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Aquatic Animal Health Subprogram: Investigation of Chlamydiales-like organisms in pearl oysters, *Pinctada maxima*

FRDC Project 2008/031

Melanie Crockford, Brian Jones

Government of **Western Australia**
Department of **Fisheries**

Australian Government
Department of Agriculture, Fisheries and Forestry

Australian Government
Fisheries Research and Development Corporation

Fisheries Research Division
Western Australian Fisheries and Marine Research Laboratories
PO Box 20 NORTH BEACH, Western Australia 6920

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Enquiries:

WA Fisheries and Marine Research Laboratories, PO Box 20, North Beach, WA 6920
Tel: +61 8 9203 0111
Email: library@fish.wa.gov.au
Website: www.fish.wa.gov.au
ABN: 55 689 794 771

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List of Abbreviations

AAHL	Australian Animal Health Laboratories
bp	Base pair
CLO	Chlamydiales-like organism
CLOs	Chlamydiales-like organisms
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribose nucleic acid
FFPE	Formalin fixed and paraffin embedded
FHL	Fish Health Laboratory
FRDC	Fisheries Research and Development Corporation
ISH	*in-situ* hybridization
MW	Molecular weight
NSW	New South Wales
NT	Northern Territory
OIE	Office International des Épizooties (World Organisation for Animal Health)
OOD	Oyster Oedema Disease
ORF	Open reading frame (a DNA sequence that codes for a protein)
PCR	Polymerase chain reaction (a patented method of copying DNA)
QLD	Queensland
RC	Reverse complement
SA	South Australia
TAS	Tasmania
TEM	Transmission electron microscopy
VIC	Victoria
WA	Western Australia

Contents

1.0 Non-technical summary

2008/031 Aquatic Animal Health Subprogram: Investigation of Chlamydiales-like organisms in pearl oysters, *Pinctada maxima*

Principal investigator: Dr Brian Jones
Address: Department of Fisheries
39 Northside Drive
Hillarys WA 6025
Telephone: 08 9368 3649
Fax: 08 9474 1881

Co-investigator: Dr Melanie Crockford
Department of Fisheries
39 Northside Drive
Hillarys WA 6025
Telephone 08 9368 3205
Fax 08 9474 1881

Objectives:

- To further develop the current conventional PCRs being used to investigate two CLO's in pearl oysters and use these PCRs in an attempt to gain further sequence data. An expansion of the current known sequence data will be used to develop a real-time PCR that is specific and sensitive enough to detect and differentiate between the two CLO's in pearl oysters. The real-time PCR will be validated at two independent laboratories using known OOD-positive and negative control samples.
- To test healthy versus OOD-affected pearl oysters to determine if the presence of either or both CLO's plays a role in the onset of OOD. Pinctada maxima samples from Queensland will be tested as negative control animals to determine the prevalence of the two CLO's. This study will determine if there is a link between the presence of these CLO's and the onset of OOD.
- To survey non-maxima shellfish associated with pearl farms to determine the prevalence of these organisms in molluscs in Australian waters, and whether there are further molluscan reservoir hosts. Any positive samples obtained will be confirmed by sequencing the PCR product.

Outcomes achieved to date

The project outputs have contributed to, or will lead to the following outcomes:

1. Optimised conventional PCRs for the detection of the each of the two CLOs in *P. maxima* oysters and optimised and validated real-time PCRs for the detection of the two CLOs in *P. maxima* oysters, allowing rapid and specific detection of each CLO. This will enable industry to test for and manage the presence of the CLO's, particularly the one associated with OOD.
2. Sequence data obtained from the two CLOs discovered in *P. maxima* oysters, has allowed preliminary taxonomic classifications.
3. This report documents the prevalence of these organisms in *P. maxima* oysters and other molluscs and provides a better understanding of their link to OOD. This provides industry with information on the risk posed by other shellfish due to the presence or absence of these CLOs.
4. The project results have been communicated to industry and were also communicated through the FRDC Australasian Aquatic Animal Health Scientific conference 5-8 July 2011.

During the initial disease outbreak investigation into Oyster Oedema Disease (OOD), staff from the Department of Fisheries WA visualized CLOs in OOD-affected animals by transmission electron microscopy (TEM), suggesting a link between the presence of these CLOs and OOD-affected pearl oysters. Initial molecular research confirmed that two CLOs were present in OOD-affected pearl oysters, one of which had sequence similarity to *Simkania negevensis*, the other was new and uncharacterised (referred to as *maxima*-CLO).

The conventional PCR for the detections of the two CLOs was developed as much as possible.

A multiplex real-time PCR was attempted, which targeted the two CLOs in the one PCR. However, this lacked sensitivity and was ineffective. Separate real-time PCRs were developed. One real-time PCR targeted *S. negevensis*; the other real-time PCR was designed for the detection of *maxima*-CLO.

There appeared to be a high prevalence of *S. negevensis* in pearl oysters. However, results showed that *S. negevensis* was prevalent in many samples including both OOD affected animals and healthy oysters. Furthermore, *S. negevensis* was not detected in shellfish sampled during the initial mortality investigation at the index site (Whalebone Island), suggesting that this organism is a commensal and not associated only with OOD- affected oysters.

The uncultured CLO PCR produced different results. Even though this organism appeared to be less prevalent, there appeared to be an association between the presence of this organism and sick oysters, although it is not clear that this organism alone is sufficient to cause OOD. An AusVet report into the epidemiology of OOD (AusVet 2007) suggested that the cause of OOD is most likely multi-factorial, so it remains a possibility that other factors are also required for the onset of clinical OOD.

Keywords: Oyster Oedema Disease, Chlamydiales, *Simkania negevensis*, real-time PCR

2.0 Acknowledgements

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- David Mills of Paspaley Pearling Company, Darwin, NT, for the supply of pearl oysters from Darwin and Broome, WA, for this research;
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- Amber Howard, Department of Fisheries WA, for her laboratory contribution to this project.
- Sheila Mortimer-Jones, past employee of Department of Fisheries WA, for her laboratory contribution to this project.

3.0 Background

Department of Fisheries WA staff visualized CLOs in OOD-affected animals by electron microscopy, suggesting a link between the presence of these CLOs and OOD-affected pearl oysters. Molecular research confirmed that two CLOs were present in OOD-affected pearl oysters.

Molecular research involved the use of a published universal PCR (Ossewaarde and Meijer, 1999) for the detection of Chlamydiales in various specimens. This PCR was used for the detection of two CLOs in *P. maxima* oysters. Sequence data indicated that one organism was *Simkania negevensis*, an organism belonging to the Order Chlamydiales, whose DNA is often identified in environmental samples from sewage and waste water treatment plants (Horn and Wagner, 2001). Data suggests that *S. negevensis* may have a wide geographical and environmental distribution and has been associated with respiratory and other health problems in humans. This organism has not been found before in pearl oysters and was detected in almost all *P. maxima* samples tested, although the significance of finding this organism in pearl oysters was unclear when the project commenced.

The second organism discovered in *P. maxima* oysters produced a PCR product that was very similar in size to *S. negevensis*, which led to difficulty in differentiating the two organisms. Sequence data from this second organism was only approximately 75% similar to uncultured *Chlamydia* organisms – clearly different to *S. negevensis* and also significantly different to any other organism in the database. A recent paper by Israelsson (2007) tells of the discovery of numerous Chlamydiae related to Simkaniaceae in *Xenoturbella*, an enigmatic marine animal that has few features and only one distinct organ. This report appears to be very similar to the discovery of two CLOs in pearl oysters. The significance of the presence of this *Chlamydia*-like organism was unclear but there was a trend in the *P. maxima* samples tested for this organism to be present in OOD-affected oysters and not present in "healthy" oysters. Further investigation of this trend was initially limited due to a focus on a virus-like agent in oysters. It is possible that the *Chlamydia*-like organism together with other agents, such as the putative virus play a significant role in the onset of OOD and further investigation is required.

The characterisation of *Chlamydia*-like organisms is not yet settled. Taxonomic classification of Chlamydia is based on 16S rRNA gene sequence identity, whereby Chlamydiaceae have 16S rRNA gene sequences that are >90% identical and *Chlamydia*-like organisms that have reticulate bodies and elementary bodies have >80% 16S rRNA gene sequence identity (Draghi et al., 2004). Interestingly, the chlamydia-like agents of epitheliocystis from fish have never been successfully cultured *in vitro* to facilitate genetic studies, and neither antigenic reactivity nor 16S sequence data have been obtained to further a molecular characterisation of a chlamydia-like organism from a salmonid host (Draghi et al., 2004). Organisms of the Order Chlamydiales continue to be under review based on data resulting from further investigation of these organisms and the discovery of new similar organisms. *Chlamydia pneumoniae*, a frequent cause of pneumonia and bronchitis in humans, is still under scrutiny with regard to persistent infections due to the lack of evidence confirming that the presence of this organism is the cause or a triggering cofactor, or a commensal (Kutlin et al., 2001). Interestingly, the Phylum Chlamydiae is part of a group known as the PVC superphylum, comprised of Planctomycetes, Verrucomicrobia and Lentisphaerae and these organisms possess dramatically different lifestyles, some of which can alter the marine nitrogen levels, known as "annamox" bacteria (Kirkpatrick et al., 2006). Planctomycetales have been isolated from diverse aquatic ecosystems, including aquatic freshwater, brackish water, marine and hypersaline habitats, including isolation from *Penaeus monodon* prawns (Fuerst et al., 1997).

Chlamydiales-like organisms are common in the epithelia of bivalves and these organisms rarely cause serious disease in their molluscan hosts. However, there have been reports of mortalities caused by CLOs in giant clams (Norton et al., 1993) and serious disease in scallops (Leibovitz et al., 1984). Chlamydiales bacteria are known to cause problems in other aquatic animals, such as epitheliocystis in Leafy Seadragon, Silver Perch, Barramundi (Meijer et al., 2006) and Sea Bream (Crespo et al., 1999). Meijer et al. (2006) suggest that lymphocystis cysts that are caused by iridovirus infection could be co-infected with Chlamydiales bacteria to cause epitheliocystis. The same could be true for OOD-affected pearl oysters. With a need for further investigation of these two CLOs present in *P. maxima* oysters and how they fit into the environment, this research project was initiated.

4.0 Need

In October 2006 unexplained high mortalities of *P. maxima* oysters were reported. Many farms were affected and the cause is not known. The disease is termed Oyster Oedema Disease (OOD) and it is likely that an infectious agent is involved. The disease spread rapidly and there are no known control measures, no knowledge of a causative agent, no understanding of how widespread the disease was and no way to test for it. Molecular and electron microscope research into the mortalities resulted in the discovery of two undescribed CLOs associated with affected shell, and their involvement in OOD is unclear but it is considered unlikely that they are by themselves the major pathogen. Preliminary research in WA suggested a link between OOD-affected animals and the presence of at least one of these CLOs. An independent report detailing the OOD mortality event (AusVet, 2007) concluded that it is likely that more than one factor contributes to the onset of OOD. Therefore it is possible that these organisms are contributing to the onset of OOD but further work was required to eliminate or confirm these organisms as a factor in OOD. Though "rickettsia-like" bodies have been described from *P. maxima* based on histology, the discovery of CLOs in pearl oysters is a new finding.

Mortalities continue to be reported and OOD still represents a high risk to surviving and young oysters. The work forming this report continues that started at the Department of Fisheries WA in 2007 with the discovery of two CLOs in *P. maxima* oysters. There was a need to further characterise these two organisms in relation to each other and to similar organisms worldwide, determine if they play a role in the onset of the fatal OOD, and represent a risk to surviving pearl oysters.

5.0 Objectives

- To further develop the current conventional PCRs being used to investigate two CLO's in pearl oysters and use these PCRs in an attempt to gain further sequence data. An expansion of the current known sequence data will be used to develop a real-time PCR that is specific and sensitive enough to detect and differentiate between the two CLO's in pearl oysters. The real-time PCR will be validated at two independent laboratories using known OOD-positive and negative control samples.
- To test healthy versus OOD-affected pearl oysters to determine if the presence of either or both CLO's plays a role in the onset of OOD. *Pinctada maxima* samples from Queensland will be tested as negative control animals to determine the prevalence of the two CLO's. This study will determine if there is a link between the presence of these CLO's and the onset of OOD.
- To survey non-maxima shellfish associated with pearl farms to determine the prevalence of these organisms in molluscs in Australian waters, and whether there are further molluscan reservoir hosts. Any positive samples obtained will be confirmed by sequencing the PCR product.

6.0 Methods

6.1 Samples used for PCR

From November 2006, *P. maxima* samples deemed healthy or OOD-affected were sent to the Fish Health Laboratory for testing. These samples were used for testing for the presence of the two CLOs. Available samples include frozen *P. maxima* "piggyback" spat collected annually by Department/Industry wild shell surveys in 2007 through to 2010.

In July 2009, Melanie Crockford and Amber Howard went to northern Queensland to collect *P maxima* samples that were deemed to be unaffected by oyster oedema disease. The farm had never received spat from either WA or NT staff from the farm had worked only at that location since before the OOD outbreak in WA and all equipment had only been used at the one location. These samples are of great importance as it is not known how widespread OOD is in WA and samples to be used as a negative control in molecular work needed to be sourced from outside WA.

The positive control sample, in the absence of a definitive agent for OOD, was tissue from Exmouth Gulf oysters (Case number FH07-06) submitted from the index site with gross signs of being sick.

Non-maxima samples included *P. margaritifera* (black-lipped pearl oyster, Geraldton), *P. albina* (penguin oyster, Denham), *Crassostrea gigas* (rock oyster), *Mytilus edulis* (WA mussel), *Perna canaliculus* (New Zealand mussel), *Placopecten* sp (WA scallop). *Penaeus esculentus* (prawn) and *Sardinops sagax neopilchardus* (fish) were used as outliers, particularly since Planctomycetales have prevously been isolated from *Penaeus monodon* (Fuerst et al., 1997).

6.2 DNA Extraction

A modified DNAzol (Life Technologies) procedure was used for DNA extractions (Crockford et al., 2005). Briefly, oyster gill and mantle tissue (25 to 50 mg) was added to a 1.5 ml microcentrifuge tube containing 0.7 ml of DNAzol reagent (Molecular Research Center, Inc), homogenised using a hand-held disposable pestle (Scientific Specialties) and centrifuged at 10 000 x g for 10 min at ambient temperature. The supernatant was transferred to a fresh microcentrifuge tube, and 0.4 ml of 100% ethanol (Sigma) was added. The tubes were inverted several times to allow a precipitate to form, before being centrifuged at 10 000 x g for 5 min at ambient temperature. The supernatant was decanted, leaving a visible pellet at the bottom of the tube. The pellet was washed twice by resuspending the pellet in 0.6 ml of 75% ethanol, centrifuging at 10 000 x g for 5 min at ambient temperature and decanting the supernatant after each wash. Any remaining ethanol was removed using a pipette, and the pellet was dried on a heating block at 50°C. TE (Tris-EDTA) buffer pH 8.0 (Amresco) was added to the DNA and the tube was placed on a heating block at 55°C for up to 1 h to resuspend the DNA. The concentration of the DNA sample was determined by spectrophotometry. Aliquots were stored at -20°C.

6.3 Conventional universal PCR for the detection of Chlamydiales

A published universal PCR for the detection of Chlamydiales in various specimens was used (Ossewaarde and Meijer, 1999). A 25 µl reaction mixture was prepared with 0.5 µl of each of the 8 primers, 12.5 µl of Biomix Red, 1 µl of genomic DNA and 7.5 µl of sterile distilled water. Amplifications were performed using a 'touch-down' protocol, with an initial denaturation of 95°C for 1 min, followed by 16 cycles at 95°C for 20 s, 65°C for 30 s – decreasing 1°C every 2 cycles to 57°C, and 72°C for 40 s. The expected amplification product was 270 bp.

Following amplification, PCR products (9 µl), along with a 25-bp DNA ladder (5 µl), were loaded horizontally onto a 2% ethidium bromide stained agarose gel and subjected to electrophoresis for 65 min at 95 V in 1x TAE buffer. Gels were photographed under UV-light trans-illumination. Primers from the universal PCR were tested in combinations of two to determine if the PCR could be improved for the detection of the CLOs in pearl oysters. All possible combinations of forward and reverse primers were tested. For the best combination, primers F1 and R1, optimisation of the PCR include testing magnesium concentrations of 1.5 mmol, 2.0 mmol and 2.5 mmol. Primer optimisation included evaluation of 0.5 µmol, 1.0 µmol and 1.5 µmol concentrations. Gradient cycling with parameters of 50°C to 62°C was used to determine the best annealing temperature.

6.4 Conventional PCR for the detection of the two CLOs in pearl oysters

A 25 µl reaction mixture was prepared with 1.5 µl of primer F1 and R1 @ 25 µmol each from the universal PCR described above, 12.5 µl of Biomix Red, 1 µl of genomic DNA and 8.5 µl of sterile distilled water. Amplifications were performed as follows: initial denaturation of 95°C for 1 min, followed by 40 cycles at 95°C for 20 s, 56°C for 30 s, and 72°C for 40 s, then a final extension of 72°C for 5 min, and held at 10°C. The expected amplification product was 240 or 260 bp, depending on the CLO amplified.

Following amplification, PCR products (9 µl), along with a 25-bp DNA ladder (5 µl), were loaded horizontally onto a 3% agarose gel containing ethidium bromide and subjected to electrophoresis for 65 minutes at 95 V in 1x TAE buffer. Gels were photographed under UV-light trans-illumination. Various agarose gel percentages and running conditions were tried to allow optimal separation of PCR products, should both the 240 bp and 260 bp products be amplified from the same sample.

6.5 Freeze-squeeze method for extracting DNA from agarose gel

The PCR product was run on an agarose gel, the desired band cut out and placed in a 1.5 ml microcentrifuge tube, and frozen at –20ºC immediately for at least 30 min. The gel slice was squeezed between a parafilm sheet that had been folded in half, and all of the liquid that appeared as the gel slice thawed was extracted and placed in a sterile microcentrifuge tube. One tenth of the extract volume of 3 M sodium acetate pH 5.2 and double the extract volume of 70% ice-cold ethanol were added, and gently agitated using a vortex mixer. The preparation was centrifuged at 10,000 x *g* for 5 min, the supernatant discarded, and the pellet vacuum-dried. Twelve µl of sterile water was added to dissolve the pellet, and 5.75 µl of this was used in a sequencing reaction.

6.6 Sequencing

PCR products or plasmids were purified using DNAce Quick-Clean (Bioline) and manufacturer's instructions, or bands extracted using a freeze-squeeze gel extraction method (described above) and were sent to Australian Genome Research Facility, Adelaide node, for sequencing. Sequence analysis was performed using the BioEdit sequence analysis program and Blastn non-redundant database.

6.7 Development of real-time PCR for the detection of uncultured CLO in pearl oysters

Real-time PCR was carried out on an iCycler real-time thermalcycler (Bio-Rad) using 0.2 ml thin-walled PCR tubes (Bio-Rad). The protocol is based on the method described by Everett et al. (1999) Test 2 with modifications. A master mix was prepared as follows: 1x IQ Supermix (Bio-Rad), 250 nmol each primer TQF and TQR, 250 nmol dual-labelled probe, sterile water (to make volume up to 19 µL). Reaction volume was 20 µL per tube, consisting of 19 µL of master mix and 1 µL of template. A known positive control, as well as a negative control consisting of 1 µL water and master mix was included with each real-time PCR. Cycle conditions: 95ºC x 10 min, followed by 40 cycles of 95ºC x 15 s, 55ºC x 20 s, and 72ºC x 25 s. Data collection and real-time analysis was enabled at the annealing step of each cycle (55ºC). Some real-time PCR products were run on a gel and sequenced as described previously, to confirm that the correct target product of 132 bp was amplified.

6.8 Validation of the CLO real-time PCR at two independent laboratories

Positive and negative samples and primers were sent to the Virology Laboratory, Department of Agriculture and Food WA and to the Plant Health Laboratory, Department of Agriculture and Food WA to independently validate the real-time PCR. These two laboratories are operated independent of each other and of the Fish Health Laboratory.

6.9 Development of real-time PCR for the detection of *Simkania* sp. in pearl oysters

Primers SimF and SimR were designed on the nucleotide region of the 23S rRNA gene of *S. negevensis*, with a target amplicon of 794 bp. For increased sensitivity, a hemi-nested real-time PCR was also used. Primer SF and a dual-labelled probe were designed for use with primer SimR, with a target amplicon of 121 bp. All primers and probe were designed using Integrated DNA Technologies (IDT) and are shown in Table 1. GeneWorks synthesized the primers. The probe was synthesized by IDT.

Table 1. Sequences of primers and probe. The position of the primers is based on GenBank accession numbers U68460.2[a], EZ420093[b]. BHQ, black hole quencher.

Name	Sequence 5'-3'	Size (bp)
SimF	ATCCATGACCAGGATGAAGCACGA	794
SimR	AGCGGCCTCATAGAGAAACGAACA	
SF	AAATGCGTAGGCGATGGAAAGCAG	121
Sprobe	6FAM-ACGACGAAGTATGTTAAGCACGCGA-BHQ-1	

For the primary reaction for the detection of *S. negevensis* a 25 µl reaction was set up as follows: 12.5 µl Biomix Red (Bioline), 400 nmol each of primers SimF and SimR, sterile water and 2 µl DNA. The thermal cycling conditions were: 95ºC for 10 min, followed by 30 cycles of 95ºC for 30 s, 57ºC for 30 s and 72ºC for 1 min. The PCR product was diluted 1:100 with nuclease-free water and 2 µl of this was used as the template for the real-time hemi-nested reaction. The optimum annealing temperature for TaqMan® real-time PCR is 60ºC. To test the efficiency of the real-time PCR at 60ºC, the PCR was run at 58-60ºC in a conventional PCR. Specificity was tested using *C. psittaci* as a control. The components for the hemi-nested real-time assay in a 25 µl reaction were: 12.5 µl JumpStart Taq Ready Mix (Sigma), 2 µl $MgCl_2$ (final concentration 5.5 mmol), 150 nmol each of primers SF and SimR and 100 nmol Sprobe. The thermal cycling conditions were 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 60 s. All real-time assays were performed in triplicate.

6.10 Culture of the CLOs in chicken eggs

Fifteen whole oysters from case FH07-19A (oysters with OOD, less than 2 cm in size) were ground using a mortar and pestle. Viral transport media that consisted of Hanks media concentrate, yeast and lactalbumin hydrolysate concentrate, no antibiotics, was added to approximately 7 ml of the oyster material. The homogenate was centrifuged at 2000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.8 µm filter and then a 0.45 µm filter. Five day old eggs were candled. Eight eggs had holes drilled after being swabbed with 70% alcohol. A 23-gauge needle was inserted into the hole in 7 of the 8 eggs, and 200 µl of inoculum was inserted into each of the 7 eggs. The 8th egg was kept as a control. The hole in the eggs were sealed with a hot glue gun and returned to the incubator (37-39°C). Eggs were examined at 24 h intervals. One egg was discarded due to death of the embryo. One egg was removed at 11 days old, 6 days after inoculation, and the yolk sac, embryo and chorioallantoic membrane were harvested. Another egg, 8 days old, from another batch of eggs that had been in the incubator was also harvested as a negative control. When the eggs were 18 days old, all eggs were harvested, including the original control. Embryos were killed by chilling, the area over the air sac was disinfected with 70% alcohol and the shell was removed with sterile instruments. The egg contents were poured into a sterile petri dish and yolk sac, embryo and chorioallantoic membrane were harvested. DNA was extracted using an Axyprep Multisource Genomic DNA Miniprep kit (Axyprep), which purifies genomic DNA from animal tissues.

6.11 *In-situ hybridisation*

The method for construction of a CLO riboprobe was based on the method of Crockford et al. (2008). Briefly, 4 µl of CLO PCR product was made up to 9.5 µl with sterile water and used in a riboprobe reaction mix, consisting of 4 µl of 5× transcription buffer, 2 µl of 100 mmol dithiothreitol (DTT), 3 µl RNA labeling mix (Boehringer-Mannheim), 1 µl of SP6 RNA polymerase, and 0.5 µl of RNAsin. The reaction mix was incubated at 37°C for 2 h, followed by the addition of 2 µl of 0.2 M EDTA (pH 8.0) to stop the reaction. Labeled RNA was incubated at –80°C for 30 min with 2.5 µl of 4 M lithium chloride and 75 µl of 100% ethanol to precipitate the labeled RNA. Following centrifugation at 10 000 × *g* for 20 min, the resulting pellet was washed in 70% ethanol, centrifuged at 10 000 × *g* for 15 min, and dried following decanting of the supernatant. The pellet was then re-suspended in 100 µl of sterile water. Ten µl of the preparation were run on a 1.5% agarose gel at 90 volts for 50 min to check the quality of the riboprobe. One µl of RNAsin was added to the re-suspended labeled RNA, and the preparation was stored in 5 µl aliquots at –80°C. Methodology for the preparation of sections and ISH is described in Crockford et al. (2008).

7.0 Results

7.1 Adoption of the conventional PCR for the detection of *Simkania* sp. and *maxima*-CLO

Earlier attempts at amplification of Chlamydiales-like organisms in pearl oysters had resulted in two products of similar size (246 and 260bp respectively), using the F1/R1 primer set. These products were difficult to separate during electrophoresis making it difficult to analyse data or excise bands for sequencing. As this project began, many different gel conditions were tried in order to get sufficient separation of the Chlamydia bands (see Figures 1 and 2). Sequencing was conducted to determine the specificity of amplification of Chlamydia 23S rDNA genes

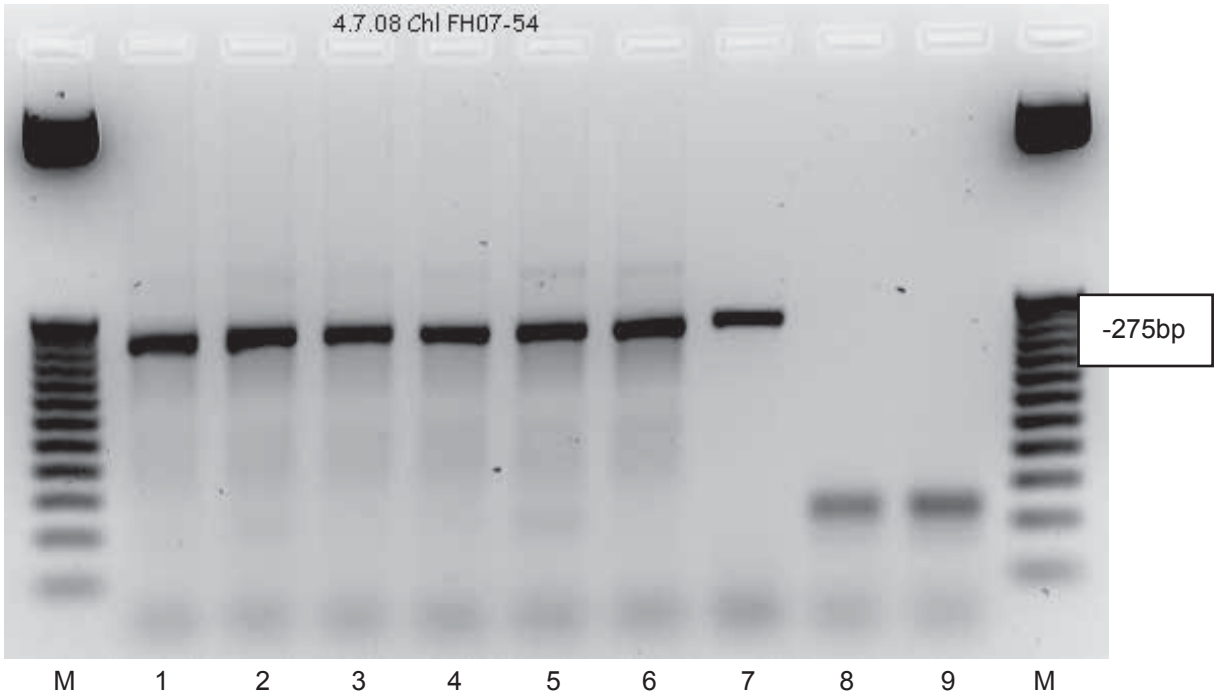

Figure 1. Attempt to get better separation of PCR products using a low melting point agarose gel. The band in lane 7 is not differentiated enough when compared to the bands in lanes 1-6. Most samples tested to date have shown the two bands merged together, hence the need for better size separation. Lanes 1-6 = lower band consistent with previously sequenced *Simkania* sp. CLO; Lane 7 = top band consistent with previously sequenced uncultured CLO; Lanes 8 and 9 = negative control; M = 25 bp marker.

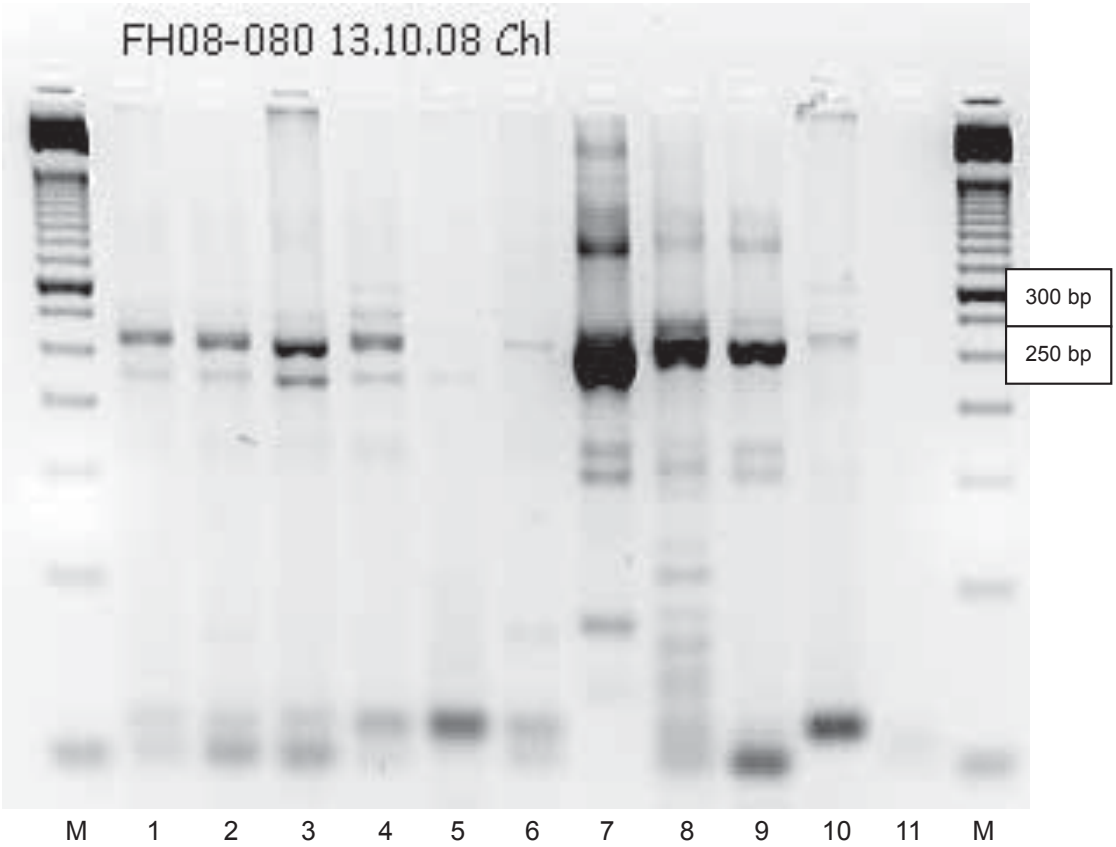

Figure 2. Attempt to get better separation of PCR products using a 4% agarose gel. PCR products of interest are approximately 260 bp and 240 bp. Some samples are producing both PCR products, while others are producing only 1 product. Lanes 1-11 = various samples from batch FH08-080; M = 50 bp marker.

7.2 Confirmation of specificity of the 246bp product

The CLO lower band (246 bp) sequence originally obtained using the F1R1 PCR suggested an unclassified Simkaniaceae and most likely a symbiont. The sequence was determined as follows:

CCTATTTGTTGATGAGGCATGCGAGTCGAACGAAGTAGCTTGCTACTTAGTGGCGAAAGGGTT
AGTAATACATGAGTAACGCWCCTCTTTCCTGGGGATAACGGTTGGAAACGACCGCTAATACCG
AATGAGGAGATCTGGGGTAGCCCGATATCTTCAAAGTAGGGGATCCTTCAGGACCTTACGGAG
AGAGAGcGGcTCATGGGATATCAGCTTGTTGGTGTGGTAAAGGCGCACCCAAAAGGC

This sequence fragment is most closely aligned with *Simkania negevensis* (Figure 3).

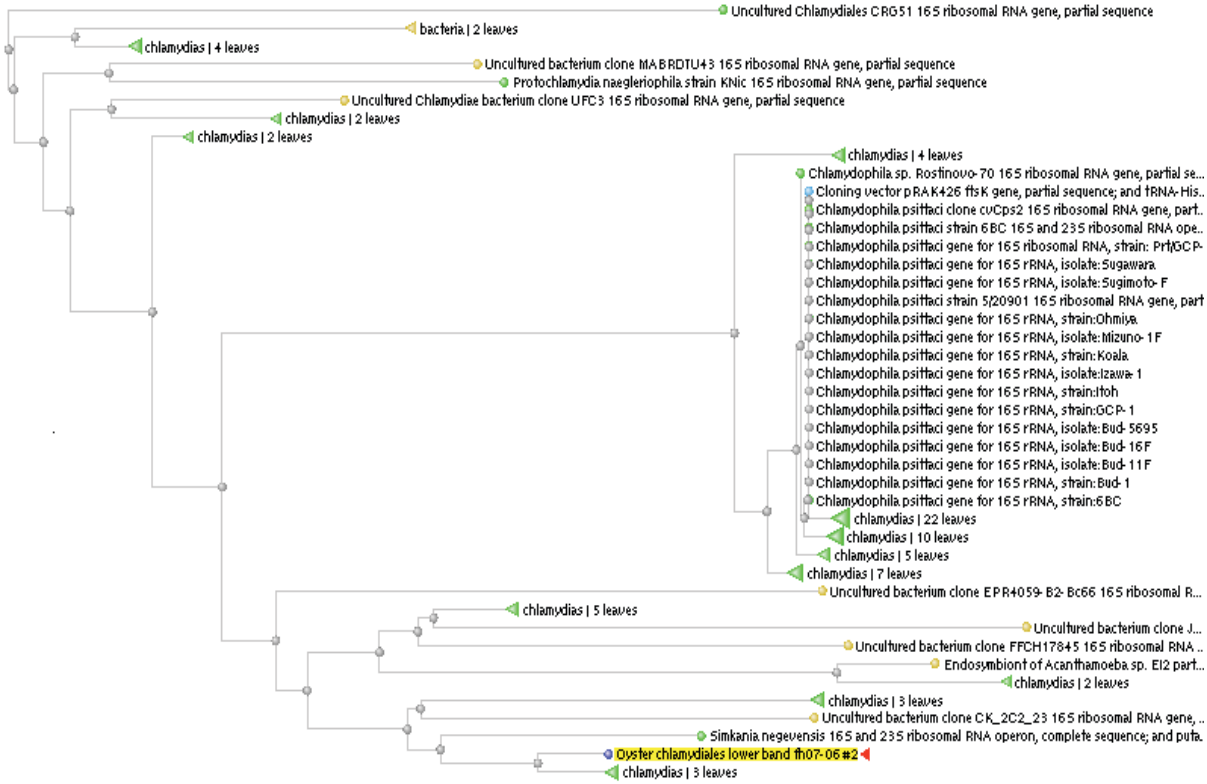

Figure 3. Dendrogram of the CLO in pearl oysters producing a PCR product of 246 bp, showing matches with other CLOs. Using NCBI Blast the sequence from the CLO lower band 246bp revealed 90% sequence identity with uncultured Chlamydiae bacterium clone UMAC8 16S Ribosomal RNA gene, partial sequence (genbank accession number FJ817591.1) matching 92% of the 390 nucleotide query with an E value of 2e-105.

7.3 Confirmation of specificity of the 260bp product

The CLO band of approximately 260 bp produced sequence data that suggests an uncultured chlamydiales organism. Optimal primer concentrations and magnesium concentrations were determined, as shown in Figures 4 and 5.

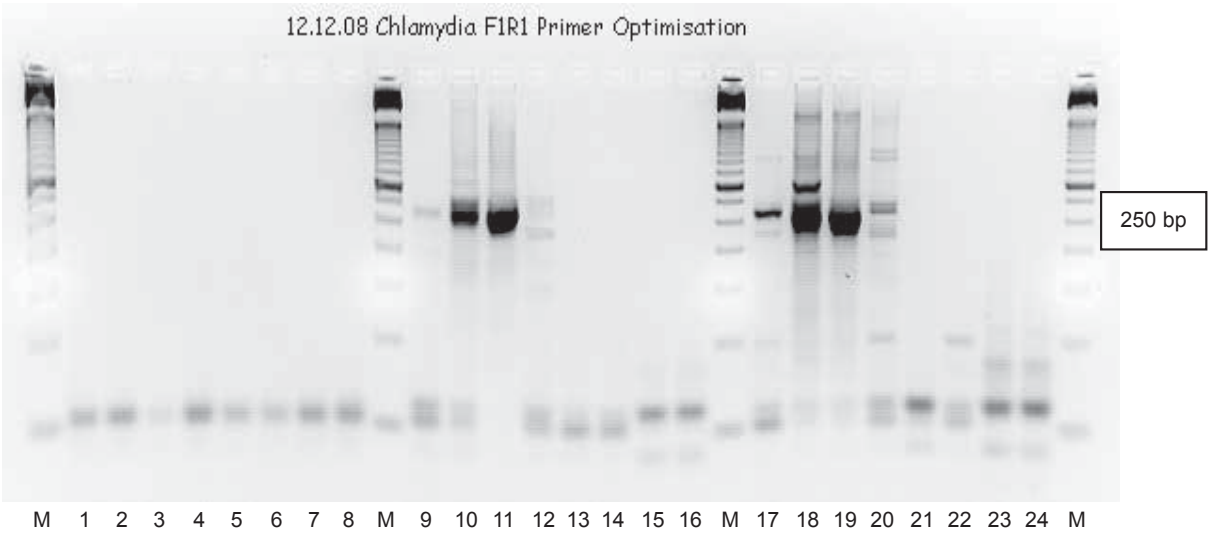

Figure 4. Primer concentration optimisation. Primer concentrations of 0.5 µM, 1.0 µM and 1.5 µM were used in the PCR. A primer concentration of 1.5 µM of each primer produced the best reaction. Lanes 1-8 = primer concentration of 0.5 µM; Lanes 9-16 = primer concentration of 1.0 µM; Lanes 17-24 = primer concentration of 1.5 µM; M = 50 bp ladder.

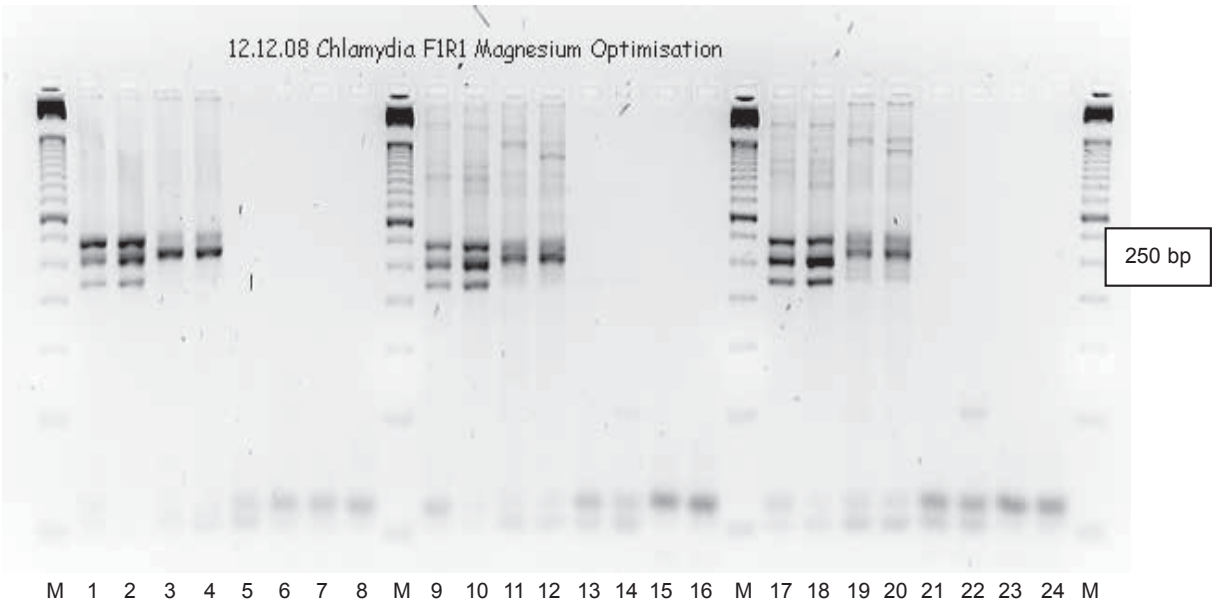

Figure 5. Magnesium concentration optimisation. Magnesium concentrations of 1.5 mmol, 2.0 mmol and 2.5 mmol were used in the PCR. A magnesium concentration of 1.5 mmol produced the best reaction. Lanes 1-8 = magnesium concentration of 1.5 mmol; Lanes 9-16 = magnesium concentration of 2.0 mmol; Lanes 17-24 = magnesium concentration of 2.5 mmol; M = 50 bp ladder.

The sequence was determined as follows:

KTKGATGAGGCATGCGAGTCG.AACGAAGTGCAAAGCTTGCTTTGTACTTAGTGGCGGACGGG
TTAGTAATACATGGGTAACTTACCTTTTACTTGGGAATAACAACTGGAAACGSTTGCTAATACCTA
ATGAGGTACACGGAAGGCATCTTCTGTGTATCAAAGCGGGGGACGAGAGAGATCTTGCCTCG
TGGTAAAAGATAGGCTCATGCGATATCAGCTTGTTGGTGTGGTAATGGCACAACCAACGCC

This PCR product matched best with other uncultured CLOs, not including *Simkania* sp. (see Figure 6) and was subsequently labelled, for convenience, "*maxima*-CLO".

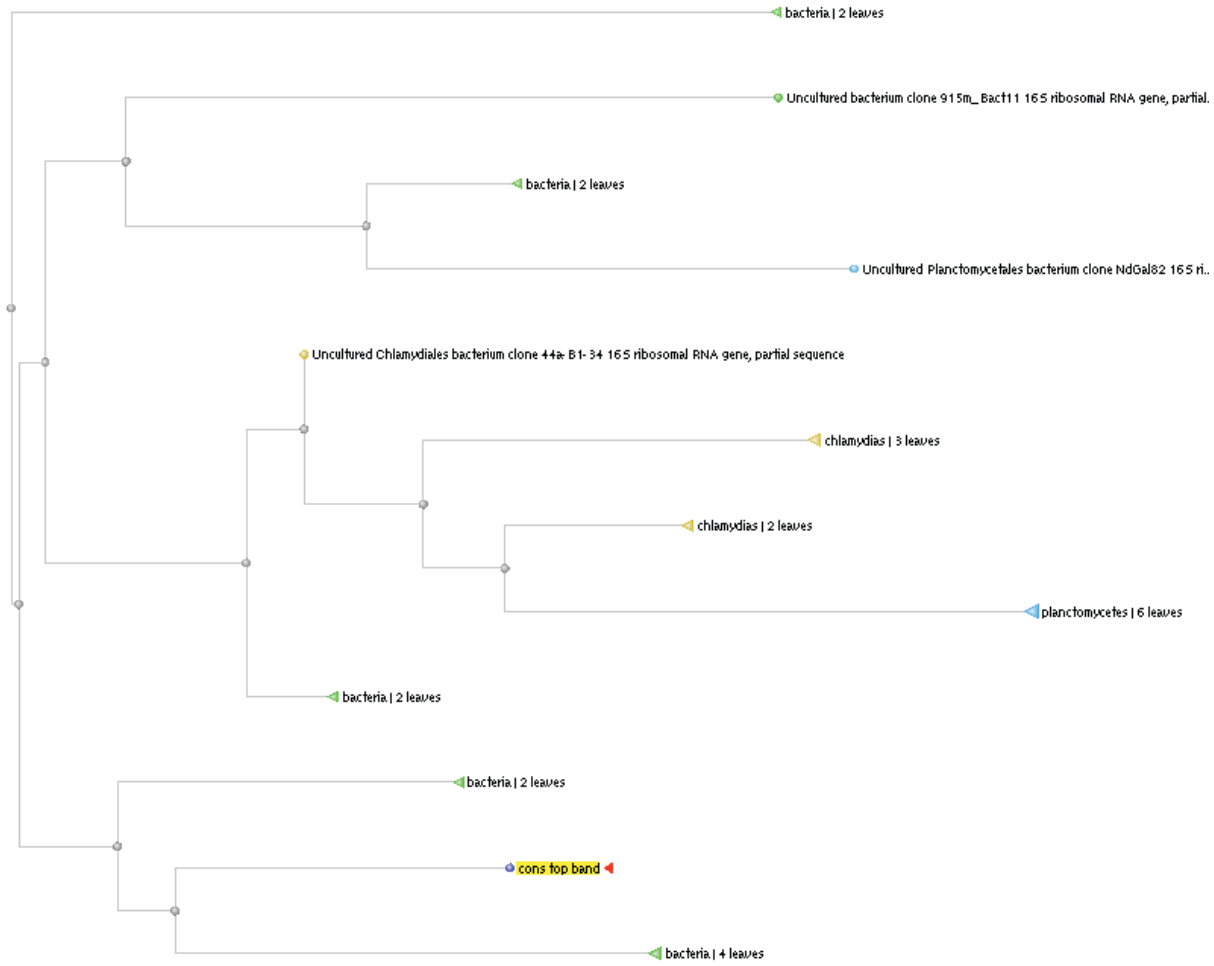

Figure 6. Dendrogram of the CLO in pearl oysters producing a PCR product of 260 bp, showing matches with other CLOs. The cons top band highlighted in yellow represents the 260 bp sequence.

7.4 Screening of *P. maxima* samples with the optimised Chlamydiales 23S rDNA PCR

A number of *P. maxima* samples were screened using the PCR for detection of Chlamydiales and generated either 246 or 260bp products. Sequencing of the 260 bp PCR products using the conventional, and other, PCRs is listed in Table 2.

All PCR products of 246 bp matched uncultured chlamydiales or *Simkania negevensis*, and all PCR products of 260 bp matched *maxima*-CLO or similar.

Table 2. Sequencing of the two PCR products produced using the CHL F1R1 conventional PCR. All PCR products of 246 bp matched uncultured chlamydiales or *Simkania negevensis*. All PCR products of 260 bp matched *maxima*-CLO or similar organisms.

Sample Name	Matched	Primers in Master Mix
Fish # 6	Uncultured chlamydiales/*Simkania negevensis*	F1R1
MC 1	Uncultured bacterium	F1R1
MC 2	Uncultured bacterium	F1R2
MC 3	Maxima-CLO	F1R5
FH07-06 2	Uncultured chlamydiales/*Simkania negevensis*	F1R1
FH07-23 7	Maxima-CLO	F1R1
FH07-19 A 13 lower band	Uncultured bacterium/*Simkanianegevensis*	F1R1
FH08-53 2 (Nov 08)	Maxima-CLO	F1R1
FH08-53 2 (Mar 09)	Maxima-CLO	F1R1
FH08-53 2 (Apr 09)	Maxima-CLO	F1R1
FH07-06 1+2 lower	Maxima-CLO	F1R1
FH07-17 13	Uncultured chlamydiae bacterium	F1R1
FH07-17 51	Uncultured chlamydiae bacterium	F1R1
FH07-17 52	Maxima-CLO	F1R1

7.5 Specificity testing of optimised conventional PCR across a broad range of molluscan and non-molluscan hosts

Using this conventional optimised Chlamydiales PCR that was developed, testing began on pre OOD *P. maxima* from 2005, other species of mollusc, as well as avian and bovine samples that were known to be positive for various species of Chlamydiales, to determine the specificity of the PCR and its suitability for wider screening (see Table 3). Some non-*P. maxima* samples produced a PCR product of approximately 260 bp. However, numerous sequencing attempts did not produce consistent sequence data. The sensitivity of the PCR cannot be determined satisfactorily as these organisms are unable to be cultured.

Table 3. Results of non-*P. maxima* samples tested using the F1R1 PCR. Some samples produced a band of the expected size but sequencing attempts failed to produce any valid data.

Species	Identification	Results for F1R1 PCR	Notes
Avian sp	AA08-2671	+/-	Positive for C psittaci via histology
Avian sp	AA08-2672	+/+	Positive for C psittaci via histology
Bovine sp	AS05-663 B8	+	Positive for C pecorum via histology
Bovine sp	AS05-663 B10	-	Positive for C pecorum via histology
Crassostrea sp (Oyster)	FH08-058 B1	-	
Crassostrea sp (Oyster)	FH08-058 B2	Sequencing inconclusive	260 bp band
Crassostrea sp (Oyster)	FH08-058 B3	Sequencing inconclusive	260 bp band
Mytilus edulis (Mussel)	FH08-080 1A	Sequencing inconclusive	260 bp band
Mytilus edulis (Mussel)	FH08-080 6A	Sequencing inconclusive	260 bp band
Mytilus edulis (Mussel)	FH08-080 1Ii	Sequencing inconclusive	260 bp band
Mytilus edulis (Mussel)	FH08-080 1Iii	Sequencing inconclusive	260 bp band
Mytilus edulis (Mussel)	FH08-080 1Iiii	-	
Mytilus edulis (Mussel)	FH08-080 1Iiv	Sequencing inconclusive	260 bp band
Perna sp (Mussel)	FH08-058 C1	Sequencing inconclusive	260 bp band
Perna sp (Mussel)	FH08-058 C2	Sequencing inconclusive	260 bp band
Perna sp (Mussel)	FH08-058 C3	Sequencing inconclusive	260 bp band
Pinctada margaritifera (Oyster)	FH07-169 1	Sequencing inconclusive	260 bp band
Pinctada margaritifera (Oyster)	FH07-169 2	Sequencing inconclusive	260 bp band
Pinctada margaritifera (Oyster)	FH07-169 3	Sequencing inconclusive	260 bp band
Placopecten sp (Scallop)	FH08-058 A1	-	
Placopecten sp (Scallop)	FH08-058 A2	Sequencing inconclusive	260 bp band
Placopecten sp (Scallop)	FH08-058 A3	-	

The conventional PCR for the two CLO's was optimised as much as possible but due to issues with apparent specificity the focus was directed to the development of real-time PCR tests targeting the two known species of Chlamydiales identified in pearl oysters.

7.6 Real-time PCR Assays

A real-time duplex PCR was attempted for the detection of the CLOs, but unfortunately it lacked specificity and sensitivity. For this reason, 2 separate real-time PCRs were adapted, one for *Simkania sp*. and the other for Chlamydiales.

Maxima-CLO PCR

A real-time PCR for the detection of CLOs was based on the method by Everett et al. (1999). Sensitivity testing detected down to approximately 1 x 10^5 copy numbers. Specificity testing was performed on a range of samples with results presented in Table 4. Only the WA *P. maxima* sick animal gave a Ct value of less than 30.

Table 4. Specificity testing for the CLO real-time PCR. The P. maxima sample from WA collected as a sick animal tested positive with a Ct less than 30. All other samples were negative, with the exception of 1 mussel and 1 P. margaritifera sample that had a Ct greater than 30 and are considered to be borderline positive. The Ct cut-off value was based on results of a 10 fold series dilution using the positive control (FH07-06). Ct values for these these positive controls average around 29.

SAMPLE TYPE	Ct VALUE	REAL TIME PCR RESULT
P. maxima pearl oyster (WA) sick	28	Positive
Prawn	Negative	Negative
P. maxima pearl oyster (WA) Hillarys collected 2005 healthy	Negative	Negative
Mussels	1/6 Ct 35; 5/6 negative	1/6 borderline positive; 5/6 negative
Scallops	Negative	Negative
P. margaritifera pearl oyster (WA)	1/6 Ct 40; 5/6 negative	1/6 borderline positive; 5/6 negative
Fish	Negative	Negative
P. maxima pearl oyster (Qld)	Negative	Negative

The real time PCR was validated at two different laboratories using selected positive and negative samples, as determined by previous testing (see Table 5). PCR reagents and primers were provided along with the samples. The two laboratories, although part of the Department of Agriculture and Food WA, are independent of each other and of the Fish Health Laboratory where this CLO research was performed. Results show that only the sick *P. maxima* samples from WA produced a positive Ct. All other samples were negative.

Table 5. Results of the CLO real-time PCR performed at two different laboratories in WA for validation of the PCR. Both laboratories produced results as expected that were consistent with the history of the samples and previous testing at the Fish Health laboratory. Only sick *P. maxima* samples tested positive.

SAMPLE TYPE	LABORATORY 1 Virology Laboratory – Department of Agriculture and Food WA	LABORATORY 2 Plant Health Laboratory – Department of Agriculture and Food WA	EXPECTED RESULT
P. maxima pearl oyster (WA) FH07-23 sick	Positive	Positive	Positive
P. maxima pearl oyster (WA) FH07-20 Hillarys routine collected	Negative	Negative	Negative
P. maxima pearl oyster (WA) FH08-53 sick	Positive	Positive	Positive
Prawn FH08-141	Negative	Negative	Negative
P. maxima pearl oyster (Qld) FH08-134 healthy	Negative	Negative	Negative
No template control	Negative	Negative	Negative

Simkania PCR

The PCR using the SimF-SimR primers targeting the 23S rRNA gene of *S. negevensis* produced expected bands of 794 bp. Agarose gel bands produced from three samples from the OOD-affected areas Broome and Exmouth Gulf were sequenced and found to have 97-98% identity with the type strain. The sequences were submitted to GenBank and allocated accession numbers HQ684730, HQ684731 and HQ684732.

The hemi-nested primers SF-SimR were tested at temperatures 58-60ºC in conventional PCR and were found to be efficient at 60ºC. The PCR produced the expected band size of 121 bp and did not amplify *C. psittaci* DNA (see Figure 7).

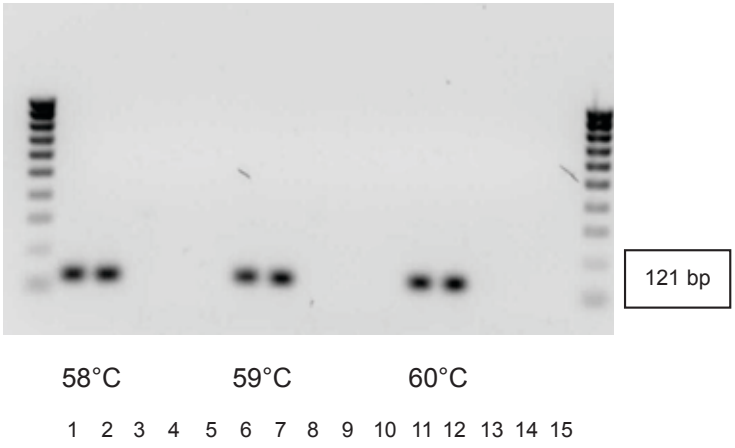

Figure 7. 1.5% agarose gel of hemi-nested PCR products using SF-SimR primers, annealing temperatures 58, 59 and 60ºC. Lanes 1,6,11, OOD Exmouth Gulf; Lanes 2,7,12, OOD Broome; lanes 3,8,13, healthy 80 mile beach, Lanes 4,9,14, *C. psittaci*; Lanes 5,10,15, no template control (NTC). (100 bp ladders, Bioline).

The real-time hemi-nested PCR using the SF-SimR primers gave Ct values <30 for samples from OOD-affected areas and >30 for samples from unaffected areas with the threshold set at 0.03 (see Figure 8).

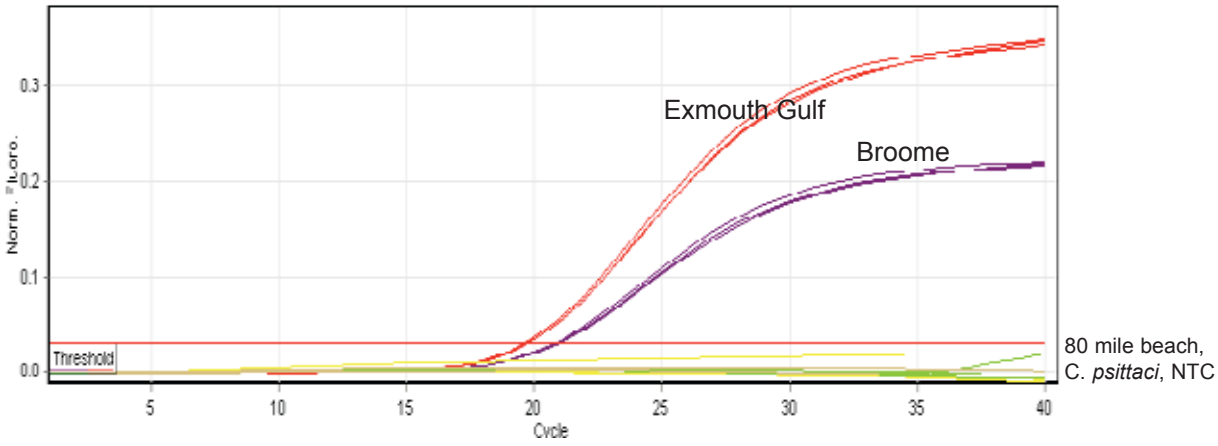

Figure 8. Normalized fluorescence showing detection of S. negevensis in two triplicate samples from Exmouth Gulf and Broome. The samples from 80 mile beach and C. psittaci had Ct values >30.

The primers BAF-BAR targeting the □-actin gene of *P. maxima* amplified 124 bp in pearl oyster tissues with an annealing temperature of 60ºC (see Figure 9).

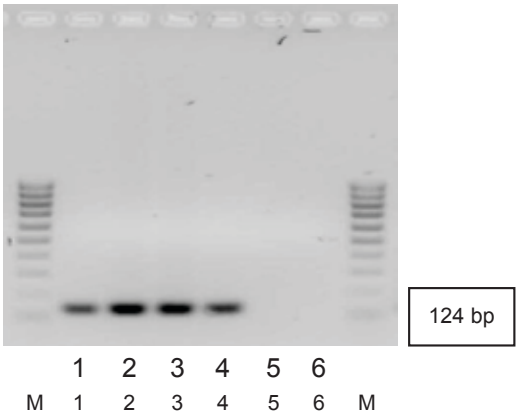

Figure 9. 1.5% agarose gel of PCR products using BAF-BAR primers, annealing temperature 60ºC. Lanes 1-3 = OOD *P. maxima* species; Lane 4 = healthy *P. maxima* species; Lane 5 = Avian species infected with *C. psittaci*; Lane 6 = no template control; M = 100 bp marker (Bioline).

The AF-BR primers targeting the intron within the 23S rRNA gene of *S. negevensis* did not produce the expected band of 1099 bp, but amplified a 400 bp band. Sequencing of this band in the reverse direction was not achievable, however, sequencing in the forward direction revealed amplification of *Pinctada* DNA.

The CCF-CCR/ZPF-ZPR nested primers targeting the 16S rRNA gene of *S. negevensis* amplified a 405 bp region as expected. Sequencing of the bands in the reverse direction was not achievable. Sequencing in the forward direction was successful and revealed amplification of the 16S rRNA gene of *S. negevensis*. Using the CCF-CCR/ZpF-ZpR primers specific for the 16S rRNA gene, 124 samples from OOD-affected areas were found to be positive and 105 samples were negative. Eight samples from unaffected areas were positive and 53 were negative. The unaffected black-lipped pearl oysters and penguin oyster spat were also positive for the 16S rRNA gene.

The results of the real-time PCR for the detection of the *Simkania*-like organism are shown in Table 6.

Amplicons from selected positive samples were submitted for sequence analysis, with sequence data entered into the NCBI Blastn non-redundant database. The sequence data produced from the selected positive samples showed 98% similarity to *Simkania negevensis*. The consensus sequence of the *Simkania*-like organism detected is as follows:

CATGACCAGGATGAAGCACGAGTAACATCGTGTGAAGGTCCGAACCAATGTATGTTGAAAAATACTTGGAT
GAGTTGTGGATAGGGGTGAAAGGCCAATCAAACTTGGAGATATCTTGTTCTCTCCGAAATAACTTTAGGGTT
AGCCTTGACTTACCACCTTTTGGGGGTAGAGCACTGGATTCCCGCGGGGGCCTACCGGCCTACCAACGGA
AACCAAACTCCGAATACTAAAAGGAAAGTCAGGAGATAGACAGTGGGGGATAAGCTTCATTGTCAAGAGGG
GAACAGCCCAGATCGTCGATTAAGGCCCCTAATTCTATGCTAAGTGTGTAAGGATGTGAAGTTTCACAGACA
GTTGGAATGTTGGCTTAGAGGCAGCCACCATTTAAAGAGTGCGTAACAGCTCACCAACCGAGAAATTTTGC
GCCGATAATAAACGGGACTAAGCATAGAGCCGAAATCACGGGTGCTATACTTTTGTATAGCGCGGTAGGAGA
GCGTAGTATGTGCGGTGAAGATATACCGAAAGGAGTGTTGGAGCGCATACTAGTGAGGATGCATGGCATGA
GTATACGATAAAGGAGGTGAGAATCCTCCTCGCCGAAAGCCTAAGGTTTCCAGGGTAAAGCTCGTCTTCCC
TGGGTTAGCCGGCCCCTAAGCCGAGGCAGAAATGCGTAGGCGATGGAAAGCAGGTTAAATATTCCTGCGC
CACCTAAAACTATGGTGATGGGACGACGAAGTATGTTAAGCACGCGGACGATTGGATGTTCGTTTCTCTATG

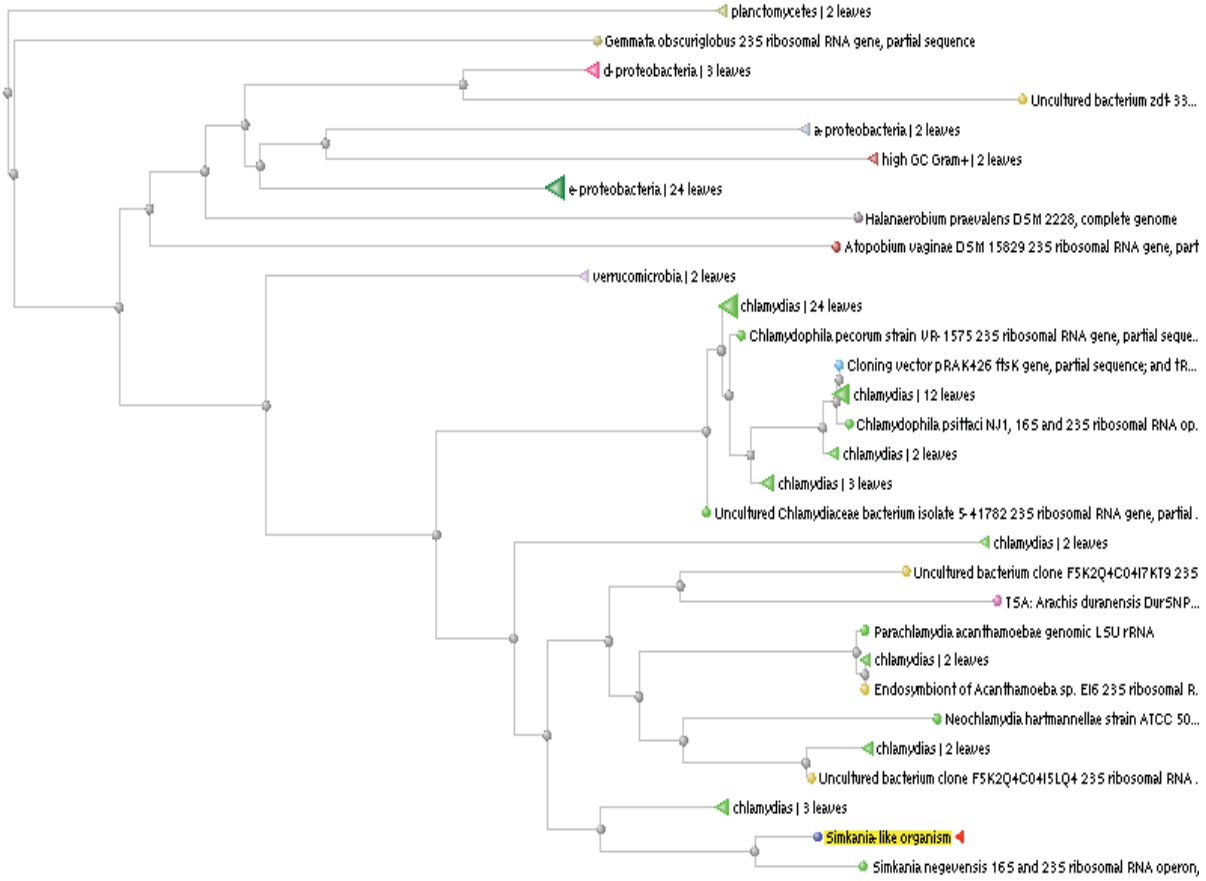

Figure 10. Distance tree of *Simkania*-like organism to other highly similar organisms. The *Simkania*-like organism detected originally in pearl oysters is most closely related to *Simkania negevensis*.

With the optimisation of the real-time PCRs for the detection of *Simkania negevensis* and *maxima*-CLO in pearl oysters, all available samples were tested. These included *P. maxima* oysters and some non-*P. maxima* samples.

As shown from Table 6, some samples that were deemed to be healthy amplified a PCR product, and some samples deemed to be sick and affected did not produce any PCR product amplification (see points below). Two real-time PCRs were used for the detection of an uncultured CLO and *Simkania sp*. in various samples with results shown in Table 6. Samples listed in Table 6 are

batches of at least 6 animals per batch. Some areas represent several batches of samples. The number of individual samples tested is several hundred.

Table 6. Real-time PCR results for the detection of *Simkania sp.* and an uncultured CLO in various sick or healthy *P. maxima* and non-*P. maxima* samples. Some results for the *Simkania sp.* PCR were unexpected based on the history of the sample but suggests that the *Simkania sp.* organism is not associated with oyster oedema disease affected areas or to *P. maxima* pearl oysters, and that the uncultured CLO has low prevalence but may still be associated with sick oysters.

Sample origin	History of sample	Results Simkania PCR (pos/neg)	Results *maxima*-CLO PCR (pos/neg)
Broome	Sick	Pos	Pos
Exmouth	Sick	Pos	Pos
Gales Bay (Exmouth Gulf)	Sick	Neg	Pos
Admiral Bay (near Broome)	Sick	Neg	Pos
Carnarvon	Sick	Pos	Pos
Whalebone Island (Exmouth Gulf)	Sick	Neg	Pos
Whitmore Island (near Carvarvon)	Sick	Pos	Pos
80 mile beach, two batches (near Broome)	Healthy	Pos	Neg
80 mile beach, 9 batches (near Broome)	Healthy	Neg	Neg
Lacepede Island (near Broome)	Healthy	Neg	Neg
Murdoch transmission trial tank 1	Healthy	Pos	Neg
Murdoch transmission trial tank 2	Sick	Pos	Pos
Murdoch transmission trial tank 3	Sick	Pos	Pos
Cygnet Bay, 2 batches (near Broome)	Healthy	Neg	Neg
Knocker Bay (Northern Territory)	Healthy	Pos	Neg
Port Bremer (Northern Territory)	Healthy	Pos	Neg
Friday Island (Queensland)	Healthy	Neg	Neg
80 mile beach 2005 (pre-OOD)	Healthy	Neg	Neg
Geraldton P. margaritifera (non-*P. maxima*)	Healthy	Pos	1/3 Pos
Denham Penguin oyster (non-*P. maxima*) (near Carvarvon)	Healthy	Pos	Neg
WA mussels (non-*P.maxima*)	Healthy	1 weak pos	5/5 weak pos
Prawn (non-*P. maxima*)	Healthy	Neg	Neg
Fish (non-*P. maxima*)	Healthy	Neg	Neg

7.7 In-situ hybridisation

A CLO riboprobe has been prepared for in situ hybridization (ISH). Initial attempts were difficult to interpret due to the amount of artefact. Further optimisation is required for this method to be acceptable.

7.8 Culture

For the attempt to culture the CLOs in chicken eggs, only embryo liver and chorioallantoic membrane samples resulted in a product being seen on an agarose gel after the F1R1 Chlamydia PCR had been run. Two bands were seen of approximate sizes 260 bp and 210 bp. Sequencing of these bands was unsuccessful and the many attempts to purify the bands still returned mixed sequence results. It is suggested that the 260 bp band may be from the inoculum itself, not from culture of the organism.

8.0 Discussion

Rickettsia-like and Chlamydia-like organisms (RLO's) have been frequently identified in association with molluscs, including *Pinctada* spp. In all cases they appear as basophilic inclusions, and the classification of such inclusions into one of the two groups cannot be achieved by light microscopy. Most of these records have not been associated with pathology. A rickettsia-like organism has been associated with mortalities of *P. maxima* and *P. fucata* in China. However, a problem with the Chinese reports is that images show, and text describes, the organisms as being "eosinophilic" which would indicate that the images are not of RLOs. (Wu and Pan 1999a-d; Wu et al. 2001).

Chlamydiales are a unique obligate intracellular bacterial group, first classified as an order in 1971 (Storz and Page 1971). Five chlamydial families are currently recognized (Chlamydiaceae, Parachlamydiaceae, Rhabdochlamydiaceae, Simkaniaceae, and Waddliaceae) (Everett et al. 1999, Rurangirwa et al. 1999) though recently it has been proposed that Criblamydiaceae be added to accommodate two species found in river water; and *Piscichlamyida salmonis* associated with epitheliocystis in fish gills, remains unclassified (Draghi et al. 2004, Thomas et al. 2006, Lienard et al. 2011). Most members of the first five families have been implicated in important human and animal infections (Leiberman et al. 1997, Kahane et al. 1998, Wyrick 2000, Corsaro and Greub 2004, Sachse et al. 2009, Lamoth et al. 2011).

The main difficulty experienced with matching CLO sequences with other CLOs is that there are likely many species that are still unclassified are still being added. It appears that the Chlamydiales group are still being organised into families and genera and the taxonomic classifications are still under active review (see Figure 11).

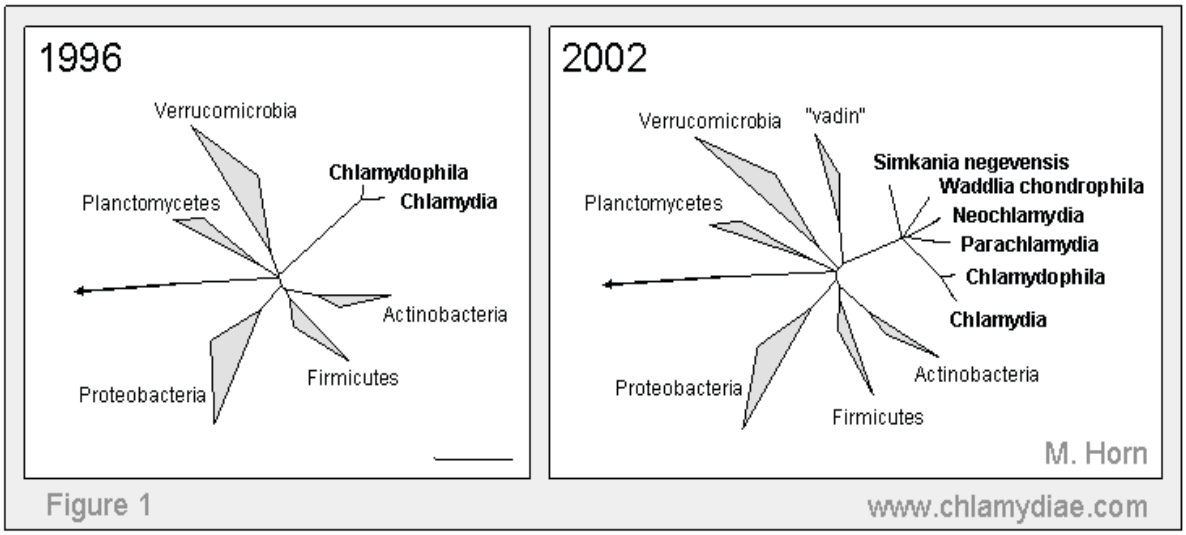

Figure 11. Phylogenetic tree based on comparative 16S rRNA gene sequence analysis showing the recognised diversity of chlamydiae in 1996 and 2002. (Source www.chlamydia.com)

All Chlamydiaceae share common features: They are obligate intracellular bacteria; their primary host cell is a mucosal epithelial cell; growth in the host cell involves two distinct morphological types, an elementary body and a reticulate body; development cycle takes place within a membrane bound vacuole. The development cycle can cause little damage to the host cell so most infections are chronic and expression of disease in hosts is immune modulated (Wyrick 2000). Interestingly, the encoding gene for nonmitochondrial ADP/ATP translocase is

found only in Rickettsiales, Chlamydiales, and plant and alga plastids leading Greub and Raoult (2003) and Smitz-Esser et al. (2004) to suggest that the gene was horizontally transferred from ancestral Chlamydiales to plants.

Chlamydia or Rickettsia-like organisms were first reported from molluscs in 1977 and have subsequently been identified in many mollusc species (Harshbarger et al. 1997; Fryer and Lannan 1994). Identification up until the mid 1990's was based on histology and transmission electron microscopy so that distinctions between Chlamydia and Rickettsia were seldom made. In addition, most reports are not associated with significant pathology or mortalities leading to the assumptions that these organisms were incidental findings (Romalde and Barja 2010). Exceptions to this observation are a chlamydia-like agent in Bay Scallops *Argopecten irradians* where they cause larval mortalities in hatchery stock (Morrison and Shum 1982, Leibovitz 1989); a chlamydia-like organism associated with visible gill lesions and occasional mortalities in *Crassostrea gigas* from the Atlantic French coast in 1992-1993 (Renault and Cochenne 1994, 1995); a "rickettsiales-like organism in Australian giant clams *Hippopus hippopus* (Norton et al. 1993); and a rickettsia-like organism causing high mortalities in Chinese *Pinctada fucata* and *P. maxima* (Wu and Pan 1999a, b).

Chlamydiales are difficult to separate except by molecular methods. All members of the order share >80% 16S rRNA or 23S rRNA gene sequence identity with other members of Chlamydiales (Draghi et al. 2004). This is reflected in the difficulty we encountered in separating species based on the conventional PCR and gel electrophoresis given that PCR products of the two CLOs are almost identical in size. From the sequence data obtained it appears that one of the CLOs in pearl oysters is highly similar to *Simkania negevensis* (see Figure 2).

The family Simkaniaceae currently includes two genera: *Simkania* and *Fritschea.* The type species is *Simkania negevensis,* initially isolated as a contaminant of cell cultures and its natural host is not known (Everett et al. 1999). It is readily grown in monolayers of eukaryotic Vero cells. Serological evidence and PCR indicate that *S. negevensis* has a widespread geographical distribution among humans (Friedman et al. 2003). It has been found in waste water and reclaimed water supplies in Israel (Kahane et al. 2004) and is able to grow and survive in protozoa, such as *Acanthamoeba polyphaga* (Kahane et al. 2001). The Simkaniaceae are not recognized by monoclonal antibodies that are specific for Chlamydiaceae lipopolysaccharide (Kahane et al. 1999). However, reactivity against anti-Chlamydiaceae LPS antibodies has been reported for the fish pathogen *Piscichlamydia salmonis* and for at least two uncharacterized organisms in fish and an oyster, *Crassostrea gigas* (Draghi et al. 2004, Groff et al. 1996, Renalt and Cochennec 1995).

Using the real-time PCR, *Simkania negevensis* was detected not only in *P. maxima* oysters but also in non-*P. maxima,* including *P. margaritifera* from Geraldton, *P. albina* (penguin oysters) and WA mussels (1 sample borderline positive). As shown from Table 1, some samples that were deemed to be healthy amplified a PCR product when tested with the *Simkania* PCR, and some samples deemed to be sick did not produce any PCR product. This suggests that the *Simkania sp*. organism detected in *P. maxima* oysters is not a significant factor in oyster health. For example, samples from Knocker Bay and Bremer Bay (both Northern Territory), *P. margaritifera* (black pearl oyster) from Geraldton, WA mussels and *P. albina* (penguin oysters from Denham, WA) were healthy upon collection but have produced a positive result with this PCR. Samples from Gales Bay, Admiral Bay and Whalebone Island in WA were heavily affected by OOD and Whalebone Island was the index site for the OOD outbreak, however all of these samples are negative with the *Simkania* PCR.

The presence of *Simkania negevensis* in pearl oyster samples is interesting finding. However, caution must be exercised in interpreting the results. Since *Simkania negevensis* is an environmental contaminant, false-positive amplification products may result from *S. negevensis* (or of part of its genomic material) in PCR reagents (water, deoxynucleoside triphosphates, or enzyme stocks). Corsaro and Greub (2006) point out that the presence of target DNA in laboratory reagents has caused false-positive results for Chlamydiae in at least two studies (Maraha et al. 2004, Meijer and Ossewaarde, 2002) and that false-positive results may occur due to the presence of *S. negevensis* in the noses of healthy staff, as has been reported to have occurred for Parachlamydiae (Aman et al. 1997). It is also significant that the *S. negevensis* was not associated with *Pinctada maxima* material from the index case, nor was it grown on Vero cells innoculated with material from the index site in Exmouth Gulf in 2006 (unpublished results), given that according to the literature, *S. negevensis* was originally isolated as a contaminant living in Vero cell lines.

Inconsistency in detection of the *Simkania*-like organism between batches of animals from the same location was noted. Samples in this category include those from 80 mile beach and Cygnet Bay, WA. There were 2 batches positive for *Simkania*-like organism from 80 mile beach, and 9 negative. Cygnet Bay had 1 batch that was positive and 2 batches that were negative. This could be due to the time of collection of batches.

It appears that the *Simkania*-like organism in pearl oysters is not confined to *P. maxima* and has no clear association with OOD. This is consistent with the literature for *S. negevensis,* which shows it has been widely isolated from the environment, including drinking water and its occurrence in pearl oysters may simply reflect its acquisition by the oysters from the environment.

By contrast, the non-characterised "maxima-CLO" does seem to have a strong association with affected samples. The real-time PCR used to detect this species was developed based on the PCR designed by Everett et al. (1999), was optimised and then used to test for *maxima*- CLO in *P. maxima* and non-*P. maxima* samples.

Samples tested with the *maxima*-CLO PCRs, both conventional and real-time, demonstrated a possible link with oyster health. *Pinctada maxima* samples that had a history of being sick were positive with these PCRs, and samples that had a history of being healthy were negative, with only a few exceptions. One *P. margaritifera* sample was positive although these samples were a different species to *P. maxima* and healthy. All 5 WA mussels that were tested showed slight amplification of PCR product. The real-time PCR is designed to be specific for CLOs, so this result may warrant further investigation.

Additional work was attempted to isolate one or both of the CLOs. An attempt was made to culture the CLOs in chicken eggs. Initial results suggested a successful culture of at least one of the CLOs in chicken eggs, however sequencing was unsuccessful. Five-day-old chicken eggs were inoculated with CLO-positive pearl oyster material, as determined by PCR. Yolk sac, chorioallantoic membrane and the embryo of each egg were harvested.

Following DNA extraction, the samples underwent PCR to determine if a CLO could be detected, representing successful culture in the chicken egg. Some samples were PCR-positive, however further analysis by way of sequencing was unsuccessful. On another approach, bands of the expected size were cut from the agarose gel, purified and cloned into a pCR2.1 vector. Again, sequencing did not produce any confirmatory results. It is possible that the PCR positives were from the original material used in the chicken egg culture rather than growth of the organisms in the eggs.

It is unfortunate that the Rickettsia-like organisms in *Pinctada maxima* from China have not been sequenced. The pattern of mortalities is consistent with that observed in Western Australia and it is possible that the same organisms are involved.

9.0 Benefits and adoption

The beneficiaries of the project are the pearling industry and the state government agencies in the Northern Territory and Western Australia

The project has identified a potential qPCR marker associated with OOD (the *maxima*-CLO). This will assist with the detection of putative OOD in hatcheries and in translocation of oysters. However, there is considerably more work to do to both to characterise the organism and to establish the aetiology of OOD.

It is difficult to quantify the research in terms of value to the industry; however, given that OOD is unable to be detected by histology, the ability to detect an organism associated with OOD gives the industry some ability to manage this economically devastating disease, both in the hatchery and on the farm.

10.0 Further development

The presence of the *maxima*-CLO in sick and dying oysters fitting the case definition for OOD does not, of itself, indicate causality. Further work is required to:

- Obtain further sequence
- Determine the relationship of the *maxima*-CLO to the epithelial lesion described by Jones et al. (2010), and to the CLOs seen by TEM.
- Determine the relationship of the chlamydia to the rickettsia-like bodies previously described in *P. maxima* by light microscopy and TEM
- Fulfill Henle-Koch postulates

11.0 Planned outcomes

In terms of practical outcomes for industry, the project has delivered:

- Sequence data demonstrating the presence of *Simkania negevensis* in pearl oysters in Australia.
- Sequence data demonstrating the presence of a Chlamydia like organism associated with oysters dying of OOD (the *maxima*-CLO).
- The development of a qPCR test for the *maxima*-CLO associated with OOD which can be used by Industry and the state health laboratories to test oysters for OOD (by proxy – while the CLO is not known to cause OOD, it is associated with the disease).

12.0 Conclusions

This project has shown that there are two organisms belonging to the Chlamydiales in *Pinctada maxima*. One is *Simkania negevensis* and the other is an as yet uncharacterised species we have tagged "*maxima*-CLO".

The finding of *S. negevensis* is of interest because this organism has been associated with respiratory conditions in humans, and this may be the mechanism by which it has spread across the industry. This is the first record of the organism in Australia and the first record of its association with bivalve shellfish.

The discovery of maxima-CLO is also of interest in that it appears to be associated with pearl oysters dying of oyster oedema disease. While there is an association, it is not yet clear what causes OOD and whether the CLO is the only pathogen present. This will require further study.

13.0 References

AusVet 2007. An investigation into a pearl oyster (*Pinctada maxima*) mortality event. AusVet Animal Health Services.

Amann RI, Springer N, Schönhuber W, Ludwig W, Schmid EN, Müller KD and Michel R. 1997. Obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydia* spp. Applied Environmental Microbiology 63:115-121.

Cajaraville MP and Angulo E. 1991 *Chlamydia*-like organisms in digestive and duct cells of mussels from the Basque coast. Jouranl of Invertebrate Pathology 58(3):381-6.

Corsaro D and Greub G. 2006. Pathogenic Potential of Novel Chlamydiae and Diagnostic Approaches to Infections Due to These Obligate Intracellular Bacteria. Clinical Microbiology Reviews 19: 283-297.

Crockford M, Jones JB, Crane MSJ and Wilcox GE. 2005. Molecular detection of a virus, Pilchard herpesvirus, associated with epizootics in Australasian pilchards *Sardinops sagax neopilchardus*. Diseases of Aquatic Organisms 68:1-5.

Crockford M, Jones JB, McColl K and Whittington RJ. 2008. Comparison of three molecular methods for the detection of Pilchard herpesvirus in archived paraffin-embedded tissue and frozen tissue. Diseases of Aquatic Organisms 82(1):37-44.

Crespo S, Zarza C, Padros F and Marin de Mateo M. 1999. Epitheliocystis agents in sea bream *Sparus aurata*: morphological evidence for two distinct chlamydia-like development cycles. Diseases of Aquatic Organisms 37:61-72.

Draghi A, Popov VL, Kahl MM, Stanton JB, Brown CC, Tsongalis GJ, West AB and Frasca S. 2004. Characterization of "*Candidatus Piscichlamydia salmonis*" (Order Chlamydiales), a Chlamydia-Like Bacterium Associated With Epitheliocystis in Farmed Atlantic Salmon (*Salmo salar*). Journal of Clinical Microbiology 42:5286-5297.

Everett KD, Bush RM and Andersen AA. 1999. "Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms." International Journal of Systematic Bacteriology 49:415-440.

Friedman MG, Dvoskin B and Kahane S. 2003. Infections with the chlamydia-like microorganism *Simkania negevensis*, a possible emerging pathogen. Microbes and Infection 5:1013-1021.

Fryer JL and Lannan CN. 1994. Review: Rickettsial and chlamydial infections in freshwater and marine fishes, bivalves and crustaceans. Zoological studies 33:95-107.

Fuerst JA, Gwilliam HG, Lindsay M, Lichanska A, Belcher C, Vickers JE and Hugenholtz P. 1997. Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn, *Penaeus monodon*. Applied and Environmental Microbiology 63:254-262.

Greub G and Raoult D. 2003. History of the ADP/ATP-Translocase-Encoding Gene, a parasitism gene transferred from a Chlamydiales ancestor to plants 1 billion years ago. Environmental Microbiology 69:5530–5535.

Groff JM, LaPatra SE, Munn RJ, Anderson ML and Osburn BI. 1996. Epitheliocystis infection in culture white sturgeon (*Acipenser transmontanus*) antigenic and ultrastructural similarities of the causative agent to the Chlamydiae. Journal of Veterinary Diagnostic Investigations 8:172-180.

Harshbarger JC, Chang SC and Otto SV. 1977. Chlamydia (with phages) mycoplasmas and rickettsia in Chesapeake Bay bivalves. Science 196:666-668.

Horn M and Wagner M. 2001. Evidence of additional genus-level diversity of Chlamydiales in the environment. FEMS Microbiology Letters 16:71-74.

Israelsson O. 2007. Chlamydial symbionts in the enigmatic Xenoturbella (Deuterostomia). Journal of Invertebrate Pathology 96:213-220.

Johnson MA and Pennec M. 1995. Association between the mollusc bivalve *Loripes lucinalis* and a Chlamydia-like organism, with comments on its pathogenic impact, life cycle and possible mode of transmission. Marine Biology 123:523-530.

Jones JB, Crockford M, Creeper J and Stephens F. 2010. Histopathology of oedema in pearl oysters (*Pinctada maxima*) – is oedema a general problem for sick bivalves? *Diseases in aquatic organisms* 91:67-73.

Kahane S, Greenberg D, Friedman MG, Haikin H and Dagan R. 1998. High prevalence of "*Simkania* Z", a novel *Chlamydia*-like bacterium, in infants with acute bronchiolitis. Journal of Infectious diseases 177:1425–1429.

Kahane S, Dvoskin B, Mathias M and Friedman MG. 2001. Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* Survival within amoebal cysts. Applied and Environmental Microbiology 67:4789–4795.

Kahane S, Platzner N, Dvoskin B, Itzhaki A and Friedman MG. 2004. Evidence for the presence of *Simkania negevensis* in drinking water and in reclaimed wastewater in Israel. Applied and Environmental Microbiology 70:3346-3351.

Kahane S, Everett KDE, Kimmel N and Friedman MG. 1999. *Simkania negevensis* strain Z: growth, antigenic and genome characteristics. International Journal of Systematic Bacteriology 49:815-820.

Kjeldsen KU, Obst M, Nakano J, Funch P and Schramm A. 2010. Two types of endosymbiotic bacteria in the enigmatic marine worm *Xenoturbella bocki*. Applied and Environmental Microbioloy 76:2657-2662.

Kirkpatrick J, Oakley B, Fuchsman C, Srinivasan S, Staley JT and Murray JW. 2006. Diversity and distribution of planctomycetes and related bacteria in the suboxic zone of the Black Sea. Applied and Environmental Microbiology 72:3079-3083.

Kutlin A, Flegg C, Stenzel D, Reznik T, Roblin PM, Mathews S, Timms P and Hammerschlag MR. 2001. Ultrastructural study of *Chlamydia pneumoniae* in a continuous-infection model. Journal of Clinical Microbiology 39:3721-3723.

Lamoth F, Jaton K,Vaudaux B and Greub G. 2011. Parachlamydia and Rhabdochlamydia: Emerging agents of community-acquired respiratory infections in children Clinical infectious diseases. 53(5):500-501.

Lieberman D, Kahane S and Friedman MG. 1997. Pneumonia with serological evidence of acute infection with the chlamydia-like microorganism "Z." American Journal of Respiratory and Critical care medicine 156:578–582.

Liebovitz L, Schott EF and Karney RC. 1984. Diseases of wild, captive and cultured scallops. Journal of World Mariculture Society 14:269-283.

Leibovitz L. 1989. Chlamydiosis: a newly reported serious disease of larval and postmetamorphic bay scallops, *Argopecten irradians* (Lamarck). Journal of Fish Diseases 12:125-136.

Lienard J, Croxatto A, Aeby S, Jaton K, Posfay-Barbe K, Gervaix A and Greub G. 2011. Development of a new *Chlamydiales*-specific real-time PCR and its application to respiratory clinical samples Journal of Clinical Microbiology 49:2637-2642.

Maraha B, Berg H, Kerver M, Kranendonk S, Hamming J, Kluytmans J, Peeters M and van der Zee A. 2004. Is the perceived association between *Chlamydia pneumoniae* and vascular diseases biased by methodology? Journal of Clinical Microbiology 42:3937-3941.

Meijer A and Ossewaarde JM. 2002. Description of a wider diversity within the order Chlamydiales than currently classified. International Chlamydia Conference, Antalya, Turkey, 16-21 June 2002. Available online at: http://chlamydiae.com/twiki/bin/view/Classification/ChlamydialesDiversity

Meijer A, Roholl PJM, Ossewarde JM, Jones B and Nowak BF. 2006. Molecular evidence for association of Chlamydiales bacteria with Epitheliocystis in Leafy Seadragon (*Phycodurus eques*), Silver Perch (*Bidyanus bidyanus*), and Barramundi (*Lates calcarifer*). Applied and Environmental Microbiology 72:284-290.

Meyers TR. 1979. Preliminary studies on a chlamydial agent in the digestive diverticular epithelium of hard clams, *Mercenaria mercenaria* (L) from Great South Bay, New York. Journal of Fish Diseases 2:179-189.

Molloy DP, Giamberini L, Morado JF, Fokin SI and Laruelle F. 2001. Characterization of intracytoplasmic prokaryote infections in *Dreissena* sp. (Bivalvia: Dreissenidae). Diseases of Aquatic Organisms 44(3):203-216.

Morrison C and Shum G. 1982. Chlamydia like organisms in the digestive diverticula of the bay scallop *Argopecten irradians* (Lmk). Journal of Fish Diseases 5:173-184.

Norton JH, Shepherd MA, Abdon-Nagutt MR and Lindsay S. 1993. Mortalities in the giant clam *Hippopus hippopus* associated with rickettsiales-like organisms. Journal of Invertebrate Pathology 62:207-209.

Ossewaarde J M and Meijer A. 1999. Molecular evidence for the existence of additional members of the order Chlamydiales. Microbiology 145:411-417.

Renault T and Cochennec N. 1994. Rickettsia-like organisms in the cytoplasm of gill epithelial cells of the Pacific oyster *Crassostrea gigas*. Journal of Invertebrate Pathology 64:160-162.

Renault T and Cochennec N. 1995. Chlamydia-like organisms in ctenidia and mantle cells of the Japanese oyster *Crassostrea gigas* from the French Atlantic coast. Diseases of Aquatic Organisms 23:153-159.

Romalde JL and Barja JL. 2010. Bacteria in mollusks: good and bad guys. In Mendez-Vilas, A (ed). Current research technology and education topics in applied microbiology and microbial biotechnology Vol 1. Formatex Research Centre http://formatex.org/microbiology2/

Rurangirwa F, Dilbeck P, Crawford T, McGuire T and McElwain T. 1999. "Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: proposal of Waddliaceae fam. nov., *Waddlia chondrophila* gen. nov., sp. nov". International Journal of Systematic Bacteriology 49 Pt 2:577-81.

Sachse K, Vretou E, Livingstone M, Borel N, Pospischil A and Longbottom D. 2009. Recent developments in the laboratory diagnosis of chlamydial infections. Veterinary Microbiology 135:2-21.

Schmitz-Esser S, Linka N, Collingro A, Beier CL, Neuhaus HE, Wagner M and Horn M. 2004. ATP/ADP translocases: a common feature of obligate intracellular amoebal symbionts related to Chlamydiae and Rickettsiae. Journal of Bacteriology 186:683-691.

Storz J and Page LA. 1971. Taxonomy of the Chlamydiae: reasons for classifying organisms of the genus *Chlamydia*, family Chlamydiaceae, in a separate order, Chlamydiales ord. nov. International Journal of Systematic Bacteriology 21:332-334.

Thomas V, Casson N and Greub G. 2006. *Criblamydia sequanensis*, a new intracellular Chlamydiales isolated from Seine river water using amoebal co-culture. Environmental Microbiology 8:2125-2135.

Wyrick PB. 2000. Intracellular survival by *Chlamydia*. Cellular Microbiology 2:275-282.

Wu X and Pan J. 1999a. Studies on Rickettsia-like organism disease of the tropical marine pearl oyster I: The fine structure and morphogenesis of *Pinctada maxima* pathogen Rickettsia-like organism. Journal of Invertebrate Pathology 73:162-172.

Wu X and Pan J. 1999b. (in Chinese) Studies on Rickettsia-like organism disease of the tropical marine pearl oyster: The morphology, morphogenesis and ultrastructure of RLO inclusions, an agent for *Pinctada maxima*. Oceanologica et Limnologia Sinica 30:73-80.

Wu X and Pan J. 1999c. (in Chinese) Studies on Rickettsia-like organism disease of the tropical marine pearl oyster *Pinctada maxima* and *P. fucata* IV: On histocytopathology of the RLO disease. Acta Oceanologica Sinica 21:93-98.

Wu X and Pan J. 1999d. (in Chinese) Studies on Rickettsia-like organism disease of the tropical marine pearl oyster V: Ultrastructural pathology and pathogenesis of rickettsia-like organism disease. Acta Oceanologica Sinica 21:113-118.

Wu X, Li D and Pan J. 2001. (in Chinese) Studies on Rickettsia-like organism disease of the tropical marine pearl oyster – epidemiological investigation of RLO disease in larval populations of maricultured *Pinctada maxima*. Acta Oceanologica Sinica 20:563-574.

14.0 Appendices

14.1 Intellectual property

No intellectual property has been generated by this project.

14.2 Staff

Staff member	Affiliation	Funding
Brian Jones	Department of Fisheries WA	FRDC and in-kind
Melanie Crockford	Department of Fisheries WA	FRDC and in-kind
Paul Hillier	Department of Fisheries WA	FRDC and in-kind
Amber Howard	Department of Fisheries WA	FRDC
Druime Nolan	Department of Fisheries WA	FRDC
Sheila Mortimer-Jones	Department of Fisheries WA	FRDC