheries and Oceans Canada laboratory and published the following year (Carnegie et al. 2003). That laboratory was subsequently appointed as the World Organization for Animal Health (OIE) reference laboratory for the disease caused by *M. mackini*. The disease agent was reportable to the OIE until 2006, and is still a disease of concern in some locations.

As a result of the 2002 preliminary investigation, a more extensive examination of oysters including managed aquaculture sites, public harvest sites, and feral oyster sites was undertaken by the Washington Department of Fish and Wildlife to evaluate the distribution, prevalence, and severity of *M. mackini* infection in Pacific oysters and Olympia oysters *Ostrea lurida*. The results of this public and private oyster bed surveillance study from 2002 to 2006 are presented in this paper and provide an estimate of baseline prevalence, severity, and distribution of *M. mackini* in Washington in Pacific oysters and native Olympia oysters.

MATERIALS AND METHODS

Sample locations

Sample locations in Washington are listed in Tables 1 & 2. Many of the Pacific oyster sample locations contain feral oysters on public oyster beds, while some were part of more highly managed commercially harvested beds as indicated in Table 1. Sites were selected to represent all hydro-geographic areas in Washington where oysters were cultured or found in harvestable numbers on private or publically owned tidelands. Older, larger oysters occurring at the lowest possible tidal level (usually between +1 and −0.3 m tidal elevation) were targeted for sampling as they have been reported to be most likely infected by the disease agent (Quayle 1961, Bower 1988). In all cases, either histology or PCR were used as screening tests. One case (Deer Harbor, Orcas Island, 2002) was excluded from this report, as histology and PCR were only run on animals with gross lesions which biased the associated prevalence.

Test methods and necropsy

From 2002 to 2004, samples were examined using histology as the primary method, and the qualitative PCR method based on Carnegie et al. (2003) was used for confirmation, with the exception of the 2002 Dungeness Bay sample in which all individuals were processed by both methods. From 2004 to 2006, the qualitative PCR method was used as the primary method. All oysters were transported live to the laboratory, and during necropsy the shell length and whole wet body weight were measured and recorded for each specimen.

Histology

A transverse section was cut through the anterior portion of the visceral mass from each oyster including mantle, gonad, stomach, digestive gland, and labial palps. Any raised lesions, typical of active infections, or depressed gray appearing ulcers, typical of resolving lesions, on other parts of the body were also excised and included in the histology sample. Tissue samples were preserved in Davidson's solution and processed using routine histological techniques for paraffin embedment, and sections were stained with hematoxylin and eosin.

PCR

Oysters for PCR analysis were opened in sets of 4 (brood stock) and placed on clean sodium hypochlorite-rinsed polyethylene trays. Each oyster shucking knife and set of dissection tools used for a single

Table 1. Sample locations, dates, and results of surveys for *Mikrocytos mackini* in Washington State (USA) Pacific oysters *Crassostrea gigas*, 2002 to 2006. Prevalence estimates are adjusted to account for imperfect test sensitivity and pooling. Sample locations are designated as public (P), managed (M), or unmanaged (U). Public and unmanaged beds are likely to have a greater proportion of larger, older oysters than beds managed for commercial aquaculture on which oysters are systematically planted and harvested at 2 to 3 yr of age. Except for the first listed Dungeness Bay sample, which was conducted on individual samples, all PCR testing was done in pools of 4. For the Dungeness Bay sample, both histology (H) and PCR (Carnegie et al. 2003) confirmed 3 positive individuals. Prevalence for all pooled PCR samples was estimated as described in the 'Materials and methods', according to Cowling et al. (1999). G: gross examination

Sampling location	Management type	Sample date	Oysters sampled (n)	Method	Number (H) or pools (PCR) positive	Estimated prevalence (%)	
						Mean	95th percentile
Dungeness Bay, Clallam County	U	13-May-02	50	H & PCR	3	6.7	18.2
Blind Bay, San Juan County	U	27-May-02	60	H	0	0.0	9.3
Deer Harbor, San Juan County	U	26-May-02	45[a]	G/H/PCR			
Semiahoo Spit, Whatcom County	P	30-May-02	60	H	0	0.0	9.3
Duckabush River mouth, Jefferson County	P	11-Jun-02	60	H	0	0.0	9.3
Willapa Bay, Pacific County	P	3-Jun-02	60	H	0	0.0	9.3
Samish Bay, Skagit County	M	19-Apr-03	59	H	2	6.8	19.4
Mystery Bay, Jefferson County	U	20-Apr-03	60	H	0	0.0	9.3
West Sound, San Juan County	U	17-May-03	60	H	3	10.0	24.4
Westcott Bay, San Juan County	M	17-May-03	60	H	1	3.3	14.4
Portage Bay, King County	U	18-May-03	60	H	1	3.3	14.4
McMicken Island State Park, Mason County	P	20-May-03	60	H	1	3.3	14.4
Minter Creek, Pierce County	M	12-Jun-03	60	H	0	0.0	9.3
Big Beef Creek, Kitsap County	U	12-Jun-03	60	H	0	0.0	9.3
Willapa Bay, Pacific County	P and M areas	18-Apr-03	60	H	0	0.0	9.3
Deer Harbor, Orcas Island, San Juan County	U	26-May-05	60	PCR	4	8.41	18.90
Westcott Bay, San Juan County	M	26-May-05	60	PCR	1	1.91	8.87
Samish Bay, Skagit County	M	27-May-05	60	PCR	1	1.91	8.87
Purdy Lagoon, Pierce County	M	22-May-05	60	PCR	1	1.91	8.87
Illahee State Park, Kitsap County	P	23-May-05	60	PCR	2	3.93	12.13
Penrose Point State Park, Pierce County	P	23-May-05	60	PCR	1	1.91	8.87
Frye Cove County Park, Thurston County	P	25-May-05	60	PCR	3	6.09	15.44
Tolmie State Park, Thurston County	P	25-May-05	60	PCR	0	0.0	5.5
Point Whitney, Jefferson County	P	24-May-05	60	PCR	0	0.0	5.5
Willapa Bay, Pacific County	P and M areas	26-May-05	60	PCR	0	0.0	5.5
Sequim Bay State Park, Clallam County	P	29-Apr-06	60	PCR	0	0.0	5.5
Discovery Bay, Jefferson County	U	17-May-06	60	PCR	0	0.0	5.5
Totten Inlet, Thurston County	M	27-Apr-06	60	PCR	0	0.0	5.5
Hood Head, Jefferson County	P	30-Apr-06	60	PCR	1	1.91	8.87
Dosewallips River mouth, Jefferson County	P	19-May-06	60	PCR	0	0.0	5.5
Kitsap Memorial State Park, Kitsap County	P	17-May-06	60	PCR	4	8.41	18.90
Seabeck Scenic Beach State Park, Kitsap County	U	19-May-06	60	PCR	1	1.91	8.87
Big Beef Creek, Kitsap County	U	13-Jun-06	60	PCR	0	0.0	5.5
Quilcene Bay, Jefferson County	U	13-Jun-06	60	PCR	1	1.91	8.87
Zelatched Point, Jefferson County	P	12-Jun-06	60	PCR	0	0.0	5.5
Triton Cove, Jefferson County	P	25-May-06	60	PCR	1	1.91	8.87

[a]The Deer Harbor, Orcas Island, sample was positive by histology and PCR, but the 45 sampled oysters were screened grossly, with histology and PCR only used as confirmatory tests. Thus the results are not well suited to a comparative prevalence estimation and were excluded from the statistical analysis

Table 2. Locations in Washington State (USA) for sampling of native Olympia oysters *Ostrea lurida*. All PCR samples were analyzed in pools of 4, and PCR test results showed no positive individuals out of 60 sampled at each location

Sampling location	Sample date
Discovery Bay	23-May-04
Clam Bay	26-May-04
North Bay	10-Apr-04
Oakland Bay	08-May-04
Willapa Bay	18-May-04

pool of oysters was cleaned in running water, immersed in 15% sodium hypochlorite for a minimum of 5 min, rinsed in tap water, immersed in 95% ethanol, then flamed and cooled prior to use. Tissue samples for determination of *Mikrocytos mackini* infection by PCR consisted of routine collection of ca. 2 mm^3 sections of labial palp placed in non-denatured 95% ethanol. Samples from oysters showing no clinical signs of infection were collected in pools of 4 oysters each; for those showing gross signs of disease, lesions were excised and preserved separately. PCR amplification of 546 bp of 18S-rDNA used primers MIKROCYTOS-F and MIKROCYTOS-R, which were designed by Carnegie et al. (2003) to be *M. mackini* specific. PCR reaction mixtures of 15 µl included 5 PRIME HotMaster *Taq* Buffer at 1× concentration (with weakly chelated 'self-adjusting' $MgCl_2$ at 2.5 mM), dNTPs at 0.2 mM, primers at 0.2 µM, 5 PRIME HotMaster *Taq* DNA Polymerase at 0.02 U $µl^{-1}$, and template DNA at 3.5 ng $µl^{-1}$). Template DNA consisted of genomic DNA from either the *M. mackini*-infected or -uninfected *Crassostrea gigas* or *Ostrea lurida*, or a no-template water control. The temperature cycling profile was an initial denaturation at 94°C for 2 min; 40 cycles of 94°C for 1 min, 60.5°C for 1 min, and 70°C for 1 min; and a final extension at 70°C for 10 min. Products were electrophoresed on a 1.0% agarose gel containing 1× concentration of Sybr®Safe.

Prevalence estimates

True prevalence estimates (mean and 95th percentiles) were calculated using interactive calculators available on-line through AusVet Animal Health Services (http://epitools.ausvet.com.au/content.php?page=home) according to reviewed methods for individual or pooled samples (Cowling et al. 1999, Sergeant 2015, Reiczigel et al. 2010). Selected methods assume exact knowledge of the sensitivity and specificity of the test methods, and a fixed pool size. The pooled prevalence calculator, designed for surveys with 1 or more positive results, adjusts prevalence estimates to account for pooled samples commonly used to facilitate testing of large numbers of individuals (Sergeant 2015). For pooled sample sets with 0 positives, we used the disease freedom sample size calculators. To determine the 95th percentile of these (0 positive) prevalence estimates, we set the desired herd sensitivity at 95% and entered different design prevalence values until the calculated sample size described our sample.

Definitive sensitivity and specificity estimates were not available for the PCR or histology methods used for *M. mackini*. Therefore, we used expert estimates (by R.E.) of 90% sensitivity for PCR and 50% sensitivity for histology in order to obtain an approximation of prevalence from the individual and pooled values. Although we recognize that neither qualitative PCR nor histology eliminates the possibility of false positives, we assigned 100% specificity to both tests to ensure that our calculations are conservative (i.e. over-estimate, rather than under-estimate, prevalence). All histology samples, as well as the PCR from one of the collections (Dungeness) were run individually (pool size of 1). All PCR samples, except from Dungeness, were run in pools of 4. All 50 Dungeness samples were run individually by both PCR and histology. We used results from this initial sample (May 2002) in which histology was 67% as sensitive as PCR (*x* positive by histology out of *y* positive by PCR) to inform histology sensitivity. Carnegie et al. (2003) reported that the qualitative PCR method was 3 to 4 times more sensitive than histology. We settled on an estimate of 50% (between the 2 provided), as our protocols for histology also include categorization of typical focal areas of hemocytosis from susceptible oysters as suspicious, and indicate confirmatory testing if the lesion in a susceptible species of oyster has a characteristic focal necrotic zone with radiating hemocytosis.

RESULTS

Table 1 shows the sample locations in Washington and results for each Pacific oyster sample. Prevalence estimates (mean values) ranged from 0 to 10.0% (95th percentiles estimated at 9.3 to 24.4%) by histology, and prevalence estimated from pooled PCR samples ranged from 0 to 8.41% (95th percentiles estimated at 5.5 to 18.9%). Table 2 shows the sample locations in Washington for native oysters. We found

no evidence of *Mikrocytos mackini* in these native oyster samples using the PCR method.

Gross lesions consisted of gray ulcers (Fig. 1), but active lesions consisting of yellow-green pustules were only observed in a few instances. When parasites were observed histologically, the individual parasitic cells had a typical appearance as reported previously (Fig. 2). However, we found very few cases in which the parasitic cells were common in the lesions. In many cases which were PCR positive, distinctive *M. mackini* were extremely rare or could not be detected at all by histology; rather, areas of hemocytosis containing host cell and parasite cell fragments were the usual finding (Fig. 3).

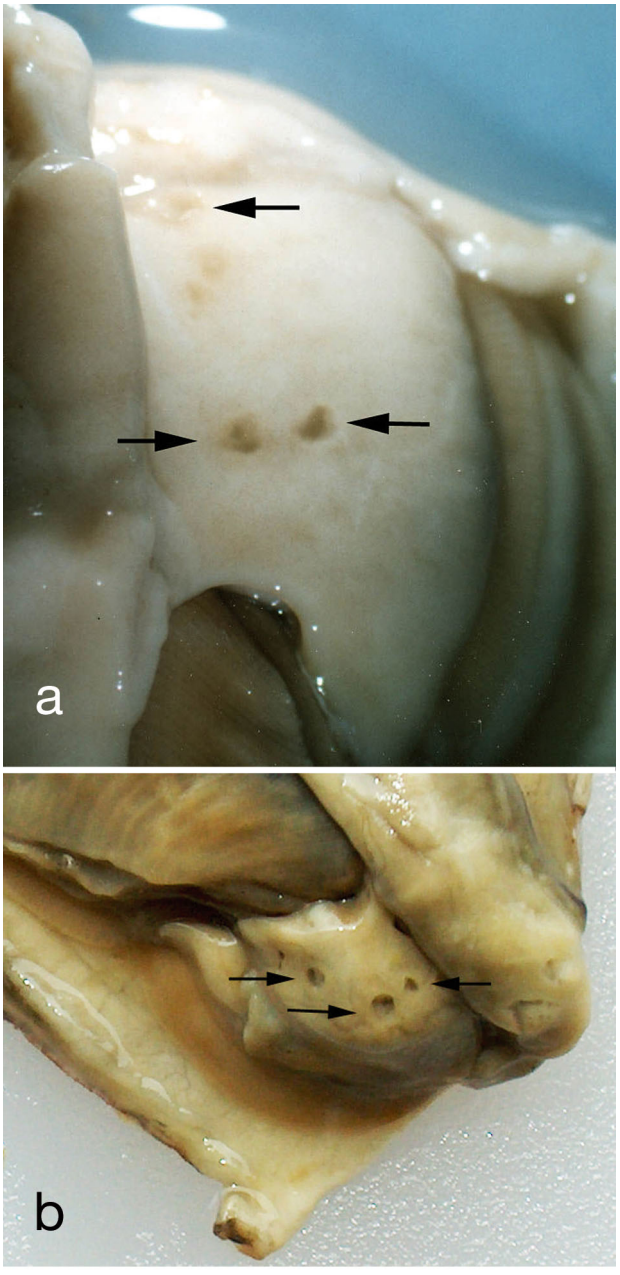

Fig. 1. Pacific oyster *Crassostrea gigas*, showing clear ulcerated lesions in the labial palps, typical of a resolving *Mikrocytos mackini* infection (arrows) sampled from (a) Dungeness Bay, Washington (USA), on 13 May 2012, and (b) Deer Harbor, Washington (USA), on 26 May 2005

DISCUSSION

Infection with *Mikrocytos mackini* was listed as a notifiable disease of mollusks by the OIE until 2006, when it was delisted (Meyer et al. 2008). Nonetheless,

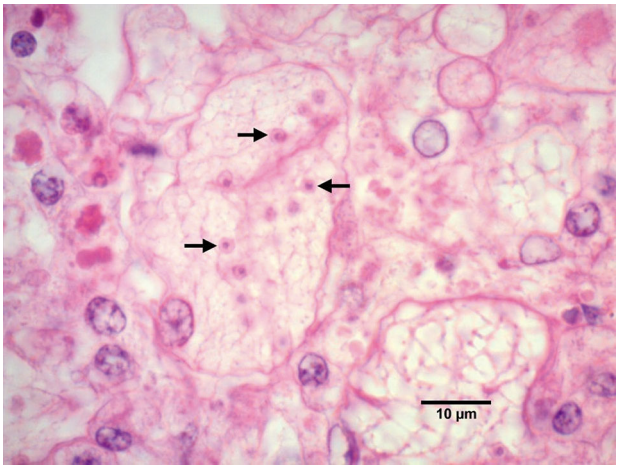

Fig. 2. Histological section of a Pacific oyster *Crassostrea gigas*, showing an active infection. Multiple *Mikrocytos mackini* (arrows) can be observed within connective tissue cells

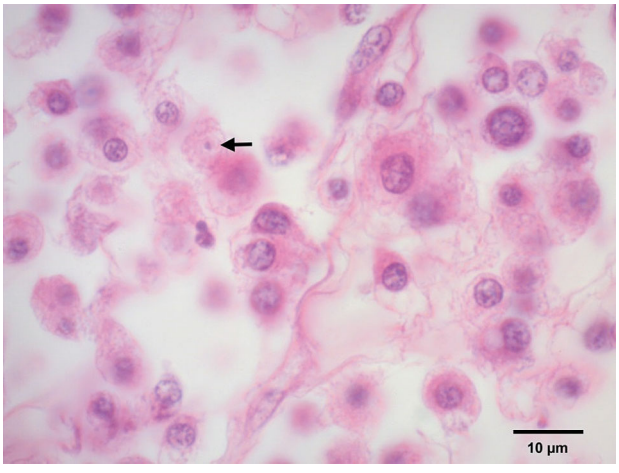

Fig. 3. Histological section of a Pacific oyster *Crassostrea gigas*, showing a late-stage resolving infection. Very few *Mikrocytos mackini* are present and are difficult to discern, as they often appear to be phagocytized or free among lysed necrotic host cells (arrow)

the disease is still one of regional concern (Abbott et al. 2011) and of concern outside of its known range where Pacific and other oyster species are farmed.

Following the initial findings of DID, as reported by Quayle (1961), little was published about the disease and causative agent until 1988 when the agent was named (Farley et al. 1988) and a paper recommending methods to manage the severity of the disease was published (Bower 1988). A variety of additional studies were conducted over the following 25 yr (e.g. Hervio et al. 1996, Bower et al. 1997, 2005, Meyer et al. 2005, Abbott et al. 2011). These subsequent studies in British Columbia, the northernmost jurisdiction in the known range of *M. mackini*, established that the disease occurred with greater frequency in older oysters and that disease expression was confined to cold temperature seasons, but could, in some cases, cause morbidity and mortality in shell stock oysters raised for human consumption harvested at less than 3 yr of age.

DID in Washington compared to British Columbia

Relationships with mortality are unclear, due to the difficulty of assigning cause of death and differentiating single and multiple causative factors. However, the relatively low prevalence of DID in oyster populations in Washington, compared to historical prevalence reported in British Columbia (BC), is notable. Population prevalence of the pathogen in Henry Bay of Denman Island was reported to vary from 11 to 39% from 1960 to 1987 (Bower et al. 1997), with mortality attributed to the disease reported up to 30% by Quayle (1982), who also estimated that in Henry Bay in 1960, 55% of all the oysters had 'typical symptoms.'

More recent observations from the OIE reference laboratory (Pacific Biological Station, Fisheries and Oceans Canada) for *M. mackini* in Nanaimo, BC, suggest that the disease has a sporadic outbreak occurrence. Characteristics of the outbreaks are summarized as follows with regard to *M. mackini* infection in Pacific oysters, the primary oyster of commerce in that region:

(1) Over the past 10 yr, *M. mackini* has only caused sporadic mortality and reduced marketability (due to presence of visible lesions) of BC oysters.

(2) Typically, the disease is not a problem when oysters are harvested at <3 yr old.

(3) Prevalence of infection is usually <5% during spring surveys; however, there have been several notable exceptions:

(a) spring of 2002, 2004, and 2011, when up to 40% of the older oysters (4+ yr) from 1 location on West Coast Vancouver Island (near Tofino, BC) showed gross signs of the disease and mortality was estimated at 10 to 20%.

(b) spring of 2010 and 2011, when up to 20% of young oysters (<2 yr old) from 1 location in the Strait of Georgia (Baynes Sound) showed gross signs of the disease and mortality was estimated at 10 to 20%.

It is important to note that our Washington estimates account for imperfect test sensitivity which will raise values over those that report apparent prevalence (without sensitivity correction). Further, our estimates are based on a targeted selection of older animals which will also magnify prevalence over that expected from a random sample. Even with this caveat, however, the prevalence estimates in Washington, using histology, never exceeded 10% in mean value (95th percentile, 24%), and the estimated prevalence from pooled PCR samples did not exceed 8.4% (95th percentile, 18.9%; Table 1). Further, the high end of our estimate range derives from samples evaluated by histology. This questions whether our estimate of histology sensitivity (50%) is overly conservative (i.e. too low).

As noted in the 'Results', we found very few cases in which the parasitic cells were common in the lesions, unlike previous reports of the disease in Pacific oysters from BC and in the recent report of *M. mackini* infection in Kumamoto oysters *Crassostrea sikamea* (Elston et al. 2012) as well and markedly reduced prevalence compared to that observed in BC. The lower observed prevalence in Washington, as determined by histology, could be argued to be due to the fact that the samples were collected in late spring (end of May and early June), when DID infections are often resolving or already in remission. However, spring sampling should not have affected the PCR prevalence and does not explain the lower occurrence of reported and observed lesions of the disease in the more intensively managed (aquaculture) oyster beds in which oysters are systematically harvested at 2 to 3 yr of age (Table 1). From 1985 to 2002, when routine oyster disease surveillance targeted brood stock oysters from managed culture areas rather than feral and older animals from unmanaged areas, no evidence of DID was found. The disease appears to be even less common, or nonexistent, in brood stock animals. These observations may be explained by the fact that oysters cultured for food production are generally harvested by 3 yr of age and that brood stock are generally 2 to 4 yr old oysters that are periodically culled, cleaned, and held

in specified areas. These are factors which reduce the risk of infection, particularly given the relatively low infection rates generally in Washington. We conclude that DID occurrence in Washington primarily affects the older oyster populations, such as those found on public and unmanaged oyster beds, although it occurs in some extensively cultured oyster beds.

Temperature effect on disease prevalence and severity

Hervio et al. (1996) systematically addressed the effect of temperature on development of the disease. They also showed that inoculated oysters held above the permissive temperature for disease development would still develop the disease, if placed in a permissive water temperature (10°C) after 3 mo at a high, non-permissive temperature. However, for 'development of the disease, exposed oysters required prolonged incubation at low temperatures (about 10°C)' (Hervio et al. 1996, p. 72). Thus it seems likely that water temperatures are generally not cold enough for a long enough period to allow expression of the disease in most or all of Washington oyster culture waters. Further, this generally non-permissive condition for disease expression may also tend to repress the transmission and thus overall prevalence of the disease, as well as reduce the risk of infection of native oysters.

We conclude that Washington is likely in the peripheral part of the range of the disease in Pacific oysters, probably as a result of somewhat warmer average winter seawater temperatures than experienced in BC and differing host susceptibility to the disease under various conditions. However, disease range and prevalence is species specific, depending on host factors as well as availability of the infectious agents, as shown in the recent study reporting DID in Kumamoto oysters from California (Elston et al. 2012).

Host range

M. mackini is infective for the following species of oysters: Pacific oysters *C. gigas*, eastern oysters *C. virginica*, flat oysters *Ostrea edulis*, and Olympia oysters *O. conchaphila* (= *lurida*) (Bower et al. 1997). Interestingly, we did not find any evidence of infection of Olympia oysters in Washington, during this study, while Bower et al. (1997) reported that over 23% of Olympia oysters were infected after being brought into the laboratory and placed in tanks at 10°C for 15 wk. Although Olympia oyster populations have historically declined in Washington, substantial populations still exist. The absence of DID could result from sufficient geographic isolation of the 2 species in combination with low source prevalence as reported here in Pacific oysters, but our study was not designed to determine the reason for absence of the disease in Olympia oysters. In another report, Elston et al. (2012) found that Kumamoto oysters are susceptible to DID and may be more susceptible to infection with *M. mackini* than Pacific oysters.

Hatchery and nursery status

Hatchery populations appear to maintain a distinct DID-free health status. We believe that the low prevalence of the disease in oyster populations on tidelands in Washington is also a contributing factor to the disease-free status of hatchery and nursery stocks of Pacific oysters in in this state. Pacific oyster seed from hatchery stock has been shipped from Washington to Alaska for many years, but there have been no reported cases of *M. mackini* infection in Alaska, which has a rigorous shellfish disease control policy and surveillance program, requiring all seed sources, including brood stock, to be tested at least annually. More detailed explanation and understanding of the disease transmission dynamics of *M. mackini* will require further examination and study.

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