

Denman Island disease (causative agent *Mikrocytos mackini*) in a new host, Kumamoto oysters *Crassostrea sikamea*

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ABSTRACT: *Mikrocytos mackini*, causative agent of Denman Island disease in Pacific oysters *Crassostrea gigas* and other oyster species, was found in 2011 in a previously unreported host, the Kumamoto oyster *C. sikamea*, in Humboldt Bay, California, USA. The detection was also the first reported finding of *M. mackini* in California. Prevalence was estimated as high as approximately 27% from pooled samples analyzed by PCR. Higher prevalence appeared related to longer residence time in the bay and somewhat colder than typical winter seawater temperatures. No *M. mackini* was detected in Humboldt Bay juvenile Kumamoto oysters or Pacific oyster brood or seed stock in 2011 or 2012.

KEY WORDS: *Mikrocytos mackini* · Denman Island disease · Kumamoto oyster · *Crassostrea sikamea*

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INTRODUCTION

Denman Island disease (DID) is an infectious disease causing morbidity and mortality of Pacific oysters *Crassostrea gigas* and was first described by Quayle (1961, 1982, 1988) in the vicinity of Henry Bay of Denman Island, which lies on the eastern aspect of Vancouver Island, British Columbia, Canada. The causative agent is an amitochondriate protistan parasite of undetermined taxonomic affinity (Abbott et al. 2011, cf. Carnegie et al. 2003), commonly referred to as a 'microcell' based on its histological presentation. These microcells, which are 2 to 3 µm in diameter, primarily infect vesicular connective tissue and were designated *Mikrocytos mackini* by Farley et al. (1988). Infections may be manifested by grossly visible raised yellow to green pustules on the body surface or within the adductor muscle and are known to be transmissible directly from oyster to oyster (Bower et al. 1997). Disease expression requires prolonged

environmental seawater temperatures in the range of 10°C or lower, while higher temperatures (>15°C) are not permissive for disease expression (Hervio et al. 1996, Bower et al. 1997). Mortality from the disease has been observed in British Columbia but not elsewhere. Prior to this study, the known geographic range of *M. mackini* included British Columbia, Canada, adjacent areas of Washington State, USA, and one site in Oregon, USA, on the Pacific Northwest coast (Farley et al. 1988, Bower et al. 2005). The only previously known wild host species of *M. mackini* are Pacific oysters *Crassostrea gigas* and the Olympia oyster *Ostrea conchaphila*, but laboratory experimentation has shown that Eastern oysters *C. virginica* and European flat oysters *O. edulis* are also susceptible (Bower et al. 1997).

Based on the Shellfish High Health Program of the Pacific Coast Shellfish Growers Association (PCSGA 2010), routine disease surveillance is conducted on brood and seed stock populations of participating

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shellfish farms, consisting of independent sampling conducted by US Department of Agriculture accredited veterinarians. As a result of such routine surveillance in 2011, *Mikrocytos mackini* infections were discovered in a previously unrecognized host species, Kumamoto oysters *Crassostrea sikamea* in Humboldt Bay, California, a location previously unknown to harbor the disease agent.

MATERIALS AND METHODS

Sample collection

Kumamoto and Pacific brood and seed stock oysters were initially sampled ($n = 600$) from the northern arm of Humboldt Bay, in February 2011 as part of annual routine brood stock disease surveillance (Table 1). Samples are collected by an independent veterinarian accredited by the US Department of Agriculture, Animal and Plant Health Inspection Service. Following the detection of *Mikrocytos mackini* from the February sampled group, additional Kumamoto and Pacific oyster brood and seed stock were sampled from March to May 2011 by producer collections from Humboldt Bay ($n = 600$, Table 1). Except as noted in Table 1, brood stock was held on long lines and seed stock was held in floating upweller systems (FLUPSY). In addition to results of testing on samples collected in 2011, we also report results from samples collected by an accredited veterinarian for disease surveillance in 2012 ($n = 600$, see Table 1).

Histological testing

All oysters were processed for histological examination using routine methods. A transverse section of oysters was made in the dorsal region through the digestive gland, gills and connective tissue, including the labial palps in some cases. This was placed in a histological cassette and fixed in seawater formalin (10% v/v). Histological preparations were processed routinely for paraffin embedment and sections were stained with hematoxylin and eosin. In addition to the examination of tissue for hemocytosis and infectious agents, the height of the epithelium of the digestive gland tubules was rated on a basis of 1 to 4 (low to high), with ratings of 3 and 4 considered to be in the normal active feeding range and ratings of 1 or 2 indicating low height of digestive gland epithelium resulting from limited food availability and starvation.

Molecular testing

Oysters for PCR analysis were opened in sets of 4 (brood stock) or 6 (juvenile stock) and each set of tissue was preserved separately in non-denatured 95% ethanol. For brood stock oysters, tissue samples for determination of *Mikrocytos mackini* infection by PCR consisted of routine collection of ca. 2 mm³ sections of labial palp. Samples from oysters showing no clinical signs of infection were collected in pools of 4 oysters each; for those showing gross signs of dis-

Table 1. Prevalence of *Mikrocytos mackini* infection in *Crassostrea* spp. oysters examined from Humboldt Bay, CA, USA, in 2011 and 2012

Species	Sample location	Life stage	Sample date	Analysis method	Number examined (pool size)	Number positive	Prevalence estimate (%)
<i>C. sikamea</i>	Bird Island, South	Brood stock 2008	21 Feb 2011	Histology	150	30	20.0
<i>C. sikamea</i>	Bird Island, South	Brood stock 2008	21 Feb 2011	PCR	150 (4)	13 pools	11.6 ^a
<i>C. sikamea</i>	Mad River Slough	Brood stock 2009	26 Mar 2012	PCR	150 (4)	0	
<i>C. sikamea</i>	East Bay	Brood stock 2008	29 Mar 2011	PCR	150 (4)	24 pools	27.3 ^a
<i>C. sikamea</i>	Bird Island	Brood stock 2009	29 Mar 2011	PCR	150 (4)	2 pools	1.5 ^a
<i>C. sikamea</i>	Mad River	Brood stock 2008	11 Apr 2011	PCR	150 (4)	24 pools	27.3 ^a
<i>C. sikamea</i>	Arcata Channel	Brood stock 2009	11 Apr 2011	PCR	150 (4)	1 pool	0.8 ^a
<i>C. sikamea</i>	Fairhaven nursery	Juvenile stock 2010	21 Feb 2011	PCR	150 (6)	0	
<i>C. sikamea</i>	Fairhaven nursery	Juvenile stock 2011	26 Mar 2012	PCR	150 (6)	0	
<i>C. sikamea</i>	Mad River Slough	Juvenile stock 2011	13 Jun 2011	PCR	150 (6)	0	
<i>C. gigas</i>	Bird Island, South	Brood stock 2008	21 Feb 2011	PCR	150 (4)	0	
<i>C. gigas</i>	Sand Island	Brood stock 2009	26 Mar 2012	PCR	150 (4)	0	
<i>C. gigas</i>	Fairhaven nursery	Juvenile stock 2010	21 Feb 2011	PCR	150 (6)	0	
<i>C. gigas</i>	Fairhaven nursery	Juvenile stock 2010	26 Mar 2012	PCR	150 (6)	0	
<i>C. gigas</i>	Mad River Slough	Juvenile stock 2011	17 May 2011	PCR	150 (6)	0	

^aPrevalence estimated from pooled PCR results, based on the cited statistical method and assumptions

ease, 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 specific for *M. mackini* (but see next paragraph). PCR reaction mixtures of 15 μ l included 5 PRIME HotMaster *Taq* Buffer at 1 \times concentration (with weakly chelated 'self-adjusting' $MgCl_2$ at 2.5 mM), dNTPs at 0.2 mM, primers at 0.2 μ M, 5 PRIME HotMaster *Taq* DNA Polymerase at 0.02 U μ l⁻¹, and template DNA at 3.5 ng μ l⁻¹. Template DNA consisted of genomic DNA from either the *M. mackini*-infected or -uninfected *Crassostrea sikamea* or *C. gigas*, 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 \times concentration of Sybr[®]Safe.

Upon generating positive results for *Mikrocytos mackini* using the PCR test just described (see 'Results'), we sequenced the complete ITS1-5.8S-ITS2 rDNA region for confirmatory purposes, as the PCR test is known to also amplify another *Mikrocytos* sp. from multiple geographic regions (Gagné et al. 2008, Wang et al. 2010, Abbott et al. 2011). PCR primers Mm18S_1435F and pro28S-R were used to amplify and sequence a ca. 700 bp product following methods in Abbott et al. (2011). This sequence is known to be invariant within *M. mackini* but is substantially divergent from the only other *Mikrocytos* species that has been sequenced at this region (*Mikrocytos* sp.-BC; Abbott et al. 2011). Sequence data were viewed, edited, and compared against the published *M. mackini* sequence (GenBank HM563060) using Sequencher v4.8 (Gene Codes).

Prevalence estimates

Statistical tools are available for estimating prevalence from pooled sample analyses (Cowling et al. 1999), and such estimates assume a fixed pool size and exact knowledge of the sensitivity and specificity of the test methods. These methods were developed due to the use of pooled samples to provide economy when testing large numbers of individuals and are available on an interactive online pooled prevalence calculator (Sergeant 2009). Exact sensitivity and specificity is not known for the PCR method we used for *Mikrocytos mackini*. Therefore, we made estimates in order to obtain an approximation of prevalence from the pooled values and also varied the

sensitivity and confidence levels in the statistical analysis. Using the pooled prevalence calculator, we evaluated the pooled data using the starting assumptions of 0.90 for sensitivity and 1.0 for specificity, with upper and lower confidence limits set at 0.025 and 0.975. We set the number of pools at 37 because there were 37 full pools of 4 ind. and one half-sized pool of 2 ind. for the 150 pooled oysters.

Humboldt Bay sea surface temperature

We made an evaluation of Humboldt Bay winter sea surface temperature over a 7 yr period ending in 2011 using data obtained online from NOAA buoy 46212 (NOAA 2011) located near South Spit, Humboldt Bay. To obtain an annual winter average temperature, we averaged all hourly or half-hourly temperature readings, as available, for each reported winter season from October 1 through March 31.

RESULTS

The first diagnosis of *Mikrocytos mackini* infection in *Crassostrea sikamea* was made by histology during the routine annual screening of brood stock oysters for a variety of disease conditions. Initial rapid screening examination revealed foci of infected cells surrounded by a hemocytic reaction (Fig. 1) in 11 of 150 Kumamoto oyster brood stock sampled in February 2011 (Table 1). Subsequent, more detailed histological examination revealed a total of 30 clearly positive individuals. These infected connective tissue foci contained typical microcells identical by histological comparison with previously known *M. mackini* infection in Pacific oysters (Figs. 2 & 3). Following the preliminary histological diagnosis, we proceeded to test 3 of the PCR pools corresponding to the histologically positive animals, all of which returned strongly positive results. Subsequently, DNA sequence of the complete ITS1-5.8S-ITS2 region from these sample pools further confirmed the presence of *M. mackini*: the sequence was 100% homologous with the published *M. mackini* sequence, which is known to be invariable within the species based on sequences from over 70 samples collected throughout British Columbia and Washington State (Abbott et al. 2011). We later tested all PCR pools from the initial positive group in order to estimate prevalence. From the additional subsequent 2011 brood stock samples, we found 4 other groups of Kumamoto oyster brood stock from Humboldt Bay positive for

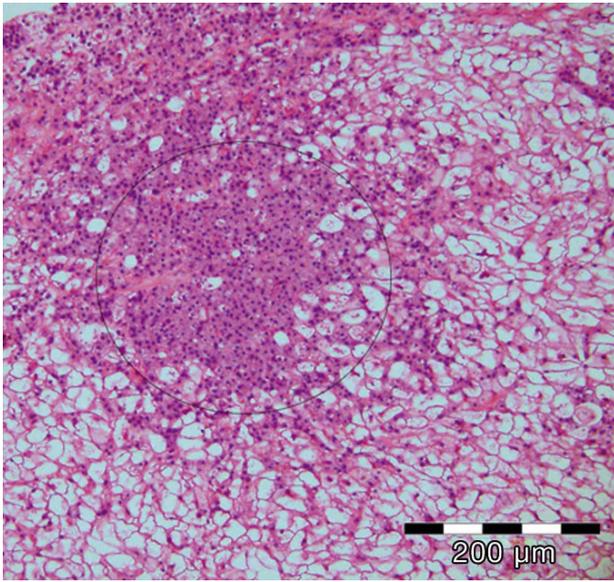


Fig. 1. *Crassostrea sikamea*. Area of vesicular connective tissue of Kumamoto oyster showing intense hemocytosis reaction to *Mikrocytos mackini* infection (area outlined by circle)

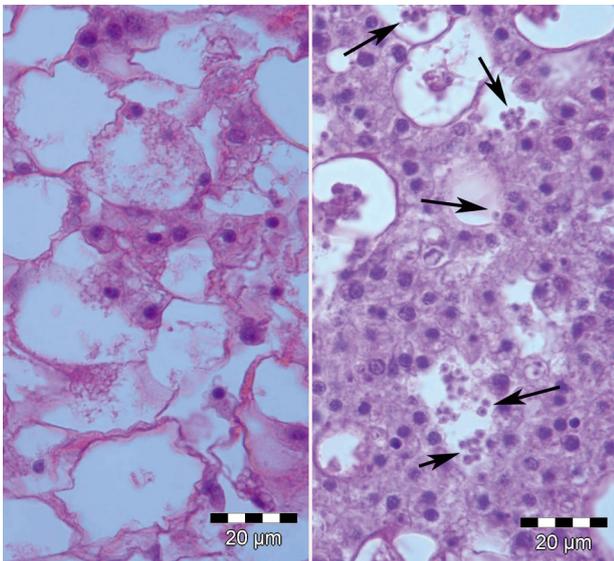


Fig. 2. *Crassostrea sikamea*. Comparison of uninfected vesicular connective tissue of Kumamoto oysters (left panel) with *Mikrocytos mackini*-infected tissue (right panel), with examples of the infectious microorganism indicated by the arrows

M. mackini using PCR (Table 1). The 2 tested brood stock groups originating from 2008 seed had a relatively high proportion of positive PCR pools (24/38 each) compared with 2 brood stock groups originating from 2009 seed (1/38 and 2/38 positive pools). All juvenile Kumamoto oysters and all Pacific oyster brood and juvenile stock from Humboldt Bay tested

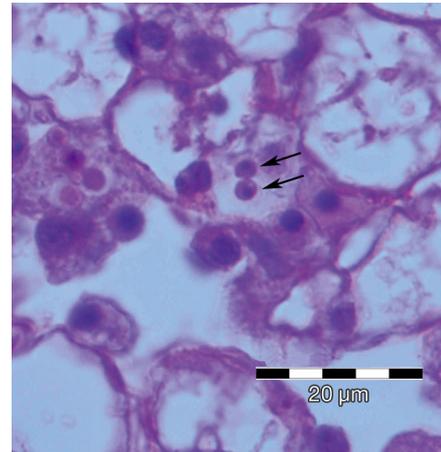


Fig. 3. *Crassostrea sikamea*. *Mikrocytos mackini* cells in vesicular connective tissue of Kumamoto oysters (examples indicated by arrows, with additional microcells to the left of those designated by arrows)

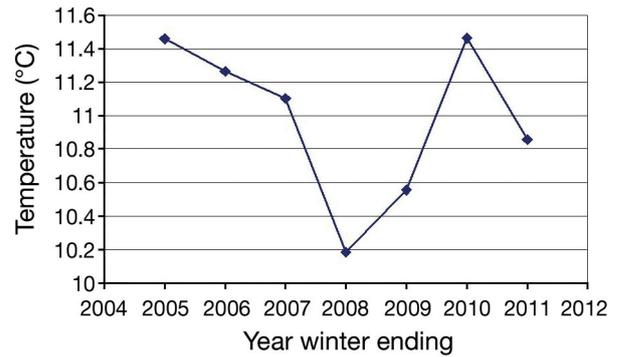


Fig. 4. Surface seawater temperature from October 1 through March 31. The year indicates the March date for each successive data series. Data are from the National Oceanic and Atmospheric Administration, US Department of Commerce buoy located near South Spit, Humboldt Bay. Hourly or half-hourly data, as available, were averaged for each time period indicated

negative for *M. mackini* in 2011 using the PCR method, as did all brood stock and seed stock samples from both species tested collected in 2012 (Table 1).

The digestive gland condition of the 21 February 2011 sample of Kumamoto brood stock was generally poor, with 61 % of all individuals rated 1 or 2. The ratings for *Mikrocytos mackini*-infected oysters (by histology) had 63 % rated as 1 or 2 while the uninfected oysters had 60 % of individuals rated as 1 or 2. Thus, there was no difference between infected and non-infected individuals. Two of 150 oysters from this group had focal bacterial abscesses, typical of nocardiosis. Humboldt Bay winter sea surface temperature data (Fig. 4) showed average temperatures in 2008

and 2009 known to be sufficiently low to exacerbate *Mikrocytos mackini* infection in Pacific oysters from other locations (Hervio et al. 1996; see 'Discussion')

DISCUSSION

DID is caused by the protozoan parasite *Mikrocytos mackini*, first discovered in Pacific oysters *Crassostrea gigas* in British Columbia in the 1960s. The disease can cause visible pustules on the skin surface in Pacific oysters in colder water areas. However, the prevalence of such lesions in Pacific oyster growing areas further south, in Washington State, for example, is generally less than 5% and the disease is considered to be of incidental health consequence to cultured oysters. *Mikrocytos mackini* was delisted by the World Animal Health Organization (OIE) as an internationally reportable disease in 2007. Prior to this study, its known geographic range in North America was restricted to British Columbia, Canada, adjacent areas in Washington State, USA (Bower et al. 2005), and one area in Oregon, USA (Farley et al. 1988). Whether it is truly endemic only to this region or whether it may have been introduced to this region with Pacific oysters is not known (Abbott et al. 2011). In the currently reported case, the discovery of DID in Kumamoto oysters was made during routine surveillance and testing, not as a result of morbidity and mortality reports. Thus, we do not know the numerical value for morbidity and mortality that may be attributed to DID in Kumamoto oysters.

This is the first report of *Mikrocytos mackini* infection in Kumamoto oysters *Crassostrea sikamea* and the first report of *M. mackini* in any species of oyster in California. We found the infection first by histology in brood stock oysters from Humboldt Bay in February 2011. By histology, prevalence was 20% in the first population examined. Upon using PCR to evaluate pooled samples from additional source groups of Kumamoto brood stock oysters from various locations in Humboldt Bay, the prevalence was estimated to range from ~0.8% up to ~27% in 2011 (see discussion below of prevalence estimates from pooled samples). Identity of the 2011 positive samples with west coast *M. mackini* was further confirmed by internal transcribed spacer (ITS) sequence comparison. Evaluation of brood and seed stock Pacific and seed stock Kumamoto oysters from Humboldt Bay by PCR revealed no additional positive samples in 2011 and no brood or seed stock were positive in 2012. However, Humboldt Bay is a relative large body of water and the 2012 negative sam-

ples are not interpreted to claim that the bay is free of *M. mackini* following the 2011 detection. The lack of infection of the known host Pacific oysters is interesting and somewhat unexpected, considering the relatively high prevalence in Kumamoto oysters.

Estimation of prevalence from pooled PCR samples is based on knowledge of certain statistical characteristics of the PCR test (Cowling et al. 1999, Sergeant 2009). To estimate prevalence of *Mikrocytos mackini*, we made clearly stated assumptions regarding the undetermined specificity and sensitivity of the PCR test. Our prevalence numbers are thus estimates but our purpose is to suggest that there may be a need for a more definitive determination of the comparative sensitivity of PCR and histology. We found an apparently greater relative sensitivity of histology compared to PCR in our study, compared to the Carnegie et al. (2003) study, although we acknowledge that the Carnegie et al. (2003) study utilized a higher sample number for comparison. In the case which initiated this study, for which we have both histology and PCR samples, the prevalence by histology was 20.0% while the estimated prevalence by PCR was 11.6% using assumptions of 0.90 PCR sensitivity and perfect specificity (i.e. 1.0). In order to return a prevalence by PCR (19.7%) approximating that derived by histology, we had to reduce the assumed sensitivity of the PCR test to 0.60. In all cases of estimated PCR prevalence shown in Table 1, we used assumptions of sensitivity of 0.90 and specificity of 1.0. Reducing the confidence interval to 0.05 and 0.95 made no difference in prevalence outcome. Carnegie et al. (2003) reported that the PCR test had 3 to 4 times greater sensitivity for *M. mackini* detection than did histology. While our results suggest that the differential sensitivity of histology and PCR might not be as great as that reported by Carnegie et al. (2003), in all cases, a more substantial and systematic comparison should be undertaken. In their study, sample prevalence was lower. If lower prevalence was linked to lower intensity, this might account for lower detection sensitivity using histology. While it initially seems that PCR should be more sensitive, the method utilizes a very small tissue sample and could lose sensitivity when the distribution of the parasite in the tissue is particularly patchy, such as in early or resolving infections. In the population examined by histology and PCR, the estimated prevalence by PCR (11.6%) is closer to the histology initial rapid screening examination prevalence (7.3%) than the more detailed histological examination result (20%), suggesting that PCR may give false negative results when few organisms are present. However, our pri-

mary point is that it is important to develop a more rigorously defined sensitivity relationship between the 2 methods.

The higher prevalence of *Mikrocytos mackini* infection in the two 2008 seed origin brood groups, compared to the 2009 seed origin brood groups, is consistent with the longer residence time of the 2008 seed origin oysters in Humboldt Bay. In addition, the outbreak in general, as well as the higher 2008 seed origin prevalence, could have been exacerbated by the drop in surface seawater temperature in Humboldt Bay in 2008 and 2009. Although the 2008 seed origin brood stock was not placed in the bay until after the 2008 winter, the density of infective organisms already in infected hosts in the bay could have increased as a result of low 2008 winter temperatures, thus providing a higher risk of infection. Subsequently, infection of the 2008 seed could have been further enhanced by the still relatively cooler 2009 winter, consistent with the known propensity of this pathogen to infect Pacific oysters more aggressively as seawater temperatures decrease to around 10°C (Hervio et al. 1996, Bower et al. 1997).

In addition, the fact that we found significant infections in Kumamoto oysters but no infections in the known susceptible and closely related Pacific oysters in Humboldt Bay suggests that Kumamoto oysters may be more susceptible to infection by *Mikrocytos mackini* than Pacific oysters. A low digestive gland condition of the Kumamoto oysters sampled in winter from the west coast of North America is a typical finding, as was shown in the evaluation of the initial positive sample from this report. This indicator of poor feed availability, which leads to poor body condition, along with occasional bacterial abscesses, further suggests that the oysters may be more susceptible to any infection during winter, and particularly to infection by *M. mackini*, which requires cold winter temperatures for infection and replication (Hervio et al. 1996).

As a result of the February 2011 finding of *Mikrocytos mackini*, the California Department of Fish and Game undertook certain legislatively mandated prescriptive actions because of the lack of prior knowledge of this oyster pathogen in California. These actions included an immediate holding action on movement of Kumamoto oyster stocks. After consideration of the emerging data, the following actions were imposed in May 2011. Transfer of Kumamoto oyster stock greater than 20 mm shell height to other bays in California was prohibited, but transfer of Kumamoto oysters less than 20 mm was permitted if a PCR test performed 2 to 4 wk prior to the transfer

was negative. These requirements will remain in effect until testing is negative for the presence of *M. mackini* in adult Kumamoto oysters for 2 consecutive years.

In addition, growers using Kumamoto oyster brood stock from the infected areas voluntarily established procedures to more frequently test for presence of the disease agent, optimize brood stock holding conditions, screen and hold potential brood stock in an isolation facility prior to spawning and continue testing seed oyster stock for DID freedom. Using these procedures, all tested hatchery-produced Kumamoto seed stock in 2011 and 2012, derived from Humboldt Bay brood stock, was PCR test negative for *Mikrocytos mackini*.

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