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Understanding of spatial extent, infection window and potential alternative hosts for the oyster disease QX in Port Stephens

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Abbreviations

BE fixative – Buffered ethanol fixative

Ct – Cycle threshold

IMT - Incident management team

PBS – phosphate buffered saline

PCR – polymerase chain reaction

qPCR – quantitative polymerase chain reaction

QX – Queensland unknown

SRO – Sydney rock oyster

Executive Summary

What the report is about

This report details an investigation by the NSW Department of Primary Industries into QX disease in Sydney Rock Oysters (*Saccostrea glomerata*; SROs) in Port Stephens during the 2022. This followed from the first incursion of this disease in this estuary in August of 2021. QX disease has devastated SRO production in other major growing regions in NSW and Southern Queensland and currently threatens the viability of SRO production in Port Stephens, the State's second largest producer of SROs after Wallis Lake. Using a newly developed quantitative PCR assay alongside traditional cytological methods, we undertook geospatial and temporal investigations of this disease and advanced research into potential transmission pathways to assist oyster growers with future disease management.

Background

The SRO industry comprises approximately 40% of total national oyster production and is the largest aquaculture industry in NSW with an estimated value of $50 million per annum. QX disease specifically affects the SRO and was first described approximately 50 years ago. Despite being a long-standing burden and the major threat for this industry, the drivers for disease are poorly understood. Transmission does not occur from oyster to oyster but has a seasonal pattern and occurs via an (unknown) intermediate host(s). While molecular evidence of the causative agent, *Marteilia sydneyi*, is apparently present across NSW estuaries, clinical disease, involving the sporulating form of the organism and mass SRO mortalities, only occurs in some areas. Where disease does occur, it can be devastating to production and therefore biosecurity policy currently prevents movement of oysters and aquaculture equipment from high QX risk areas (where disease is expressed) to lower risk areas (the QX biosecurity zones for NSW SRO estuaries can be found at https://www.dpi.nsw.gov.au/fishing/aquatic-biosecurity/aquaculture/aquaculture/qx-oyster-disease). Prior to 2021, Port Stephens was considered a "low risk" estuary with respect to QX disease. However, in August of 2021, QX disease was detected for the first time in oysters from cultivation areas within the inner port. As Port Stephens is a major SRO production area, including hatchery facilities, and a major supplier of oysters to other estuaries for grow out, this incursion has significant implications for the SRO industry.

Aims/objectives

This project aimed to undertake a geospatial survey of Port Stephens to identify areas affected (and those unaffected) by QX disease to enable oyster growers to make informed decisions about where to farm in the coming season/s. A temporal investigation of disease transmission within the Port (window of infection study) was also undertaken using sentinel oysters to determine when the season for transmission closes. Finally, we aimed to progress research on intermediate hosts for disease transmission to begin closing knowledge gaps around the lifecycle of *M. sydneyi*, which is considered critical for understanding how best to manage and prevent further spread of QX disease.

Methodology

This project involved the use of a newly developed quantitative PCR (qPCR), as well as traditional cytological examination of oyster tissues to monitor for disease and the presence of *M. sydneyi*. The qPCR assay was validated using a panel of oyster samples known to be positive or negative for *M. sydneyi*

and a cross reactivity panel of protozoans and common marine bacteria. The analytical sensitivity of the assay was determined using a standard series.

For the geospatial survey, 300 SROs were collected across 10 zones within Port Stephens including areas of the inner and outer port. A total of 30 oysters were tested per zone, and where possible included a mix of wild and cultivated SROs. Oysters were processed in the laboratory at Elizabeth Macarthur Agricultural Institute and gross observations of oyster condition and digestive gland colour were recorded. Cytological imprints from the digestive glands were examined to determine disease status (presence/absence of sporulating organisms), and DNA extracts of the digestive glands were used to test for PCR status.

For the window of infection study, oysters were deployed weekly from early May to early June into two areas of Port Stephens (Tilligerry Creek and Karuah River) that were severely affected by QX disease. Oysters were exposed for a period of 4 weeks and then removed for testing as described above for the geospatial survey. Water temperature data was collected from nearby sensors over the study period.

To investigate potential intermediate hosts for transmission of QX disease, we focussed on polychaete worms of the species *Aglaophamus* (formerly *Nephtys*) *australiensis*, which were previously shown to harbour *M. sydneyi*, and plankton samples due to copepods being implicated in the transmission of *Marteilia refringens* in Europe. Polychaete worms were collected from QX-affected areas of Port Stephens for molecular testing and naïve polychaetes from QX-free Wallis Lake were subjected to laboratory transmission trials under controlled temperature and salinity to determine if *M. sydneyi* could be transmitted from affected oyster tissue to *A. australiensis.* All polychaete worms were tested using qPCR to determine the presence of *M. sydneyi*.

Finally, plankton was sampled from 6 of the 10 zones used in the geospatial survey to determine if *M. sydneyi* could be detected. Lateral surface plankton tows and vertical hauls with a plankton net were performed and control water samples were collected from the same sites to distinguish between *M. sydneyi* suspended in the water column and parasites associated with plankton. DNA extracts of plankton were tested by molecular methods and fixed samples were examined microscopically to determine the types of planktonic organisms present.

Results/key findings

The qPCR assay developed in this project was found to be 100% specific for *M. sydneyi* and also highly sensitive, with an analytical sensitivity down to 1 gene copy per µL of DNA extract. Comparisons of qPCR and cytology results indicated that a cycle threshold (Ct) value of 20 is an approximate cut-off for the detection of sporulating QX, indicating that very high burdens of the parasite are present by the time disease is evident.

The geospatial survey determined that QX disease (i.e., as indicated by oysters with sporulating parasites from cytology) was present in the areas of inner Port Stephens that were affected by the 2021 incursion, including Tilligerry Creek, Karuah River and Oyster Cove, with Upper Tilligerry Creek and Upper Karuah River the most severely affected. However, over the course of this project, mortalities in Cromarty Bay and Bundabah were also confirmed to be due to QX disease, indicating that the disease spread to new areas of the Port later in the season.

All oysters collected from the outer Port were negative for QX disease. However, low level detections of *M. sydneyi* were found across all zones in the outer Port using qPCR.

One way analysis of variation (ANOVA) indicated that there was a statistically significant difference in mean load of *M. sydneyi* in oysters across the sampling sites with very high infectious load (and prevalence of *M. sydneyi*) in Upper Tilligerry Creek and Upper Karuah River.

Wild oysters had statistically lower levels of QX disease compared to cultivated oysters, although the load of *M. sydneyi* was not statistically different, suggesting that wild oysters are less prone to developing disease.

The window of infection study indicated that the window for transmission of QX disease had effectively closed by early May in both Tilligerry Creek and Karuah River, with no sporulating QX detected in any oysters deployed between early May and early June. Oysters deployed in the first week of May only, and only at the Tilligerry Creek site, yielded a relatively high number of qPCR positive oysters suggesting that some transmission occurred at this time. The nearby temperature sensor indicated that the water temperature was over 21.5°C, a temperature previously determined to be permissive for transmission. Nonetheless, data indicated that the temperature dropped below 21.5°C by the second week of the study suggesting that *M. sydneyi* infection was unable to progress.

Investigations into potential intermediate hosts for disease transmission were unable to confirm *A. australiensis* polychaetes as an intermediate host for *M. sydneyi* with all samples collected from QX-affected areas of Port Stephens testing negative via qPCR and with experimental transmission experiments failing to demonstrate transmission of *M. sydneyi* from QX-affected oyster tissue to *A. australiensis*.

Surface plankton tows from Upper Tilligerry Creek and Upper Karuah River tested positive for *M. sydneyi* indicating that the parasite may be associated with plankton. Control water samples from these same sites tested negative suggesting that *M. sydneyi* was concentrated in the plankton rather than free in the water column. Microscopic examination of plankton samples indicated that calanoid copepods were present and were also the most abundant component of the zooplankton.

Implications for relevant stakeholders

This study has defined the current extent of spread of QX disease in Port Stephens and provides information for growers on areas that can currently be farmed without major risk of disease transmission. While *M. sydneyi* was detected in some samples from the outer Port, levels of the parasite were extremely low compared to areas of the inner Port which have high burdens of both parasite and disease. Spread to the outer Port is still possible over subsequent seasons but this may depend on whether hydrogeographical features of the outer Port will support disease and whether these conditions favour the intermediate host for transmission. It is important to note the risk of QX disease within estuaries does change over time and the disease can spread to other areas and areas further downstream as has occurred in the Georges and Hawkesbury Rivers.

This project also provides temporal information regarding the closure of the window of infection for transmission of QX disease. In 2022, disease was not observed in sentinel oysters deployed in early May through to early June. PCR testing suggests that transmission may still occur if the temperature is over 21.5°C but new infection followed by disease progression is unlikely to occur once the temperature drops below this level.

This study advances our knowledge on the intermediate host transmission of QX disease and provides clues for where ongoing research in this area should be directed.

Finally, this project has provided some preliminary information for policy makers about where Port Stephens lies with respect to the current risk ratings.

Recommendations

QX disease remains the largest known threat for SRO production. The disease, particularly outside of the oyster host, is poorly understood and warrants further research given the value of the SRO industry and

the size of the industry as a proportion of oyster production nationally. Furthermore, the SRO industry is our native oyster fishery and has cultural, historical and ecological significance. Despite 50 years having elapsed since QX disease was first recognised, large knowledge gaps remain in our understanding of this disease. Disease spread is likely to continue to occur into other key growing areas if these questions remain unanswered.

Specifically in Port Stephens, the geospatial survey should be repeated in 2023 to determine disease risk across zones in the next season and to determine if disease is likely to be ongoing each season or sporadic. The commencement of the window of infection in this estuary is also yet to be determined but is key to restocking and preserving that stock throughout its grow out cycle.

General research on QX applicable across estuaries includes applying findings from this study on potential intermediate hosts for transmission and closing the parasite lifecycle. This is critical to understanding infection dynamics and preventing disease spread.

Finally, further research is essential to shape evidence-based biosecurity policy around the QX disease risk ratings of NSW estuaries due to the impact of closures and subsequent movement restrictions on existing oyster businesses. This research would entail determining whether *M. sydneyi* strain variation plays a role in disease outbreaks or whether environmental factors are the major drivers of disease.

Keywords

Sydney Rock Oyster, *Marteilia sydneyi*, QX disease, Port Stephens, qPCR, cytology

Introduction

The Sydney Rock Oyster (SRO) is an edible mollusc native to NSW estuaries and comprises the State's most valuable aquaculture product valued at over $50 million during 2019-20 [1]. The seasonal disease termed "Queensland Unknown" (QX disease) caused by the parasite *Marteilia sydneyi* [2], can result in mass mortalities of oysters during outbreaks [3-5] and is the most significant disease affecting this important commercial species. This disease has effectively ended commercial cultivation of SROs in the Hawkesbury and Georges rivers [5], and for the first time in 2021, was detected in Port Stephens. This recent outbreak is a major biosecurity concern due to the potential disease impact upon Port Stephens industry, the volume of SRO seed stock that is sent to other estuaries in NSW for grow out, and the need to ensure appropriate biosecurity risk management is implemented to minimise risk of spread to other oyster growing areas.

Port Stephens is the second largest oyster producing estuary of NSW, and also contains the State's largest commercial hatchery facility for producing oyster spat (juvenile oysters), a dedicated oyster research hatchery facility at the NSW DPI Port Stephens Fisheries Institute, and oyster nursery facilities. Port Stephens is a major supplier of oysters for grow out in other estuary systems within NSW. Port Stephens is also the location of the NSW DPI SRO breeding program operations where QX resistant broodstock are produced. There are limited numbers of QX resistant broodstock held outside of Port Stephens to supply hatcheries in other locations, however, this stock must be managed carefully until new broodstock are produced to replace it. The estuary also contains extensive natural SRO reefs.

In August 2021, a NSW DPI incident management team (IMT) was established to coordinate the response to the initial detection of QX disease in Port Stephens. The IMT intent was to contain, and establish the extent of the spread, of QX disease following detection of sporulating *M. sydneyi* by cytology in oyster samples collected from Port Stephens for the first time on 27/8/2021. The QX disease classification of the Port Stephens estuary remains under investigation with control measures and movement restrictions remaining in place through the Biosecurity (QX Disease) Control Order 2021. The initial surveillance performed in the estuary during 2021 revealed a total of 7/180 QX positive oysters in the three western waterways of Port Stephens (Karuah River, Oyster Cove/Swan Island; Lemon Tree Passage/Tilligerry Creek). However, this sampling occurred at a point in the season in which disease expression is rarely observed in other estuaries where QX is known to occur. Therefore, the need for additional sampling within an optimal window of disease expression (late Summer/Autumn 2022), was deemed essential.

Even though it has been 50 years since *M. sydneyi* parasite was first observed [3], key knowledge gaps around this parasite remain. These include the drivers for the development of disease in new estuaries and which hosts are responsible for supporting the parasite's lifecycle [5]. The polychaete worm, *Aglaophamus australiensis*, has been shown to harbour *M. sydneyi* [6], but experimental transmission work has not been attempted to confirm the role of this worm in the lifecycle. European studies on a related parasite, *Marteilia refringens,* have implicated a copepod as an intermediate host for transmission [7, 8] but this possibility has never been investigated for *M. sydneyi*. Limited scientific data on the epidemiology of this pathogen means that devising effective, evidence-based management strategies, including biosecurity policies, remains difficult.

The aim of this project was to provide information to oyster growers around the geospatial distribution of QX disease in the Port Stephens estuary, and the timing of closure of the window for transmission of QX to assist with stock management in the coming seasons. This project also aimed to extrapolate on prior research pointing towards polychaete worms and/or copepods as intermediate hosts for QX transmission.

Objectives

This project aims to:

- Complete analysis of all samples collected in the 2022 QX survey
- Undertake additional sampling of oysters introduced into Port Stephens to better define the window of infection
- Collect additional oyster and biota samples to expand our knowledge of the secondary host Develop a template for an ongoing longitudinal sampling program to address industry questions regarding QX disease.

Method

Geospatial survey of QX disease in Port Stephens

Sample collection

A geospatial survey of QX disease in Port Stephens was undertaken in March of 2022 following original detections of QX disease in the Port in August of 2021. Ten collection sites comprising the main cultivation areas within Port Stephens were chosen for the survey (Table1). A total of 30 oysters were randomly sampled within each site with the exception that (where possible) an equal mix of wild and cultivated oysters were sampled. Thus, the total number of oysters surveyed was 300. A sample size of 30 per site was chosen to provide 95% confidence of detecting at least one affected individual assuming a 10% disease prevalence at any given site.

Table 1: Sample collection sites for geospatial survey

Zone	Site name	Samples collected
1	Lower Tilligerry Creek	15 × cultivated SROs 15 × wild SROs
2	Oyster Cove	15 × cultivated SROs 15 × wild SROs
3	Lower Karuah	15 × cultivated SROs 15 × wild SROs
4	Upper Karuah	15 × cultivated SROs 15 × wild SROs
5	Bundabah	15 × cultivated SROs 15 × wild SROs
6	Soldiers Point	15 × cultivated SROs 15 × wild SROs
7	Salamander Bay	30 × wild SROs*
8	Corrie Island	15 × cultivated SROs 15 × wild SROs
9	Tea Gardens	15 × cultivated SROs 15 × wild SROs
10	Upper Tilligerry	29 × cultivated SROs† 1 × wild SRO

*non-cultivation area, spat harvesting site only

†the majority wild oysters collected from this site were Pacific oysters and therefore were not tested. The total sample number for this site was made up to 30 with cultivated oysters.

Oyster processing

Oyster samples were labelled according to sample site and type and placed into mesh bags. Samples were transported to the laboratory in eskies the following day and were processed immediately upon arrival for cytological examination and molecular testing. Oysters were shucked aseptically using sterile shucking knives and a cross section was taken using a sterile scalpel blade. Gross observations of oyster condition and digestive gland colour were recorded according to the criteria in Tables 2 and 3 respectively.

Table 2: Criteria for oyster condition scoring

Score	Description
1	Oyster is thin lacking gonad
2	Gonad present but in small amounts, digestive gland is clearly visible
3	Moderate amount of gonad surrounding the digestive gland
4	Well-developed gonad, digestive gland not visible
5	Oyster is ripe with gonad and ready to spawn, digestive gland hidden

Table 3: Criteria for scoring of oyster digestive gland colour

Score	Description
1	Colour of the digestive gland is pale (yellow-brown to white)
2	The digestive gland is medium brown in colour
3	Dark brown or greenish brown digestive gland (normal healthy colour of an oyster that has been feeding well)

For cytological examination, a 1.5 mm cube of digestive gland from each oyster was used to prepare a tissue imprint on a glass slide which were allowed to air dry. A further 1.5 mm cube of digestive gland was removed to a sterile microfuge tube for molecular testing. A cross section of each oyster was archived in 10% neutral buffered formalin and the remaining oyster tissues stored archived at -80°C.

Cytological examination

Slides with digestive gland imprints were fixed with methanol and stained with Diff-Quik stain according to the manufacturer's instructions (Sigma Aldrich). For the geospatial survey, each slide was examined for the presence of sporulating forms of *M. sydneyi* as this defines the disease for biosecurity purposes. For the window of infection study, cytological smears were examined for both sporulating forms of M. sydneyi as well as the earlier nurse or daughter cell forms to maximise sensitivity.

Molecular testing

Molecular testing was performed on all samples using DNA extracted with the MagMAX™ Core nucleic acid purification kit on the Kingfisher™ Flex system (ThermoFisher Scientific). Existing methods for molecular detection of *M. sydneyi* rely on conventional PCR [9] and thus only provide a qualitative presence/absence result. Because *M. sydneyi* appears to produce subclinical infections (such as in "low risk" estuaries) as well as clinical disease, we developed a quantitative PCR assay for use in this study to assist in providing information on the burden of *M. sydneyi* infection.

The qPCR assay for *M. sydneyi* was designed based on alignments of the intergenic spacer (ITS) region from *M. sydneyi* isolates from a range of estuaries (Figure 1) and incorporated a TaqMan™ 5' FAM-labelled probe. Each reaction contained 10 µL of Environmental Master Mix (Applied Biosystems), 0.5 µM of each primer, 0.2 µM probe, 2 µL of DNA template and molecular grade water with a total reaction volume of 20 µL. All PCR reactions were performed on a QuantStudio5 thermal cycler using the following cycling conditions: 95°C for 10 min (1 cycle); 95°C for 15 s, 60°C for 60 min (45 cycles).

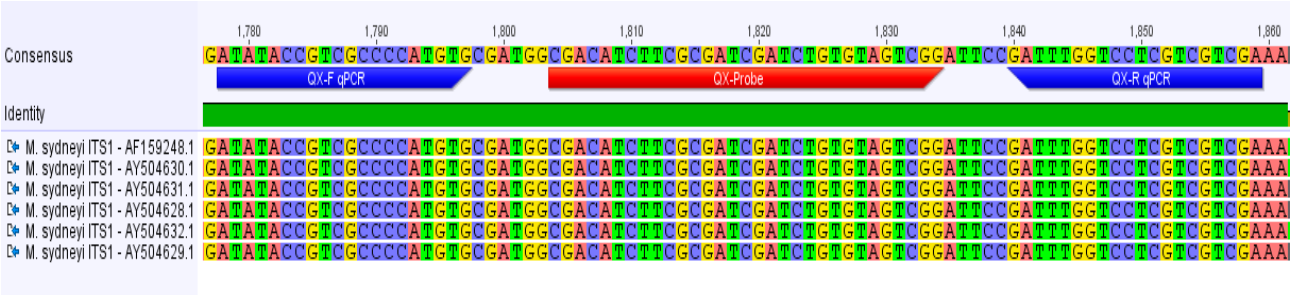

Figure 1: *M. sydneyi* qPCR assay design.

Plasmid standards were prepared by cloning the QX PCR product using the TA cloning kit (ThermoFisher Scientific) according to the manufacturer's instructions. A qPCR standard series was prepared by making 10-fold serial dilutions of the plasmid in transfer RNA (Sigma Aldrich).

The assay specificity was determined using a panel of oysters known to be negative for QX disease, samples of non-target protozoa and bacteria (cross-reactivity panel), and oysters that were known to be positive for QX disease by cytology and/or conventional PCR (Table 4) and then applied to the test samples. The analytical sensitivity of the assay was determined by testing 8 replicates of the standard series. The point at which 50% of the samples tested positive was deemed to be the limit of detection of the assay.

Table 4: Panel of samples used for qPCR specificity and sensitivity testing.

Sample description	**Organism**	**No. samples tested**
Cross reactivity panel	*Perkinsus* sp.	3
	Vibrio alginolyticus	1
	Vibrio vulnificus	1
	Vibrio parahaemolyticus	1
	Babesia bovis	3
	Theileria orientalis	3
	Tritrichomonas foetus	6
	Pentatrichomonas hominis	1
	Tetratrichomonas gallinarum	3
Known negative oyster samples	*M. sydneyi* negative *S. glomerata*	25
	Crassostrea gigas	10
Known positive oyster samples	*M. sydneyi* positive *S. glomerata*	16
Total		**73**

Statistical analysis

All statistical analyses were performed using GraphPad Prism v9.2.0. A chi square test was used to compare cytology positive detections across sites, while Fisher's exact test (with a 2-tailed P value) was used to test differences in detection of sporulating QX disease using cytology and *M. sydneyi* infection with qPCR and whether there was a difference in the proportion of positive detections in wild vs cultivated oysters. In the latter comparison, only sites where equal numbers of both wild and cultivated oysters were collected were examined to avoid statistical bias in the data based on sampling site. Cycle threshold (Ct) values from PCR testing were compared across sites using analysis of variance (ANOVA) with Tukey's post hoc test to compare the means of individual groups. Pearson's correlation coefficient was used to determine whether there was a statistical correlation between oyster condition or digestive gland colour and positivity for *M. sydneyi.*

Window of infection study

To determine whether *M. sydneyi* was still actively being transmitted to oysters in QX-affected areas of Port Stephens, approximately 35 × naïve sentinel SROs sourced from Wallis Lake were deployed weekly for 5 weeks to each of the Upper Karuah River and Upper Tilligerry Creek from early May to early June (Table 5). Oysters were maintained within the estuary for a period of 6 weeks to allow *M. sydneyi* transmission to occur and disease to develop [10]. After 6 weeks exposure in the estuary, oyster mortality was assessed, and 30 oyster samples were collected for submission to the laboratory for cytological examination and qPCR testing. Oysters were processed as above for cytology and PCR.

Table 5: Dates of deployment and retrieval of sentinel SROs for window of infection study

Deployment Week	Deployment Site	Date	Sample Date
1	Karuah	4/5/22	22/6/22
1	Tilligerry	4/5/22	22/6/22
2	Karuah	12/5/22	28/6/22
2	Tilligerry	11/5/22	28/6/22
3	Karuah	19/5/22	6/7/22
3	Tilligerry	19/5/22	6/7/22
4	Karuah	27/5/22	11/7/22
4	Tilligerry	27/5/22	11/7/22
5	Karuah	9/6/22	18/7/22
5	Tilligerry	8/6/22	19/7/22

Plankton sampling

To investigate the possibility that components of the zooplankton, such as copepods may be involved in QX disease transmission, plankton was sampled from seven sites across Port Stephens corresponding to a number of zones sampled in the geospatial study (Table 6). All plankton tows were performed with a 100 µm plankton net (Figure 2) with attached collection container. Duplicate surface lateral tows were undertaken for 2 min per tow at each site to sample the surface waters, and duplicate vertical hauls were also undertaken to sample zooplankton across the entire water column. Within Port Stephens at Salamander Bay, which was unaffected by QX disease, but had weak qPCR detections of *M. sydneyi* in wild oysters, 2 min oblique tows were performed to maximise plankton collection and increase sensitivity of detection. Oblique tows combine lateral and vertical sampling in a single collection. After each tow, the plankton net was washed to concentrate the sample in the collection jar and each sample was transferred to a labelled 500 mL sample jar. Unfiltered water samples were also taken from each site to control for the presence of *M. sydneyi* parasites free in the water column. Temperature and salinity measurements were taken at each site using a portable Horiba sensor. Measurements were recorded at both the water surface and at a depth of 1 m.

Following collection, all plankton samples were filtered further in the laboratory using filter paper to reduce the sample volumes and duplicate filtered samples from each site were fixed with either 10% neutral buffered formalin or buffered ethanol fixative (BE fixative: 70% ethanol; 1% glycerol; 0.5% glacial acetic acid; 28.5% phosphate buffered saline [PBS]).

Plankton processing

Plankton samples fixed in ethanol were centrifuged and the pellet was resuspended in 400 µL phosphate buffered saline (PBS). DNA was extracted from the zooplankton that had been fixed in BE fixative using the same methods described above for oyster tissue, with the exception that a bead beating step was added to the beginning of the workflow assist with lysis of chitinous organisms. All steps were performed according to the MagMax™ Core workflow recommended by the manufacturer.

Both ethanol and 10% formalin-fixed samples were examined by microscopy (Olympus BX53 microscope, dark field setting) to assess the range of planktonic organisms in the samples. Images were collected with an Olympus SC180 digital camera.

Table 6: Plankton sampling sites

Site number	Site name	Coordinates
1	Lower Tilligerry Creek	-32.738695, 152.050616
2	Upper Tilligerry Creek	-32.768615, 151.967833
3	Bundaba	-32.668091, 152.052973
4	Lower Karuah River	-32.653690, 151.969303
5	Upper Karuah River	-32.587566, 151.970132
6	Salamander Bay	-32.724741, 152.092630

Figure 2: Plankton net with 100 µm mesh used for zooplankton sampling.

Polychaete collection

Nephtid polychaete worms, specifically *Aglaophamus australiensis* (formerly *Nephtys australiensis*) have been proposed as an intermediate host for *M. sydneyi* [6]. To further investigate this *A. australiensis* were collected from Port Stephens to test for the presence of *M. sydneyi* .

Polychaetes were collected by taking a 10 cm diameter core of sediment approximately 20 cm deep and sieving the sediment through 500 µm metal sieves. Polychaetes morphologically identified as *A. australiensis* were collected into 5 mL sample vials containing seawater for *M. sydneyi* PCR testing. Samples were collected from Lemon Tree Passage, near the mouth of Tilligerry Creek (coordinates: -32.728453, 152.039006) as *A. australiensis* polychaetes were not detected in sediment cores taken in Upper Tilligerry Creek adjacent to QX-affected oyster leases.

For experimental transmission studies, naïve *A. australiensis* were collected from a site at Wallis Lake previously found to harbour *A. australiensis* [11], (coordinates: -32.182347, 152.497101). Polychaetes were collected into 500 mL containers with a 5 cm layer of clean coarse sediment overlayed with 10 cm of seawater from the collection site. All samples were transported to the laboratory in eskies to maintain temperature.

Transmission study

Naïve *A. australiensis* collected from Wallis Lake were maintained in a similar manner as described as King et al. [12]. Briefly, individual polychaetes were placed in separate 500 mL containers with a 1 cm overlay of clean coarse sediment. Polychaetes were maintained within their individual containers within larger plastic tanks (1 × control and 1 × test tank) containing 20 L of fresh seawater (Figure 3) at a constant salinity of 33 ppt (the salinity of the collection site) and temperature of 22°C with continuous water aeration and filtration. Test polychaetes (n = 13) were maintained for a total 6 weeks and fed every 2 weeks with 1 mm^3 pieces of digestive gland (approximately 25 mg per 500 mL container) derived from oysters with sporulating QX. Control worms (n = 11) were fed a commercial diet of Sera Vipagran fish food as described in [12]. Seawater was checked twice weekly for ammonia levels using an API NH_3/NH_4 test kit and if the ammonia levels exceeded 1.0 ppm, the seawater was exchanged.

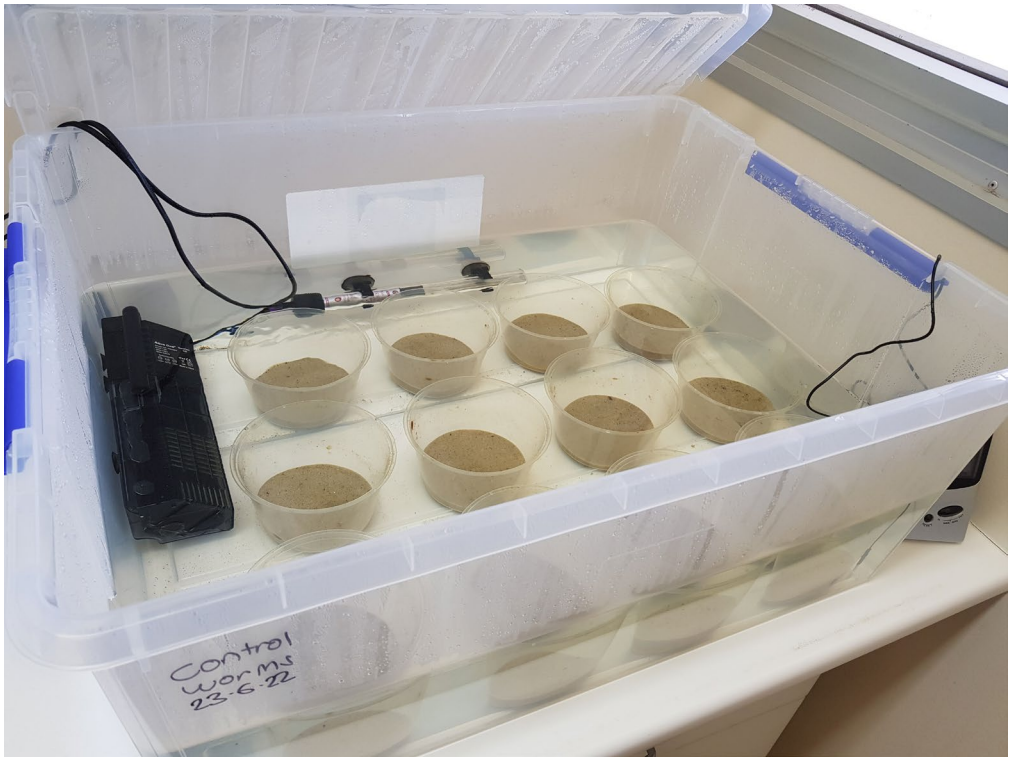

Figure 3: Polychaete worms were maintained under controlled conditions in a seawater tank within individual containers with a sediment overlay.

Polychaete processing

Polychaete worms collected from Port Stephens, and control and test worms from the transmission trial, were all processed by bisecting the worms, archiving half in 10% neutral-buffered formalin and reserving the other half for molecular testing. DNA was extracted from polychaetes and tested using the same methods described above for oyster tissues.

Results and Discussion

qPCR validation

The *M.sydneyi* qPCR was shown to be both sensitive and specific for *M. sydneyi* detection. All known positive oyster tissues (n = 16) were detected as positive and all known negative oyster tissues (n = 35) were detected as negative. Known negative oysters included SROs sourced from estuaries unaffected by QX disease (n = 25) and negative on conventional PCR and Pacific oysters (*Crassostrea gigas*) which are not affected by QX disease (n = 10). Furthermore, none of the non-target species in the cross-reactivity panel tested positive. Based on this panel of 73 samples, the QX qPCR had a specificity of 100%. The analytical sensitivity of the assay determined using 8 replicates of a plasmid standard curve, was determined as approximately 1.02 gene copies μL^{-1} of DNA extract (or around a Ct value of 40), indicating a highly sensitive test for *M. sydneyi* detection. This assay was used for PCR detection of *M. sydneyi* throughout the remainder of the study with a Ct value of 40 as a cut-off for test positivity.

Geospatial distribution of QX disease in Port Stephens

Results from cytological and qPCR testing of 300 oysters collected for the geospatial survey are shown in Table 7. Detections of sporulating QX disease (cytology positive detections) are also shown in Figure 4. Upper Karuah River, Upper Tilligerry Creek and Oyster Cove were the sites with the highest levels of sporulating QX. Oysters from Lower Karuah River and Lower Tilligerry Creek were also affected, but in lower numbers than those the upper reaches of these rivers. Cytology positive oysters were also detected at Bundabah and Soldiers Point; however, these positive detections were both traced to translocation of stock from Tilligerry Creek, suggesting that the oysters may have been infected prior to movement (Figure 4). Oysters from Bundabah and Soldiers Point that were not sourced from Tilligerry Creek were negative for sporulating QX disease. All sites in the outer estuary (Salamander Bay, Corrie Island and Tea Gardens) were negative for sporulating QX.

qPCR detected a higher number of *M. sydneyi* positive oysters compared to cytology and this was statistically significant ($p = 0.0001$), although the sensitivity of cytology was likely to be lower in this study as only sporulating forms of the parasite were considered. There was also a statistically significant difference in the mean Ct value of cytology positive vs negative oysters within the PCR positive group ($P < 0.0001$) (Figure 5). The mean Ct value of an oyster with sporulating QX disease was 16.4. Sporulating *M. sydneyi* was only detected in 10% of oysters with a Ct > 20, while 94% of oysters with a Ct > 20 were negative for sporulating QX. This data indicates that QX disease is associated with very high burdens of *M. sydneyi* and demonstrates the utility of qPCR as a diagnostic test for detecting high parasite loads where sporulating forms of the parasite are not evident on cytological smear.

Table 7: Cytology and qPCR results from geospatial survey

Zone	Site name	No. cytology positive*	No. qPCR positive
1	Lower Tilligerry Creek	4/30	11/30
2	Oyster Cove	8/30	19/30
3	Lower Karuah River	2/30	23/30
4	Upper Karuah River	17/30	27/30
5	Bundabah	2/30	4/30
6	Soldiers Point	2/30	7/30
7	Salamander Bay	0/30	3/30
8	Corrie Island	0/30	2/30
9	Tea Gardens	0/30	1/30
10	Upper Tilligerry Creek	16/30	25/30

*A positive detection is defined in this case by the presence of sporulating forms of *M. sydneyi* only.

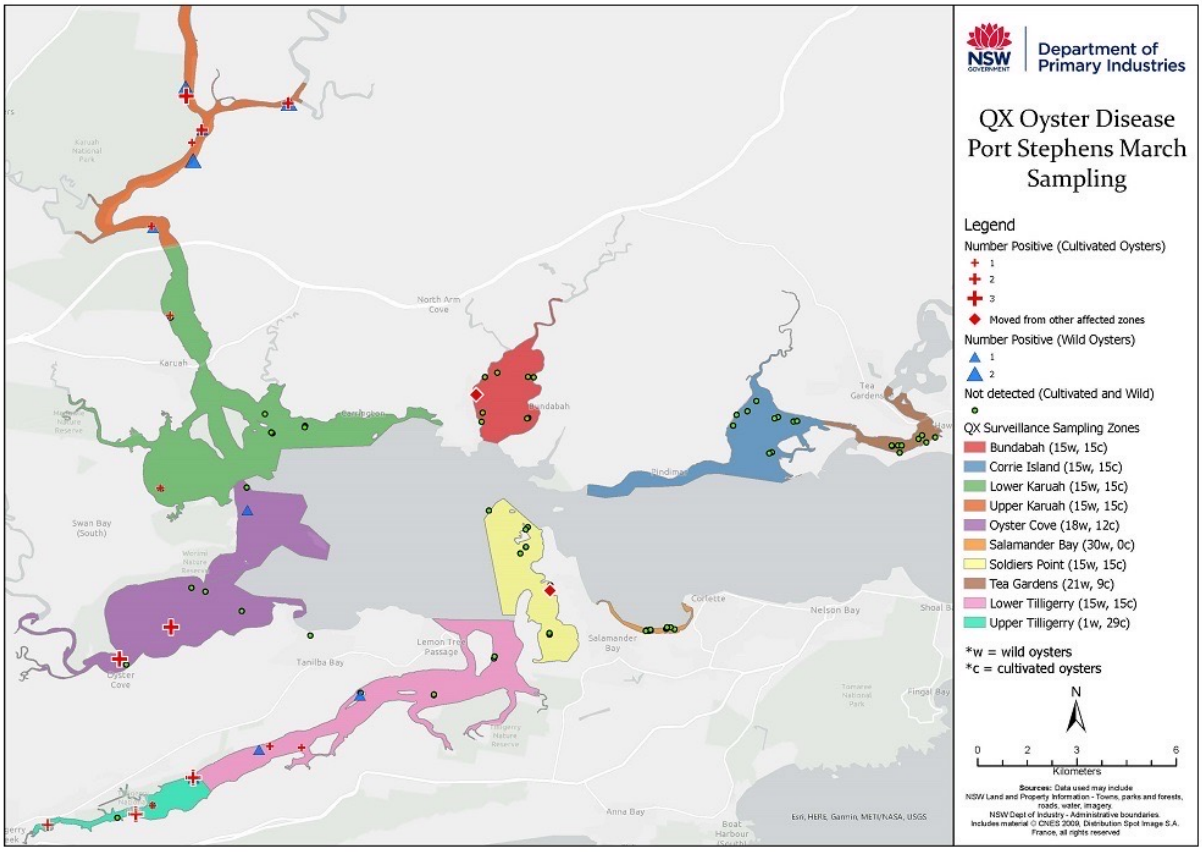

Figure 4: Map of sites sampled and areas where sporulating QX disease was detected (via cytology) in wild and cultivated oysters.

Across all oysters, there was a significant but weak positive correlation between the oyster condition score and the Ct value (Pearson's r = 0.258; P <0.0001) and a significant moderate correlation between the digestive gland score and Ct value (Pearson's r = 0.574; P <0.0001). These data demonstrate that the gross pathological features observed were related to the *M. sydneyi* infectious load and this is consistent with a prior study of QX disease in the Hawkesbury River [10].

Unsurprisingly, the mean Ct values of oysters collected from each site were statistically different (P < 0.0001) (Figure 6). The lowest mean values were observed at Upper Karuah and Upper Tilligerry Creek, consistent with the detection of sporulating QX at those sites. Interestingly, while there were a similar number of qPCR positive detections at Oyster Cove and Lower Karuah with a similar mean Ct value, detections of sporulating QX were 4 × higher at Oyster Cove. One limitation of this survey was the collection of samples at a single time point, therefore whether the lower number of detections of sporulating QX at Lower Karuah were due to the timing of collection, environmental, or other reasons is unclear. Furthermore, PCR detections of *M. sydneyi* were made across all sampling sites, including those in the outer estuary which were free from sporulating QX (Table 7). All PCR detections in the outer estuary had Ct values in the high 30s indicating a low burden of *M. sydneyi*. PCR positive oysters from Bundabah and Soldiers Point (apart from oysters that were translocated from Tilligerry Creek) had similarly high Ct values. Both Bundabah and Soldiers Point (Cromarty Bay) went on to experience QX-related mortalities later in the season (data not shown), while sites in the outer estuary remained QX disease-free. The hydrogeography of the outer estuary may make it less conducive to QX outbreaks due to higher salinity and greater oceanic exchange. However, the infectious dose of *M. sydneyi* required to progress to the sporulation phase is also currently unknown. This is difficult to determine in the absence of an experimental infection model; however, a longitudinal study in the field with the aid of qPCR testing may assist in understanding the clinical threshold for disease, and potentially provide a means of monitoring for impending outbreaks, particularly in areas that are known to be at risk of disease based on this geospatial survey.

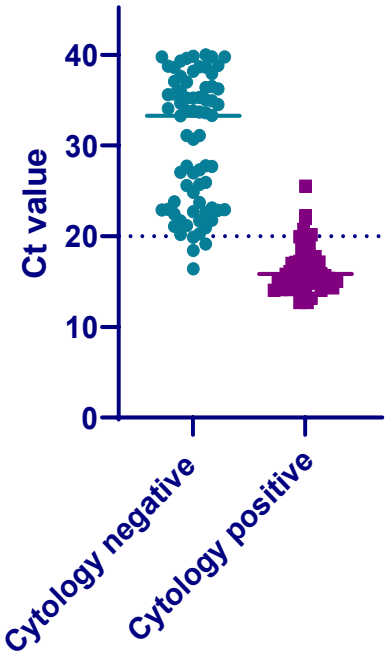

Figure 5: Comparison of Ct values of cytology positive and negative oysters within the PCR positive group. Solid horizontal lines indicate the mean Ct for each group. The dotted line indicates an approximate Ct cut-off value for detection of sporulating QX disease.

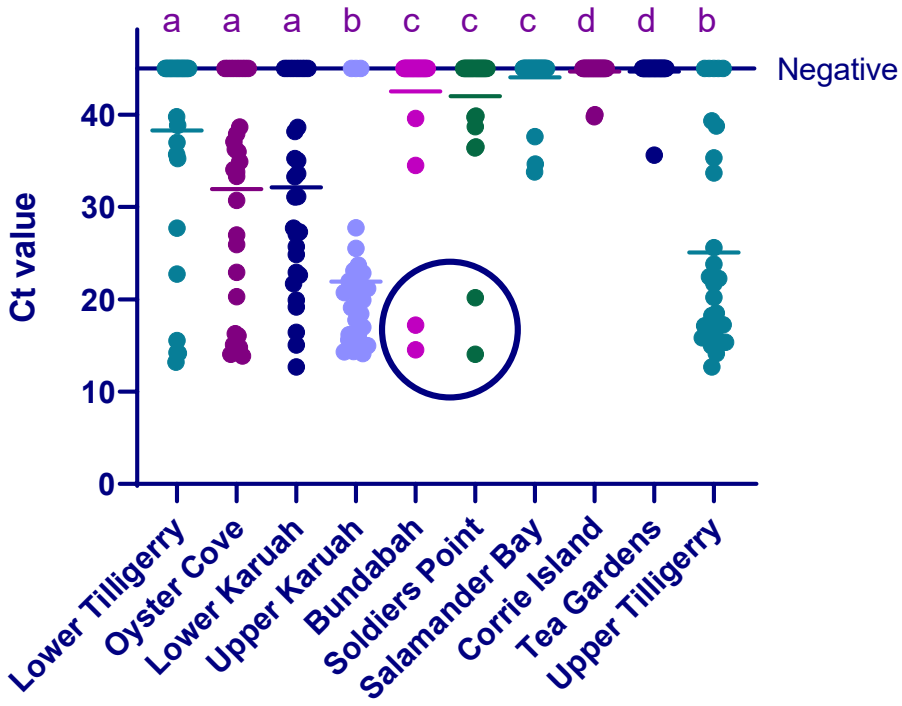

Figure 6: Mean Ct values by sampling site. A Ct of 45 indicates a negative PCR result. Closed circles indicate the Ct values of individual samples and horizontal lines the mean Ct at each site. The open circle indicates the samples that were translocated from Tilligerry Creek to Bundabah and Soldiers Point (suggesting that they were infected prior to translocation). Groups with significantly different means are indicated by the superscript above each column ($p < 0.05$).

In areas where sporulating QX disease was prevalent, (Tilligerry Creek, Karuah River and Oyster Cove), positive detections were made in both cultivated and wild oyster populations (Figure 4); however positive detections were statistically higher in cultivated oysters (P = 0.041). There was also a numerically (but not statistically) higher number of PCR positive cultivated vs wild oysters (P = 0.085). However, there was no statistical difference in mean Ct values of PCR positive cultivated and wild oysters (P = 0.567) (Figure 7). These results suggest that wild oysters were just as susceptible to infection with *M. sydneyi* as cultivated oysters, but sporulating QX disease was more likely to occur in cultivated oysters. This may be due to a slightly greater resistance to QX disease in wild oysters, perhaps due to a prior year of exposure (2021) of wild oysters in some areas of Port Stephens, differences in population density, or other factors (for example age, hydrodynamic, level in the water column) or stressors imposed on cultivated oysters.

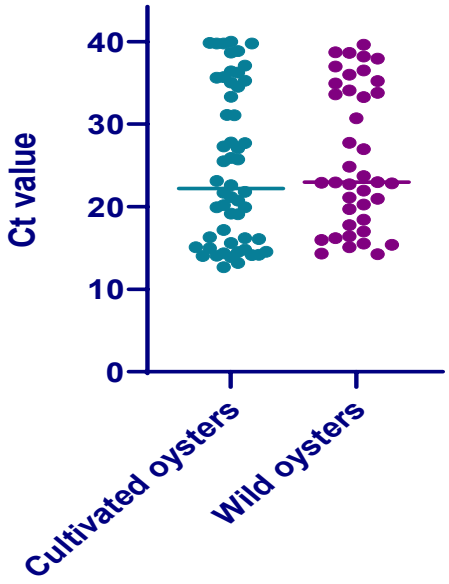

Figure 7: Comparison of *M. sydneyi* qPCR Ct values in cultivated vs wild oysters collected from the same sampling sites. Closed circles indicate the Ct of individual samples. Mean Ct indicated by horizontal lines.

Window of infection

Results from the window of infection study show that while some mortalities were observed in the deployed oysters (Table 8), no oysters from either site displayed gross signs of QX disease (all had normal condition and digestive gland colour). Furthermore, no oysters from either site contained sporulating QX organisms over the course of the experiment, therefore the observed oyster mortalities are more likely due to translocation stress related to deployment. While no oysters tested positive for QX on cytology, there were a number of PCR positive oysters detected. A substantial number of oysters (11/30) deployed at Tilligerry Creek on week 1 tested positive for the presence of *M. sydneyi*, but detections declined thereafter with only 2/30 detected from week 2 oysters and 2/30 detected from week 5 oysters. At Karuah, only one oyster tested PCR positive from the week 1 deployment. A prior study conducted in the Hawkesbury River [10] suggested that once the water temperature is below 21.5°C, no infection occurs. Interestingly, the temperature sensors closest to the Tilligerry Creek and Karuah sites indicated that there were different temperature profiles at the deployment sites. While the sensor closest to Karuah never exceeded 21.5°C, the Tilligerry Creek sensor indicated that temperatures were above 21.5°C at the time of the week 1 deployment and fell below 21.5°C 2 days after the week 2 deployment.

The PCR results suggest that oysters deployed at Tilligerry Creek on week 1 became infected with *M. sydneyi* while the water was above the permissive temperature, but that progression of disease was halted soon after when the water temperature dropped below 21.5°C. Indeed, Ct values of positive oysters from week 1 were all above 30, indicating a low burden of *M. sydneyi*. The low number of detections later in the study period are consistent with a low infection rate due to the decline in water temperature. Based on the

data from this study, the window for infection (and progression) of QX disease had already closed by early May. This is consistent with findings from the Hawkesbury where the window of infection was shown to close by early-late April [10].

Table 8: qPCR and cytology results from the window of infection study

Deployment Site	Week of Deployment	% mortality	Positive cytology*	Positive PCR
Karuah River	1	0%	0/30	1/30
	2	3.1%	0/30	0/30
	3	9.1%	0/30	0/30
	4	0%	0/30	0/30
	5	0%	0/30	0/30
Tilligerry Creek	1	0%	0/30	11/30
	2	2.9%	0/30	2/30
	3	16.7%	0/30	0/30
	4	2.9%	0/30	0/30
	5	2.9%	0/30	2/30

*Included examination of smears for sporulating and non-sporulating forms of *M. sydneyi*.

Investigation of potential intermediate host for M. sydneyi transmission

Polychaete worms collected from the Hawkesbury River were previously shown to harbour *M. sydneyi* using a combination of conventional PCR and *in situ* hybridisation (ISH) techniques [6]. While positive PCR detections were made in a variety of polychaete genera, only *A. australiensis* (formerly *Nephtys australiensis*) was confirmed by ISH to harbour *M. sydneyi*, suggesting that it could represent a potential intermediate host for QX disease transmission. To determine whether *A. australiensis* may act as an intermediate host for QX disease transmission in Port Stephens, we specifically focussed on collecting *A. australiensis* polychaetes from a site in Lemon Tree Passage, near the mouth of Tilligerry Creek. Polychaete collection was initially attempted in Upper Tilligerry Creek adjacent to QX-affected oyster leases, however we were unable to detect any polychaetes in the sediment in this area. However, data from the geospatial survey suggested that oysters in Lower Tilligerry Creek (near Lemon Tree Passage) were also affected by QX disease, albeit less severely. A total of 23 *A. australiensis* as well as 3 phyllodocid polychaetes (paddle worms) were collected and tested by qPCR for the presence of *M. sydneyi*. None of the polychaete samples were found to be positive for *M. sydneyi,* therefore we were unable to demonstrate the presence of *M. sydneyi* in this species. While sample numbers were low, in the prior study in the Hawkesbury River, approximately 20% of *A. australiensis* were shown to be PCR positive [6], thus some positive samples may have been expected from the samples collected.

To further investigate the role of polychaete worms in supporting the lifecycle of *M. sydneyi*, we subjected naïve *A. australiensis* (sourced from Wallis Lake) to an experimental transmission trial. Polychaetes maintained in the laboratory were fed every 2 weeks with oyster tissue containing QX sporonts. In the study of *M. sydneyi* in *A. australiensis* from the Hawkesbury River, primordial and plasmodial cells were identified in the intestinal epithelium of *A. australiensis* [6], suggesting that ingestion *M. sydneyi* may be the route of exposure. Worms were maintained for a total period of 6 weeks to allow *M. sydneyi* infection to develop. To prevent false positive detection of *M. sydneyi* from the environment, the polychaetes were starved for the final 2 weeks of the trial, as *M. sydneyi* sporonts are not known to persist in seawater for longer than 9 days [13]. Two weeks after the commencement of the study 3 polychaetes died (1 × control

worm and 2 × test worms); however all remaining polychaetes (n = 21) survived for the full 6 weeks. All worms were tested by qPCR at the completion of the study; however no positives were detected, suggesting that *M. sydneyi* sporonts may not be readily transmissible to *A. australiensis*. This does not preclude *A. australiensis* as an intermediate host for QX disease, as the laboratory conditions under which the polychaetes were maintained (33 ppt salinity and 22°C) may not have been conducive to transmission. Furthermore, the ability of *A. australiensis* to transmit *M. sydneyi* to SROs was not tested.

Clues to the lifecycle of *M. sydneyi* can also be derived from studies of the related paramyxean parasite, *Marteilia refringens* which infects European flat oysters (*Ostrea edulis*) and various species of mussel [14]. Studies on the *M. refringens* lifecycle were greatly simplified by the presence of this parasite in Claires oyster ponds in the Marennes-Oleron Bay, France which have limited biodiversity and therefore limited possibilities with respect to intermediate hosts for disease transmission [7]. Systematic studies in the Claires using PCR for detection, revealed that a calanoid copepod, *Paracartia grani* becomes infected by *M. refringens* sporonts with the parasite retained in the copepod ova. To address the possibility that components of the zooplankton, such as copepods may act as an intermediate host for *M. sydneyi*, we undertook plankton sampling in Port Stephens, following the peak in QX infection of oysters, to determine whether organisms such as copepods within the zooplankton may be involved in the next stage of the *M. sydneyi* lifecycle. Sites sampled included those that had experienced heavy oyster losses from QX disease and those with no apparent disease. Water samples that had not been filtered through the plankton net were also taken to control for the presence of QX sporonts free in the water column. All samples were tested by PCR and the results are presented in Table 9.

Table 9: Results of qPCR testing of plankton samples and corresponding controls collected from various sites across Port Stephens

Collection site	Temperature (surface/1 m depth)	Salinity (surface/1 m depth)	qPCR result (control)	Plankton collection method	qPCR result (plankton)
Lower Tilligerry Creek	17.7°C/17.6°C	25.8ppt/26.2ppt	-	2 min Surface tow	-
				Vertical haul	-
Upper Tilligerry Creek	19.2°C/18.2°C	15.5ppt/25.3ppt	-	2 min Surface tow	+
				Vertical haul	-
Bundabah	20°C/18.4°C	25.8ppt/29.6ppt	-	2 min Surface tow	-
				Vertical haul	-
Lower Karuah River	18.3°C/18.8°C	16.8ppt/17.6ppt	-	2 min Surface tow	-
				Vertical haul	-
Upper Karuah River	17.3°C/16.9°C	7.2ppt/8.2ppt	-	2 min Surface tow	+
				Vertical haul	-
Salamander Bay	18.9°C/18.7°C	31.6ppt/31.6ppt	-	2 min Oblique tow	-
				2 min Oblique tow	-

Only surface tow samples collected from Upper Tilligerry Creek and Upper Karuah River were positive for *M. sydneyi.* These sites corresponded to the areas of Port Stephens with the most severe QX disease according to the geospatial survey. Interestingly, only surface plankton collections were positive from these two sites. *M. sydneyi* sporonts are negatively buoyant and are not expected to remain in the upper portion of the water column [7], suggesting that *M. sydneyi* was associated with something in the surface waters. Furthermore, all control water samples from the collection sites were negative for the presence of *M. sydneyi*, suggesting that *M. sydneyi* was not free in the water column, and was concentrated by use of a plankton net with a mesh size of 100 µm, pointing to an association of this parasite with plankton >100 µm. Microscopic examination of plankton samples positive for *M. sydneyi* revealed the presence of phytoplankton, cnidarians, calanoid copepods and polychaete larvae, with calanoid copepods the most frequently observed in all samples examined apart from those collected from Salamander Bay. Figure 8 depicts some of the planktonic organisms observed in samples from the Upper Karuah River.

While preliminary, the results from this study combined with those from prior work on *M. refringens* suggest that a more detailed investigation of the role of zooplankton, and particularly calanoid copepods, as an intermediate host for *M. sydneyi* transmission is warranted. Calanoid copepods experience population fluctuations driven by environmental factors, such as temperature, that may explain the limited infection window of *M. sydneyi* [8, 10], but they also have strategies for surviving suboptimal conditions between seasons including overwintering of eggs in the sediment [8, 15].

Polychaete larvae were only rarely observed in plankton samples collected in this study but further investigation of the larval polychaetes as potential reservoirs of *M. sydneyi* is also warranted given that adult *A. australiensis* have been shown to harbour *M. sydneyi* [6]. While no evidence for a role for *A. australiensis* polychaetes in *M. sydneyi* transmission was found in this study, a lifecycle with more than one intermediate host is a possibility. Indeed, while *M. refringens* is readily transmitted from *O. edulis* to *P. grani* copepods, transmission from *P. grani* to oysters has not yet been demonstrated [16]. A further possibility is that the lifecycle of *M. sydneyi* is driven by the lifecycle of the intermediate host, with only some host life stages supporting *M. sydneyi* forms that are infectious to oysters.

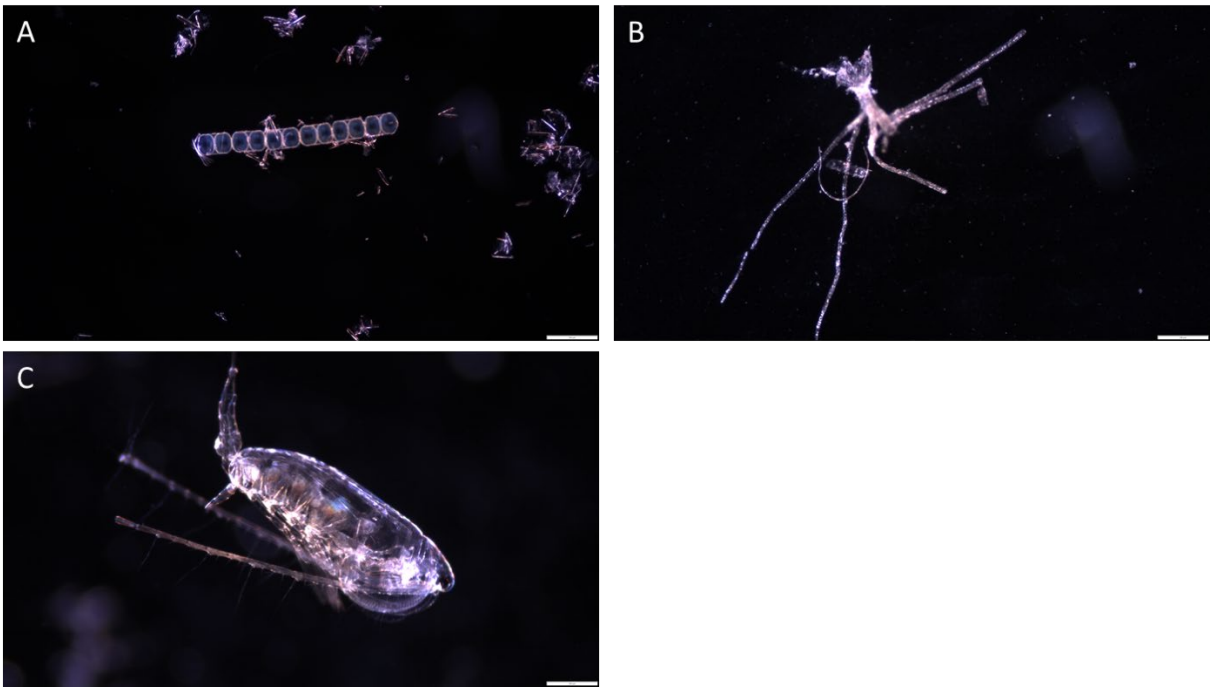

Figure 8: Planktonic organisms identified in samples collected from the Upper Karuah River. A) phytoplankton, B) cnidarian and C) calanoid copepod.

Conclusion

The aims of this study were to define the geospatial distribution of QX disease and the causative agent, *M. sydneyi* in the Port Stephens estuary, define the window of infection for transmission to oysters in this estuary and investigate potential intermediate hosts. Results from cytological testing of 300 oysters across the estuary indicated that during the 2022 season, QX disease was confined to the inner estuary (west of Soldiers Point). At the first detection in August of 2021, reports of disease were initially confined to the Karuah River, Oyster Cove and Tilligerry Creek regions and the geospatial survey revealed that these areas were the most severely affected in the 2022 season. During the course of this project, further reports of mortalities at Bundabah and in Cromarty Bay were confirmed as being due to QX disease, indicating further spread of QX within the inner estuary since the initial sampling was conducted in March. Spread of disease to the outer estuary has not yet occurred and it is currently unclear whether the hydrogeographical differences between the inner and outer estuary including differences in salinity and oceanic exchange will preclude spread of disease to the outer estuary. Quantitative PCR testing indicates that the parasite is also

present in low levels in the outer estuary and therefore, ongoing monitoring for the spread of QX disease over future seasons is warranted.

The window of infection study indicated that the window for QX disease transmission had substantially closed in the two most severely affected locations (Upper Tilligerry Creek and Upper Karuah River) by early May. However, qPCR testing indicated that infection of oysters was likely still occurring in the Tilligerry Creek region in the first 1-2 weeks of May when the water temperature exceeded 21.5°C. The subsequent decline in water temperature in the creek appeared to halt the progression of infection. In 2023, a similar study is recommended to define the commencement of the infection window.

Preliminary investigations into intermediate hosts for *M. sydneyi* failed to confirm *A. australiensis* polychaete worms as the next host in the lifecycle of this parasite, however given that the permissive temperature as well as other conditions required for transmission of sporonts is unknown, this needs to be confirmed with further studies. Conversely, our results from the testing of zooplankton are consistent with a potential role for calanoid copepods in the transmission of *M. sydneyi*, as demonstrated for *M. refringens* in the European flat oyster. These preliminary investigations provide an avenue for future investigations into the lifecycle of *M. sydneyi,* which is considered essential for understanding the infection dynamics of this parasite and improving disease management.

Implications

This project defined the current extent of spread of QX disease in Port Stephens and has provided information on the seasonal closure of the window of infection for disease transmission in key areas. Therefore, this project has implications for oyster growers in terms of stocking decisions for the coming growing season. While *M. sydneyi* was detected in some samples from the outer Port, levels of the parasite were extremely low compared to areas of the inner port which have high burdens of both parasite and disease. Spread to the outer Port is still possible over subsequent seasons but this may depend on whether hydrogeographical features of the outer Port will support disease and whether these conditions favour the intermediate host for transmission.

This project also provides temporal information regarding the closure of the window of infection for transmission of QX disease. In 2022, disease was not observed in sentinel oysters deployed in early May through to early June. PCR testing suggests that transmission may still occur if the temperature is over 21.5°C but disease progression is unlikely to occur once the temperature drops below this level.

This study advances our knowledge on the intermediate host transmission of QX disease and provides clues for where ongoing research in this area should be directed. Elucidating the lifecycle is critical to informing good disease management into the future and preventing further disease spread.

Finally, this project has provided some preliminary information for policy makers about where Port Stephens lies with respect to the current risk ratings for QX disease. Whether these risk ratings continue to be meaningful requires closure of some of the knowledge gaps around this disease.

Recommendations

This project was intended as a short-term project to assist oyster growers with stock management decisions for the coming season. However, QX disease is poorly understood and warrants further research given the value of the SRO industry and the size of the industry as a proportion of oyster production nationally. Furthermore, the SRO industry is a native oyster fishery with cultural, historical and ecological significance. Despite 50 years having elapsed since QX disease was first recognised, large knowledge gaps remain in our understanding of this disease. Disease spread is likely to continue to occur into other key growing areas if these questions remain unanswered.

Specifically in Port Stephens, the geospatial survey should be repeated in 2023 to determine disease risk across zones in the next season and to determine if disease is likely to be ongoing each season or sporadic. The commencement of the window of infection in this estuary is also yet to be determined but is key to restocking and stock management.

General research on QX applicable across estuaries includes applying findings from this study on potential intermediate hosts for transmission and closing the parasite lifecycle. This is critical to understanding infection dynamics and preventing disease spread.

Finally, further research is essential to shape evidence-based biosecurity policy around the QX disease risk ratings of NSW estuaries due to the impact of these risk ratings on oyster translocations. This research would entail determining whether *M. sydneyi* strain variation plays a role in disease outbreaks or whether environmental factors are the major drivers of disease.

Extension and Adoption

Outcomes from this project have been communicated to producers principally via the Port Stephens QX Working Group (PSQXWG) and a presentation was made to growers on Wednesday 27th of July. With final results to be presented at the next meeting on September 14th. Findings have also been presented on the NSW DPI website (https://www.dpi.nsw.gov.au/fishing/aquaculture/permit-holder-information/latest-news/port-stephens-qx-information-for-port-stephens-oyster-permit-holders) and this will continue to be updated. Presentation of results at oyster field days is also planned.

This work will also be published in the scientific literature in peer-reviewed journals.

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