THE DEVELOPMENT, OPTIMISATION AND EVALUATION OF MOLECULAR METHODS TO DIAGNOSE ABALONE TUBERCLE MYCOSIS (ATM) CAUSED BY *HALIOTICIDA NODULIFORMANS* IN SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*.

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Dissertation Submitted in fulfilment of the requirements for the degree of

***Magister Scientiae* in the Faculty of Natural Science,**

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Declaration

I declare that this is my own work, that **The development, optimisation and evaluation of molecular methods to diagnose Abalone Tubercle Mycosis (ATM) caused by *Halioticida noduliformans* in South African abalone, *Haliotis midae*** has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name.. Date.................................

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Keywords

Abalone

Disease

Tubercle mycosis

Peronosporomycete

Halioticida noduliformans

DNA extraction

Quantitative real-time polymerase chain reaction

Nested polymerase chain reaction

ABSTRACT

Land-based abalone aquaculture in South Africa started in the early 1990s and is based on the local species *Haliotis midae*. This industry expanded with great success over the last decade. In 2006 abalone exhibiting typical clinical signs of tubercle mycosis was discovered for the first time in South African abalone culture facilities, posing a significant threat to the industry. *Halioticida noduliformans,* a fungus belonging to the Peronosporomycetes (formerly Oomycetes), has been identified as the causative agent of abalone tubercle mycosis (ATM). While diagnoses of this disease are currently done by gross observation and histopathology, these methods fail to be sensitive enough to identify the causative agent accurately and reliably. Molecular confirmation could provide for quicker more accurate diagnostic information. The aim of this study was to develop a DNA based molecular diagnostic test. Polymerase chain reaction (PCR) has been used to rapidly detect, characterise and identify a variety of organisms. Nucleotide sequences of the small- and large-subunit ribosomal ribonucleic acid (rRNA) and mitochondrial cytochrome oxidase subunit II (cox2) genes of *H. noduliformans* were compared with closely related Peronosporomycete gene sequences to identify potential PCR primer sites. *H. noduliformans* specific real-time quantitative PCR (Q-PCR) primer sets were designed and optimised for each of the selected genes. Results indicate that, although all tested primers sets could amplify fungal DNA, only the LSU and cox2 primer sets

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demonstrated no cross-amplification with the closely related Peronosporomycete and non-fungal DNA tested in the present study. The *H. noduliformans* specific LSU primer set was chosen for further analysis and used for all subsequent real-time PCR assays. The lowest detection limit for the LSU primer set was evaluated by running Q-PCR on serial dilutions of known quantities of extracted *H. noduliformans* DNA. Serial dilutions were made in PCR grade water as well as in an abalone tissue matrix. The sensitivity of the Q-PCR reaction was determined to be 266 pg of *H. noduliformans* DNA per 25 µL reaction volume. However, inclusion of a nested PCR step, utilising universal fungal outer primers, followed by Q-PCR with the *H. noduliformans* LSU specific primers improved sensitivity to 0.266 pg of *H. noduliformans* DNA per 25 µL reaction volume. This equates to approximately 2.4 spores per 25 µL reaction volume. DNA extraction protocols were optimised to ensure efficient and repeatable extraction of high quality fungal DNA from pure fungus and tissue samples spiked with known quantities of fungal DNA. PCR amplification efficiency and potential inhibition were examined for each extraction method. Results suggest that real-time PCR has great potential in monitoring and quantifying *H. noduliformans* on abalone culture facilities in South Africa.

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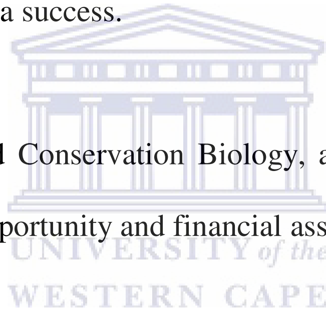

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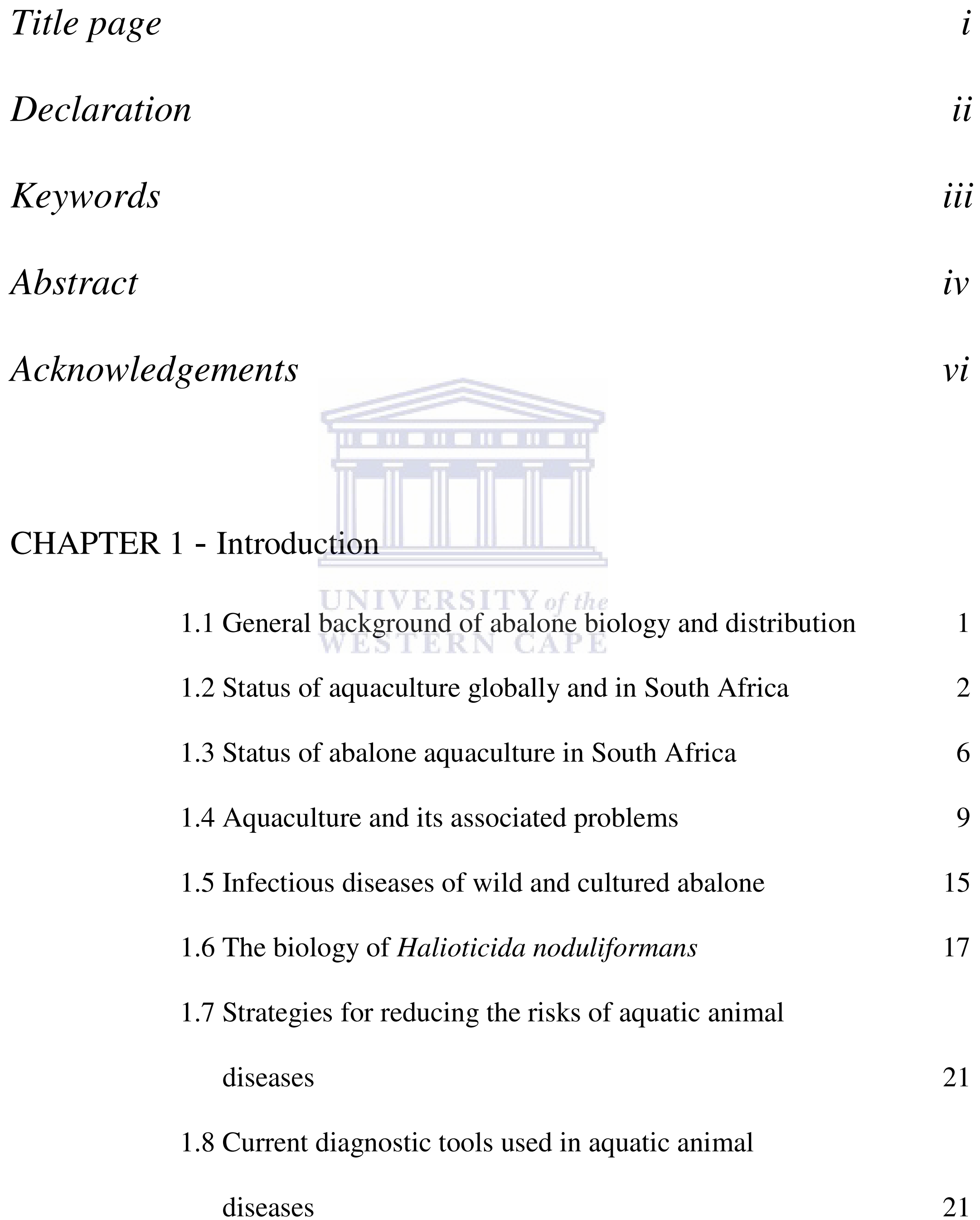
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CHAPTER 1

Introduction

1.1 General background of abalone biology and distribution

Abalone play a significant role in the ecosystem and are also seen as a valuable commodity and impact the economy because of their high import and export value worldwide. These globally important animals are marine gastropods belonging to the Phylum Mollusca (Archaeogastropoda:Haliotidae). Different abalone species are found worldwide in both tropical and temperate waters along most coastlines, except around South America and the Atlantic coast of North America (Najmudeen and Victor, 2004; Degnan *et al.*, 2006). None of the abalone species identified are distributed globally (Geiger, 2000). Five endemic species of *Haliotis*, namely *Haliotis midae, H. parva*, *H. spadicea, H. queketti*, *H. speciosa* and *H. pustulata* occur along specific regions of South African coastline (Fig. 1.1). *Haliotis midae* is however the only species that is commercially exploited in South Africa. *H. midae* is distributed from Port St. Johns on the East coast right through to St. Helena Bay on the West coast of Southern Africa (Fig. 1.1) (Branch *et al.*, 2010). Differences in water temperatures and seaweed preference are some of the main factors determining the geographical distribution of each of these species along the coastline.

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A variety of different seaweed species occur along the South African coastline, forming the principal component of the herbivorous diet of abalone. For example, on the Southern West Coast of South Africa *H. midae* are often found in the unique ecosystems created by kelp forests (Branch *et al.*, 2010). Abalone have a large muscular foot (Fig. 1.3) that enables them to grip tightly to rock surfaces (Branch *et al.*, 2010) commonly found in the intertidal zone that is characterised by strong wave action (Geiger, 2000; Degnan *et al.*, 2006). The consequent dependence of a solid substrate and macro-algal food preference increases the probability for the sharing of a common niche or microhabitat by sympatrically occuring species.

1.2 Status of aquaculture globally and in South Africa

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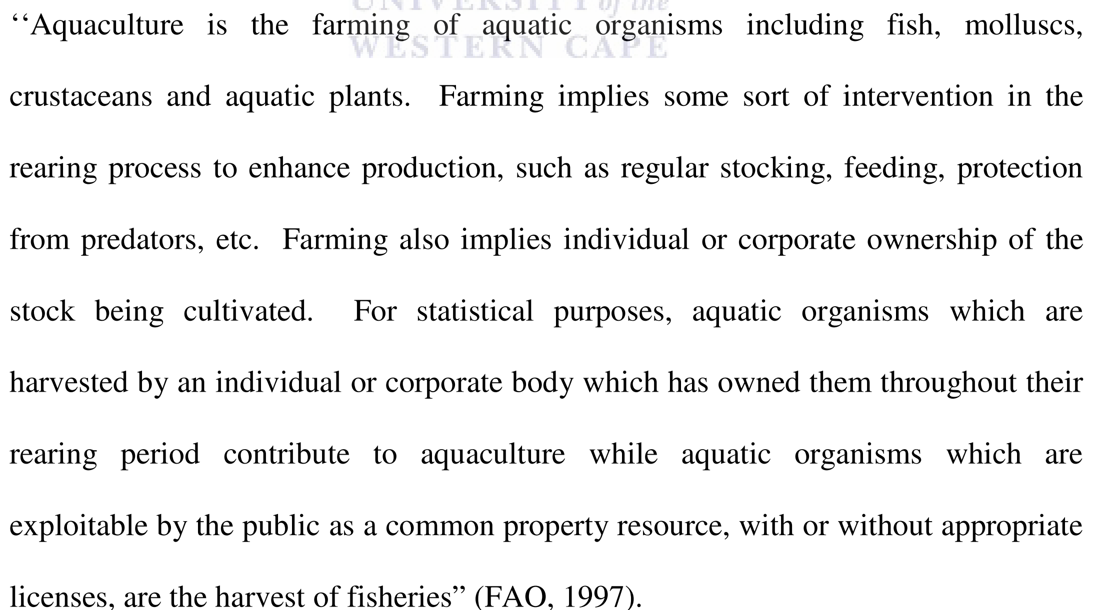

‘‘Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated. For statistical purposes, aquatic organisms which are harvested by an individual or corporate body which has owned them throughout their rearing period contribute to aquaculture while aquatic organisms which are exploitable by the public as a common property resource, with or without appropriate licenses, are the harvest of fisheries” (FAO, 1997).

Aquaculture originated as early as 1100 B.C. in the People's Republic of China (PR China) with common carp raised in fresh water to help address the human demand for protein food security. Thereafter, other kinds of aquaculture rapidly developed (Bondad-Reantaso *et al.*, 2005), resulting in the global mega-food production industry it is today. Seafood is seen as an excellent source of high quality animal protein and highly digestible energy, of which abalone is seen as one of the most highly valued seafood commodities in the world. In Asian countries abalone products form part of the traditional cuisine and ceremony. Collectively, these factors have contributed to a substantial increase in consumer demand for abalone products. Demand has also been closely related to human population growth, diminishing wild stocks due to habitat loss, over-fishing and poor stock management, and an increase in consumer demand for selected species (Macey, 2005). Approximately three quarters of the major marine fish stocks are presently harvested to their maximum limit and the global contribution of capture fisheries to food security and employment has been in decline since the 1950s (Macey, 2005). In South Africa, this decline has resulted in severe job loss and economic hardship. Consequently, South Africa, together with many other foreign countries, regard aquaculture as the only real solution to help sustain a growing demand for both

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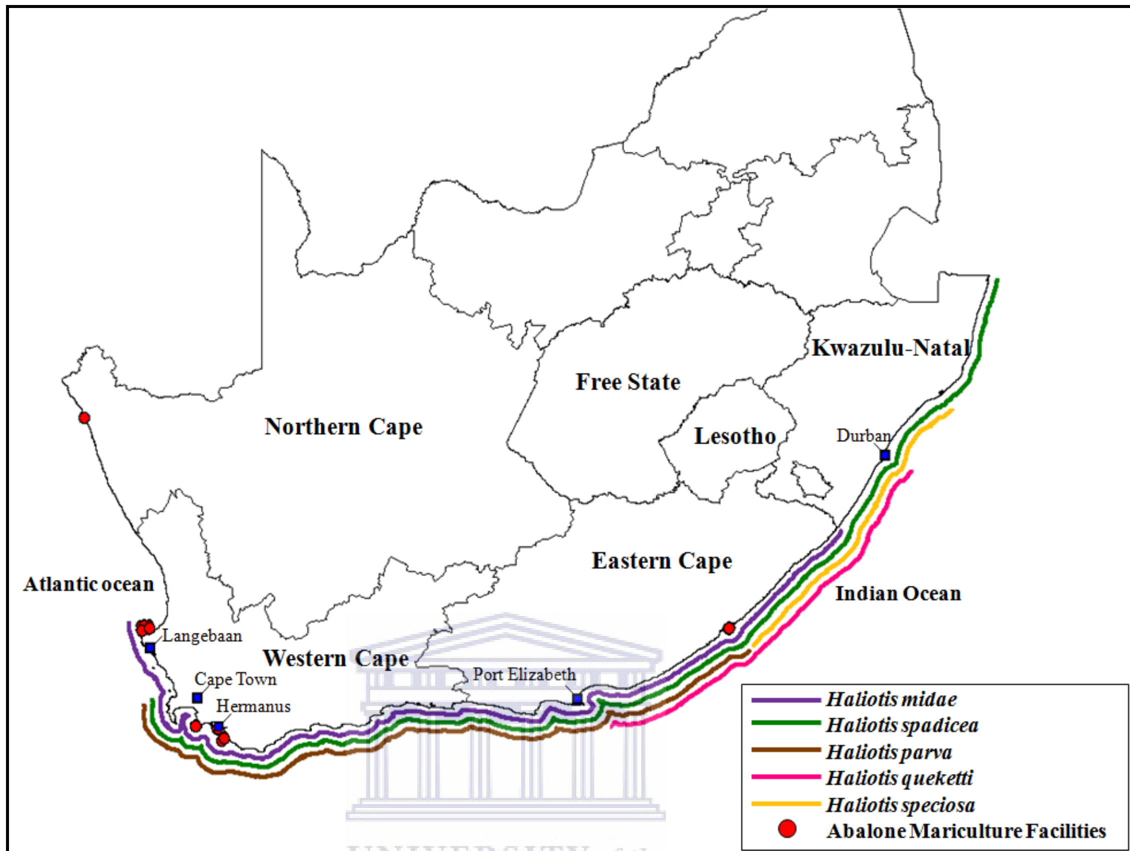

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Figure 1.1: Map showing the distribution of endemic Haliotid species along the coastline of Southern Africa and the location of the various commercial abalone farms. There are approximately 14 commercial farms in production with the majority occurring in the Western Cape Province (Hijmans *et al.*, 2002; Branch *et al.*, 2010).

freshwater and marine organisms; to help supplement wild stocks; and to help serve as a producer of low-cost, high-protein food (Macey, 2005).

According to the FAO, (2008a) global aquaculture production increased substantially over the last decade and amounted to 51.7 million tons for 2006, with marine aquaculture contributing 20.1 million tons of the total production. These figures exclude the aquatic plants component. Of this, production in Africa contributed 1.5% towards the total aquaculture production in 2006 and South Africa contributed less than 1% towards Africa's total aquaculture production (DEAT, 2009). Since the aquaculture industry is small in South Africa compared to other countries such as China, Chile and Norway, it has been identified as a sector requiring immediate attention in respect of promoting expansion and diversification of activities. The South African coastline does however offer a small amount of protected sites that are suitable for ranching and cage farming and there is also intense competition for these sites between industry and recreational users. Consequently, marine aquaculture activities in South Africa are primarily confined and focused on land based operations. This being said, exciting new industry initiatives are presently underway that are exploring the culture of indigenous marine finfish species in net-cage farms in the sea. According to a recent report by DEAT (2009), there has been an overall increase in South African aquaculture production from the year 2000 to 2008. The total aquaculture production figure of 2,014 tons recorded in 2008 was almost double the amount produced in 2000, namely 1,055 tons. In 2008 the total value of the

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marine aquaculture sector was estimated to be R 307,597,338 (DEAT, 2009). Aquaculture is one of the fastest growing food-production sectors worldwide. Carps are the most cultured species in the world with 39% of production volume. Some other major cultured shellfish species include oysters, clams, mussels and scallops (FAO, 2008b). Relative to the rest of the world South Africa is the second largest abalone producing country and within in South Africa abalone aquaculture is the largest contributor toward overall aquaculture production (Fig. 1.2).

1.3 Status of abalone aquaculture in South Africa

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Abalone fisheries in South Africa first started in 1949, but high demand for and extensive poaching of wild abalone stocks has led to the virtual collapse of the wild abalone fishery and subsequent expansion of the abalone aquaculture industry. South Africa has a very young abalone aquaculture industry and successful cultivation only started in 1981 after spat and juveniles were successfully produced from spawning wild caught specimens (Sales and Britz, 2001). Abalone are slow-growing (Branch *et al*., 2010) and easy to catch, making them vulnerable to overfishing. Therefore, it has been necessary to cultivate abalone to meet the growing demand for abalone products worldwide (Troell *et al*., 2006). Commercial abalone farming essentially began in the 1990s when collaborative programmes were initiated by the University of Cape Town, the Council for Scientific and Industrial Research and Rhodes University

(Sales and Britz, 2001). From these modest beginnings the industry has grown to where it is today.

Today, the South African abalone (*Haliotis midae*) industry is recognised as one of the largest producers of farmed abalone in the world outside of China. In fact, South Africa produced 783 and 1,037 tons of abalone for 2007 and 2008, respectively. With a contribution of 94.4% (Fig. 1.2) to the total value of the South African marine aquaculture industry in 2008, cultured abalone is the most developed and successful sector of the South African aquaculture industry (DEAT, 2009). This industry has an important economic impact on South Africa as its export earnings support both local and regional economies. Furthermore, abalone farms provide much needed employment among poor communities along the coast of South Africa (Troell *et al.*, 2006).

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Reductions in the total allowable catch (TAC) for abalone, temporary closures (Hauck and Sweijd, 1999) of the fishery in the 2004/5 season (Raemaekers *et al.*, 2011), and continued success and development of the abalone aquaculture industry have allowed for cultured abalone to dominate the abalone export market in South Africa, and this market is expected to expand even further (Troell *et al.*, 2006). The development of abalone culture technology has been almost entirely industry driven and funded and with the success rate still growing abalone farming could be described as the pioneer industry of marine aquaculture in South Africa. This success

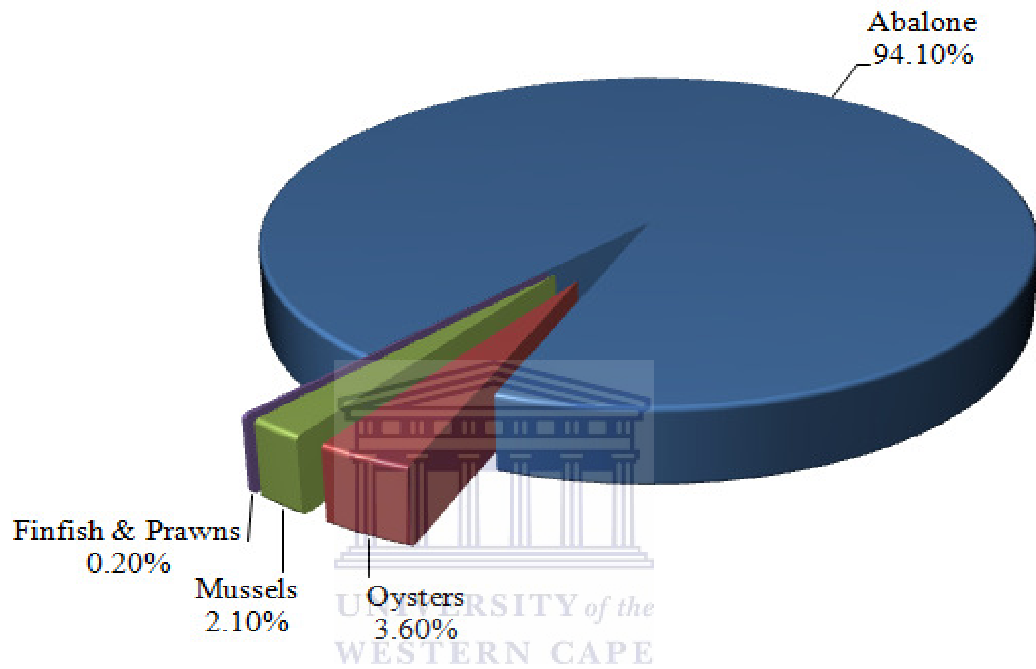

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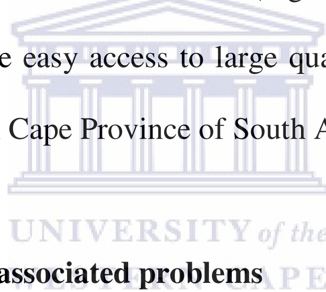
Figure 1.2: Contribution toward the overall aquaculture production, (in biomass of each subsector) of the South African aquaculture industry (total aquaculture contribution of 2,014 tons), indicating the dominance of the abalone sector (DEAT, 2009)."

rate can be attributed to the good collaboration between the industry and science community, but although much research has been done on various aspects of H. midae biology and physiology, apart from Botes (1999) and Mouton (2010), very little research has been dedicated to diseases (Sales and Britz, 2001).

Currently the abalone aquaculture industry in South Africa consists of 14 land based facilities, stretching from Port Nolloth on the Atlantic coast to East London on the Indian Ocean (Fig. 1.1), and includes 12 hatcheries and one experimental abalone sea cage farming operation (Mouton, 2010). All commercial abalone farms have been established along the South African coastline (Fig. 1.1), and are situated close to the shoreline, where they have easy access to large quantities of seawater. Most farms are situated in the Western Cape Province of South Africa (Fig. 1.1).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1.4 Aquaculture and its associated problems

Current aquaculture development is geared towards increased intensification and commercialisation of aquatic production. As aquaculture activities intensify and expand diseases and their associated problems, some of which are non-infectious diseases and others which are infectious diseases, are bound to increase. Nevertheless, when a disease increases mortality among animals or perhaps slows down the overall growth of animals it is considered as a significant problem. Reduced production figures may result in a chain reaction of problems, impacting not

only the local and international sector and food supply, but also having negative consequences socially and financially (Bondad-Reantaso and Subasinghe, 2008).

1.4.1 Non-infectious diseases

Non-infectious diseases are caused by environmental disorders, such as nutritional deficiencies, or genetic anomalies and are not contagious (Leatherland, 2010). Just like in the wild, cultured animals are still under the influence of a number of environmental parameters, such as temperature, high ammonia and nitrite, pH and dissolved oxygen (Cheng *et al.*, 2002). However, high stocking densities typically associated with most aquaculture operations tend to exacerbate these problems.

1.4.1.2 Culture techniques in Abalone aquaculture

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Abalone cultured in South Africa are primarily grown-out in large flow-through concrete raceway systems and to a lesser extent in recirculation systems where they are housed in baskets that are pre-moulded or made from oyster mesh (Mouton, 2010). To prevent overcrowding in the baskets animals are routinely graded by removing and redistributing them on the farm once every four to six months. If animals are not split into more baskets it could slow down growth and cause deterioration of water quality and shell structure (Mouton, 2010). In the flow-through

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Figure 1.3: A) Typical abalone farm in the Western Cape Province of South Africa. B-C) Healthy abalone found on the abalone farms.

systems, seawater is constantly being replaced in the tanks and seawater exiting the tanks is released back into the ocean. The quality of the water in these systems is therefore directly depended on the incoming water. In contrast to the flow-through system, the recirculation systems rely on the treatment and reuse of water (Macey *et al.*, 2011) and therefore water in these systems needs to be carefully monitored and controlled. Water replacement is minimal in these systems and oxygen is constantly replaced during water treatment. Treatments also remove suspended matter and dissolved toxic metabolic by-products (Mouton, 2010).

Previous studies have suggested that temperature and salinity are the two primary physical factors affecting the health of molluscs (Cheng *et al.*, 2004a). During summer increased water temperatures have been associated with increased stress and decreased immuno-competence of hetero-thermic animals, due to their inability to regulate their body temperatures, thereby making them more susceptible to opportunistic infectious agents (Lee *et al.*, 2001; Cheng *et al.*, 2004a; Malham *et al.*, 2003). Similarly the susceptibility of the host to pathogens is affected by, changes in salinity, high pH levels, high ammonia and low dissolved oxygen (Cheng *et al.*, 2002; Cheng and Chen, 2002; Cheng *et al.*, 2004a). Furthermore it has also been reported that the immuno-competence of farmed invertebrates are affected by factors such as, food intake, pollutants and physical stress resulting from routine husbandry practices (Persson *et al.*, 1987; Diaz *et al.*, 1992; Malham *et al.*, 2003).

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1.4.2 Infectious diseases

Infectious diseases are caused by pathogenic agents present in the environment or carried by other animals in the direct environment (Leatherland, 2010). Disease outbreaks are facilitated by the interaction of susceptible host and infectious agents. The likelihood of these interactions is increased by the reduction in the relative space between these two entities (Reno, 1998). As with most farming practices, susceptible hosts are typically much easier to find in aquaculture facilities than in the wild due to high stock intensities. This increases the likelihood for the pathogenic agent to infect a susceptible host. This likelihood may be further exacerbated by the use of recirculation systems. Macey *et al.* (2011) observed that the Peronosporomycete *Halioticida noduliformans,* the causative agent of abalone tubercle mycosis, was more prevalent in recirculation systems and suggested that infection pressure in the recirculation system may have been further elevated due to an increased surface area for attachment provided by the filtration substrate (Macey *et al.*, 2011).

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The global aquaculture industry has experienced numerous diseases caused by viruses, bacteria, fungi, parasites and other emerging pathogens (Bondad-Reantaso *et al.*, 2005; Leatherland, 2010; McGladdery, 2011). The Office International des Épizooties (OIE – World Organisation for Animal Health) lists 29 diseases of finfish, molluscs and crustaceans which meet the criteria of OIE as being of significant economic importance. Diseases listed by the OIE are to be of socio-economic and/or public health importance within countries and are significant in the international trade

of aquatic animals and aquatic animal products. In addition to OIE-listed diseases, many more diseases have significant impacts on aquaculture (Subasinghe *et al.*, 2000).

Diseases are a significant limitation to the culture of many aquatic species, impacting both economic and social development in many countries (Bondad-Reantaso *et al.*, 2005). The spread of disease is facilitated by multiple pathways, such as trade in live aquatic animals and their products, misunderstanding and misuse of specific pathogen free (SPF) stocks, unanticipated negative interactions between cultured and wild populations (Reno, 1998; FAO, 2007), poor or lack of effective bio-security measures, slow awareness of emerging diseases, climate-change and all other human mediated movements of aquaculture commodities (Bondad-Reantaso *et al.*, 2005). Movement of aquacultured products, organisms and equipment provides opportunities for the spread of pathogens (Bondad-Reantaso and Subasinghe, 2008). Molluscs may provide substrate for a variety of micro- and macroscopic organisms that could serve as possible carriers of diseases. The potential of this disease transmission pathway is emphasized by the recent outbreak of abalone viral ganglioneuritis in Western Australia (Hooper *et al.*, 2007; Day and Prince, 2007). Similar risks for disease transmission between wild and farmed abalone in South Africa exist since these farms are located within the natural distribution range of abalone (Fig. 1.1). These risks are further elevated by the sympatric occurrence of other *Haliotis* species and other possible disease vectors.

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1.5 Infectious diseases of wild and cultured abalone

Various diseases have been reported globally for a number of cultured abalone species (Friedman *et al.*, 2000; Lee *et al.*, 2001; Cheng *et al.*, 2004b; Mouton, 2010) including various fungal diseases such as foot tubercles and shell mycosis (McGladdery, 2011). Bacteria are known to cause systematic infection of the soft-tissue of cultured juvenile abalone, resulting in tissue necrosis and death. Most of these infections are caused by bacteria within the genus *Vibrio* or by other bacteria closely related to this group (Nicolas *et al.*, 2002).

More globally, an intracellular Rickettsiales-like prokaryote, *Candidatus Xenohaliotis californiensis*, has severely impacted a variety of abalone species in different parts of the world. This organism is the causative agent of Withering Syndrome disease in abalone (Friedman *et al.*, 2000). This disease affects abalone of all sizes and causes lethargy, retracted visceral tissues, and progressive weakening of the foot muscle (Day and Prince, 2007). Reduced feeding, adverse affects on metabolism and a reduced ability to adhere to substrates have caused mortality in affected abalone. Although Mouton (2010) has reported the presence of Rickettsia-like organisms in cultured South African abalone (*Haliotis midae*), it has not been associated with pathology.

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Various Digeneans, within the family Allocreadiidae and possibly Opeocoilidae, have been reported in Southern Africa (Botes *et al.*, 1999). However, the encystment of

these parasites in different organs does not appear to affect the survival of infected abalone (Botes *et al.*, 1999). Polychaete species in the family Spionidae are parasites found in the shells of abalone (Simon *et al.,* 2006). On some farms in South Africa, spionid polychaetes occur in high numbers and cause severe shell damage and stress to *H. midae* by penetrating the mantle cavity (Simon *et al.*, 2006). Indeed, the main disease problem encountered so far by the South African abalone aquaculture industry has been by the sabellid polychaete worm *Terebrasabella heterouncinata* (Ruck and Cook, 1998).

More recently, outbreaks of Abalone Tubercle Mycosis (ATM) have occurred in South Africa since 2006. Muraosa *et al.* (2009) originally reported on mortalities that occurred between 2004 and 2006 of three different abalone species infected with the fungus-like organisms *Halioticida noduliformans* at a holding facility in Japan. One of these species was reported to be the South African abalone, *Haliotis midae.* Since 2006, outbreaks of ATM in South Africa have been reported for five abalone culture facilities and one public aquarium (Macey *et al*., 2011). These facilities have suffered significant losses in production caused by severe pathology and mass mortality attributed to ATM. Up to 90% mortality among spat and up to 30% among older animals has been recorded on some of the affected farms (Macey *et al*., 2011).

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1.6 The biology of *Halioticida noduliformans*

Halioticida noduliformans is a marine Peronosporomycete (formally Oomycete). The Peronosporomycetes group are probably the largest and the most diverse monophyletic class of flagellate fungi (Dick, 2002) and form part of a class of heterotrophic organisms that are believed to have originated from heterokont algal ancestry (Dick, 1969; Peterson and Rosendahl, 2000). These eukaryotic organisms consists of uninucleate protoplasts bounded by cell walls in their assimilatory states and are therefore obligate osmotrophic heterotrophs (Dick, 2002). The cell walls of these organisms lack chitin but posses a fibrillar carbohydrate component (Dick, 1969). Even though there has been wide debate over the classification of Peronosporomycetes (Peterson and Rosendahl, 2000; Sekimoto *et al.*, 2007), more recently Dick (2002) proposed that both morphological and molecular characteristics are required for correct classification of organisms within this group. One of the key criteria that was used for classification include sexual and asexual reproductive structures (Dick, 1969). However these sexual structures have not been identified from Peronosporomycetes isolated from marine organisms (Muraosa *et al.*, 2009). Therefore, Sekimoto *et al.* (2007) and Muraosa *et al.* (2009) have since suggested that molecular phylogeny is equally important for the correct classification of organisms within this group. The basic morphological characteristics of *H. noduliformans* show closest affinity to organisms within the genus *Haliphthoros* (Sekimoto *et al.*, 2007). *Halioticida noduliformans* is characterised by highly branched aseptate hyphae with numerous protoplasmic oil drops (Muraosa *et al.*, 2009; Macey *et al.*, 2011) (Fig.

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1.4B). Colonies on PYGS agar are yellowish (Fig. 1.4A), flat and filamentous with irregular edges (Muraosa *et al.*, 2009) and appear to be sticky in PYGS broth. *Halioticida noduliformans* releases copious amounts of biflagellate zoospores through one or more discharge tubes (Muraosa *et al.*, 2009) approximately 24 - 30 h after hyphae are transferred to a nutrient poor media, such as sterile natural seawater (Macey *et al.*, 2011). Spores of *H. noduliformans* are 6.0 – 8.0 μm in diameter (Macey *et al.*, 2011). Motility of zoospores is crucial in order to find a susceptible host. Nevertheless, spores are able to re-encyst and germinate again at a later stage should they not find a suitable host and/or substrate and in so doing persist in the environment for extended periods of time (McGladdery, 2011). Up to date the survival time of *H. noduliformans* spores in the environment has not been documented. Infection is direct and hence no secondary hosts appear to be required for disease transmission, making this pathogen particularly problematic in the aquaculture industry.

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Halioticida noduliformans is physiologically well adapted to survive within the natural distribution range of *Haliotis midae*, which occur in the cold water regions along the Western and Eastern Cape coast of South Africa which has a seawater temperature range of 17 – 24 °C. Although *H. noduliformans* has an optimum growth rate at temperatures between 20 - 25 °C, this pathogen grows in a wide range of temperatures, 10 – 25 °C (Muraosa *et al.*, 2009; Macey *et al.*, 2011). It will not grow in the absence of seawater/ NaCl and is therefore considered to be an obligated marine Peronosporomycetes (Macey *et al.*, 2011).

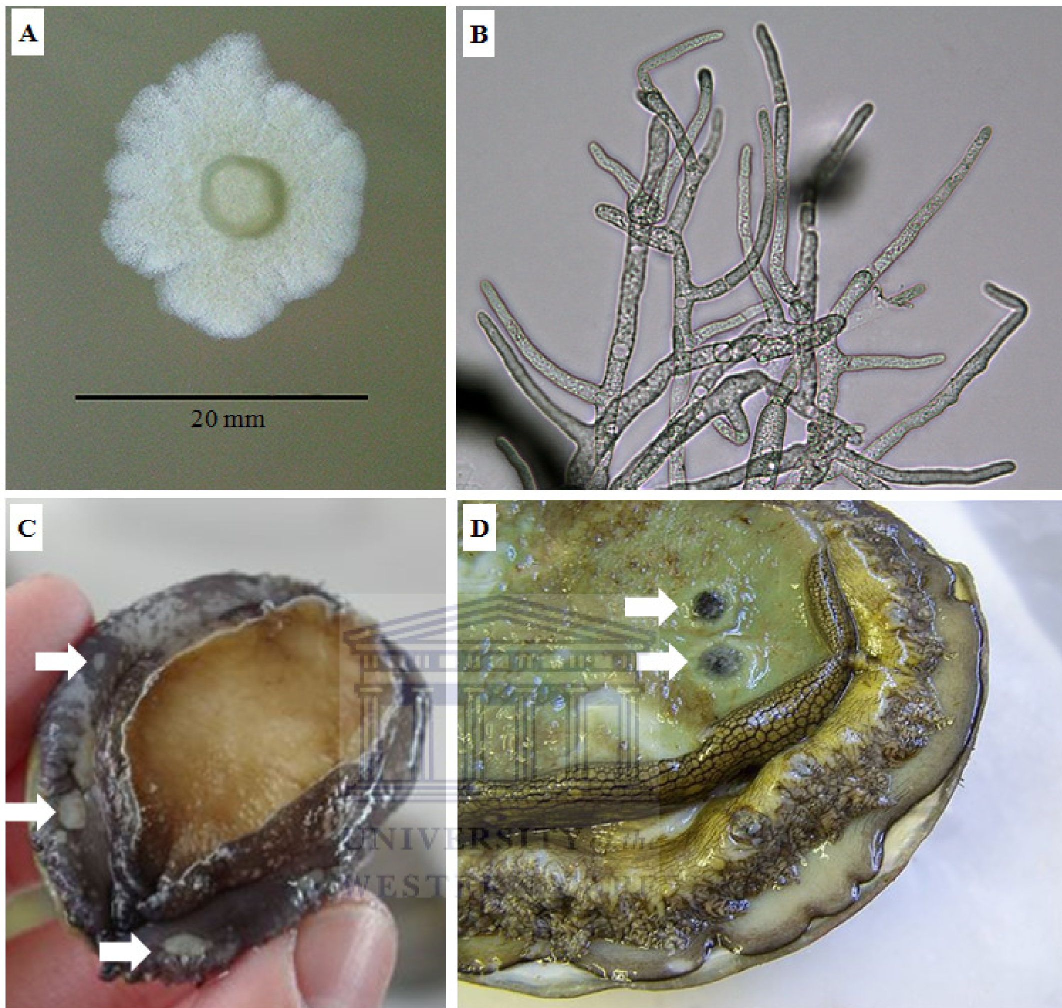

Figure 1.4: *Halioticida noduliformans* (A) Colony of actively growing mycelia on PYGS agar. (B) Light micrograph (400 x magnification) of actively growing mycelia showing branched hyphae with numerous protoplasmic oil droplets. (C) Typical clinical lesions on the epipodium and on the (D) foot muscle of *Haliotis midae* infected with *Halioticida noduliformans.*

Haliphthoros species are well known as pathogens of a wide range of marine animals including shrimps, crabs and fish (Vishniac, 1985; Diggles, 2001). *Haliphthoros* species have been frequently isolated from diseased organisms and are regarded by Sekimoto *et al.* (2007) to be serious pathogens of economically important marine animals. Considering the close phylogenetic relationship between the genera *Haliphthoros* and *Halioticida* (Sekimoto *et al.*, 2007) and since *Haliphthoros* has a host range spanning numerous invertebrate taxa, the possibility exists that other marine animals such as crustaceans may be susceptible to *H. noduliformans* or even serve as reservoirs for this pathogen, both in the wild and in culture facilities (Macey *et al*., 2011). *Halioticida noduliformans* was isolated from the gills of *Oratosquilla oratoria* (wild mantis shrimp) in Tokyo Bay, Japan (Atami *et al.*, 2009). Considering other Haliotid species found along the South African coastline, with overlapping distribution areas (Fig. 1.1), it is most likely that all of these species may be susceptible to this pathogen, although it has only been isolated from cultured *Haliotis midae*.

Abalone infected with *H. noduliformans* are weak and have a tendency to sit at the top of baskets and may even climb out of the water (Macey *et al*., 2011). Infected abalone are characterised by multiple lesions on the foot, epipodium and mantle. Typically, these lesions are 2 – 3 mm in diameter and consist of an epithelial defect, sometimes covered in loosely associated off-white material, surrounded by a thin black reaction zone (Fig. 1.4D). In advanced cases, these lesions may coalesce to affect larger areas of tissue (Macey *et al*., 2011).

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1.7 Strategies for reducing the risks of aquatic animal diseases

There are a number of strategies proposed by Bondad-Reantaso and Subasinghe (2008) for minimising the risks of aquatic animal diseases. These include: (1) the improvement of compliance with international codes and regional guidelines through national strategies; (2) increasing the bio-security awareness at all levels, which include surveillance programmes and diagnostic services to detect and identify the emergence and the spread of diseases; (3) demand on certification schemes for aquatic animal health service providers; and (4) scientific research and advice. In each one of these it is pointed out that rapid and early detection of an infectious disease agent(s) is critical for improving overall aquatic animal health (Bondad-Reantaso and Subasinghe, 2008). More specifically, standardisation and validation of diagnostic tools are required. Early diagnoses of infectious diseases are essential where stock movements are very common. This will help to control the spread of pathogens together with their hosts, especially animals with sub-clinical infections. It can therefore be said that diagnostic tools are a key component for reducing the risks of aquatic animal diseases in aquaculture.

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1.8 Current diagnostic tools used in aquatic animal diseases

Diagnosis is a term used to describe the recognition of a disease or condition by its clinical signs and symptoms (Leatherland, 2010). This definition could be extended to include the second stage of the identification process, which is the determination of

the underlying physiological, biological, biochemical or molecular factors that are regarded as being responsible for the disease or condition (Leatherland, 2010). Based on specific clinical signs, clinical tests are then used to confirm the preliminary diagnosis and where possible, treatments and disease management strategies are developed to deal with the conditions (Leatherland, 2010). These are the first steps taken towards the management of disease outbreaks.

Diagnosing non-infectious disorders in fish has tended to be much more problematic (Leatherland, 2010). Consequently, infectious diseases are generally not only diagnosed by clinical signs but also by the source responsible for infection (Leatherland, 2010). Various techniques can be used and range from gross observation to highly technical bio-molecular based tools. Histology provides a large amount of information and is therefore recommended as a standard screening method for routine health monitoring and also when abnormal mortality outbreaks occur (OIE, 2006). However, molecular methods are increasingly being used, especially for diagnosis of known infectious diseases, due to their sensitivity, specificity and quick turnaround time.

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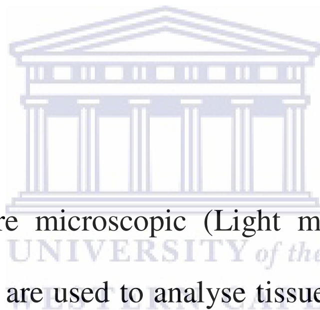
1.8.1 Gross pathology

Gross pathology of molluscs should target, as far as possible, animal behaviour, shell surface, inner shell and soft tissue (OIE, 2006). Although the observation of animals in water can have some challenges, observation of brood-stock and larvae in

hatcheries can provide useful indications of behavioural changes which may be disease-related. If signs of abnormal behaviour are noted, samples should be taken and examined for gross signs such as abnormalities, deformities and presence of fouling organisms. Animals can also be fixed for further processing. For example, in bivalves molluscs signs of weakening may include, accumulation of sand, mud and debris in the mantle and on the gills of animals and also mantle retraction away from the edge of the shell (OIE, 2006). More specifically, in abalone their ability to adhere to solid surfaces and righting reflex is affected in weakened animals and is used as a good indicator of weakness (OIE, 2006).

1.8.2 Histopathology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This is a technique where microscopic (Light microscope/ transmission electron microscope) examinations are used to analyse tissue sections (Lightner and Redman, 1998) by looking at the cell structures of tissue (OIE, 2006), in order to study the appearance of diseases caused by infectious diseases or non-infectious diseases. Different steps are required for the preparation of tissue samples. These steps include tissue fixation, dehydration, impregnation and embedding of samples, preparation of sections, staining and mounting of slides (OIE, 2006). In molluscs, standard sections will normally include the digestive gland, gills, mantle and palps (where possible) (Ifremer, 2011). Histological examination can help to determine the overall health of an animal (Robertson-Andersson *et al.*, 2008) and provide information on the tissue response of a host to infectious and non-infectious pathogens. Although

histopathology is suitable to confirm a fungus-like etiological agent from animals exhibiting clinical signs for ATM, this method lacks sensitivity to accurately identify this fungus-like organism to genus or species level. Microbiological and/or molecular methods are therefore required for the specific confirmatory diagnosis of ATM as caused by *H. noduliformans*.

1.8.3 Culture-based methods

At present, one of the most widely used methods for disease diagnosis is culture-based methods. During culture methods, total numbers of culturable microorganisms are determined by macroscopically observing and counting numbers of visible growth (colony forming units) of microorganisms (Yamamoto *et al.*, 2010). These methods are influenced by the selection of culture media and could fail to detect microorganisms and spores in a viable but non-culturable state as well as non-viable microorganisms, i.e. metabolically inactive organisms (Wu et al., 2002; Oliver, 2005; Macey *et al.*, 2008a; Yamamoto *et al.*, 2010). Microorganisms and spores have the ability to change from a viable but non-culturable state to become metabolically active (culturable) again, therefore the use of only culture based methods as diagnostic tests could fail or result in false negatives. These methods are also time consuming and laborious (Zhou *et al.*, 2000). Furthermore, there is a risk of misidentifying species due to intra- and inter-specific variations in species morphological characteristics (Wu *et al*., 2002).

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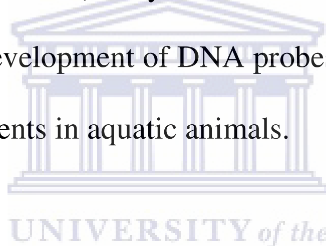
1.8.4 Molecular based diagnosis

Molecular diagnosis based on DNA techniques are routinely used to detect pathogens in tissue samples of infected animals. Molecular based techniques generally provide quick results, with high sensitivity and specificity and are particularly valuable for infections that are difficult to detect when using standard histology and tissue-culture procedures. The one outstanding difference, between the culture-based methods and PCR amplification is that culture procedures amplify is the bacterial cell itself, whereas PCR-based techniques amplify a nucleic acid sequence that is presumed to be a unique sign of that cell (Hiney and Smith, 1998). Consequently, there is increased interest in the development of DNA probes and/or PCR primers as tools for the detection of disease agents in aquatic animals.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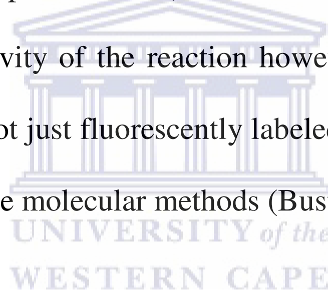
There are variety of molecular methods available with different levels of sensitivity, of which the most common method is the Polymerase Chain Reaction (PCR) technique. Well known PCR methods include (1) non-nested (conventional) PCR where a single pair of oligonucleotide primers are used to amplify a small, unique part of the genome of the infectious agent; (2) nested PCR assays which utilise two primer sets. The first primer pair amplified the target DNA as seen in any PCR experiment. Then a second step follows where a second pair of primers (nested primers) binds within the first PCR product and produces a second shorter PCR product. The reason for this second step is that if the wrong DNA region were amplified by mistake, the probability is very low that it would also be amplified, a

second time by the second pair of specific primers. This enhances the specificity and sensitivity of a reaction. Finally (3) real-time quantitative PCR (Q-PCR) where amplification of PCR products are detected directly during the amplification cycles using fluorescently-labelled probes, thereby allowing for the starting amount(s) of DNA to be accurately quantified (OIE, 2006). Since fluorescence is detected during each cycle of the Q-PCR, dynamic range of the reaction is significantly enhanced. The measured fluorescence reflects the amount of amplified product in each cycle. Furthermore Q-PCR has the potential for high-throughput application (Bott *et al.,* 2010). Results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). The fluorescently labelled probes enhance the specificity and sensitivity of the reaction however real-time PCR can also use dyes, such as SyBr, and not just fluorescently labeled probed. Real-time PCR is seen as one of the most sensitive molecular methods (Bustin 2000; Bustin & Nolan, 2004).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PCR technology is ideal for specifically recognising target DNA regardless of the life history stage of the target organism present, where other methods fail to detect these stages due to their culturability, viability and abundance (Burreson, 1999). However PCR does have shortcomings that may result in the generation of false positive and negative results (Walker *et al*., 2000). Challenges that need to be overcome when using PCR include efficient cell lysis, DNA extraction and purification, choice of primers and PCR cycling conditions. These are all factors that could influence the sensitivity and specificity of PCR based diagnostic tools (Farrelly *et al*., 1995). Given the potential and the inherent problems for the above mentioned diagnostic

techniques currently used, validation of these DNA-based techniques are of utmost importance (OIE, 2006).

1.9 Diagnosis of abalone tubercle mycosis caused by *Halioticida noduliformans*

Current diagnosis of abalone tubercle mycosis, caused by *Halioticida noduliformans*, is based on the observation of gross clinical lesions and histopathology. These methods are not specific or sensitive enough to accurately and reliably identify the *H. noduliformans* and they are only accurate when animals are presenting clinical signs. The accuracy of diagnoses of animals with subclinical infections is still questionable and may therefore increase the risk of possibly spreading the fungus due to misidentification. Confirmation of the presence of *H. noduliformans* in tissue samples currently depends on live culture of the fungus and discrimination is based on characterisation of the mode of processes involved in zoosporogenesis. However, obtaining pure cultures of the fungus and inducing zoosporogenesis can be difficult and time consuming. Macey *et al.* (2011) therefore strongly suggested that further research into the development of molecular genetic markers is essential for more accurate and rapid diagnostics of ATM.

1.10 Validation of Diagnostic tools

The sensitivity and specificity of a diagnostic tool are significant factors that require careful consideration in order to ensure accurate and reliable results. "Diagnostic

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sensitivity" is the percentage of samples that are identified by the assay as positive for a specific disease (Altman and Bland, 1994; Saah and Hoover, 1997), and "analytical sensitivity" represents the ability of an assay to accurately measure the smallest amounts of substance present in a sample (Saah and Hoover, 1997; Fegan, 1999). On the other hand, the specificity of a diagnostic tool refers to the ability of an assay to measure one particular organism, rather than others, in the sample (Saah and Hoover, 1997). Therefore, it is of great importance to validate diagnostic techniques for both specificity and sensitivity in order to avoid the incidence of false positive and negative results.

Validation is the evaluation of a diagnostic assay for the purpose of determining how suitable the assay is for a particular purpose (Hiney and Smith, 1998; OIE, 2006). PCR-based techniques can become complex, because of different experimental levels. Therefore, when validating these PCR-based techniques, there are several factors that need to be considered (Hiney and Smith, 1998; OIE, 2006; Bott *et al.*, 2010). These factors include (1) an effective and repeatable extraction method of genomic DNA from environmental samples; (2) the identification of an appropriate DNA marker that is specific to the DNA of the target organisms; and (3) avoiding PCR inhibition and false positives caused by contaminants present in samples taken directly from the environment and/or non optimal molecular methods and procedures (Bott *et al.*, 2010). Furthermore, each one of these factors can be broken up into various levels of complexity (Hiney and Smith, 1998). The first level includes *in vitro* studies, where reactions are performed *in vitro* tubes so that the basic properties of the technique can

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be established. The second level is referred to as a seeded study in which reactions are performed in an environment or matrix that is designed to be a sterile simulation of the environmental conditions. The third level is referred to as an experimentally incurred matrix where laboratory grown cells of the target organism are introduced into the environment and allowed to establish themselves, for example, an experimental infection of a host organism. The fourth and final level of validation is the evaluation of the performance of the technique in a naturally infected environment (Hiney and Smith, 1998).

One of the most important aspects for the development of a reliable PCR based method is the identification of a suitable DNA target region(s). For identification of a specific target or pathogen, the selected DNA marker should reveal minimal genetic variation within a species but differ sufficiently between species to allow for unmistakable identification (Bott *et al.*, 2010). Nuclear ribosomal DNA (rDNA) genes in a eukaryotic cell are repetitive, present in multiple copies and contain highly conserved sequences which have been successfully used as primer templates for species-specific gene amplification (Li *et al.*, 1996; Zhou *et al.*, 2000; Bott *et al.*, 2010). Mitochondria are highly abundant in cells and evolve at a quicker rate than nuclear genes and have consequently also proven to be particularly useful as species-specific markers for discriminating between closely related species. Ribosomal DNA and mitochondrial genes have been widely utilised for the development of specific, sensitive and rapid PCR-based diagnostic techniques (Zhou *et al.*, 2000; Vandersea *et*

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al., 2006). To manage abalone tubercle mycosis in South Africa effectively it will be necessary to develop techniques that will provide accurate and rapid identification of *H. noduliformans*.

1.11 Study Objectives

The primary aim of this study was to develop a practical, specific, sensitive and rapid molecular diagnostic tool to detect and identify *Halioticida noduliformans* in infected abalone tissues. In order to achieve this, the following two key objectives were investigated;

1. Establishing and optimising a method for the isolation of *H. noduliformans* genomic DNA from laboratory grown cultures, to yield high quality DNA with as little PCR inhibitors as possible.

2. To develop and provide preliminary validation results of a nested real-time PCR assay based on two of the criteria proposed by Hiney and Smith (1991) and Bott *et al.* (2010) for the validation of molecular diagnostic tools. These two criteria will be test tube (*In vitro*) and sterile seeded studies.

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CHAPTER 2

Optimisation of a DNA extraction method for the recovery of high quality *Halioticida noduliformans* DNA suitable for PCR

2.1 Introduction

The use of DNA based diagnostic methods is often constrained by the relatively small amount of target DNA in environmental samples and by the co-purification of contaminants from clinical samples that could inhibit PCR (Bott *et al.*, 2010). This technical limitation is further intensified by the inefficient extraction of target DNA required for further amplification by Polymerase Chain Reaction (PCR) (Hopfer and Amjadi, 2002). PCR is a highly sensitive tool that has the ability to amplify small amounts of DNA, even in the presence of contaminants. However, this tool cannot reach its full potential without an optimised DNA extraction method. The extraction of fungal DNA is a critical step in the process of detecting and identifying fungal pathogens by PCR. Therefore, the extraction and recovery of DNA should be carefully optimised (Cunningham, 2002) to yield large amounts of high quality DNA suitable for PCR (Karakousis *et al.*, 2006). The extracted DNA template should contain as little PCR inhibitors as possible. This component of the DNA extraction step becomes even more important when attempting to detect small quantities of

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infectious pathogen DNA material in biological samples, such as abalone tissue, where more contaminants and PCR inhibitors are expected to be present.

The initial step of DNA extraction is to break the cell walls. This step is particularly challenging when dealing with fungal cells, as fungal cell walls consist of thick layers of chitin, lipids and peptides (Karakousis *et al.*, 2006). Frequently a tough surface layer of melanin may also be present which is highly resistant to UV light, enzymatic digestion and chemical breakdown (Karakousis *et al.*, 2006). This often complicates the initial steps of DNA extractions. One of the distinguishing features of *Halioticida noduliformans* is that it has a cell wall composed of cellulose and not chitin. Cell walls need to be physically disrupted in order to allow for efficient lysis of cell membranes and organelles prior to DNA extraction. Because of the structural complexity of fungal cell walls, this step could be regarded as the most important step of the DNA extraction process. As stated previously, this step becomes more important when aiming to detect a target DNA sequence of the pathogen within tissue of animals with sub-clinical infections.

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A variety of cell wall disruption methods have been reported for different fungal DNA isolation methods (Maaroufi *et al.*, 2004; Karakousis *et al.*, 2006), all with the common goal of lysing conidia and hyphae by disrupting the cell walls. Some of these methods include, freezing in liquid nitrogen and grinding with pestle & mortar (Manian *et al.*, 2001), sonication (Karakousis *et al.*, 2006) and glass bead milling (Haugland *et al.*, 2002; Griffiths *et al.*, 2006). The use of digestive enzymes is

another successful method (Griffiths *et al.*, 2006). Incubation with digestive enzymes helps break down components of the fungal cell wall and facilitates the release of fungal DNA. With all of these available methods, there is no single method of cell lyses suitable for all fungi (Fredricks *et al.*, 2005) and each species requires a specific method to be optimised in order to efficiently extract DNA (Manian *et al.*, 2001).

Following development of a suitable cell wall disruption method, extraction and purification of DNA needs to be optimised in order to obtain the highest quantity and quality of DNA possible from a single sample. A well known DNA extraction method is the standard phenol-chloroform method. Proteins dissolve in the lower phenol phase and DNA remains in the upper aqueous phase. The DNA is then precipitated using ethanol and resuspended in an appropriate buffer. Another standard method used previously with great success is the isopropanol method (Edwards *et al.*, 1991; Karakousis *et al.*, 2006). This is a quick extraction method where samples are incubated in the presence of sodium acetate after cell wall disruption. Ice cold isopropanol is then used to precipitate the DNA. Using this method, DNA can be extracted in a short period of time and phenol or chloroform is not required. Both these two methods are very effective, but include either phase extractions or several tube transfer steps which could possibly lead to the loss of DNA. This could be particularly problematic in samples where there is only a small quantity of starting target DNA and for quantitative methods, such as real-time quantitative PCR. Conversely, heat-lysis extraction methods, including the method reported by Macey *et al.* (2008a), have been developed to allow for direct

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quantification of DNA in tissue samples. This extraction method does not require any toxic chemicals, such as phenol and chloroform, or phase extractions, which could lead to the possible loss of DNA. Cells are simply lysed in the presence of negatively charged chelax beads, through boiling, and the supernatant containing the DNA can be used directly for PCR. Chelax beads chelate metal ions which are required as catalysts or co-factors in enzymatic reactions, which helps to prevent DNA degradation. This method has been used previously for the extraction of total genomic DNA from tissue of numerous invertebrates, including oysters, crabs and shrimps (Macey *et al.*, 2008a; 2008b; Williams *et al.*, 2009).

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In order to effectively determine the efficacy of various physical disruption, DNA extraction and DNA recovery methods, both the quantity and integrity of DNA needs to be determined. Spectrophotometric quantification of DNA, determined by measuring UV light absorbance at a wavelength of 260 nm, is routinely used for this purpose, where one optical density unit at 260 nm is equivalent to 50 µg/ mL for double stranded DNA (Coyne *et al.*, 1996). However, DNA is not the only compound that absorbs UV light at a wavelength of 260 nm. Together with RNA, certain contaminants, particularly those co-purified from animal tissue, as well as compounds used during the extraction process, may absorb UV light at this wavelength. Therefore, it is suggested that alternative methods, such as agarose gel electrophoresis, are used in conjunction with spectrophotometric quantitation to determine the purity and integrity of isolated DNA.

In this study, different physical, chemical and enzymatic disruption methods were tested and compared to identify the most effective method to extract and recover high quality *H. noduliformans* DNA. Light microscopy was used to determine the most efficient method for physically disrupting cell walls, whereas spectrophotometric quantification and gel electrophoresis were used to determine DNA yield and integrity, respectively, following various enzymatic treatments and DNA extraction and/ or recovery methods. The optimal method was chosen based on the practical, safe, efficient and repeatable extraction of large quantities of high quality fungal DNA from samples.

2.2 Materials and methods

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2.2.1 Microorganisms and culture media

Aseptic techniques were used to transfer small pieces of infected tissue from the foot and epipodium of abalone (< 2 mm^3) onto Petri-dishes containing marine phycomycetes isolation agar (12.0 g Agar, 1.0 g Glucose, 1.0 g Gelatin hydrolysate, 0.01 g Liver extract, 0.1 g Yeast extract, 1 000 mL Sea water) supplemented with streptomycin sulphate and penicillin [0.05% (w/ v)] to hinder bacterial growth (Fuller *et al*., 1964). Care was taken to embed the tissue sections within the agar on plates. Inoculated plates were incubated at 20 °C and monitored daily for growth. After 7 – 10 days of incubation, cultures were aseptically sub-cultured by transferring small agar plugs (2 mm in diameter) with active growing mycelia onto fresh agar. This

procedure was repeated, where needed, until cultures were free of other fungal contaminants. Once cultures were no longer contaminated, pure *H. noduliformans* hyphae were transferred to peptone-yeast-glucose-saline (PYGS) agar (1.25 g Bacto-peptone, 1.25 g Bacto yeast extract, 3.0 g Glucose, 12.0 g Bacto agar, 1,000 mL Sea water) without any antibiotics. Cultures were maintained on PYGS agar at 20 °C and routinely sub-cultured once every three weeks.

2.2.2 Sample preparation

Large quantities of mycelia were needed in order to optimise DNA extraction methods. This was obtained as follows: agar plugs (2 mm in diameter) of actively growing mycelia from 7 day old culture plates were aseptically transferred into 5 mL of sterile PYGS broth and incubated at 20 °C. After an incubation period of 10 – 14 days, actively growing colonies were rinsed three times in autoclaved natural seawater (30 ppt salinity) and aseptically transferred to sterile 100 mm Petri-dishes. Hyphal tips (10 - 20 mg, wet weight) were removed with a scalpel blade and transferred to pre-weighed micro-centrifuge tubes. Samples were kept on ice until needed.

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2.2.3 Optimisation of DNA extraction conditions from laboratory grown cultures of Halioticida noduliformans

In order to optimise DNA extraction conditions; three different physical cell wall disruption methods were compared. Thereafter, the best cell wall disruption method was used to determine the effects of two different digestive enzyme treatments for their ability to further disrupt the fungal cell walls and release DNA. These treatments were compared to a control group with no digestive enzyme added. Finally, based on the best disruption method (see *2.2.3.1*), three different DNA extraction and recovery methods, namely phenol-chloroform, isopropanol and heat-lysis, were tested and compared for the recovery of genomic DNA.

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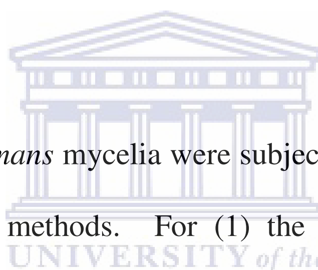
All data were expressed as µg DNA per milligram mycelium starting material. To examine the DNA yield in each extract, the isolated DNA was quantified using a Libra S12 spectrophotometer (Biochrom Ltd), at 260 nm and 280 nm. The DNA integrity was tested by agarose gel (0.8%) electrophoresis and visualised by ethidium bromide staining. Samples were treated with 0.5 µL RNase (Sigma, Cat. # R4642) for 30 minutes at 37 °C prior to spectrophotometric quantification and gel electrophoresis. A total of 6 - 10 samples were tested for each extraction method and the data recorded as the mean (± standard error [SE]) DNA concentration per mass of starting material for each treatment. Specific procedures are described below.

2.2.3.1 Fungal cell wall disruption methods

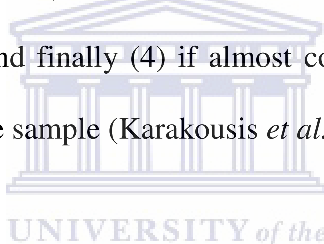
Since cell wall disruption constitutes the first step in extracting good quality DNA, three physical disruption methods were tested and compared to determine the breakage efficiencies of each on mycelium cell walls. These methods included: (1) fine glass bead homogenisation, (2) grinding samples with a conical grinder, and (3) liquid nitrogen freezing prior to grinding of samples with a conical grinder. Mycelia were prepared as described previously, and a total of six samples were tested for each disruption method. To prevent any degradation of genetic material, all procedures were performed on ice.



Pre-weighed *H. noduliformans* mycelia were subjected to the three different cell wall disruption and cell lysis methods. For (1) the fine glass bead homogenisation method, 300 µL extraction buffer and 0.3 g sterile glass beads (Sigma, Cat. # G8772) were added to each sample and vortexed for 10 minutes. For (2) the conical grinder method (without liquid nitrogen), 300 µL extraction buffer was added to each microcentrifuge tube prior to grinding the sample at 250 rpm for one minute using a Caframo BDC1850 stirrer equipped with a pellet pestle (www.caframo.com). Finally, for (3) the conical grinder method with liquid nitrogen, samples were first submerged in liquid nitrogen to flash-freeze mycelia before grinding samples with the conical grinder at 250 rpm for one minute. Care was taken to keep the latter samples frozen throughout this process. Immediately after the grinding step, extraction buffer (300 µL) was added to each sample. The same extraction buffer (17.34 mM sodium

dodecyl sulphate (SDS), 50 mM Tris, 0.4 M EDTA; pH = 8.0) was used for all samples directly after disruption.

The level of fungal cell wall disruption and cell lysis was initially assessed by observation of cell breakage. Samples were categorised according to Karakousis *et al.* (2006) under 400 x magnification using an Olympus BX51 light microscope and compared to untreated cultures as a control. The amount of cell breakage and lysis observed in samples, were expressed as percentages falling within the following categories: (1) for samples with no to minimal (0 - 25%) cell lysis; (2) if some breakage of mycelia (25 - 50%) was observed in the sample; (3) if most of the hyphae (50 - 75%) were lysed; and finally (4) if almost complete cell wall breakage (75 - 100%) was observed in the sample (Karakousis *et al.*, 2006).



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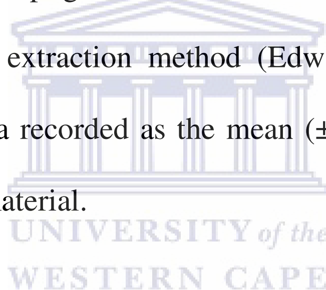
For a more quantitative comparison between the disruption methods, genomic DNA was extracted from all the samples, immediately after observing the samples under the light microscope, using a standard isopropanol extraction method (Edwards *et al.*, 1991) described in 2.2.3.3 below. The DNA yields from each sample were quantified separately and the data recorded as the mean (±SE, n = 6) DNA concentration per mass of starting material for each mechanical disruption method.

2.2.3.2 Enzymatic digestion methods

Mycelia samples were prepared as described above and grounded in 300 µL extraction buffer (17.34 mM sodium dodecyl sulfate [SDS], 50 mM Tris, 0.4 M EDTA; pH 8.0) with a conical grinder at 250 rpm for 1 minute. Immediately thereafter, 400 U lyticase (Sigma, cat. # L4025) or 10 µg proteinase K (Sigma, Cat. # P4850) was added before incubating samples for one hour at 30 °C and 55 °C, respectively. The two enzymatic treatment groups were compared to a control group which had no enzyme treatment step. All three groups consisted of 10 samples each. Following the incubation step, genomic DNA was extracted from each sample using the standard isopropanol extraction method (Edwards *et al.*, 1991), described in 2.2.3.3 below and the data recorded as the mean (±SE, n = 10) DNA concentration per unit mass of starting material.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2.2.3.3 Fungal DNA extraction and recovery methods

Following each physical disruption step, DNA extraction and recovery was optimised on the crude extracts by testing three different methods, namely (1) phenol-chloroform, (2) isopropanol and (3) heat-lysis. Specific procedures are described below.

Phenol-Chloroform method

Phenol:chloroform (50:50) (500 µL) was added to each crude extract (n = 6), finger vortexed and centrifuged at 5,000 × g for 10 minutes. Supernatants were removed with a wide-bore 1 mL tip and mixed with 500 µL chloroform and finger vortexed. Supernatants, containing the DNA, were carefully transferred into new tubes before adding 0.1 volumes of 3 M sodium acetate ($NaC_2H_3O_2$, pH = 5.2) and 1.5 volumes of ice-cold absolute ethanol. Tubes containing samples were inverted several times and incubated at -20 °C overnight. Samples were centrifuged at 13,000 × g for 10 minutes. Supernatants were carefully discarded and the pellets containing DNA were washed with 70% ethanol. Samples were centrifuged at 13,000 × g for 5 minutes before carefully decanting the supernatants and air-drying the DNA pellets for a few minutes. DNA was resuspended in 50 µL Tris-EDTA (pH = 8.0) buffer and stored at -20 °C until needed.

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Isopropanol method

The isopropanol extraction method reported by Edwards *et al.* (1991) was used, with some minor modifications. One hundred and fifty micro-litres of 3 M $NaC_2H_3O_2$ (pH = 5.2) was added to each crude extract (n = 6) and incubated at -20 °C for 15 minutes. Samples were centrifuged at 13,000 × g for 10 minutes at 18 °C. The supernatants were carefully transferred to new sterile 1.5 mL micro-centrifuge tubes and equal volumes of isopropanol were added. Samples were gently mixed by inversion and

incubated for 5 minutes at room temperature before centrifuging at 13,000 x g for 10 minutes at 18 °C. Pellets containing DNA were washed with 70% ethanol and centrifuged at 13,000 × g for 5 minutes. The supernatants were carefully decanted and pellets air-dried for a few minutes before resuspending the DNA in 50 µL Tris-EDTA buffer and stored at -20 °C**.**

Heat-lysis method

To minimise labour intensive extraction steps, eliminate the use of toxic chemicals (e.g. phenol and chloroform) and facilitate the development of a fast and easy DNA extraction method, the simple heat-lysis extraction method reported by (Macey *et al.*, 2008a) was tested with some minor modifications. Crude extracts from mycelia (as prepared above) were centrifuged at 17,000 × g for 10 minutes at 4 °C. The resulting supernatants were removed and the pellets resuspended in an equal volume (300 µL) of sterile distilled water. Samples were homogenised for a further 1 minute using a handheld motorized pellet pestle (Sigma Aldrich, Cat.# Z359971) before transfer to new sterile 1.5 mL microcentrifuge tubes containing ± 0.04 g Chelax beads (Sodium form, Cat.# 7901). The samples containing Chelax beads were briefly vortexed and incubated for 20 minutes at 56 °C in a Stuart S1500 orbital incubator set to rotate at 80 rpm. This incubation step helps soften plasma membranes, release clumps of cells from one another, and denature enzymes such as DNases. Following incubation, the samples were vortexed briefly before incubating the samples for a further 30 minutes at 95 °C to lyse all of the cells. The lysed samples were allowed to cool rapidly on

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ice for 5 minutes, vortexed briefly and centrifuged at 17,000 × g for 10 minutes at 4 °C. The resulting supernatants (~250 µL), containing genomic DNA were transferred to new 1.5 mL microcentrifuge tubes and stored at -20 °C until needed.

2.2.4 Statistical analyses

SigmaStat 3.1 software was used to perform all statistical analyses. To determine if there was significant difference in DNA yield between the three different physical disruption methods tested, namely glass bead homogenisation, grinding with a conical grinder and liquid nitrogen freezing prior to grinding with a conical grinder, a one-way analysis of variance (ANOVA) was performed. Since all tests for equal variance failed for these data sets, a Kruskal-Wallis one-way ANOVA on ranks was performed to test for significant differences between treatment groups. A Tukey test was used for all *post hoc* multiple comparisons between treatments. To test for significant differences in the yield of DNA between the DNA extraction and recovery methods tested (phenol-chloroform-, isopropanol-, and heat-lysis method) and enzyme treatments (proteinase K, lyticase, no enzyme), a one-way ANOVA was performed. These data sets were parametric and a Holm-Sidak method was used for all *post hoc* multiple comparisons between extraction methods. Significance was assigned to p-values <0.05 for t-test, one-way ANOVA, Tukey and Holm-Sidak analysis.

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2.3 Results

2.3.1 Fungal cell wall disruption

Homogenisation using fine glass beads was shown to be the least effective (Fig. 2.1A) at physically disrupting cell walls, as all the samples fell within the first category, representing no or minimal lysis (0 – 25%) of cells (Fig. 2.2A). The mycelia that were ground with a conical grinder immediately after being flash-frozen in liquid nitrogen showed a higher degree of cell lysis, 50% of these cells was categorised into the second category (25 – 50% lysis) and a few of these cells showed almost complete lysis (75 – 100%). On the contrary, cell walls that were physically disrupted by using the conical grinder method, without liquid nitrogen pre-treatment, was shown to be the most effective (Fig. 2.1B) method for physically disrupting *H. noduliformans* cell walls. Following this treatment, most samples fell within the forth category, representing 75 – 100% cell wall disruption and cell lysis (Fig. 2.2A).

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Overall, (for all physical disruption methods) DNA yields ranged from 0.08 – 2.7 µg DNA per milligram of starting hyphae mass (wet weight). Using the conical grinder without the liquid nitrogen pre-treatment showed the highest DNA yield per starting hyphal mass compared to any of the other physical disruption methods tested in this study (Fig. 2.2B). The DNA yield obtained using the latter method was significantly higher (Kruskal-Wallis one-way ANOVA on ranks; $p < 0.05$), when compared to the glass bead method. There was however no significant difference ($p > 0.05$) in the

yield of DNA between samples physically disrupted using the conical grinder method with or without the liquid nitrogen pre-treatment. Based on these results it was decided that the conical grinder method without pre-treatment with liquid nitrogen is the most practical and efficient physical disruption method for all subsequent optimisations.

2.3.2 Enzymatic digestion

Two different enzyme treatments were compared to a control group (no enzyme added) to see if this additional step could improve the yield of DNA following physical disruption with the conical grinder method. DNA yield varied significantly between the treatment groups (Fig. 2.3, one-way ANOVA P = 0.023). Samples treated with proteinase K yielded significantly higher quantities of total DNA than the lyticase treatment, with 4.85 and 3.39 µg DNA/mg hyphae obtained from each of these treatment gropups, respectively (Fig. 2.3, one-way ANOVA p = 0.009). However, these two enzyme treatment groups did not vary significantly from the control group. Consequently, the additional step of enzyme treatment was omitted.

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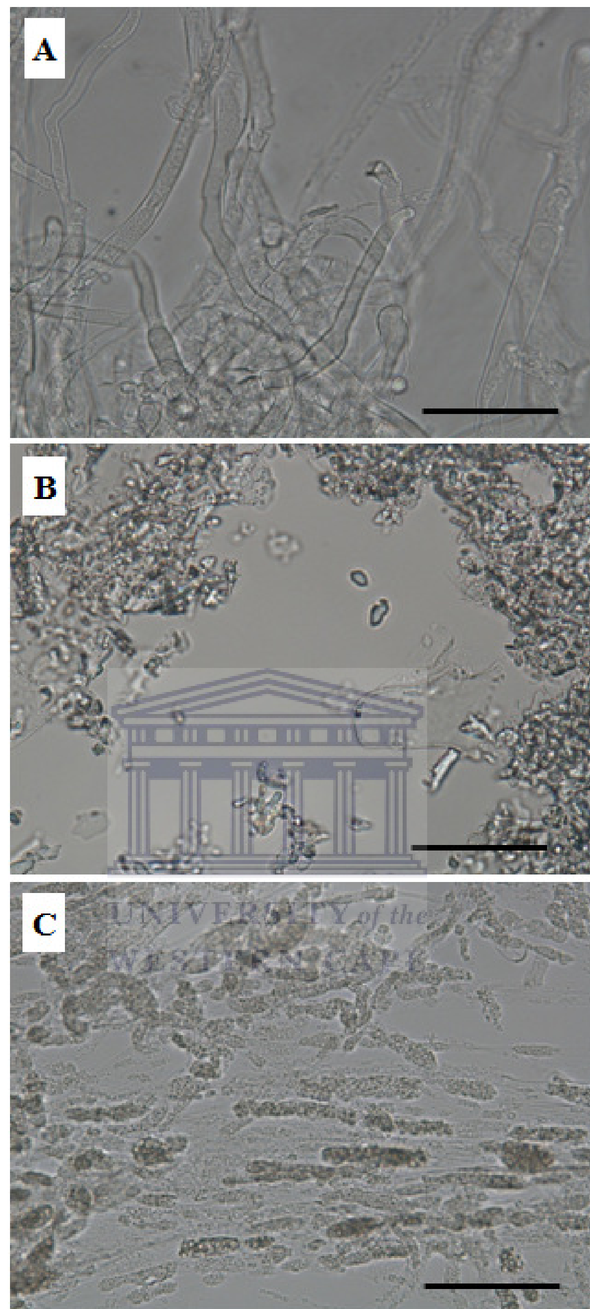

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Figure 2.1: Light micrographs (400 x magnification) of *Halioticida noduliformans* cells comparing the least effective physically disruption method, namely (A) disruption with fine glass beads (category 1; 0 – 25%), with (B) the most effective disruption method which used a conical grinder without liquid nitrogen pre-treatment (category 4; 75 – 100%). (C) Control sample showing the morphological characteristics of *H. noduliformans* hyphae, which are aseptate, highly branched and fragmented by protoplasmic constrictions. The scale bar represents 50 µm.

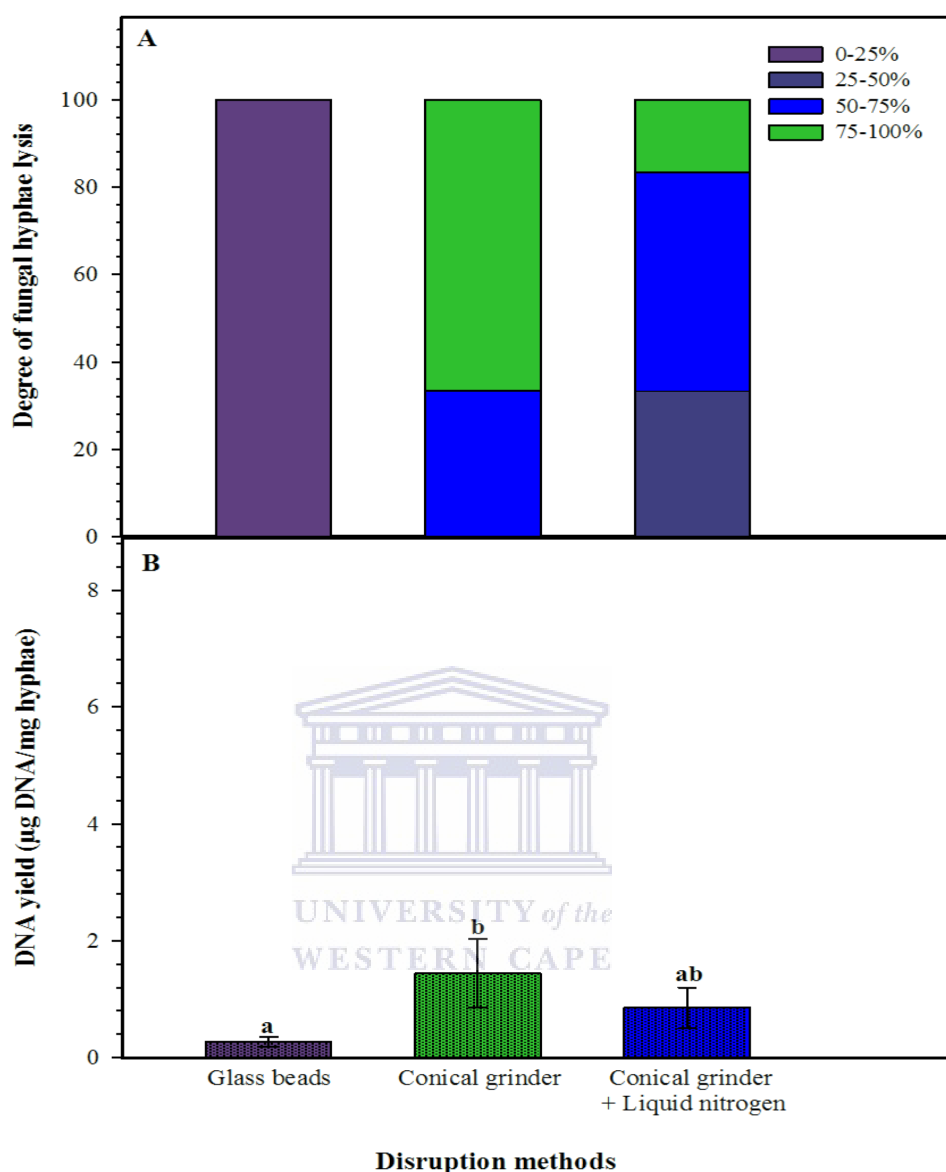

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Figure 2.2: Assessment of three different cell wall disruption methods. (A) The degree of cell wall lysis was categorised according to Karakousis *et al.* (2006) by observing mycelia from each sample under a light microscope and comparing to untreated cultures as controls. Categories: 1 - samples with no to minimal (0 - 25%) cell lysis; 2 - some breakage of mycelia (25 - 50%) was observed in the sample; 3 - most of the hyphae (50 - 75%) were lysed; and finally 4 - almost complete cell wall breakage (75 - 100%) was observed in the sample (B) The yield of DNA (mean ± SE, n = 6) was extracted from 1 mg (wet weight) of pure *Halioticida noduliformans* hyphae following each of the physical disrupted methods. Mean values (columns) with different letters were significantly different (Kruskal-Wallis one-way ANOVA on ranks; $p < 0.05$).

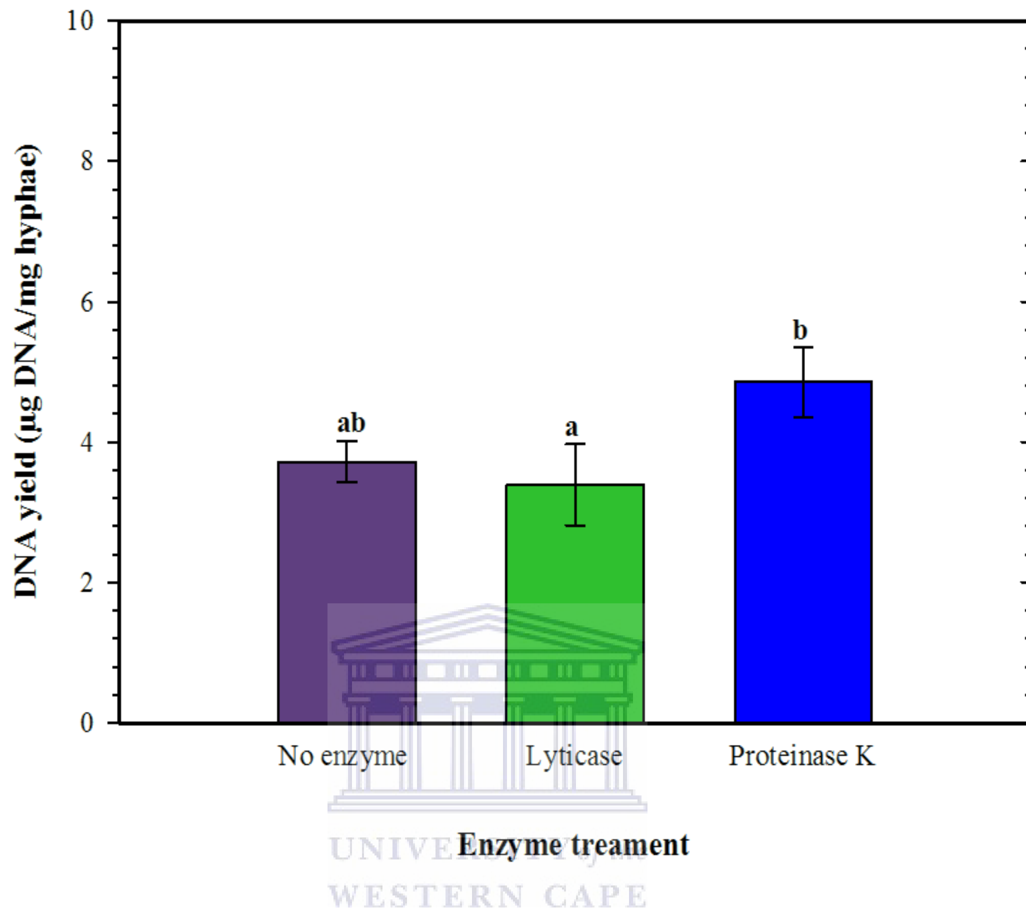

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Figure 2.3: Yield of DNA extracted and recovered from pure *Halioticida noduliformans* cultures physically disrupted with a conical grinder and then digested with either lyticase or proteinase K, and compared with a control group (no enzyme added). Values represent the mean of 6 samples (±SE). Yields were expressed in micrograms of DNA per milligram starting hyphae mass (wet weight). Mean values (columns) with different letters were significantly different (one-way ANOVA on ranks; $p < 0.05$).

2.3.3 Fungal DNA extraction and recovery

Following physical disruption of cell walls using the conical grinder method, without liquid nitrogen pre-treatment, and without addition of digestive enzymes, two organic extraction methods were tested and compared to one another and a simple heat-lysis method for the recovery of total genomic DNA. DNA yield did not vary significantly between the standard phenol-chloroform and the isopropanol extraction methods (Fig. 2.4, one-way ANOVA $p = 0.963$). However, significantly higher amounts of DNA were extracted and recovered from cells when using the heat-lysis extraction method, compared with the phenol-chloroform and isopropanol methods (Fig. 2.4, one-way ANOVA $p = 0.0049$ and 0.0055, respectively). The average yield obtained using the heat-lysis method was 7.2 µg DNA/mg hyphae, compared to an average of 3.1 µg DNA/mg hyphae for samples subjected to the other two recovery methods. Appart from the higher yields of DNA obtained using the heat-lysis method, a clear difference could also be seen in the integrity of DNA following 0.8% agarose gel electrophoresis (Fig. 2.5), keeping in mind that the DNA from using the isopropanol and phenol-chloroform methods were resuspended in a final volume of 50 µL whereas DNA extracted using the heat-lysis method is suspended in a final volume of 250 µL. Based on these results, the heat-lysis extraction method was used throughout the rest of this study.

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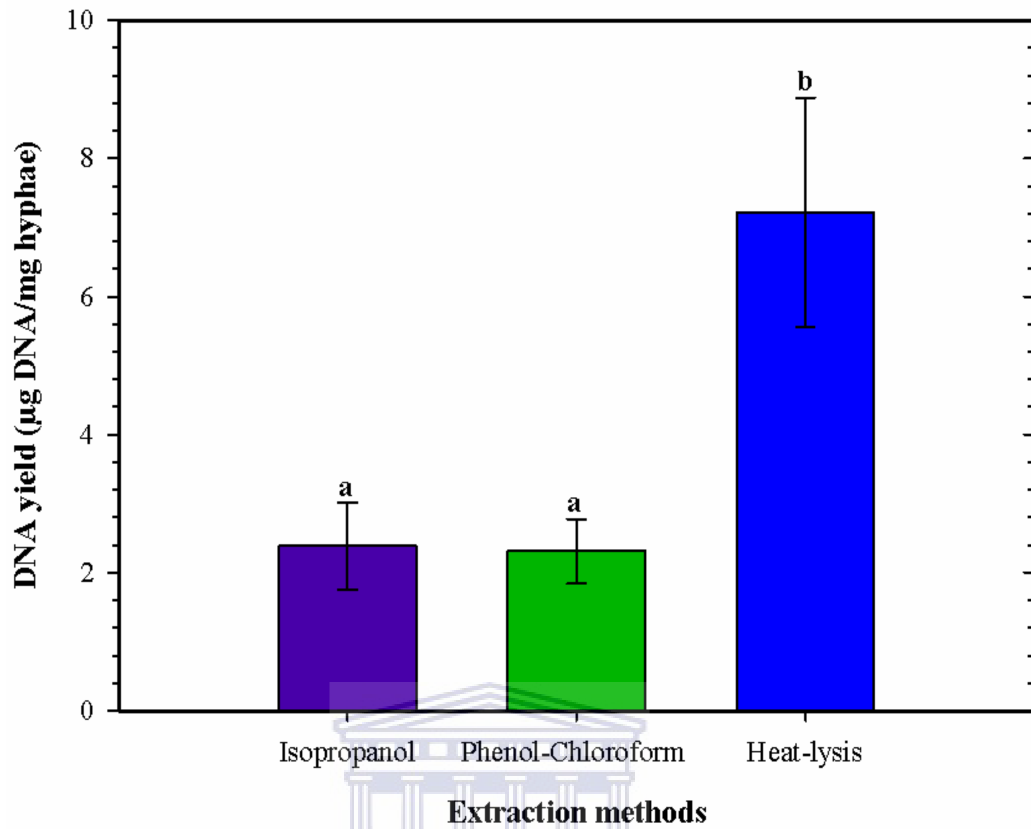

Figure 2.4: Yield of DNA extracted and recovered from pure *Halioticida noduliformans* cultures using the isopropanol, phenol-chloroform and heat-lysis methods. Values represent the mean (±SE) of six samples for each extraction method. DNA yields were expressed in micrograms of DNA per milligram starting hyphae mass (wet weight). Mean values (columns) with different letters were significantly different (one-way ANOVA on ranks; $p < 0.05$).

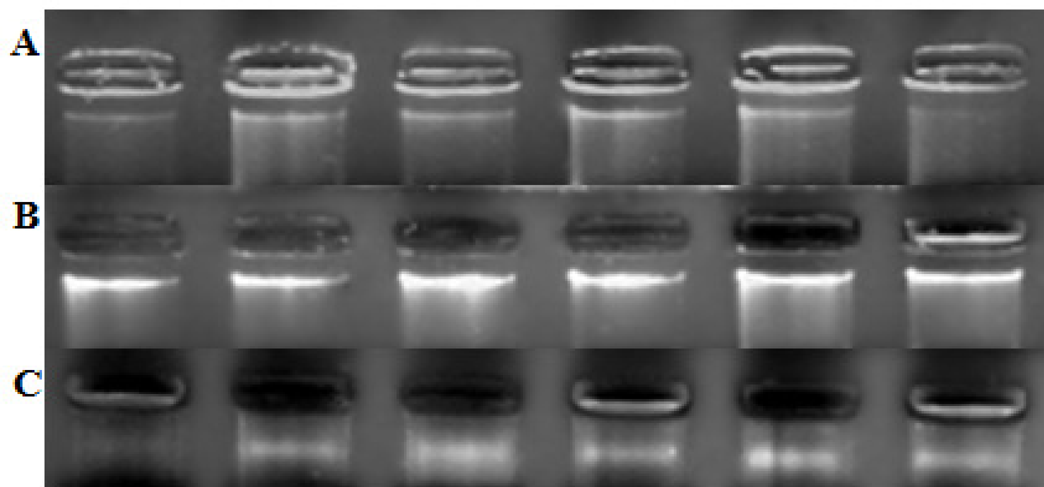

Figure 2.5: Agarose gel electrophoresis (0.8%) of *Halioticida noduliformans* genomic DNA (n = 6) extracted from pure cultures using three different extraction methods. Row (A), Phenol-chloroform extraction method; (B) isopropanol extraction method; and (C) heat-lysis method. (Although 2 µL of all samples were run in each of these lanes, the dilution factor for each of the methods differed, as the resuspended volumes of the phenol-chloroform and isopropanol methods were 50 µL, whereas the heat-lysis method was 250 µL (see section 2.2.3.3)

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2.4 Discussion

Since current diagnostic methods to diagnose abalone tubercle mycosis are not molecular based and there are no published methods for the extraction of total genomic DNA from *Halioticida noduliformans*, a DNA extraction method suitable for sensitive and precise quantification of specific DNA fragments by the PCR technique needed to be optimised. The recovery of large amounts of high quality genomic DNA needs to be carefully optimised in order to ensure sensitive and precise quantification of specific DNA fragments by the polymerase chain reaction (PCR) technique. In this study, a simple heat-lysis method, previously reported by Macey *et al.* (2008a), was tested and optimised to extract high quality genomic DNA from pure *H. noduliformans* cultures. This method included the use of a conical grinder to break up the cell walls prior to the recovery of DNA by heat-lysis extraction. When comparing this method to two routine DNA extraction and recovery methods, namely isopropanol and phenol-chloroform, we demonstrated that the simple heat-lysis method yielded the highest amount of high quality DNA per mg starting hyphae material.

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Fungal cells have tough cell walls and ordinary cell lyses treatments, such as chemical digestion and speed freezing, often do not effectively disrupt the cell walls to release DNA (Zhou *et al.*, 2000; Maaroufi *et al.*, 2004; Karakousis *et al.*, 2006). *Halioticida noduliformans* cells are no different and share this characteristic with fungi. Therefore, the first objective of this study was to find the most effective method of physically disrupting *H. noduliformans* cell walls in

order to release the cellular contents. In a previous study, Karakousis *et al.* (2006) demonstrated using microscopic examination that the most efficient physical disruption methods for fungal cells are sonication, pestle grinding and liquid nitrogen freezing followed by grinding. In two other studies glass beads and zirconia beads have both been shown to be highly efficient methods for physically disrupting fungal cell walls (Zhou *et al*., 2000; Yamamoto *et al.*, 2010). This was not the case in the present study. In this study, homogenisation with glass beads was shown to be the least efficient cell wall disruption method. This result is not surprising as previous studies have shown that the success of each method depends on the fungal species tested (Karakousis *et al.* 2006). This may be explained by the many different ways cell walls are constructed in different species and therefore highlights the fact that DNA extraction methods need to be optimised for specific species. For *H. noduliformans*, we demonstrated that the most efficient cell wall disruption was obtained following physically disruption of cells with a conical grinder, without pre-treatment with liquid nitrogen, supporting Karakousis *et al.* (2006). Almost complete lysis was observed in more than 66% of samples physically disrupted using the conical grinder method (Fig.2.2). Karakousis *et al.* (2006) explained that the increase in deposition of melanin in cell walls of some fungi could be the reason for his results. However this could not be the same reason for *H. noduliformans* as cell walls don't contain melanin, but do consist of fibrillar carbohydrates and cellulose (Dick, 1969), also causing cell walls to be very tough and hard to break. Furthermore the hyphae itself is very sticky when grown in PYGS broth (Muraosa *et al.*, 2009). These aspects combined could be the reason why the conical grinder worked best for this

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specific study. A conical grinder is a convenient, rapid, easy and very effective device for disrupting the cell walls of *H. noduliformans*, and was therefore adopted as the method of choice for all subsequent DNA extraction and recovery experiments.

In order to enhance the recovery of DNA and at the same time reduce the presence of PCR inhibitors, which are often co-purified from environmental and animal tissue samples, various DNA extraction and recovery methods were tested in the present study. Polymerase chain reaction (PCR) assays can be inhibited by the co-purification of contaminants from the environment and animal tissue samples, causing false positives, therefore results require careful consideration for the design of all PCR based procedures (Fredricks *et al.*, 2005; Bott *et al.*, 2010). Some of these inhibitors include organic and phenolic compounds and other salts that are in high abundance in marine samples; constituents of microbial and fungal cells; an over-abundance of non-target DNA; as well as other contaminants (Bott *et al.*, 2010; Yamamoto *et al.*, 2010). DNA extraction and recovery methods need to be carefully assessed and optimised to reduce the presence of any of these PCR inhibitors and also to enhance the recovery of DNA. Ma and Michailides (2007) reported that boiling mycelia and tissue samples in order to obtain template DNA is a sensitive and reproducible method for the detection of pathogens. However, the phenol-chloroform and isopropanol methods have been widely and successfully used in the past for the extraction and recovery of high quality genomic DNA from a number of different sample types (Zhou *et al.*, 2000; Oidtmann *et al.*, 2004).

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The standard phenol-chloroform method does however involve a phase extraction and subsequent tube transfer steps, potentially resulting in DNA loss and consequent reductions in DNA yield. Phenolic compounds can also be carried over into the PCR mixture and are known to cause PCR inhibition (Wilson, 1997; Cunningham, 2002). Furthermore, this method includes the use of harmful chemicals, such as phenol and chloroform, and this method is labour intensive. Although this method has been widely used with great success for extracting DNA (Zhou *et al.*, 2000; Oidtmann *et al.*, 2004), results presented here suggest that this method may not the best method for extracting *H. noduliformans* DNA. Similarly, the isopropanol extraction method has been used extensively for extracting total genomic DNA from fungal species (Cenis, 1992), as well as from other pathogens (Magalhães *et al.*, 2008), and has been shown to yield large amounts of DNA. However, as previously stated for the phenol-chloroform extraction method, the isopropanol extraction method also requires several tube transfer and washing steps, which could influence the DNA yield. In the current study, we found no significant difference in the yield of DNA obtained from samples subjected to the latter two methods, but both of these methods yielded significantly lower amounts of DNA when compared with samples subjected to heat-lysis. The heat-lysis extraction method yielded approximately twice as much DNA than the other two methods tested in this study. This method yielded an average of 7.218 µg DNA/mg (wet weight) starting hyphae (Fig. 2.4). Similar methodologies have been used before with great success to quantify *Vibrio campbelii* cells in tissues of the Atlantic blue crab, *Callinectes sapidus* (Macey *et*

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al., 2008b), and Eastern Oyster *Crassostrea virginica* (Macey *et al*., 2008a), and to quantify the gene expression of lysozyme (Burge *et al*., 2007) in response to a pathogen challenge. Some advantages of the heat-lysis are that it is simple and does not require any digestive enzymes or harmful chemicals, supporting the notion of Cenis (1992) who stated that boiling extraction methods are advantageous for detection process. With no chemicals or phase extractions, this method can yield, high quality (Fig. 2.5) DNA in less than 2-3 hours.

In this study, we demonstrated that enzymatic treatment of samples physically disrupted using a conical grinder did not significantly enhance the recovery of DNA compared with samples not treated with enzyme. Karakousis *et al.* (2006) reported significant variance between different species when treated with proteinase K or lyticase. Results in the current study showed a significant difference in the yield of DNA between proteinase K and lyticase treatments, but the yield of DNA from both enzyme treatment groups was not significantly different from the control group which had no enzyme added. Based on these findings, we decided to omit the enzymatic treatment from our DNA extraction and recovery methods, which has indirectly made our method more practical and less time consuming.

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In conclusion, the best tested method for extracting total genomic DNA from *Halioticida noduliformans* pure cultures is to physically disrupt the cell walls with a conical grinder, followed by extraction and recovery of genomic DNA using a simple and safe heat-lysis method. From the data presented in this study, an

average of 7.218 µg DNA can be extracted from 1 mg of starting hyphae material. This method is reproducible and generates high quality DNA (Fig. 2.5) for molecular analysis using PCR, thus this method is used throughout the rest of this study.

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CHAPTER 3

The development and optimisation of a nested real-time polymerase chain reaction (Q-PCR) assay, for the detection of *Halioticida noduliformans* in infected abalone tissues

3.1 Introduction

Molecular based techniques are particularly useful for early detection of pathogens in the environment and may consequently help limit the spread of specific diseases, such as Abalone tubercle mycosis (ATM), which is an epizootic disease of farmed abalone caused by the invasive Peronosporomycete *Halioticida noduliformans.* Currently, the main methods for the diagnosis of ATM are the observation of gross clinical signs associated with the pathogen, the presence of fungal hyphae in histopathology and/or the isolation and identification of *H. noduliformans* directly from lesions using culture-based methods. These methods are however time consuming and are only accurate when abalone exhibit clinical signs of infection. Infected areas on abalone are well circumscribed (Macey *et al.*, 2011), but histopathology alone are not specific enough to specifically identify *H. noduliformans*. Molecular based diagnostic tools could therefore provide for a rapid, sensitive and specific diagnosis of ATM.

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The development of DNA probes and Polymerase Chain Reaction (PCR) primers as tools for detecting the presence of DNA from specific disease agents in aquatic animals has become increasingly popular within the last decade. PCR technology is ideal for specifically recognising and detecting DNA from pathogens early in the infection cycle. PCR-based assays can be used to target either DNA (the genome) or RNA (Bustin and Nolan, 2004). Molecular based diagnostic approaches are now routinely used alongside conventional microbiological and histopathological techniques for the identification of environmental and clinical pathogens (Millar *et al.*, 2000). Of the available molecular based diagnostic techniques, PCR has become a central technique in molecular biology and is currently at the forefront of molecular diagnostic technology (Ma and Michailides, 2007). This method can be highly specific and sensitive, with the capacity to amplify as little as a single copy of a specific DNA sequence even in the presence of other non-target DNA, such as in abalone tissue (Zhou *et al.*, 2000). However, PCR, as with all other methods for detection and quantification of pathogens, have potential shortfalls. PCR primers that are not specific enough to amplify the target sequence, PCR cycling conditions that have not been optimised and the presence of PCR inhibitors in DNA extracts are just some of the factors that could cause false positive or negative PCR results (Farrelly *et al.* 1995, Bott *et al.* 2010). To determine both the presence and level of infection in a sample it is however critically important to optimise and validate the specificity and determine the sensitivity of the respective assay.

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Cunningham (2002) stated that the presence or absence of a product in a PCR assay could be sufficient enough to indicate whether a sample is contaminated with a specific pathogen or not. Generally, the specificity and sensitivity of a PCR diagnostic tool can be improved by (1) manipulating conditions for the extraction of template DNA suitable for PCR, (2) identifying suitable DNA target region(s) for primer design, and (3) optimising PCR-cycling conditions (Cunningham, 2002; OIE, 2006; Bott *et al.*, 2010). When aiming to detect low infection levels of a specific pathogen in a sample, the efficacy of these steps are critical. Furthermore, when template DNA preparation is optimised correctly, possible loss of pathogen DNA from a tissue sample is reduced, thereby enhancing the sensitivity of the overall PCR technique (Cunningham, 2002). These steps have set the platform for the remainder of this study and chapter 3, which is to develop a *H. noduliformans* specific DNA primer set and to optimise the sensitivity of this assay.

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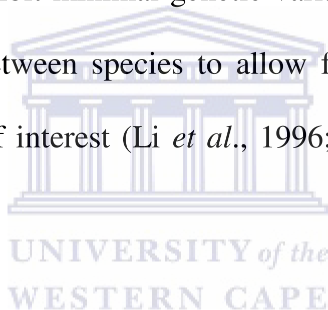
Co-purification of PCR inhibitors can also cause false negative PCR results. Furthermore, co-purification of PCR inhibitors may lead to an underestimation of the prevalence of the pathogen in a sample (Wilson, 1997; Bott *et al.*, 2010) and should be avoided as far as possible. Inhibitors that are frequently co-purified from environmental and animal samples include organic and phenolic compounds, Ca^{2+} , Mg^{2+} and Na^{2+} salts, components of microbial and fungal cells and an overabundance of non-target DNA (Bott *et al.*, 2010; Yamamoto *et al.*, 2010).

Another key step towards the development of a reliable molecular method for species-specific detection of a pathogen in environmental samples is the identification of one or more suitable DNA target regions for species-specific primer design (Bott *et al.*, 2010). Genes routinely used for this purpose include the small subunit (SSU) and large subunit (LSU) nuclear ribosomal DNA (rDNA) genes as well as the mitochondrial cytochrome oxidase subunit II (cox2) gene. These genes are highly abundant in the cell and contain highly conserved sequences that provide suitable regions for the design of species-specific probes or primers. These conserved regions are particularly useful for phylogenetic comparisons as they exhibit minimal genetic variation within a species, but they also vary sufficiently between species to allow for the selection of a region(s) specific to the species of interest (Li *et al*., 1996; Zhou *et al.*, 2000; Bott *et al.*, 2010).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Ribosomal DNA (rDNA) genes are comprised of an intergenic non-transcribed spacer (IGS or NTS) region, an external transcribed spacer (ETS) region and a transcription unit comprising two rRNA genes, namely SSU and LSU. These two rDNA genes are separated by the first and second internal transcribed spacers (Hillis and Dixon, 1991; Bott *et al.*, 2010). The SSU rDNA is the slowest evolving of the rDNA genes. Conversely, the LSU rDNA gene consists of regions that evolve faster, thus making this gene more suitable for distinguishing between very close relatives (Hillis and Dixon, 1991). Mitochondrial genes are found in the small double-stranded, circular mitochondria cell organelles (Bott *et al.*, 2010). Mitochondrial genes such as CO1 which is preferred barcoding marker

for a vast number of groups, generally evolve at a quicker rate than nuclear genes (Hillis and Dixon, 1991), meaning they are also more likely to be used for species-specific molecular detection.

Development of PCR primers specific to the target organism is also very important to achieve the best possible amplification and will influence both sensitivity and specificity of the reaction (Cunningham, 2002; Ma and Michailides, 2007). The term specificity used in this study refers to analytical specificity, which means the ability of an assay to measure a particular organism rather than others in a sample (Saah and Hoover, 1997). When aiming to design primers to be specific to a species regions that are not highly conserved should be selected for primer design. The designed primer-sets should also fulfil other criteria, such as optimum primer length, correct composition of bases (GC%), optimum annealing and melting temperatures, and finally 5' end stability and 3' end specificity (Ma and Michailides, 2007). Primer sets that facilitate maximum amplification are required for accurate quantification (Bustin *et al.*, 2005) and the specificity of the marker will ensure no cross-amplification (false positives) among heterologous species.

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Saah and Hoover, (1997) defined analytical sensitivity as "the smallest amount of substance in a sample that can accurately be measured by an assay". Optimising PCR cycling conditions will lead to optimal amplification, which in turn leads to improved sensitivity of the assay. Sub-optimal PCR reaction conditions may arise for different reasons and include factors such as incorrect annealing/ elongation

times and/ or temperatures. These factors, when not optimised correctly, can reduce the sensitivity of a primer set and also lead to the formation of primer-dimers, which is when primers bind to and amplify one another.

Strict validation of all criteria affecting specificity and sensitivity of a PCR assay also needs to be conducted at various levels of complexity in order to generate meaningful PCR data. All of the above mentioned factors need to be validated at various levels of complexity (Hiney and Smith, 1998). Validation is the evaluation of a diagnostic assay for the purpose of determining how suitable the assay is for a particular purpose (Hiney and Smith, 1998; OIE, 2006). Validation is essential for determining the performance of a diagnostic assay on environmental samples that typically contain numerous PCR inhibitors and non-target DNA. Consequently, the OIE has developed specific criteria that need to be fulfilled to ensure correct validation of diagnostic techniques. Furthermore, the establishment of quality assurance and quality control systems are required. These measures will help to ensure the development of high quality protocols, including the use of control samples to ensure that the system is working properly and to confirm data quality. The validation of molecular diagnostic assays is made up of different stages (OIE, 2006) and includes (1) feasibility studies, (2) assay development and standardisation, (3) determination of assay performance characteristics, (4) monitoring the validity of assay performance and (5) maintenance and enhancement of validation criteria.

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Feasibility studies are regarded as the preliminary step in the validation of new assays. The main goal of this step is to determine whether or not the new assay can detect a range of target concentrations. Infectious and non-infectious samples are tested and preliminary optimisation might be needed. Thereafter, further development and optimisation of the assay may take place. In this step, primer selection is critical and the nature of the infectious agent, such as the diversity of genetic sequences among different strains, should be carefully considered (OIE, 2006). Cycle time and temperature can vary between different thermal cyclers and reaction tubes (Wilson, 1997), the type of thermal cycler and reaction tubes used must also be carefully considered and consistently used to ensure repeatability. Instruments used to perform PCR may influence the results, all thermal cyclers should be carefully monitored, serviced and calibrated regularly (Wilson, 1997; OIE, 2006). Assays developed and validated using a specific thermal cycler should be revalidated when a new instrument is used.

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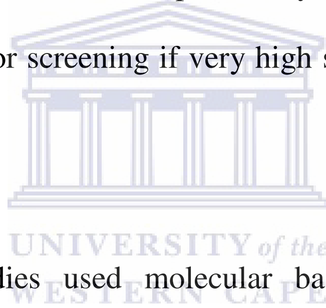
Assay development and standardisation steps are required to optimise parameters such as sample collection, preparation, transportation and DNA extraction methods. Optimisation and standardisation of a assay is very important to ensure the same results for a given sample when repeated (OIE, 2006). Excessive variability between replicates has to be prevented to ensure reliable results.

As mentioned previously, determination and optimisation of analytical sensitivity and assay specificity are essential for the development of a meaningful PCR diagnostic tool. Analytical sensitivity is also known as the limit of detection or

the smallest number of genome copies of the infectious agent (Saah and Hoover, 1997; Cunningham 2002; OIE, 2006). This can be determined by using an end-point dilution until the assay can no longer detect the target DNA (OIE, 2006). The range of the assay is then defined as the interval between the upper and lower concentration (Hiney and Smith, 1998) of an infectious agent in a sample in which the agent can reliably be detected (OIE, 2006).

On the other hand, specificity of an assay is assessed by using a panel of genetically related animals (OIE, 2006) or organisms in the direct environment (Hiney and Smith, 1998) that could potentially cause cross-amplification. An assay may not be used for screening if very high sensitivity and specificity is not achieved (OIE, 2006).



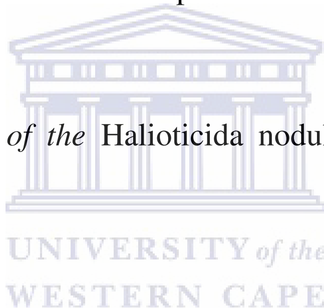
Although previous studies used molecular based methods to diagnose *H. noduliformans* from abalone showing clinical signs of tubercle mycosis (Sekimoto *et al.*, 2007; Muraosa *et al.*, 2009; Macey *et al.*, 2011), a molecular based diagnostic tool for specific detection of *H. noduliformans* in abalone has not yet been developed. Therefore, the primary objective of this chapter is to develop a PCR assay for the rapid, sensitive and specific diagnosis of *Halioticida noduliformans* in experimentally infected abalone *Haliotis midae* tissue samples. It is intended that this assay could be utilised to detect low levels of *H. noduliformans* in abalone tissue for monitoring of abalone tubercle mycosis in the local abalone aquaculture industry and environment.

3.2 Materials and methods

3.2.1 Template preparation

For the preparation of large quantities of genomic DNA for primer design and PCR optimisation, total genomic DNA was extracted from pure cultures of *H. noduliformans* grown in PYGS broth using the cell disruption and heat-lysis methods discussed in Chapter 2. The isolated genomic DNA was quantified with a Libra S12 spectrophotometer (Biochrom Ltd) and DNA quality was tested by 0.8% agarose gel electrophoresis. Samples were kept at -20 °C until needed.

3.2.2 DNA sequencing of the Halioticida noduliformans *SSU, LSU and cox2 genes and primer design*



The small subunit and large subunit ribosomal DNA (SSU and LSU rDNA, respectively) gene regions, as well as the mitochondrial DNA (mtDNA) cytochrome oxidase subunit II (cox2) gene region of *H. noduliformans* was selected as sites for the development of PCR primers specific to *H. noduliformans*. Species-specific primer sets were then designed to amplify non-conserved regions within each of the selected genes.

Initially, universal primer sets were used to amplify an almost complete sequence of each gene using Polymerase Chain Reaction (PCR). An almost complete sequence of each gene was obtained using PCR, amplification primers SR1 and

SR2 (Nakayama *et al.*, 1996) were used for the SSU rDNA gene, primers LSU-00021F (Sekimoto *et al.*, 2007) and LSU-1170R (Peterson and Rosendahl, 2000) for the LSU rDNA gene, and primers COX2-for 3 and COX2-Rev3 (Sekimoto *et al.*, 2007) for amplification of the cox2 gene. Amplification was conducted using the Labnet Multigene Thermal Cycler (Labnet International, Inc.). Each reaction was performed in duplicate in a final reaction volume of 25 µL containing 5 – 10 ng of *H. noduliformans* genomic DNA, 1 x KAPA Taq ReadyMix (Kapa Biosystems; Cat# KK1006) and 400 nM of each primer. The PCR assay consisted of an initial denaturation of 5 minutes at 95 °C, followed by 35 cycles at 95 °C for 1 minute, annealing for 1 minute, and extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. The optimal annealing temperatures for the different primer sets are listed in Table 3.1. Amplified PCR products were analysed by 0.8% agarose gel electrophoresis to verify the reaction specificity and fragment size before purification using a PCR purification kit (Roche). The purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI3730xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Both forward and reverse primers for each gene were used for cycle sequencing.

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Each sequence was edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft). Reaction specificity was verified by homology searches carried out using the BLASTN algorithm (Altschul *et al.*, 1989) provided by the Internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nml.nih.gov/BLAST). Each gene was then aligned using

the optimal alignment option of DNAMAN, with gene sequences from closely related Peronosporomycete sequences available from GenBank, particularly organisms within the Halioticida/ Haliphthoros/ Halocrusticida clade (accession numbers AB178866, AB285218, AB285222-30), in order to identify non-conserved regions for designing species-specific primers (Sekimoto *et al.*, 2007; Macey *et al.*, 2011). Standard criteria were utilised for the primer design (Bustin, 2000; 2002; Bustin and Nolan, 2004). Three or more primer sets were designed for each gene. Finally, to ensure species-specificity during the desktop phase of the study, candidate primers were blasted against the GenBank database.

3.2.3 Species-specific PCR assay development

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3.2.3.1 Optimisation of PCR cycling conditions

PCR cycling conditions were optimised for optimal detection specificity by varying the annealing temperature and the PCR cycle number for each primer set. This process is essential for preventing the occurrence of non-specific priming and to ensure that the correct product is obtained. The PCR reactions (25 µL) were carried out containing 5 – 10 ng of *H. noduliformans* genomic DNA, 1 x KAPA Taq ReadyMix (Kapa Biosystems; Cat# KK1006) and 400 nM of each primer. The Labnet Multigene Thermal Cycler (Labnet International, Inc.) was used for amplification and the PCR assay consisted of an initial denaturation of 3 minutes at 95 °C, followed by 30 - 39 cycles of denaturation at 95 °C for 15 seconds, annealing gradient from 45 °C - 65°C for 30 seconds with an increment

of 0.5 °C per cycle, and elongation at 72 °C for 30 seconds. All the amplified products were analysed by agarose gel (0.8%) electrophoresis to verify the reaction specificity and fragment size and to estimate the quantity of product produced at each annealing temperature.

3.2.3.2 Specificity of the SSU, LSU and cox2 gene primer sets

Following optimisation of PCR cycling conditions for each primer set a single primer set was chosen for each gene region (LSU, SSU, cox2) and tested for cross-amplification with DNA extracted from other closely related Peronosporomycete species, as well as from marine fungi isolated from the environment. These samples included fungi isolated from abalone not infected with *H. noduliformans*, seaweeds and crustaceans (Table 3.2). Genomic DNA was extracted from pure cultures of each isolate using the heat-lysis method described in Chapter 2. DNA (5 – 10 ng) from each sample was added to the PCR mixtures (25 µL) and amplified as described previously. Each PCR assay included a positive control that consisted of *H. noduliformans* genomic DNA in the reaction mixture and a non-template control (NTC) that substituted PCR-grade water for DNA to confirm that the reagents were not contaminated. All reactions were performed in duplicate. PCR amplification with universal fungus primers SR1 and SR12 for the SSU rDNA gene was included as an additional positive control to determine the quality and integrity of genomic DNA isolated from each of the tested fungal species. The optimal annealing temperatures for the different primer sets are listed in Table 3.1. PCR products were analysed by agarose gel

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(0.8%) electrophoresis to verify reaction specificity and fragment sizes. In addition, all amplified PCR products of the correct size (Table 3.1) were sequenced to confirm reaction specificity and sample identity.

3.2.4 Real-time PCR

3.2.4.1 Optimisation of real-time PCR conditions for the HN.LSU primer set

Halioticida noduliformans specific primers HN.LSU-F and HN.LSU-R were chosen for all subsequent experiments (Table 3.2). Since the Bio-Rad CFX96^{TM} real-time PCR detection system and C1000^{TM} thermal cycler was to be used for all subsequent real-time PCR assay development and since optimal annealing temperature for this primer set could vary between thermal cyclers, we re-optimised the annealing temperature for this primer set using the Bio-Rad instrument. PCR reactions were carried out in a final volume of 25 µL containing 5 – 10 ng of *H. noduliformans* genomic DNA, 1 x Promega GoTaq® SyBr Green master mix (Promega, Cat# A6001) and 400 nM of each primer. Amplification was monitored using a Bio-Rad CFX96^{TM} real-time PCR detection system on a C1000^{TM} thermal cycler and consisted of an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing gradient from 45 °C - 65°C for 30 seconds, and elongation at 72 °C for 45 seconds, 70 cycles of 60 °C for 10 seconds with an increment of 0.5 °C per cycle.

Table 3.1: Nucleotide sequences and annealing temperatures of all the primers used in this study for real-time PCR reaction

Set No.	Primer Name	Sequence 5' – 3'	Target Region	Product size (base pairs)	Annealing Temperature (°C)	[a]Reference
Universal fungal						
1	SR1-F	TACCTGGTTGATCCTGCCAG	SSU rDNA	1781	55	1
	SR12-R	CCTTCCGCAGGTYTCACCTAC	SSU rDNA			
2	LSU-00021F	ATTACCCGCTGAACTTAAGC	LSU rDNA	1170	63	2
	LSU-1170R	GCTATCCTGAGGGAAATTTCGG	LSU rDNA			3
3	COX2-For3	GCHACHCCWGTWATGGARGG	cox2	540	40	2
	COX2-Rev3	TACATTGDCCRTAAAAAAYMCC	cox2			
Species-specific						
4	HN.SSU-F	GCTCATTATATCAGTTATAGTCT	SSU rDNA	174	52	4
	HN.SSU-R	CCGCACAGTTATTATGAC	SSU rDNA			
5	HN.LSU-F	CGTCATAGTCAGTTTGTAT	LSU rDNA	141	59.1	4
	HN.LSU-R	ATCCCATCAATACCCTTA	LSU rDNA			
6	HN.COX2-F	GTTATATGGTTCAAGAAGAT	cox2	177	55	4
	HN.COX2-R	ACATAGAGGTTTGGTTTA	cox2			

References: [1] Nakayama *et al.* (1996); [2] Sekimoto *et al.* (2007); [3] Peterson and Rosendahl (2000); [4]This study

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Table 3.2: Isolates from the environment used to test for cross-amplification to ensure primer specificity

Species	**Isolate(s)**	**Host**	**Location of sample collection**	**GenBank Number**	**[a]Reference**
Unidentified *Pleosporales*	PG170709B1	*Palinurus gilchristi*	Western Cape	JN397388	1
Acremonium sp.	PG170709B2	*Palinurus gilchristi*	Western Cape	JN397389	1
Unidentified *Pleosporales*	PG170709B3	*Palinurus gilchristi*	Western Cape	JN397390	1
Hypocreales sp.	Kelp100210A	*Ecklonia maxima*	Western Cape	JN397391	1
Hypocreales sp.	Kelp100210B	*Ecklonia maxima*	Western Cape	JN397392	1
Fusarium sp.	SC110909A	*Macrocheira kaempferi*	Western Cape	JN397393	1
Microascus sp.	SC230909	*Macrocheira kaempferi*	Western Cape	JN397394	1
Hypocreales sp.	SCG230909	*Macrocheira kaempferi*	Western Cape	JN397395	1
Aphanomyces astaci	FDL 457	Crayfish	United Kingdom	DQ 403202.1	2
Halioticida noduliformans	AF08527	*Haliotis midae*	Western Cape	GU289906	3

[a]References: [1]This Study; [2] Oidtmann *et al.*, (2004); [3] Macey *et al.*, (2011)

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Data collection and real-time analysis occurred at the annealing step of each cycle and melt curve data collection and analysis occurred at each increment in the final 70 cycles. To determine the conditions giving the highest specificity we varied the annealing temperatures and the PCR cycle number for each primer set. The optimal annealing temperature for the HN.LSU-F and HN.LSU-R primer set is listed in Table 3.1.

3.2.4.2 Sensitivity of the Halioticida noduliformans *specific LSU primer set for real-time PCR*

The lowest detection limit for the *H. noduliformans* specific primer set HN.LSU-F/R was determined by running real-time quantitative PCR's on serial dilutions made up with known quantities of extracted *H. noduliformans* DNA. PCR-grade water was used to prepare tenfold serial dilutions, in order to determine the lowest detection limit for the *H. noduliformans* specific primer set under sterile conditions. This also served as a standard curve for the quantification of unknown samples in all subsequent real-time PCR assays. DNA added for sensitivity testing ranged from 266 to 0.266 pg. Both nested and non-nested real-time PCR assays were performed on the same dilution series. For the non-nested reaction, amplification primers HN.LSU-F and HN.LSU-R were used for the PCR. In contrast, the nested PCR first utilised universal fungal outer primers LSU-0021F and LSU-1170R (Table 3.2) followed by real-time PCR amplification with the *H. noduliformans* specific primers, HN.LSU-F and HN.LSU-R. Optimal annealing temperatures for different primer sets are listed in Table 3.1.

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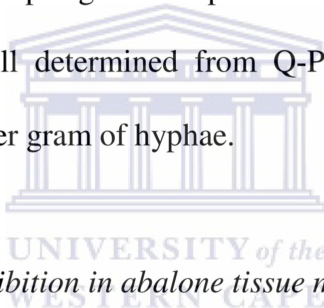
In order to estimate the number of *H. noduliformans* spores in experimentally infected abalone tissue, spore number was calculated according to Hermansson and Lindgren (2001). Since the genome size for *H. noduliformans* has not been determined, we estimated the genome size for *H. noduliformans* by averaging the oomycete genome sizes reported by Lamour *et al.* (2007). The estimated spore number was then calculated based on four assumptions. (1) The genome size of *H. noduliformans* is 108 Mb. (2) There is only 1 genome per spore. (3) One kb of dsDNA is approximately equal to 0.662×10^{6} Daltons (Coyne *et al*., 1996). (4) One dalton is equal to 1.67×10^{-24} grams (Garret and Grisham, 1995). The amount of DNA obtained per gram of spore was then divided by the estimated amount of DNA per cell determined from Q-PCR in order to determine the effective spore number per gram of hyphae.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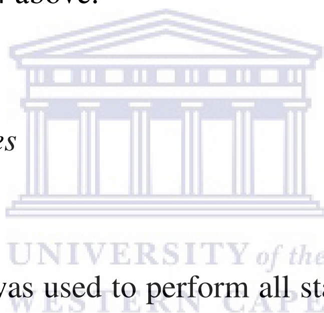
2.2.5 Real-time PCR inhibition in abalone tissue matrix

A second study was conducted to quantify the amount of PCR inhibition and the lowest detection limit for the *H. noduliformans* specific primer set under environment conditions. In order to test this, ten-fold serial dilutions were made up with known quantities of extracted *H. noduliformans* DNA in an abalone tissue matrix. The abalone tissue matrix consisted of supernatant obtained from genomic DNA extracted from abalone tissue using the heat-lysis method described in Chapter 2. To prepare abalone tissue for heat-lysis, foot muscle tissue (0.2 – 0.3 g) was aseptically transferred to a sterile 15 mL centrifuge tube containing 2 mL phosphate buffered saline (PBS: 7.3 mM monosodiumphosphate,

180 mM disodium phosphate, 0,15 M sodium chloride; pH 7.2), supplemented with 0.05% Antifoam A and 0.1% Tween 20. The sample was homogenised for 1.5 minutes using a Polytron PT 2100 tissue homogeniser and 300 µL aliquots of homogenate were transferred to separate 1.5 mL microcentrifuge tubes. Tubes containing homogenised abalone tissue were stored on ice for immediate use or at -80 °C until needed. DNA added for sensitivity testing ranged from 266 to 0.266 pg. Three independent tenfold serial dilutions were prepared in the abalone tissue matrix as well as in PCR-grade water, which served as a positive control for calculating the amount of tissue inhibition. The real-time PCR cycling conditions were as described in 2.2.4 above.

3.2.4 Statistical analyses



SigmaStat 3.1 software was used to perform all statistical analysis. To determine whether the quantification cycle (Cq) values differed between non-nested and nested PCR assays at specific template DNA concentrations, a t-test was performed. Significance was assigned to P values of <0.05.

3.3 Results

3.3.1 Species-specific PCR

A BLAST search of the GenBank database revealed that the primer sets designed for the SSU, LSU and cox2 gene sequences showed homology to *H.*

noduliformans at the time of the BLAST (15/05/2010) search. The specificity of the *H. noduliformans* specific primers HN.LSU-F/R, HN.LSU-F/R and HN.COX2-F/R were further tested and confirmed by aligning them to closely related Peronosporomycete species within the Halioticida/ Haliphthoros/ Halocrusticida clade and with *Aphanomyces piscicida* (HN.LSU-F/R: AB285225, AB285222, AB285223, AF235941; HN.SSU-F/R: AB284578, AB284576, AB178868, AB086899 and HN.COX2-F/R: DQ365736, AB160858, AF290305 AF290306). In contrast to the HN.LSU-F/R primer set, the HN.SSU-F/R and HN.COX-F primers showed homology to closely related Peronosporomycete. The CLC sequencer software programme (Version 6.4) was used to help illustrate the alignments. The *H. noduliformans* LSU-F primer differed by at least 2 bp and the LSU-R primer by at least 4 bp (Fig. 3.1) from the sequences of phylogeneticaly close relatives (Fig. 3.1). At the optimum annealing temperature of 59.1 °C the HN.LSU-F and HN.LSU-R primer set amplified a 141 bp region of the LSU gene of *H. noduliformans* (Table 3.1). At an optimal annealing temperature of 52 °C (Table 3.1) the primer set HN.SSU-F/R amplified a 174 bp region of the *H. noduliformans* SSU gene. The third primer set, HN.COX2-F/R had an optimal annealing temperature of 55 °C (Table 3.1) and amplified a 177 bp region of the cox2 gene. This primer set showed at least 2 bp differences with closely related species in the reverse primer, however the HN.COX2-F showed 100% homology with some of the tested species (Fig. 3.3).

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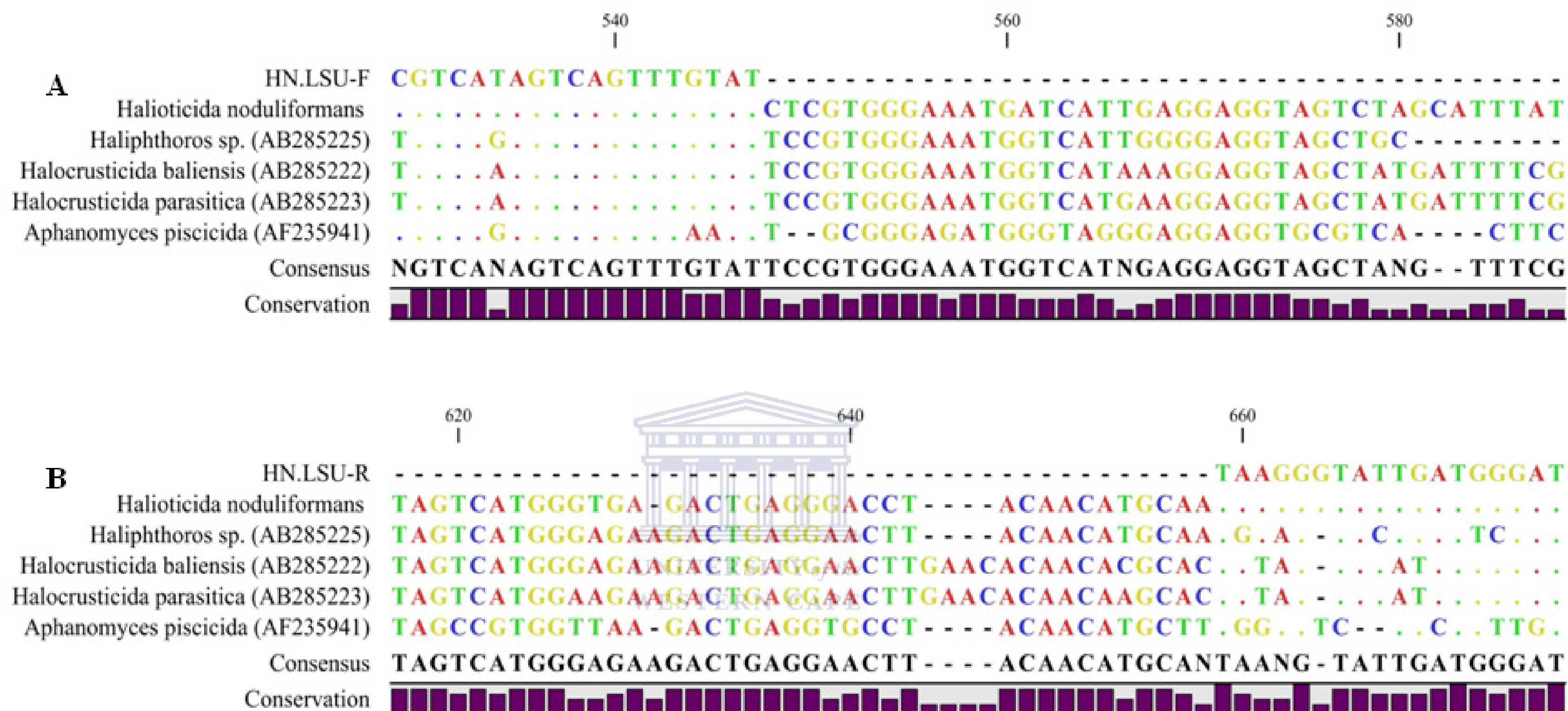

Figure 3.1: Alignment of the (A) HN.LSU-F and (B) HN.LSU-R primers to the LSU rDNA gene of *Halioticida noduliformans* and other phylogeneticaly close relatives. Identical base pairs are indicated as dots.

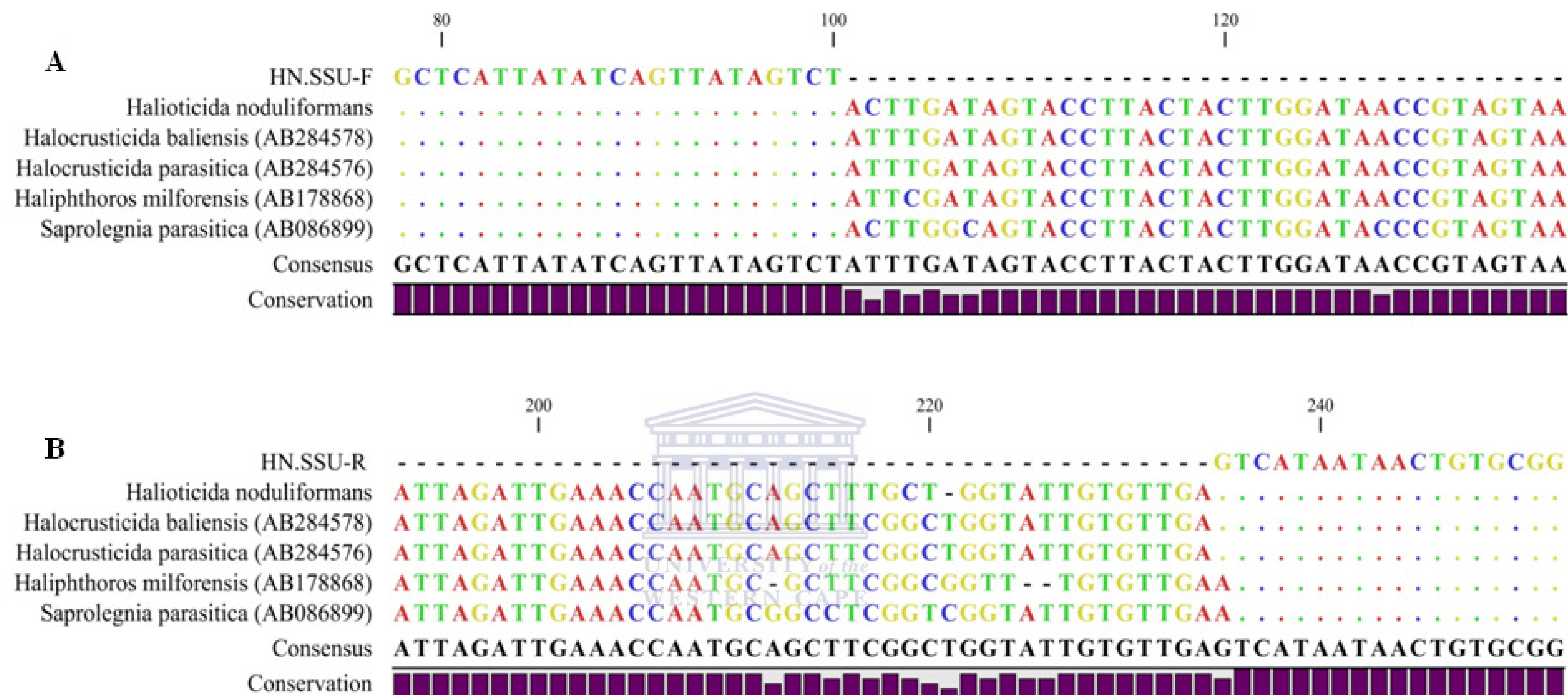

Figure 3.2: Alignment of the (A) HN.SSU-F and (B) HN.SSU-R primers to the SSU rDNA gene of *Halioticida noduliformans* and other phylogeneticaly close relatives. Identical base pairs are indicated as dots.

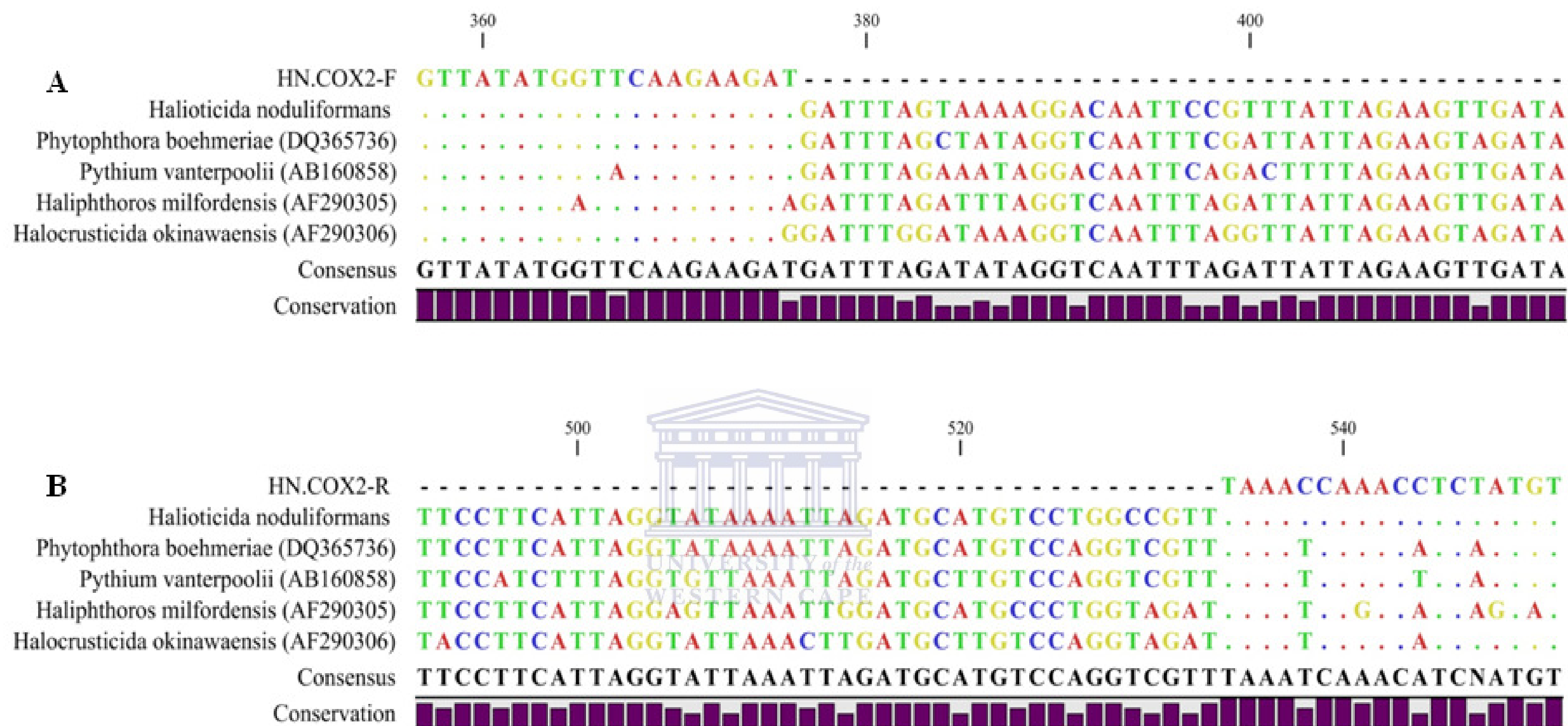

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Figure 3.3: Alignment of the (A) HN.COX2-F and (B) HN.COX2-R primers to the mtDNA gene of *Halioticida noduliformans* and other phylogeneticaly close relatives. Identical base pairs are indicated as dots.

The above mentioned BLAST searched of the GenBank database and multiple sequence alignments were done to ensure specificity of each of the designed primer pairs during the 'desktop phase' of this study. From these alignments, primer sets HN.LSU-F/R and HN.COX2-F/R were found to be specific for *H. noduliformans* when screened against a panel of closely related Peronospormycete species (Fig. 3.1). HN.LSU-F/R was selected as the most specified primer set due to no homology in either the forward or reverse primer to sequences of closely related organisms. However, another test for specificity was performed using all three of the designed primer sets to further ensure specificity. All three primer sets were tested for cross-reactivity to DNA extracted from other marine fungi isolated from the environment (Fig. 3.4) in which abalone naturally occur. Both HN.LSU-F/R and HN.COX2-F/R primer sets did not show any cross-reactivity with the closely related fungi tested in this study and only amplified DNA from *H. noduliformans* at the specified annealing temperatures (Table 3.1; Fig. 3.4). The amplified DNA was also sequenced to confirm the specificity of the PCR reactions and subsequent analysis of the nucleotide sequences showed that the PCR products, approximately 141 bp and 177 bp in length for the LSU and cox2 genes respectively, were identical to *H. noduliformans.* Amplifications using the universal small subunit primer set, SR1 and SR12, which was included as a positive control to determine the quality and integrity of genomic DNA isolated from each of the tested fungal species, produced the expected band of approximately 1781 bp in all samples, with the exception of lane 7 which contained genomic DNA isolated from *Hypocreales sp.* Furthermore, the negative control did not produce any bands, confirming that the reagents used for real-time PCR were not contaminated. In contrast, primers HN.SSU-F/R showed

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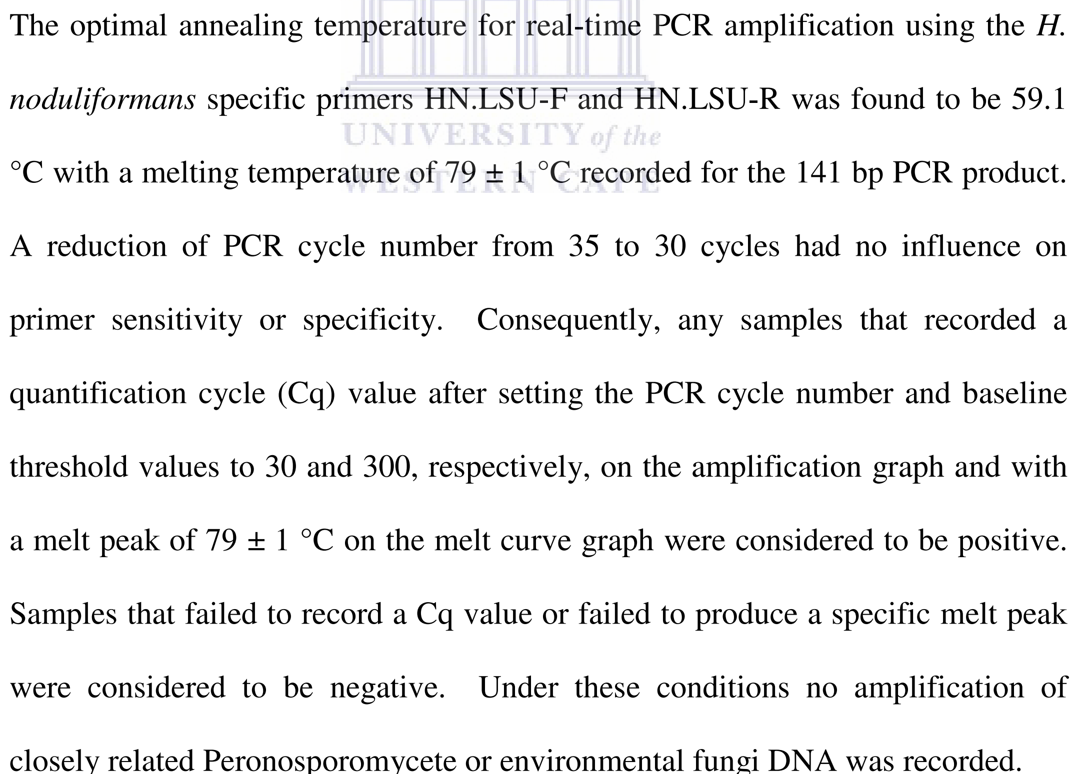
no specificity at all and cross-reacted with all tested fungi. Thus, primer sets HN.LSU-F/R and HN.COX2-F/R appeared to be good candidates for *H. noduliformans* specific primers based on their specificity. To determine why the HN.SSU-F/R primers cross-reacted with DNA from the environmental fungal isolates (Fig. 3.4), this primer set was aligned (Fig. 3.5) against the SSU gene sequences of the environmental isolates tested in this study. The resulting alignments revealed very few base pair differences. Specifically, the HN.SSU-F/R primer set shared 100% sequence identity with the *Aphanomyces astaci* SSU gene, indicating why cross-amplification occurred for this primer set (Fig. 3.5).

3.3.2 Optimisation of real-time PCR conditions for H.N.LSU-F/R primer set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The optimal annealing temperature for real-time PCR amplification using the *H. noduliformans* specific primers HN.LSU-F and HN.LSU-R was found to be 59.1 °C with a melting temperature of 79 ± 1 °C recorded for the 141 bp PCR product. A reduction of PCR cycle number from 35 to 30 cycles had no influence on primer sensitivity or specificity. Consequently, any samples that recorded a quantification cycle (Cq) value after setting the PCR cycle number and baseline threshold values to 30 and 300, respectively, on the amplification graph and with a melt peak of 79 ± 1 °C on the melt curve graph were considered to be positive. Samples that failed to record a Cq value or failed to produce a specific melt peak were considered to be negative. Under these conditions no amplification of closely related Peronosporomycete or environmental fungi DNA was recorded.

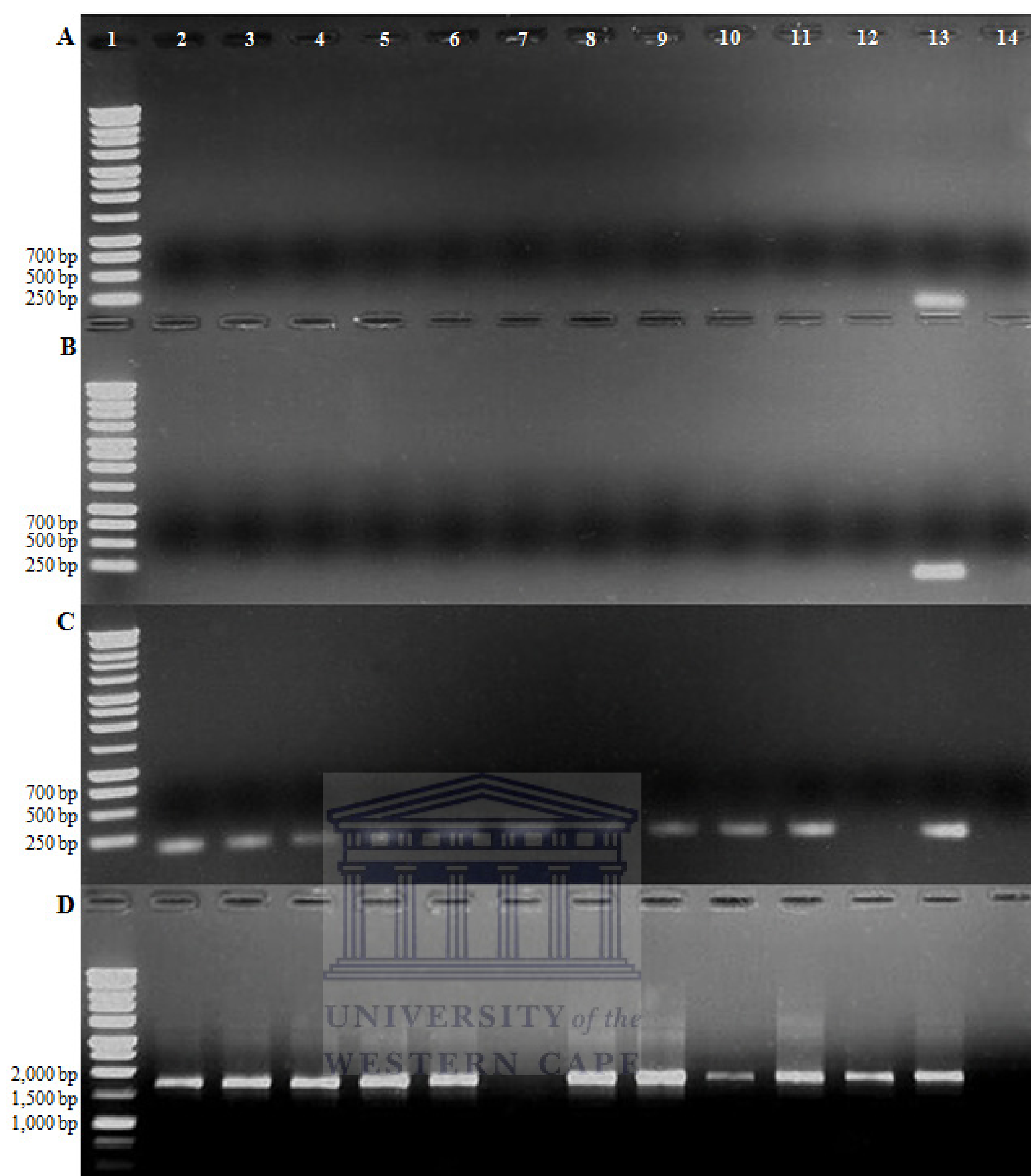

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Figure 3.4: Agarose gel electrophoresis (0.8%) of PCR products following a non-nested PCR using the different primer sets designed specifically for *Halioticida noduliformans.* Row A, HN.LSU-F/R primers, (B) HN.COX2-F/R primers, (C) HN.SSU-F/R primers and (D) universal small subunit primers (SR1, SR12) used as a positive control to verify quality and integrity of DNA isolated from each of the tested strains. Lane 1, 1kb DNA ladder, Lane 2 – *Pleosporales sp*., Lane 3 – *Acremonium sp.,* Lane 4 – *Pleosporales sp*., Lane 5 – *Fusarium sp.,* Lane 6 – *Microascus sp*., Lane 7 – *Hypocreales sp*., Lane 8 – *Hypocreales sp*., Lane 9 – *Hypocreales sp*., Lane 10 – Unknown A, Lane 11 – Unkown B, Lane 12 – *A. astaci,* Lane 13 – *Halioticida noduliformans* and Lane 14 is a non-template control.

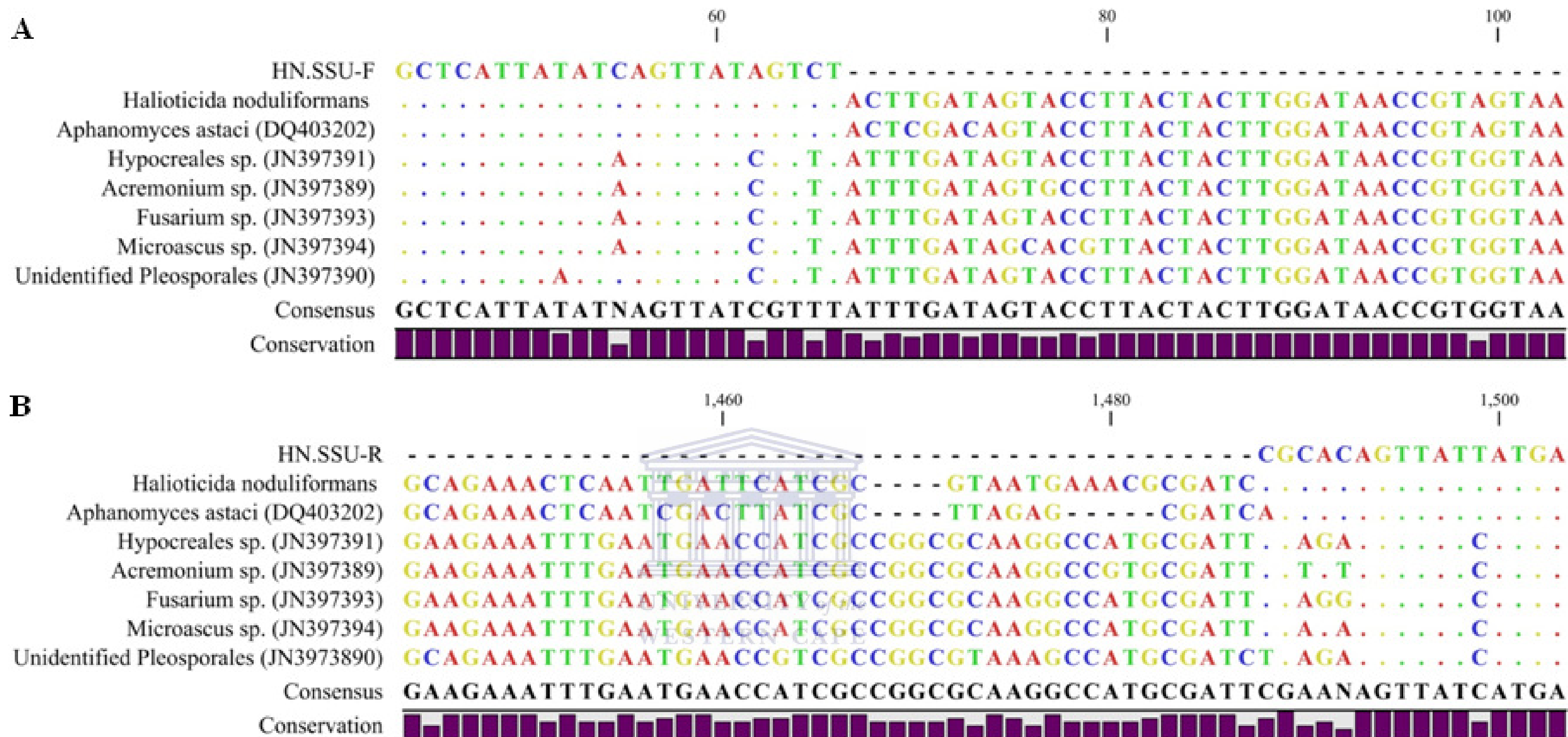

Figure 3.5: Alignment of the (A) HN.SSU-F and (B) HN.SSU-R primers to the SSU rDNA gene of *Halioticida noduliformans* and environmental isolates. Identical base pairs are indicated as dots.

3.3.3 Sensitivity of the Halioticida noduliformans *specific LSU primer set*

Following real-time PCR amplification with the HN.LSU-F/R primer set, the lowest DNA concentration (extracted from pure *H. noduliformans* cultures), that could be detected in PCR-grade water was 2.66 pg DNA at a Cq value of approximately 30. However, because of the high Cq value for this reaction and since only one out of the three replicate samples was positive for reactions containing 26.6 and 2.66 pg DNA, we regard the lowest detection limit for the HN.LSU-F/R primer set to be approximately 266 pg of DNA. The Cq values of samples containing DNA resuspended in PCR-grade water were however significantly lowered when a nested PCR was performed. PCR cycle number for samples spiked with 266 pg of DNA were significantly reduced (t-test, $P = 0.001$) from approximately 24 cycles for the non-nested PCR reactions to less than 5 cycles for the nested PCR reactions, an approximate 80% improvement in PCR sensitivity (Fig. 3.6). Consequently, the nested PCR assay could accurately detect as little as 0.266 pg of *H. noduliformans* DNA in a 25 μL reaction volume (Fig. 3.6).

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To assess the non-target DNA involvement in PCR inhibition as well as the effect of other inhibitors co-purified with the genomic DNA, PCR amplification was performed in the presence of abalone tissue matrix. This was compared to a standard curve prepared in PCR-grade water. Our results demonstrated an overall 27% reduction in nested PCR sensitivity, as indicated by the increase in PCR cycle number at each corresponding DNA concentration. However, as the DNA

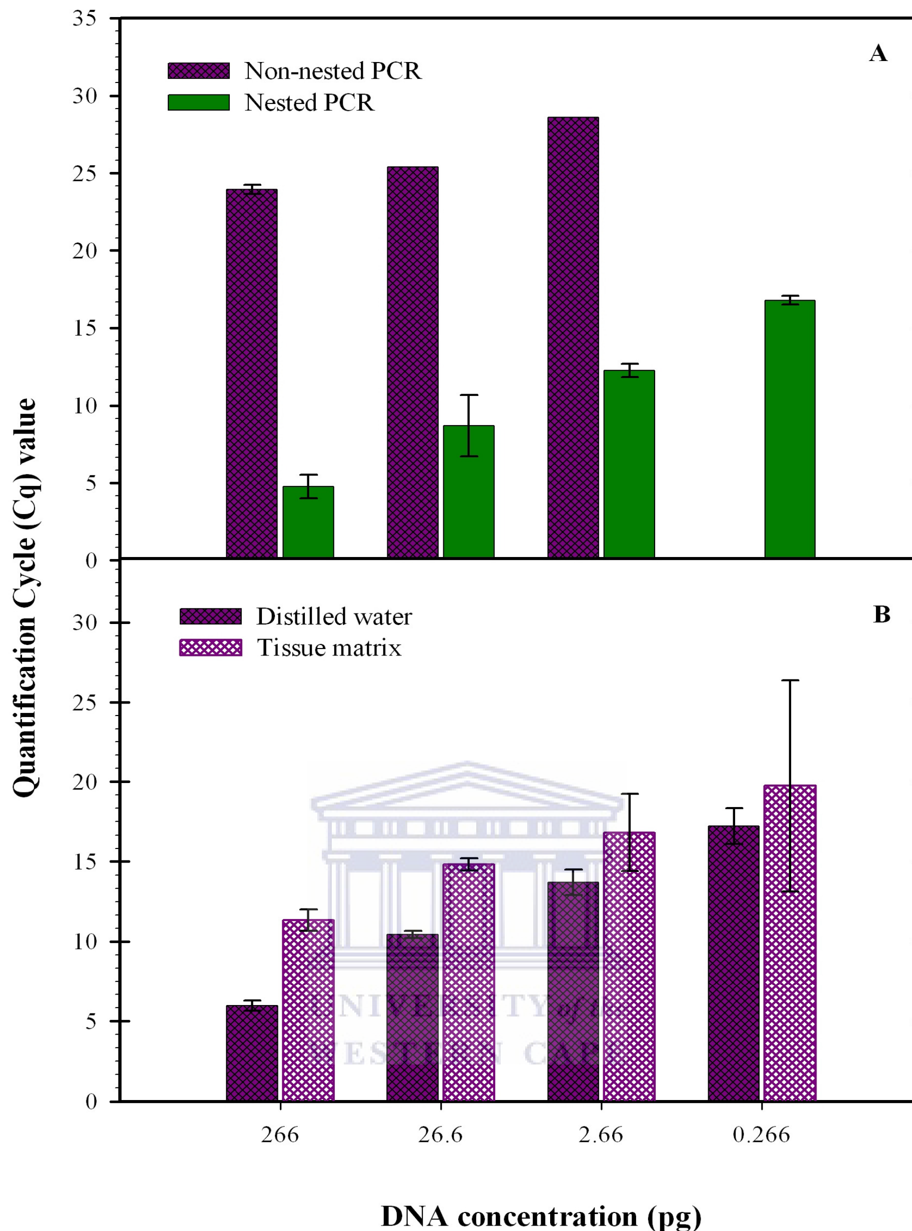

Figure 3.6: Quantification cycle (Cq) threshold values obtained following real-time quantitative PCR analysis on serial dilutions of known quantities of extracted *Halioticida noduliformans* DNA. (A) Both nested and non-nested real-time PCR assays were performed on the same dilution series made in PCR grade water. For the non-nested reaction, PCR was carried out with the *H. noduliformans* specific primers, HN.LSU-F and HN.LSU-R. In contrast, the nested PCR first utilized universal fungal outer primers LSU-0021F and LSU-1170R followed by real-time PCR amplification with the *H. noduliformans* specific primers. (B) Quantification cycle (Cq) threshold values obtained following nested PCR on serial dilutions of known quantities of extracted *H. noduliformans* DNA made in PCR grade water and in an abalone tissue matrix. This seeded study served to quantify the amount of PCR inhibition and the lowest detection limit for the *H. noduliformans* specific primer set in an environment or matrix that is designed to be a non-sterile simulation of the environmental conditions.

concentrations decreased, variance in Cq values of the technical replicates increased. Based on the calculations described in 3.2.4.2, the starting concentration of the lower detction limit for *H. noduliformans* in the pressence of abalone tissue matrix using the nested PCR assay are 0.266 pg DNA (approximately 2.37 spores) in a 25 µL reaction volume.

3.4 Discussion

A nested real-time quantitative PCR (Q-PCR) assay has been developed and optimised to accurately detect a starting concentration of as little as 0.266 pg of *H. noduliformans* DNA (approximately 2.37 spore equivalents) in a 25 µL reaction volume. The nested Q-PCR assay was designed to specifically target the *H. noduliformans* large subunit rDNA gene in a mixed genomic DNA background, specifically abalone tissue. When the LSU specific PCR primers were tested for specificity against a panel of closely related Peronosporomycetes as well as fungi isolated from the immediate environment, no false positive results due to cross-amplifications were observed. This is the first study to describe the development of a nested Q-PCR assay to specifically detect *Halioticida noduliformans* DNA in experimentally infected abalone (*Haliotis midae*) tissues. The assay in the present study has the potential for utilisation as a routine diagnostic tool for abalone tubercle mycosis surveillance and management.

Out of the three primer sets (HN.LSU-F/R, HN.SSU-F/R and HN.COX2-F/R) designed in the present study, HN.LSU-F/R was shown to be highly species-

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specific, with the most bp differences in both the forward and reverse primer, when it was blasted against DNA sequences of closely relative organisms available on the GenBank database (Fig. 3.1). In contrast, the HN.COX2-F primer showed 100% homology to some of the closely related species tested in this study, potentially causing this primer set to be less specific than the HN.LSU-F/R primer set. However, we demonstrated that both these primers are able to specifically amplify *H. noduliformans* DNA in a mixed genomic DNA background and that these primers did not cross-react with DNA isolated from phylogeneticaly related species isolated and tested in this study. In contrast, the HN.SSU-F/R primer set, cross-reacted with DNA isolated from numerous environmental samples tested in this study and in so doing did not meet the requirements stipulated in this study for a PCR diagnostic, which included specific amplification of *H. noduliformans* DNA. This finding emphasises the importance of a recommendation previously made by Hiney and Smith (1998), who suggested that in addition to conducting a Blast search for newly designed primers against sequences available on the public databases, all newly designed primers should also be screened against DNA isolated from organisms isolated from the immediate environment in which the host organisms occurs naturally.

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Although both the HN.LSU-F/R and HN.COX2-F/R primers showed high specificity for *H. noduliformans* DNA, when tested against environmental isolates, we only selected the HN.LSU-F/R primer set for further analysis, as it showed to be highly specific from the beginning during the BLAST search. This supports the findings of Hillis and Dixon (1991) and Li *et al.* (1996) stating that

the LSU rDNA are more ideal for distinguishing between very close relatives because of the different evolving rates and the numerous variable regions. Other factors that contributed to this decision (in the present study) were the more stringent criteria utilised during the design of this particular primer set (included both: 'avoid cross-homology' and 'avoid template structure' options), when compared with criteria utilised for the cox2 primer set (where sequence composition made it possible to use only one of these options at a time). Furthermore, more comparative sequence information was available on public databases for this gene at the time this work was conducted, in contrast to the mitochondrial gene. The specificity of this primer set (HN.LSU-F/R) was demonstrated by a BLAST search on the Genbank database and showed that both the forward and reverse primers differed with two or more nucleotides from all known rRNA gene sequences. In an effort to improve the sensitivity and specificity of the Q-PCR assay we performed a nested PCR. This improved the PCR sensitivity of the assay by 80%.

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According to Wilson (1997), samples obtained from the environment commonly reduce sensitivity of PCR assays due to the wide range of inhibiting substances present in these samples. Since the purpose of this study is to develop a Q-PCR assay capable of detecting *H. noduliformans* in environmental samples, the amount of inhibitory substances present in environmental samples needed to be quantified. This was investigated by comparing the PCR results from two dilution series, one made in distilled water and one in an abalone tissue matrix. This is an important step as it allowed us to evaluate the impact of the physical and chemical

properties that an abalone tissue matrix had on the performance and overall dynamics of the Q-PCR assay, as suggested by Hiney and Smith (1998). We demonstrated that substances/ compounds present in the abalone tissue matrix do interfere with the PCR reaction, resulting in an overall 27% reduction in PCR sensitivity. Previous studies have report on species specific PCR assays with a lower detection limit of 0.1 – 1 pg fungal DNA per reaction (Zhou *et al.*, 2000), whereas other have reported on even lower detection limits (Phadee *et al*., 2004; Oidtmann *et al*., 2004). Furthermore, Phadee *et al*. (2004) and Oidtmann *et al.* (2004) developed PCR assays to detect *Aphanomyces piscicida* in experimentally infected gold fish (*Carassius auratus*) and crayfish, respectively, with lower detection limits of approximately 0.5 pg DNA per reaction (Phadee *et al*., 2004) and a threshold of at least 0.1 pg of DNA after 30 PCR cycles. These two authors also reported that *A. astaci* could be detected in cuticle samples even before visible hyphae-like structures could be identified, suggesting that their PCR assay was highly sensitive (Oidtmann *et al*., 2004). However, almost all of these studies were performed in a traditional 'test-tube' or non-seeded matrix using serially diluted purified fungal DNA and did not accommodate the possible inhibition caused by substances from the environment or from the host matrix. In contrast, we accounted for possible inhibitors in abalone tissue, using abalone tissue matrix spiked with *H. noduliformans* compared to samples made up with PCR-grade water. We showed that even with a 27% reduction in sensitivity caused by abalone tissue matrix it is still possible to detect a starting concentration of approximately 2.37 *H. noduliformans* spores in a 25 µL reaction volume.

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In conclusion, the primers (HN.LSU-F/R), designed for specific detection of *H. noduliformans* DNA, have been tested and shown to be species-specific, relative to the panel of organisms we screened against. When performing a nested PCR assay, utilising universal fungal outer primers prior to PCR amplification, using the *H. noduliformans* LSU specific primers, we could accurately detect a reaction starting concentration of 0.266 pg *H. noduliformans* DNA (approximately 2.37 *H. noduliformans* spores) in an abalone matrix. This technique may be useful as a diagnostic tool in the abalone industry.

CHAPTER 4

Final discussion

Specific identification of *Halioticida noduliformans* is critical for early diagnosis of abalone tubercle mycosis and for effective management and containment of this pathogen. In the present study we report on the development of the first molecular based diagnostic tool for specific detection of *H. noduliformans* in abalone tissue. This method is a real-time nested quantitative PCR (Q-PCR) protocol, which is highly sensitive and target specific. The assay can detect a reaction starting concentration of as little as 0.266 pg of *H. noduliformans* DNA in a 25 µL reaction volume within a mixed genomic DNA background. This equates to approximately 2.4 fungal spore equivalents. This diagnostic tool has been developed and validated based on part of the framework adopted from Hiney and Smith (1998) and Bott *et al.* (2010) for the validation of PCR based techniques.

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With aquaculture currently the fastest growing food production sector in the world, effective tools for rapid and accurate diagnosis of diseases are essential for ongoing and future development of the industry. Diseases present a serious problem to many aquacultured species, regardless of whether they are infectious or non-infectious, and several studies have reported on diseases of various abalone species utilized for abalone aquaculture in different parts of the world (Lee *et al.*,

2001; Cheng *et al*., 2004b; Day and Prince, 2007). In South Africa, the main infectious disease in the local abalone aquaculture industry has been the sabellid worm *Terebrasabella heterouncinata* (Ruck and Cook, 1998). Mouton (2010) also reported on other infectious diseases agents in South African abalone, but none with major concern to the livelihood of the industry. This scenario changed recently when abalone tubercle mycosis was diagnosed in South African abalone (*Haliotis midae*) in 2006 (Macey *et al.*, 2011). This disease is an epizootic of farmed abalone caused by the invasive Peronosporomycete *H. noduliformans* (Macey *et al*., 2011). South African regulatory authorities regard it as a contagious disease that may pose a significant economic risk to the abalone aquaculture industry. This disease has been detected in two different countries (Muraosa *et al.*, 2009). In South Africa it has already impacted the abalone aquaculture industry and has resulted in the closure of three farms (Macey *et al.*, 2011). Furthermore, this disease creates a significant risk to the already vulnerable wild abalone populations, not only along the South African coast, but also throughout the world. Because of this abalone health risk, an animal health programme was initiated and implemented on many of the remaining farms within South Africa and this programme has proven to be very effective for disease management in general. The implementation of this programme has however highlighted the need for the development of a rapid and sensitive diagnostic tool for abalone tubercle mycosis. Early diagnoses will help to reduce the spread of this disease within a farm and even prevent potential spread to neighbouring farms, thus ultimately preventing the loss of stock.

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The first objective for this study was to develop and optimise a DNA extraction method to isolate *H. noduliformans* genomic DNA that can repeatedly yield high quality DNA suitable for use in a PCR assay. Karakousis *et al.* (2006) highlighted that efficient DNA extractions vary between different fungal species. Therefore, we compared different DNA extractions (Chapter 2) that have commonly been used on fungi in order to develop the most effective extraction method for *H. noduliformans*. An optimal DNA extraction protocol will ensure sensitive and precise quantification of specific *H. noduliformans* DNA fragments, ready to be used in a polymerase chain reaction (PCR) technique. DNA extraction procedures that eliminate PCR inhibitors are crucial. Dilution of DNA extracts has proven to be successful in reducing PCR inhibitors, but this process may also reduce PCR efficacy (Ma and Michailides, 2007). We demonstrated that using a conical grinder to physically disrupt the cell walls followed by a heat-lysis method to extract DNA is the most efficient method for extracting DNA from *H. noduliformans*. Although this method yielded twice as much DNA than the other two extraction methods tested in our study (Fig. 2.5), this method also has other advantages which are not necessarily limited to this study. This method has previously been reported to be an efficient method for extracting and recovering DNA from a diverse range of marine invertebrates (Macey *et al.,* 2008a; 2008b), and to quantify the gene expression of lysozyme (Burge *et al*., 2007) in response to a pathogen challenge. Collectively, our study and these previous studies highlight the success of this method. This method does not require any specialised, expensive equipment and could be used in any laboratory that is fitted with some basic instruments. The extraction method optimised in this study could

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possibly be used to extract DNA from other organisms and may therefore not be limited to extracting only fungal DNA. It may also be possible to apply this method in the field or on a farm, because the only essential instrument needed is a warm water bath. This method allows for the extraction of good quality DNA in less than three hours and the resulting supernatant contains DNA that is immediately ready to be used in a PCR reaction.

The second objective of this study was to develop a real-time PCR assay and then validate it based on some of the criteria proposed by Hiney and Smith (1991) and Bott *et al.* (2010). One of the key objectives for developing the PCR assay was to ensure that it is highly specific and sensitive enough to specifically detect *H. noduliformans* DNA in abalone tissues obtained from animals at an early stage of infection. For this reason primer sets were specifically designed to amplify only *H. noduliformans* DNA. In the present study the LSU rRNA gene region was selected to design species-specific PCR primers for *H. noduliformans* since these genes are highly abundant within the eukaryotic cell and contain sufficient variable regions to distinguish between heterlogous species (Hillis and Dixon, 1991; Li *et al.,*1996).

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Molecular based diagnostic tools can provide quick results with high sensitivity and specificity and are therefore becoming more popular. Because of the increased interest and technological developments within this field, costs are rapidly coming down. Increased sample volumes also help to lower costs even further. Nevertheless, all diagnostic tools have some advantages as well as some

shortcomings (Li *et al*., 1996). One should clearly know what you want from a specific diagnostic tool and where it will be applied before deciding on which diagnostic tool to use. Currently in South Africa, abalone tubercle mycosis is diagnosed by gross observation and histopathology. When compared to other diagnostic tools, gross pathology probably has the fastest turnaround time for results (OIE, 2009). However clinical signs are not always visible and when it is visible it is not possible to diagnose the causative pathogen to species-level. This is the problem with abalone tubercle mycosis, since by looking at an infected area it is not possible to tell if the lesion or nodule is caused by *H. noduliformans* or any other fungal-pathogen. Histopathology is a better presumptive diagnostic tool than gross pathology and is often used as a confirmatory diagnostic for certain diseases, such as epizootic ulcerative syndrome (EUS) in fish (Songe *et al*., 2011). However, Macey *et al*. (2011) stated that using histopathology infected areas tended to be focal and circumscribed but to diagnose abalone tubercle mycosis specifically caused by *H. noduliformans*, histopathology lacks specificity. In contrast to histopathology, the diagnostic tool developed in the present study is highly target specific. Alignments of the HN.LSU-F/ R primer sequences with sequences of phylogeneticaly closely related species confirmed the high level of specificity of the developed primer set, showing at least two or more base pair differences, which has been shown to be enough to avoid cross-reactivity amongst heterlogous species (Li *et al*., 1996). The primer set developed in this study will only amplify *H. noduliformans* DNA in the presence of abalone tissue matrix that contains non-target DNA and will therefore be an ideal for targeted surveillance. This was confirmed when no cross-amplification occurred when screened against

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environmental isolates or when performing PCR assays in a non-sterile abalone tissue matrix. Furthermore no amplification occurred in the negative controls (NTC) that substituted PCR-grade water for DNA.

Diagnostic tools used for presumptive diagnosis, do not necessarily need to be highly sensitive. Gross pathology is often used for this purpose. However, when information on infection intensity is required, the sensitivity of a diagnostic tool becomes just as important as the specificity. This is one of the reasons why molecular based diagnostic tools, such as Q-PCR have advanced so rapidly. These methods are highly specific and sensitive (Bondad-Reantaso *et al*., 2005). Besides normal one-step Q-PCR assays, one could further improve the sensitivity of the reaction by performing a nested Q-PCR assay. By doing this we demonstrated an 80% increase in sensitivity (Chapter 3, Fig.3.6). However, there are a variety of factors that could possibly inhibit a PCR assay, resulting in lower sensitivity. For this reason, Hiney and Smith (1998) suggested testing an assay in non-sterile conditions to evaluate the effect that the physical and chemical properties of a tissue matrix may have on the performance of the overall dynamics of the PCR assay. In this present study, the specificity of the PCR assay was tested in pure cultures and in the presence of an abalone tissue matrix. We demonstrated a 27% reduction in the overall sensitivity of the assay when performed in the presence of abalone tissue matrix compared to dilution series made up with PCR-grade water. This finding supports the importance of including this step in diagnostic tool development, yet it been left out in many previous studies (Zhou *et al.,* 2000; Phadee *et al.*, 2004; Oidtmann *et al.*, 2004).

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We therefore suspect that the lower detection limits in many of these previous studies may have been over estimated.

The molecular based diagnostic tool developed in this study was found to be practical, specific, and sensitive. The information generated in this study may assist resource managers and farmers to better predict when an outbreak of abalone tubercle mycosis will occur and consequently prevent the establishment and spread of this disease within South African aquaculture facilities and also to the rest of the world. Bondad-Reantaso *et al*. (2005) classifies diagnostic tools into three different levels according to the resource expertise and infrastructure required to diagnose a disease. Field observation of animals and the environment, together with clinical examinations are listed in the first level. Histopathology and molecular biology are listed in levels 2 and 3, respectively. However, these authors propose that many countries move to higher levels of diagnosis, ultimately level three (Bondad-Reantaso *et al*., 2005). Research done in this study can provide a platform for more research on molecular based diagnostic tools. The primers designed in this study can be used to design probes to possibly improve the sensitivity even more. There are also other DNA based diagnostic tools available, such as the LAMP (Loop-mediated isothermal amplification) assay. This is a recently developed technique that amplifies nucleic acids under isothermal conditions (Saleh *et al.*, 2008). The LAMP assay does not require a thermal cycler and relies on four specifically designed primers, two inner primers and two outer primers (Soliman and El-Matbouli, 2005; Pillai *et al.,* 2006). This test can be simplified for use as a field test kit, as this reaction can be performed

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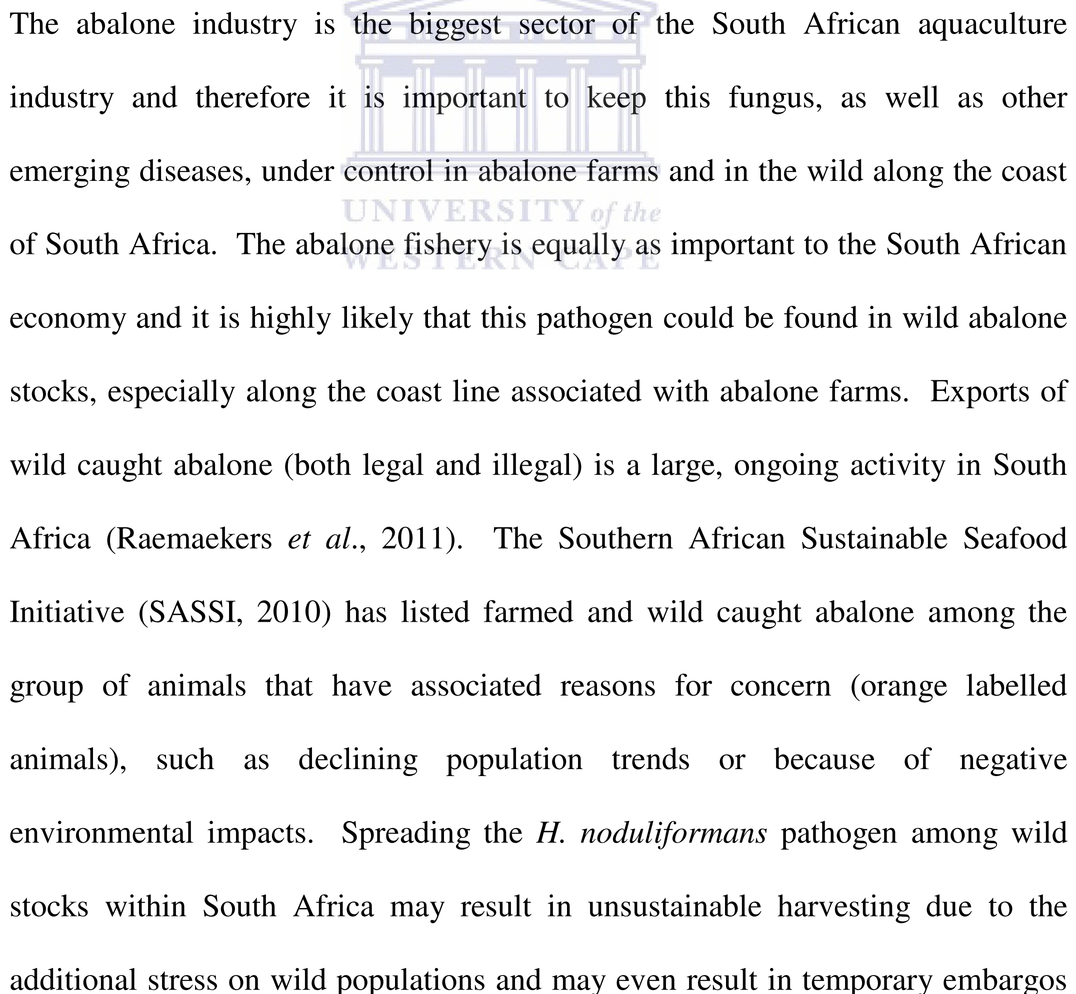
with a simple and inexpensive water bath (Pillai *et al*., 2006). Further research towards the detection of *H. noduliformans* DNA could assist in developing such a kit. Together with the DNA extraction method developed in the present study, which also requires only a water bath to extract good quality DNA, this could provide a more cost effective method to give an indication of infections caused by *H. noduliformans*. Thereafter, samples can be sent to more specialised laboratories to determine the level of infection using the molecular nested Q-PCR developed in the present study. Results from the laboratory should then be available in less than a day when the assay is followed as presented in this study.



The abalone industry is the biggest sector of the South African aquaculture industry and therefore it is important to keep this fungus, as well as other emerging diseases, under control in abalone farms and in the wild along the coast of South Africa. The abalone fishery is equally as important to the South African economy and it is highly likely that this pathogen could be found in wild abalone stocks, especially along the coast line associated with abalone farms. Exports of wild caught abalone (both legal and illegal) is a large, ongoing activity in South Africa (Raemaekers *et al*., 2011). The Southern African Sustainable Seafood Initiative (SASSI, 2010) has listed farmed and wild caught abalone among the group of animals that have associated reasons for concern (orange labelled animals), such as declining population trends or because of negative environmental impacts. Spreading the *H. noduliformans* pathogen among wild stocks within South Africa may result in unsustainable harvesting due to the additional stress on wild populations and may even result in temporary embargos

on the trade. For this reason, the conservation management system can also benefit from using the diagnostic tool developed in this study for the purpose of routine surveillance.

In conclusion, although abalone tubercle mycosis caused by *H. noduliformans* is currently under control in South Africa, it is crucial to have a reliable and rapid diagnostic tool in order to assist resource managers and farmers to better predict when an outbreak of abalone tubercle mycosis may occur. Both the objectives for this study have been met. We developed a diagnostic tool ideal for use as a confirmatory diagnostic tool specific for *H. noduliformans* which will contribute to the control and prevention of the spread of abalone tubercle mycosis, caused by *H. noduliformans,* in the South African abalone aquaculture industry and elsewhere in the world. This tool was validated based on two of the criteria proposed by Hiney and Smith (1991) and Bott *et al.* (2010) for the validation of molecular diagnostic tools. It is however suggested that an independent non-sterile field test be conducted to compare the results of the diagnostic tool developed in the present study to the results of a different diagnostic tool, such as histopathology. This will fulfil the last criteria proposed by Hiney and Smith (1998) as part of a complete validation process of a PCR based technique.

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