THE CHARACTERIZATION OF AN INTRACELLULAR PROTOZOAN PARASITE INFECTING THE DIGESTIVE GLAND OF ABALONE, *HALIOTIS MIDAE.*

BY

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Dedicated to my father Pieter Andries Cloete (11 Feb 1942- 27 Nov 2004) for always believing in me, my mother Esther Cloete for her loving support, my family and to uncle Nick Nel for teaching me about the ocean since I was little, your love and passion for marine life inspired me. Above all to God whom bestowed the privilege upon mankind to study, protect and care for al the fascinating animals on earth.

The beauty and genius of a work of art may be reconceived, though its first material expression be destroyed... but when the last individual of a race of living things breathing no more, another heaven and another earth must pass before such a one can be seen again.

William Beebe

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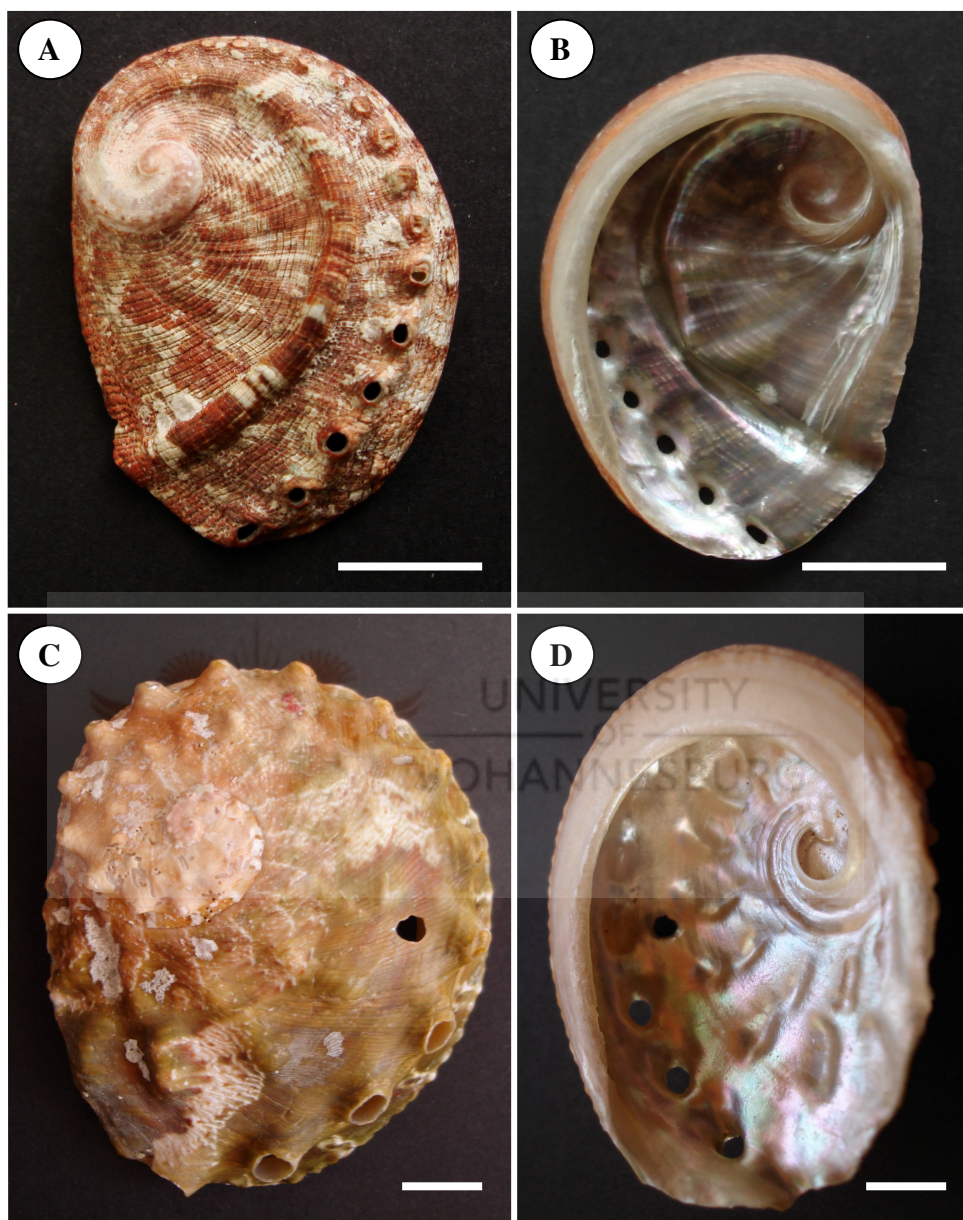
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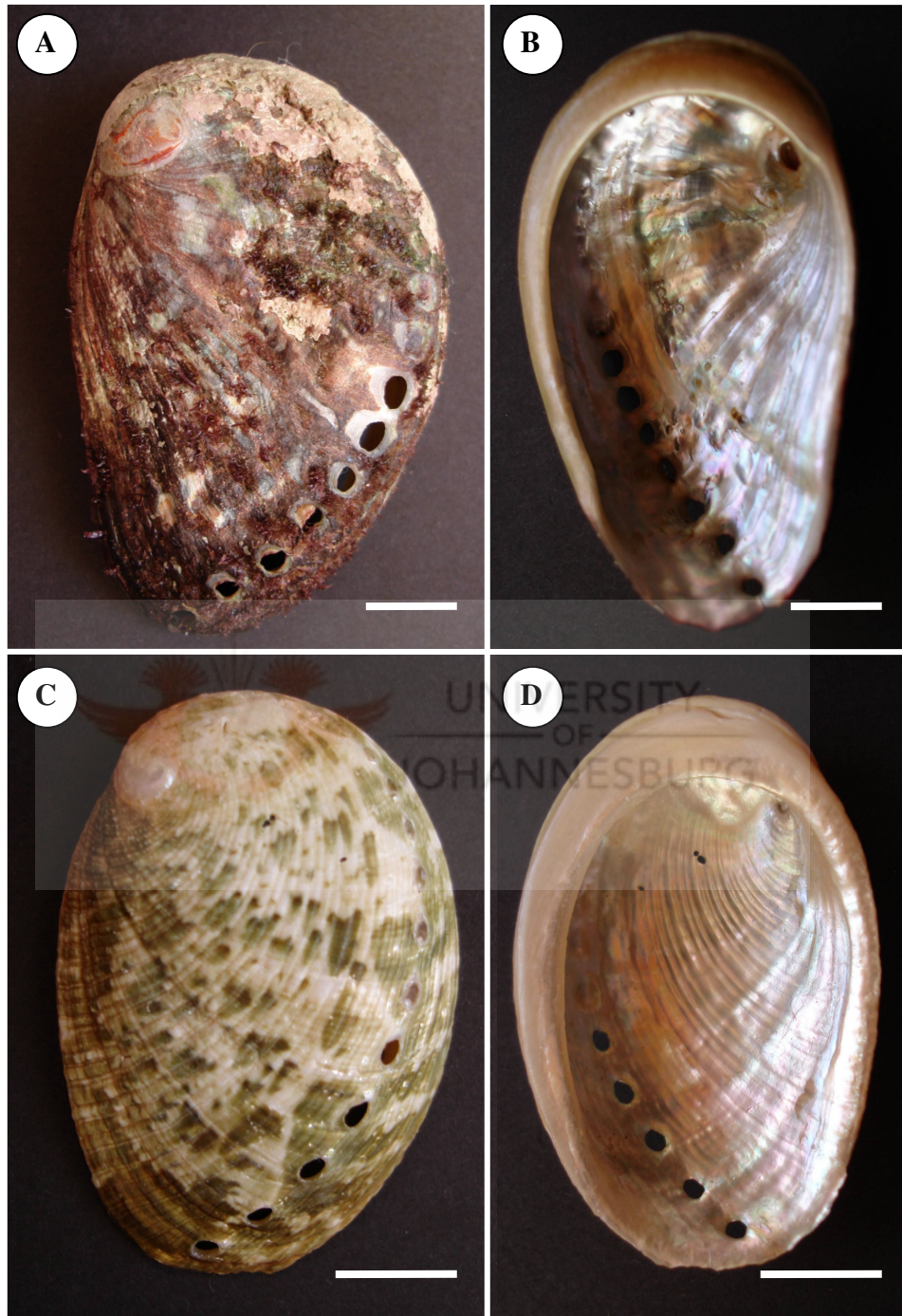
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A.

— Polar body
— Jelly coat
— Viteline space
— Viteline layer

B.

Egg
membrane
Cleavage
plane
Blastomere

C.

—Blastomere

D.

Minor cell
Major cell

E.

F.

Prototroch

Egg envelope
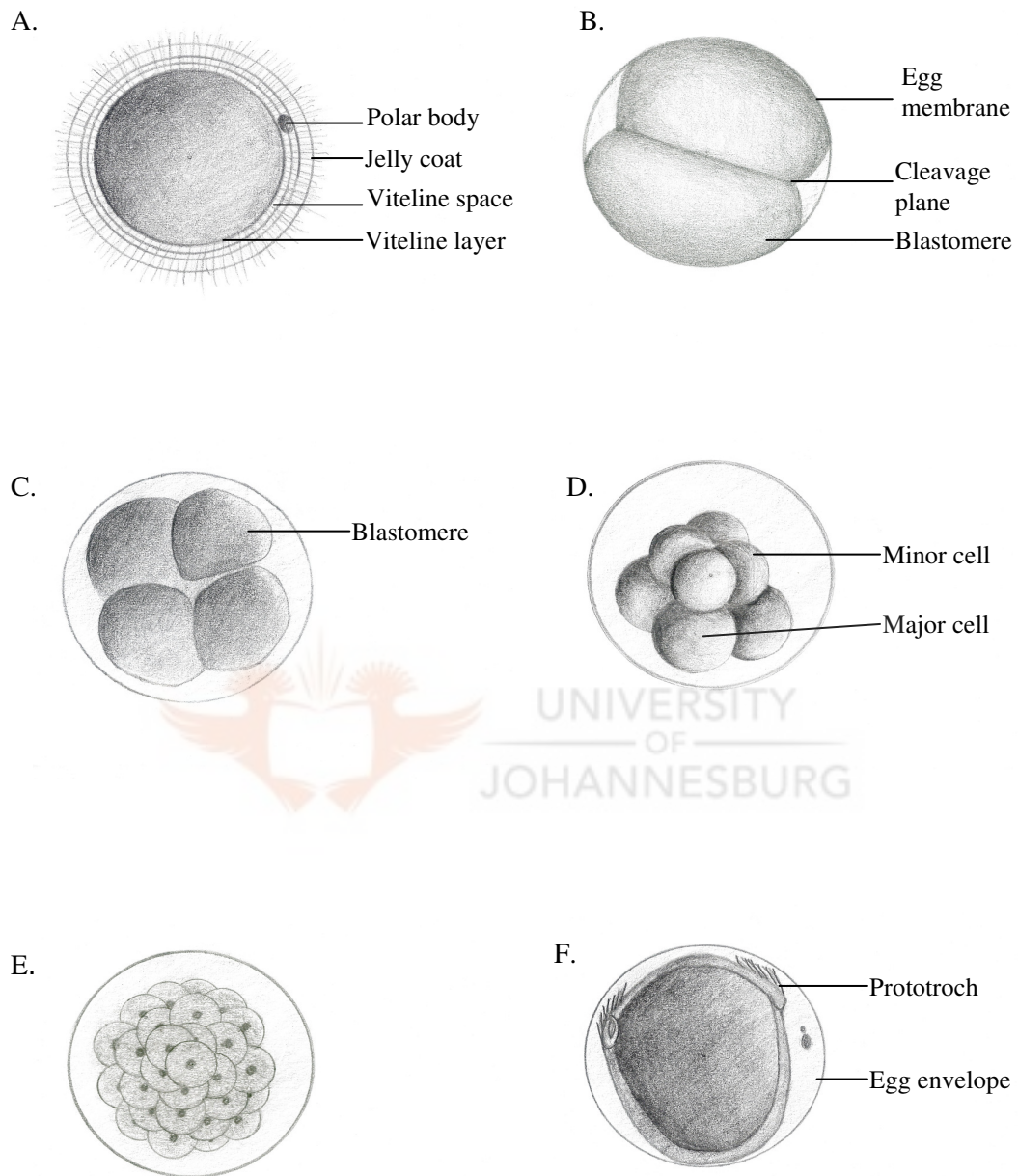
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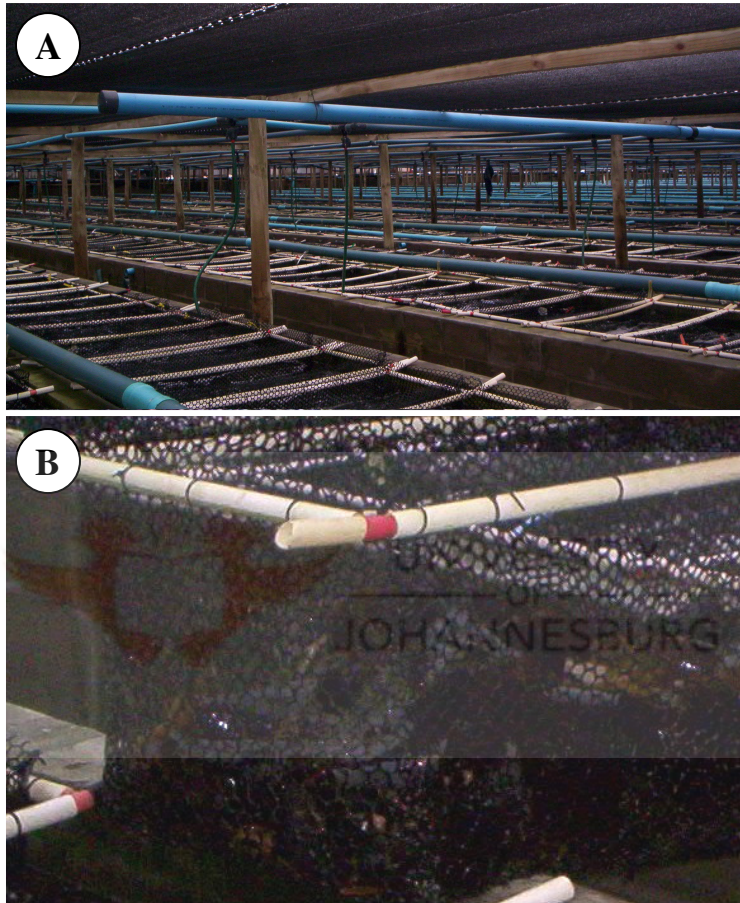
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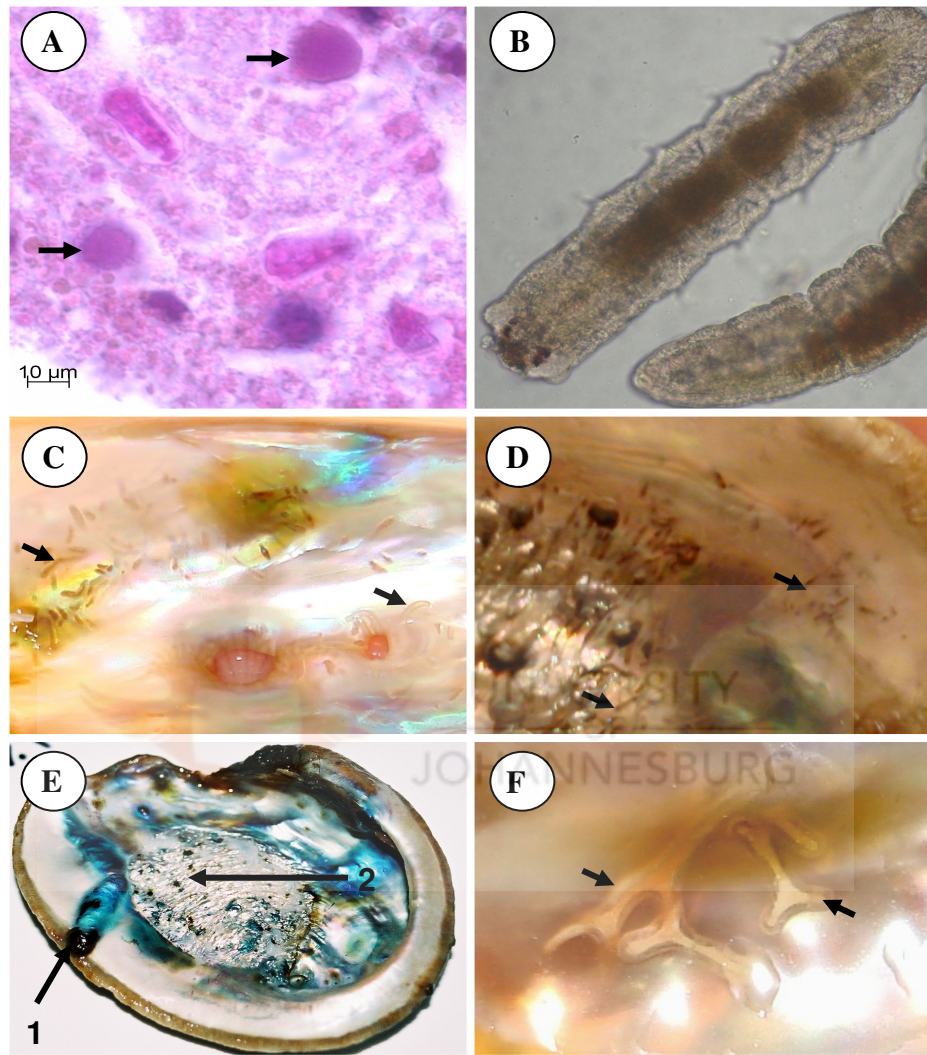
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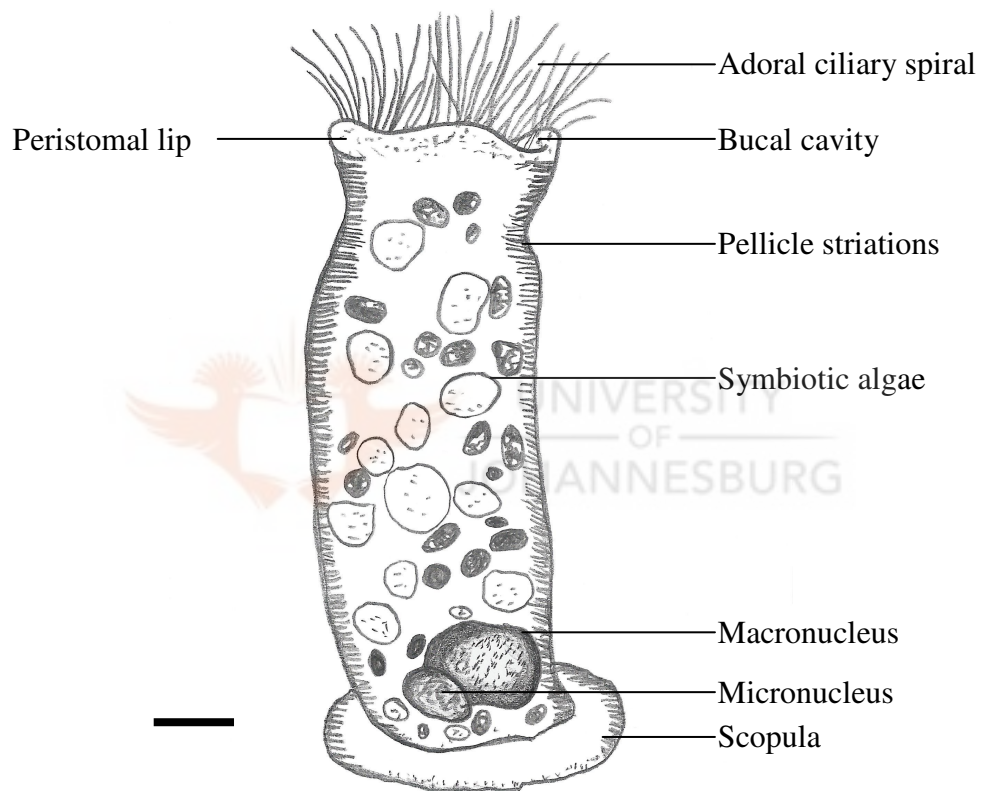
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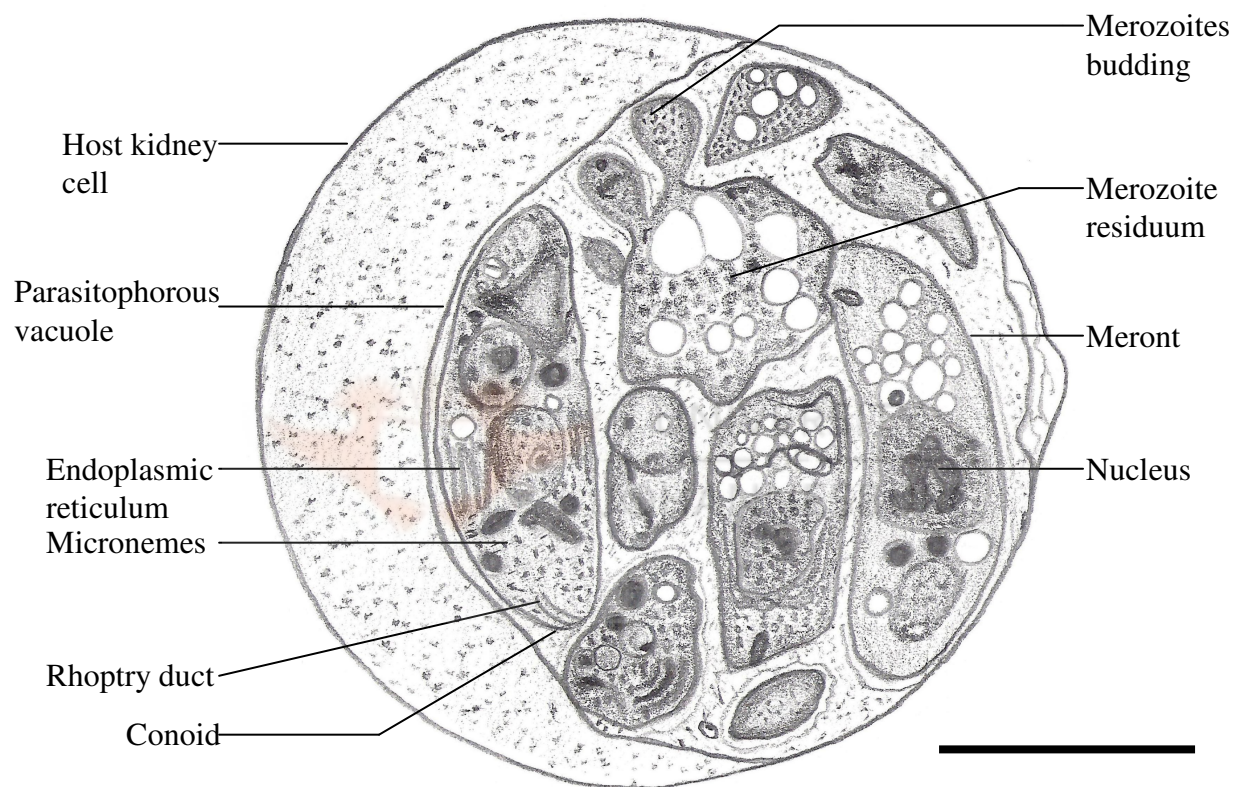
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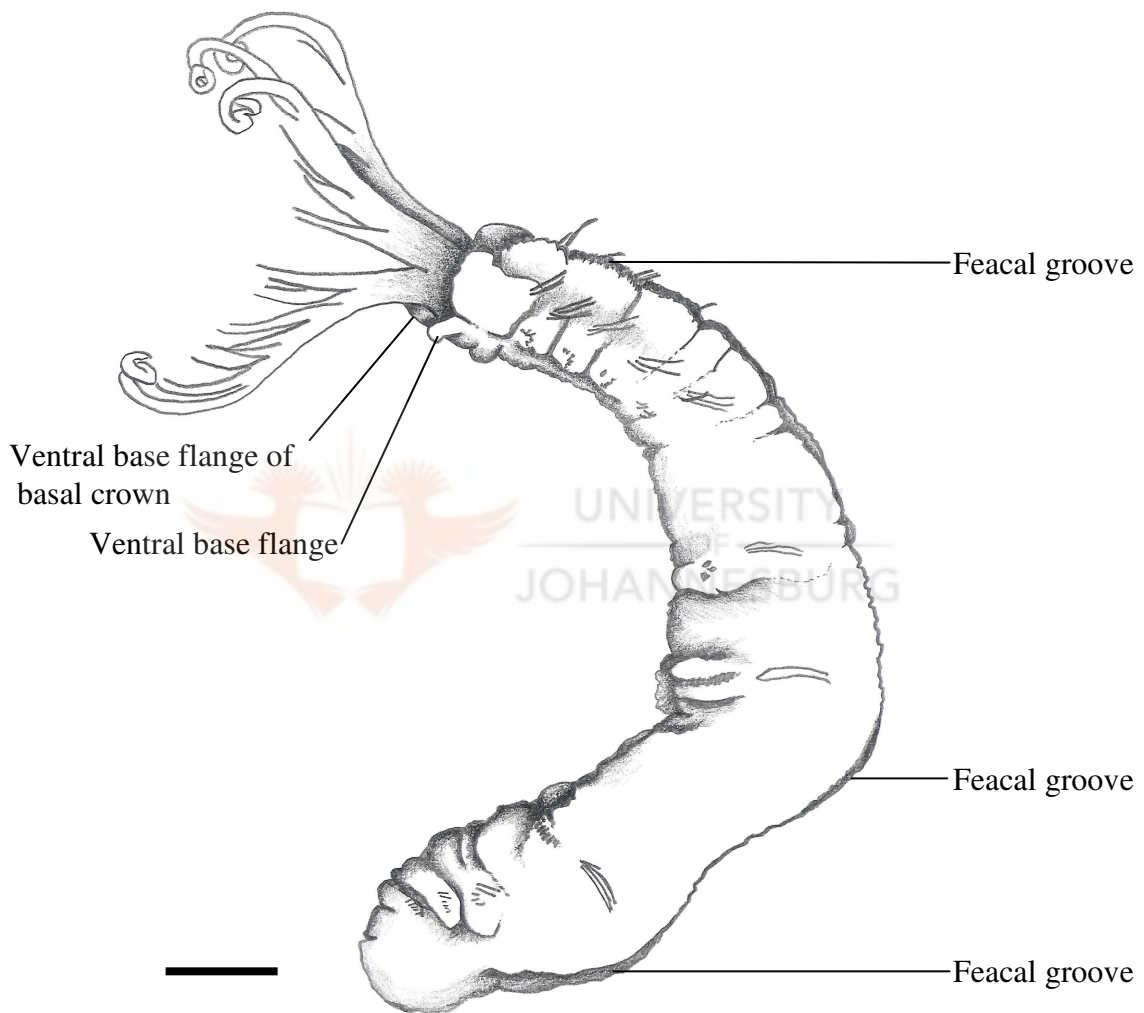
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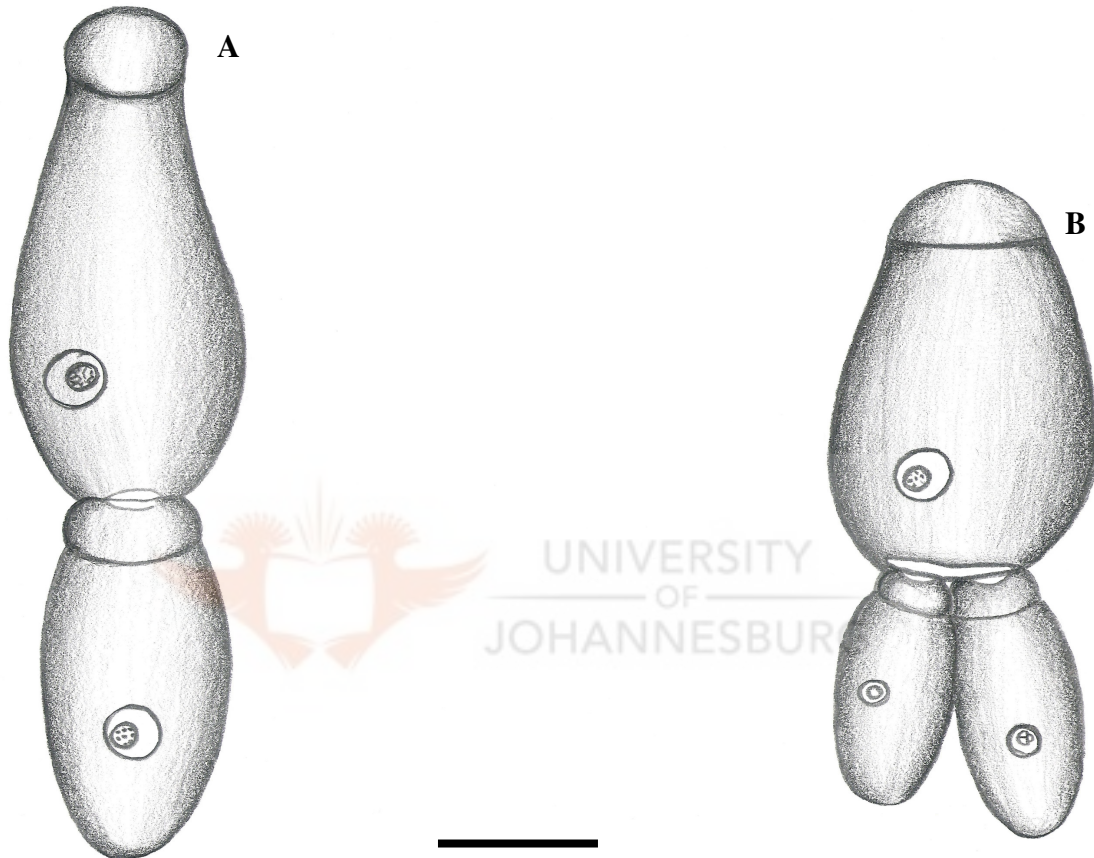
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Flange

Mitochondrion

Lid

Spherulosome

Haplosporosomes

Haplosporosome
- forming body

Epispore
cytoplasm
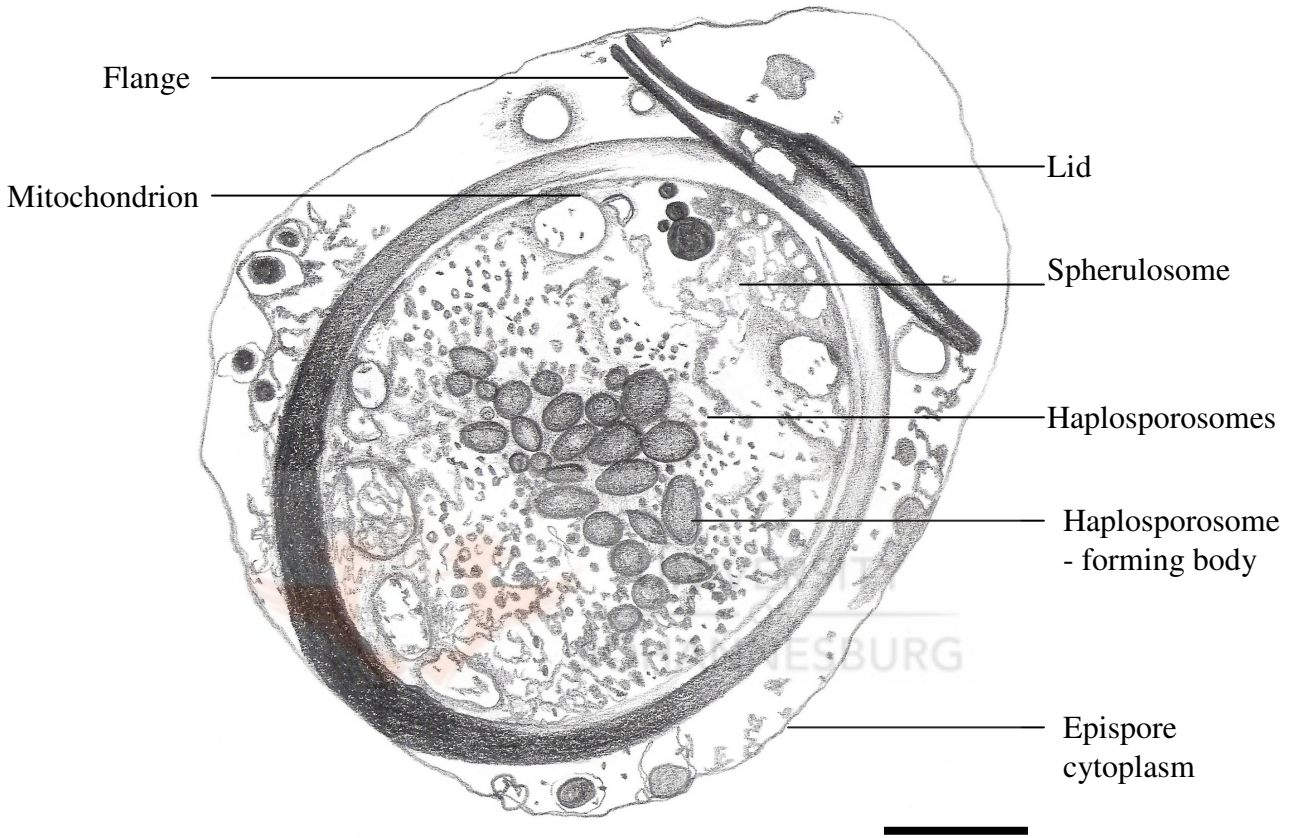
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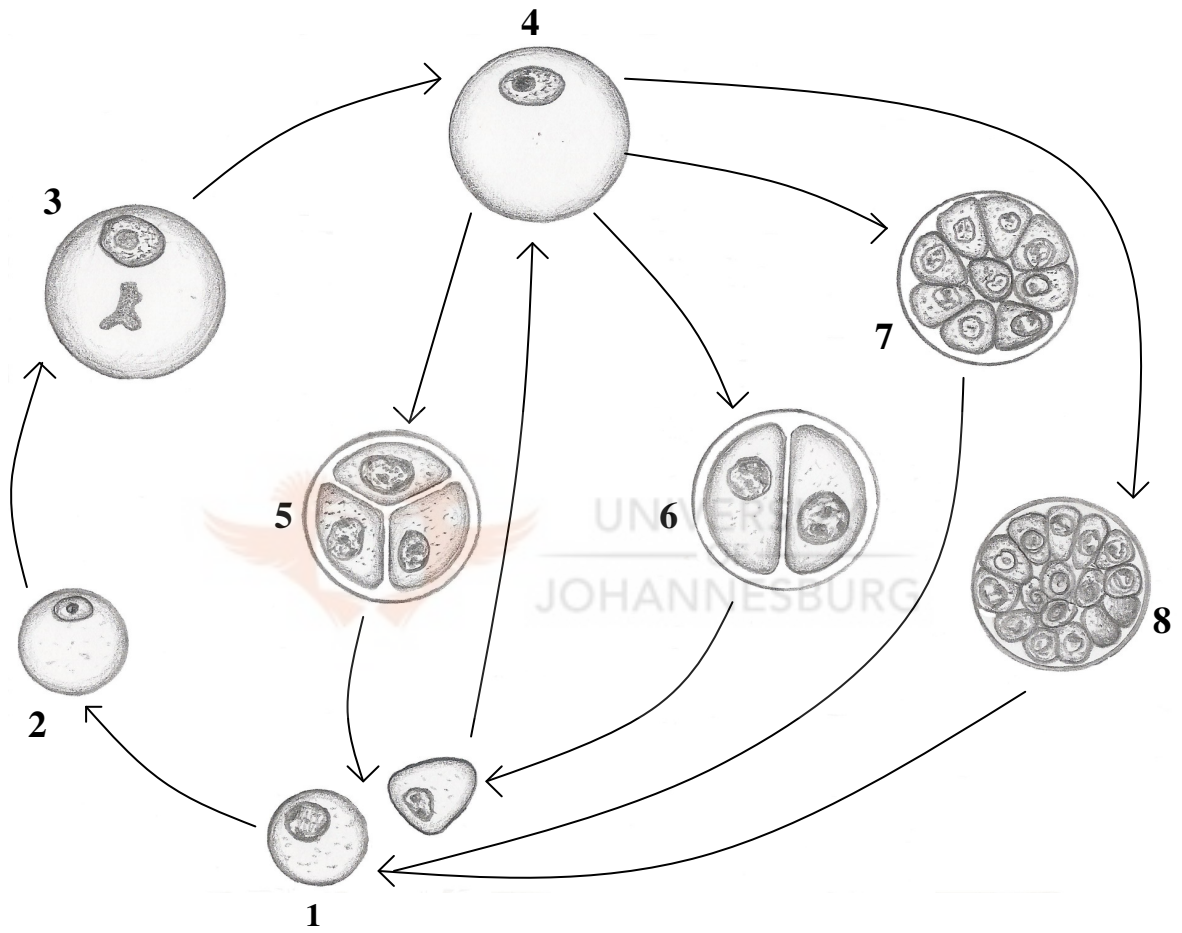
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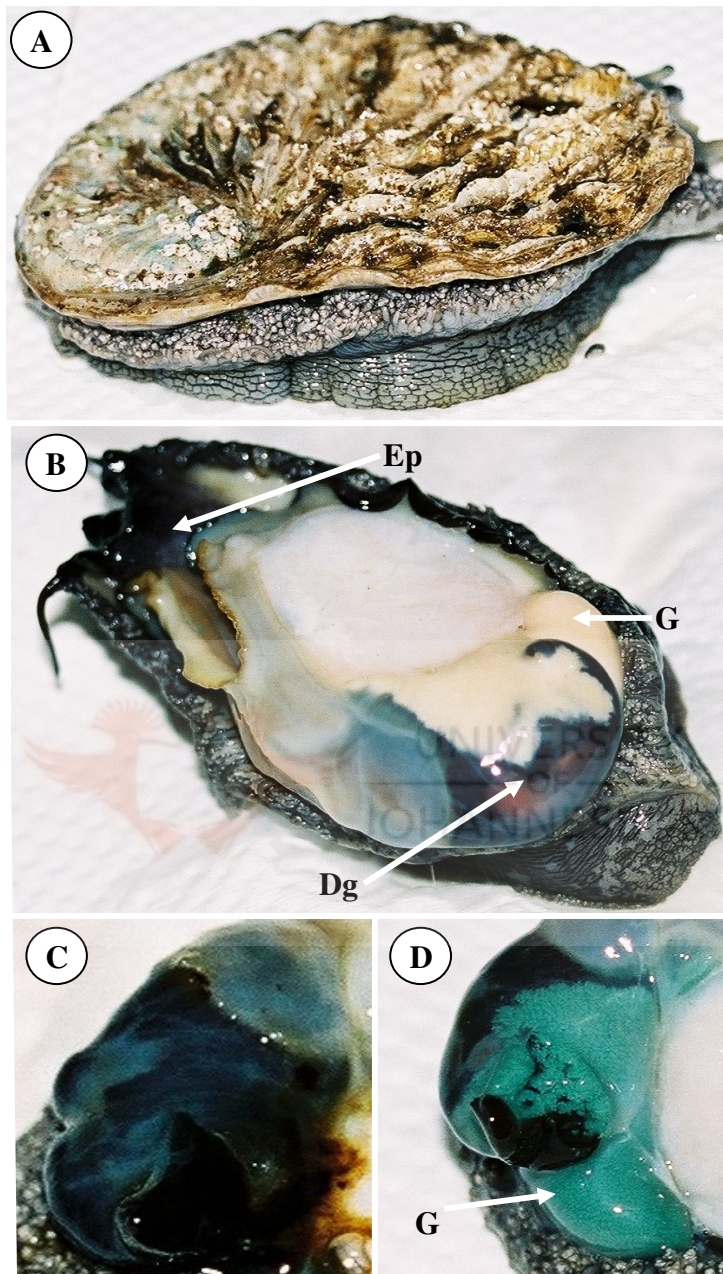
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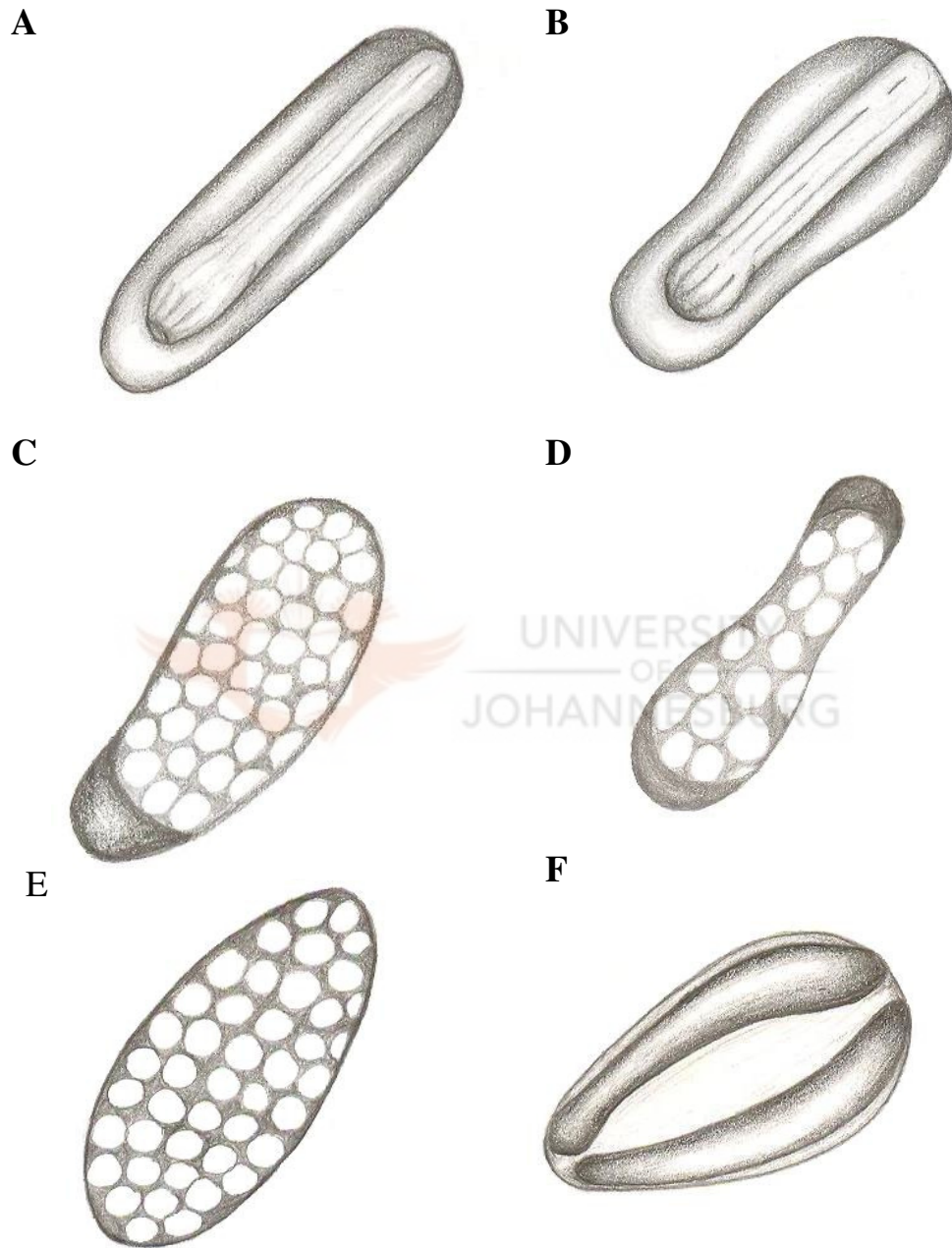
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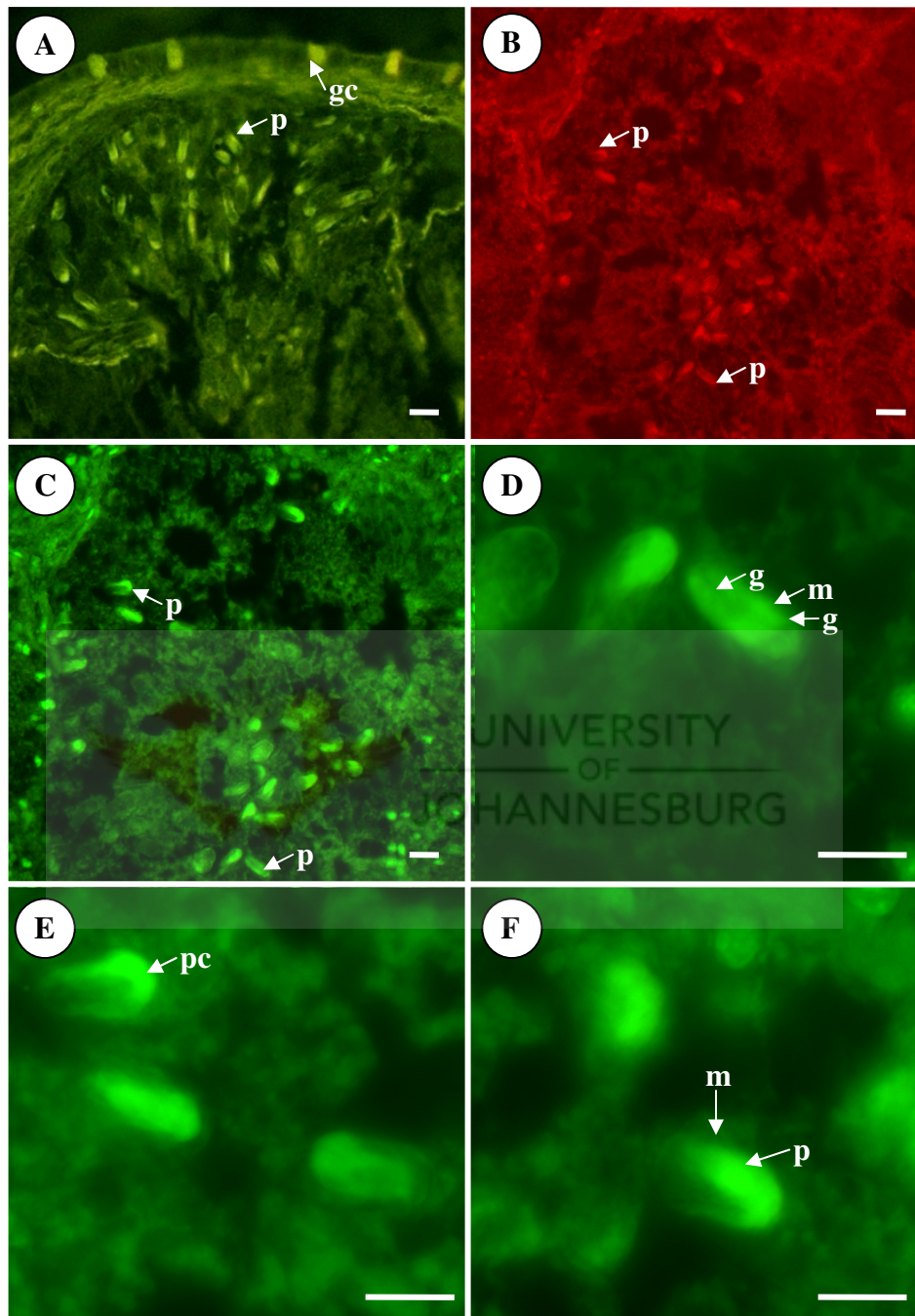
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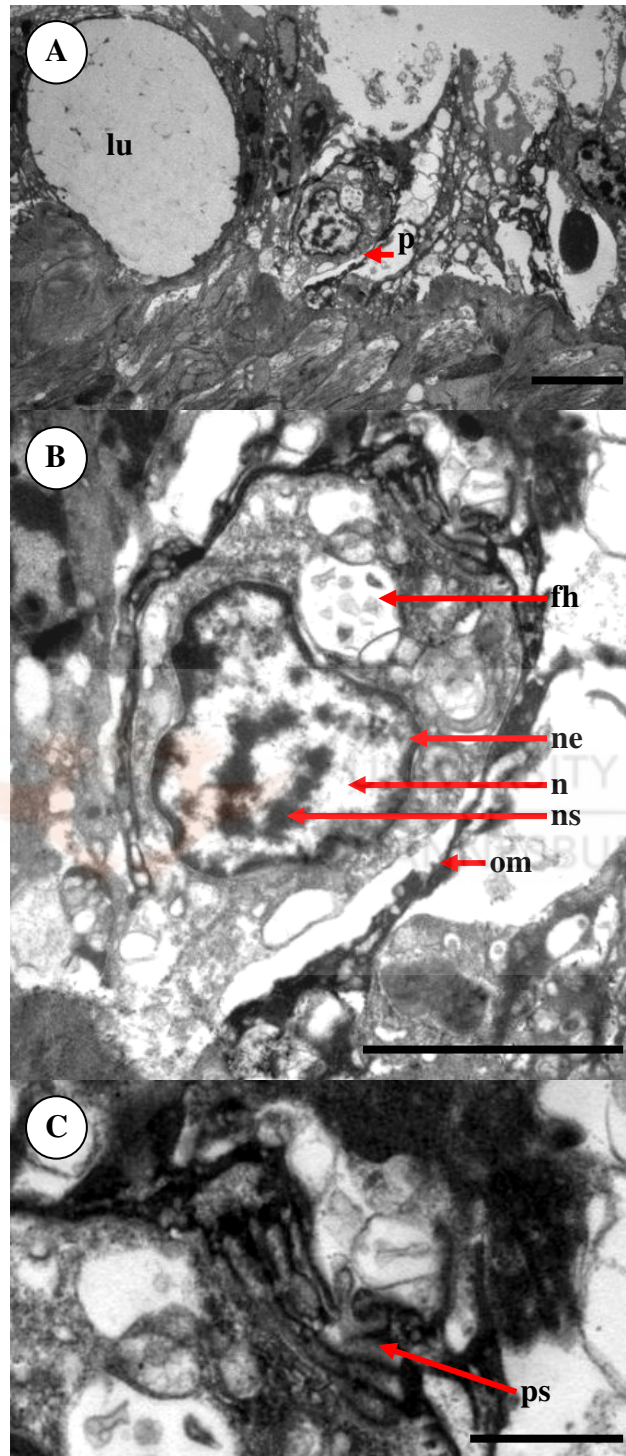
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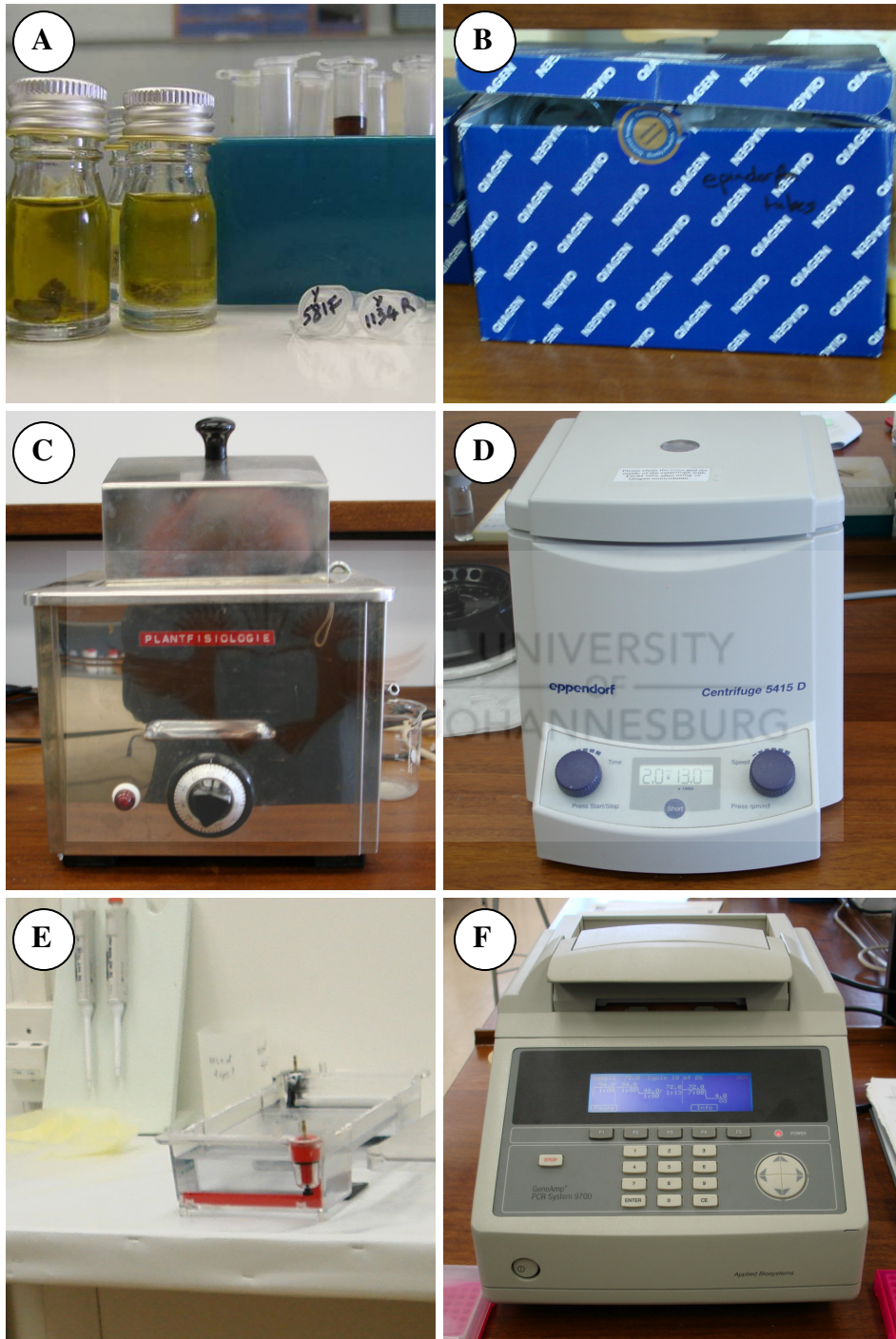
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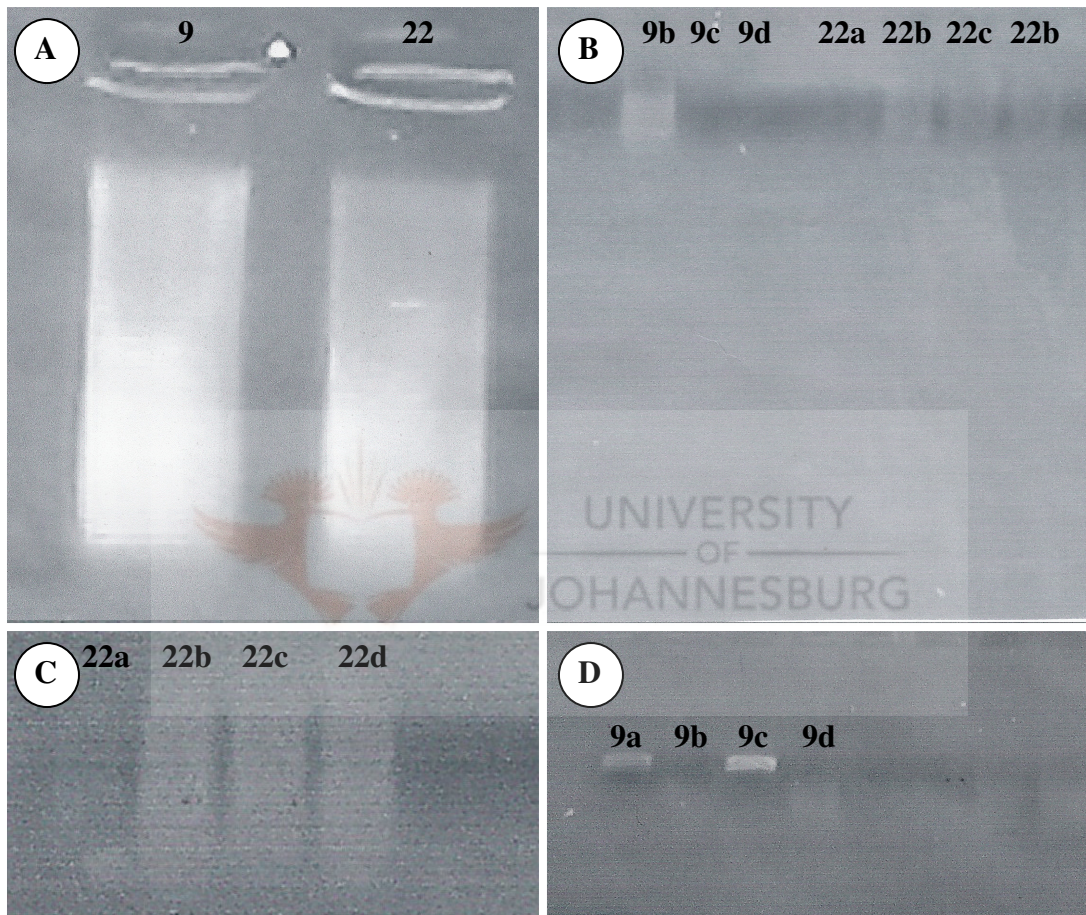
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SUMMARY

Abalone are among the world's leading shellfish consumed by human populations. Harvesting in California began in the late 1800s from intertidal zones and in the early 1900s wild abalone were collected by diving. Popular demand for abalone products in the Far East then led to extensive harvesting of wild abalone and a drastic decline in population numbers. This problem was overcome to a degree by the development of land-based abalone farms. At these farms it was possible to breed abalone on a large scale. Currently twelve abalone farms operate in South Africa and the estimated production for 2006 was 537 tons of meat, worth R 80 mil. Parasites and diseases pose threats to the production of abalone, especially under farmed conditions, and can cause considerable financial loss. *Labyrinthuloides haliotidis*, *Haplosporidium nelsoni* and *Terebrasabelle heterouncinata* are a few parasites that contribute to the above mentioned problems. Lately, a new protozoan parasite was discovered in the digestive glands of *Haliotis midae* farmed in the Western Cape Province, during routine health assessments. For the purposes of this dissertation it is designated an unidentified digestive gland parasite (UDP). The aims of this study are thus to undertake a comprehensive literature review of parasites infecting wild and farmed abalone, as well other shellfish species, describe and characterise the UDP infecting the digestive gland of *Haliotis midae* based on its structure and ultrastructure, evaluate the role of this parasite in disease by analysing data from histological studies, provide a preliminary indication of the life cycle of this parasite, attempt analysis of DNA from the UDP, and identify potential areas for further research into control of the parasite. A total of 180 abalone, (*Haliotis midae*) were collected from three abalone farms in the Western Cape during May 2005, October 2005, January 2006 and January 2007. To establish whether this parasite also occurs in wild abalone, a single sampling (six *H. midae* and 28 *H. spadicea*) took place during 2006 in Tsitsikamma National Park. Collected farmed and wild abalone were weighed and measured, removed from their shells and then killed according to accepted methods before their digestive glands were removed. Each digestive gland was cut into four pieces. The first piece of each sample was placed into a histological cassette and fixed in 10% neutral buffered formalin; the samples were then prepared for histological studies using standard

methods. The second and third pieces were fixed in Karnovsky's and 4% paraformaldehyde fixatives respectively for transmission electron microscopy (TEM). The remaining piece was fixed in 96% ethanol for DNA analysis. The presence of UDP in the digestive glands was determined by histology, and the parasite's ultrastructure was then studied by TEM. The prevalence of UDP was as follows: 10% for May 2005 (Farm A), 3.2 % for October 2005 (Farm A), 35.5% for January 2006 (Farm B) and 40% for January 2007 (Farm B). The prevalence was 0% for May 2005 (Farm B & C) and April 2006 (Tsitsikamma National Park). In histological sections the UDP was usually fiddle-shaped, or hour-glass shaped with a parasitophorous vacuole surrounding it and six presumed life stages were observed, although no obvious pathology was seen. Structures resembling those characteristic of haplosporidians (formative region for haplosporosomes and possible spherulosome) were visible in electronmicrographs. The UDP was then compared with parasites occurring in abalone throughout the world, as well as in other shellfish species. This was done mainly using its morphology, morphometrics and ultrastructural characteristics. In comparison with other parasites occurring in abalone, the UDP parasitizes the digestive gland tubules of *H. midae*, as the coccidian *Pseudoklossia haliotis* occupies the kidney tubules of the same type of host. The morphometrics of UDP and the haplosporidian *Haplosporidium nelsoni* overlap and the plasmodial stages of haplosporidian species can occur close to the basal lamina and upper gut, as does UDP. The apicomplexan *Perkinsus* species in general, from other shellfish, have life stages that divide, as do those of UDP. Microsporidians also possess a posterior vacuole and polar caps that are similar to those seen in the UDP. No exact matches could be made between the UDP and other protozoan parasites; the fit with the haplosporidians appeared closest, but unfortunately this could not be verified by DNA analyses, since attempts at these techniques were unsuccessful. However, overall the results from this study make an interesting contribution to the knowledge of farmed abalone parasites and diseases in South Africa and suggestions for extending the research appear in the latter sections of the dissertation.

OPSOMMING

Perlemoen is een van die voorste skulpvis spesies in die wêreld wat deur die menslike polulasie verbruik word. Die oes van intergetysone perlemoen in Kalifornia het in die laat 1800's begin en in die vroeër 1900's is perlemoen deur duikers versamel. Die populêre aanvraag na perlemoen produkte in die verre Ooste het gelei tot die uitgebreide oesting van perlemoen, gevolglik het die populasie getalle drasties afgeneem. Hierdie probleem is tot 'n mate oorkom deur die ontwikkeling van perlemoen plase. By hierdie plase was dit moontlik om perlemoen op grootskaal te kweek. Huidiglik is twaalf plase funksioneel in Suid-Afrika. Die geraamde produksie vir 2006 was 537 ton vleis, terwaarde van R80 miljoen. Parasiete en siektes hou 'n groot bedreigings in vir die produksie van perlemoen, veral onder plaastoestande, en kan aansienlike finasieële verlies tot gevolg hê. *Labyrinthuloides haliotidis*, *Haplosporidium nelsoni* en *Terebrasabella heterouncinata* is 'n paar van die parasiete wat bydra tot die bogenoemde probleem. Onlangs is 'n nuwe protozoa parasiet ontdek in die verteeringsklier van gekweekte perlemoen afkomstig van die Wes Kaap, gedurende 'n roetine gesondheids assesering. Vir die doel van hierdie verhandeling staan dit bekend as die ongeïdentifiseerde verteeringsklier parasiet (UDP). Die doel van hierdie studie is die volgende: om 'n deeglike literatuur oorsig van die parasiete wat wilde en gekweekte perlemoen afekteer asook die van ander skulpvis spesies te onderneem, om die UDP wat die verteeringsklier van *H. midae* infekteer te beskryf en te karakteriseer gebaseer op sy strukture en ultrastrukture, om die rol van hierdie parasiet in siektes te evalueer volgens die analise van data afkomstig van histologiese studies, om 'n voorlopige aanduiding van hierdie parasiet se lewens siklus weer te gee, om DNA analisis van die UDP te onderneem en om potensieële areas vir verdere navorsing oor die beheer van hierdie pasasiet te identifiseer. 'n Totaal van 180 perlemoen (*H. midae*) was versamel van drie perlemoen plase in die Wes Kaap gedurende Mei 2005, Oktober 2005, Januarie 2006 en Januarie 2007. 'n Enkele versameling van ses *H. midae* en 28 *H. spadicea* het plaasgevind by die Tsitsikamma Nasionale Park gedurende 2006 om te bepaal of hierdie parasiet ook in wilde perlemoen voorkom. Versamelde en gekweekte perlemoen was geweeg en gemeet, verwyder van die skulp en is dan van kant gemaak volgens aanvaarde metodes waarna

die verteeringsklier verwyder is. Elke verterings klier was dan in vier stukke verdeel. Die eerste deel van elke monster is geplaas in 'n histologiese kaset waarna dit gefikseer is in 10% nutraal gebufferde formalien en is dan voorberei vir histologie volgens standaard metodes. Die tweede en derde dele is onderskeidlik gefikseer in Karnovskies en 4% paraformaldehied vir transmissie elektron mikroskopie (TEM). Die oorblywende deel is gefikseer in 96% etanol vir DNA analise. Die teenwoordigheid van UDP in die verteeringsklier is bepaal deur histologie en die pasasiet se ultrastrukture was gebestudeer deur middel van TEM. Die persentasie besmetting van UDP was soos volg: 10% vir Mei 2005 (Plaas A), 3.2% vir Oktober 2005 (Plaas A), 35.5% vir Januarie 2006 (Plaas B) en 40% vir Januarie 2007 (Plaas B). Die persentasie besmittig was 0% vir Mei 2005 (Plaas B & C) en April 2006 (Tsitsikamma Nasionale Park). In die histologiese senee was die UDP gewoonlik viool of uurglas vormig en was omring met 'n parasitoforus vakuool. Ses moontlike lewens staduims is opgemerk al was daar geen duidelike patologie waargeneem nie. Strukture voorstellend van diè, kenmerkend aan haplosporidia (voremende area vir haplosporosome en moontlike spherolosome) was duidelik met elekton micrografieke. Die UDP was toe vergelyk met parasiete wat in perlemoen spesies van reg oor die wêreld, sowel as in ander skulpvis spesies voorkom. In die vergelykings met perlemoen parasiete, parasiteer die UDP die verteeringsklier buisies van *H. midae* net soos in die geval van die Koksidia parasiet *Pseudoklossia haliotis* wat die nierbuisies van dieselfde tipe gasheer parasiteer. Die meetings van UDP en die van die haplosporidia, *Haplosporidium nelsoni* oorvleul. Plasmodium stadia van ander haplosporidia spesies kom naby die basale lamina en die bo-derm voor net soos in die geval van UDP. Algemene *Perkinsus* spesies (Apikompleksa) van ander skulpvis het stadia wat verdeel net soos UDP. Mikrosporidia besit 'n posterior vakuool en polêre kapsels wat soortgelyk is aan die wat waargeneem word in UDP. Geen perfekte ooreenstemming kon gemaak word tussen UDP en ander protozoa nie, die ooreensteming met haplosporidia blyk die naaste, maar ongelukkig kon dit nie met die DNA analise geverifiseer word nie, aangesien die poging onsuksesvol was. Oor die algemeen maak die resultate van hierdie studie 'n interressante bydra tot die kennis van gekweekte perlemoen parasiete en siektes in Suid-Afrika. Voorstelle vir die uitbreiding van die navorsing kom voor in die laaste deel van hierdie verhandeling.

1. Introduction

Abalone is one of the most important and valuable exported shellfish products in the world (McShane 1992; Ebert & Houk 1984; Sales & Janssens 2004; Reddy-Lopata, Auerswald & Cook 2006). This popularity is due to the foot muscle meat of the abalone that is considered as a great delicacy globally and also to the shells that are used in the production of jewellery (Alvarez-Tinajero, Caceres-Martinez & Gonzalez-Aviles 2001). Abalone farming and commercial diving are globally as well as locally very lucrative businesses (Macey & Coyne 2005), contributing millions of dollars to countries exports and economy. Besides this the abalone industry creates considerable job opportunities (Macey & Coyne 2005; Cai, Chen, Thompson & Li 2006) to local people in various countries (Troell, Robertson-Andersson, Anderson, Bolton, Maneveldt, Halling & Probyn 2006).

1.1 Global abalone industry

1.1.1 The history of the abalone industry

In California, abalone were harvested from intertidal zones by native people for thousands of years (Davis, Richard, Haaker & Parker 1992; Lafferty & Kuris 1993; Davis 1993; Moore, Finley, Robbins & Friedman 2002). In the 1800s Asians harvested abalone by hand (Davis 1993), and in the early 1900s abalone were collected from subtidal regions by diving (Davis 1993). During the 1950s to 1960s abalone became a popular fishery and sport activity (Davis *et al*. 1992): the abalone was collected by scuba diving to depths of 100ft and more. Throughout 1950 to 1970 commercial divers in southern California harvested over one million pounds of abalone.

From the 1970s, intertidal abalone populations decreased (Moore *et al*. 2002) and became rare (Davis *et al*. 1992). By 1996 less than 10% of the annual take could be harvested by the commercial divers. Abalone aquaculture commenced in California in the 1960s (McBride 1998). Research on the spawning of abalone (Morse, Duncan, Hooker & Morse 1977) and the settlement of larvae were conducted successfully in

the 1970s in an attempt to cultivate abalone (Morse, Hooker, Duncan & Jensen 1979). In 1981 the first cultivated abalone from California were sold (McBride 1998).

1.1.2 Impact of parasites and diseases on the abalone industry

In the history of commercial shellfish mariculture, disease outbreaks have been common and have mostly led to mass mortalities which in turn have been responsible for the failure of farming and commercial operations (Kuris & Culver 1999; Simon, Kaiser & Britz 2004). An example of this has been the demise of abalone farming operations in British Columbia, Canada where almost all the juvenile abalone died due to a thraustochyrid parasite (Bower 2000). High abalone densities and poor husbandry are perfect combinations for overwhelming diseases and parasites to accumulate, and spread between hosts (Cook 1998). Clearly parasites and diseases of abalone can have a devastating financial impact on abalone farming and the industry.

Hine and Jones (1994) listed the following points as being of significant economical importance when looking at parasites of cultured fish and shellfish species:

- Parasites induce host mortalities leading to reduced harvest, this includes mortalities of juveniles and so reduces future recruitment to the fisheries
- Weight loss in infected hosts, which are still saleable, but represent a reduction in return to the fisher
- Parasite induced reduction in fecundity, leading to a poorer than expected spawning and a subsequent poor recruitment to the host population
- Reduced flesh quality, which results either in additional processing costs or in rejection of product. The effects can be visual, or chemical changes in the fillet such as enzyme degradation, or the public health risk associated with the presence of the parasite
- The cost of complying with regulatory requirements associated with the actual or potential presence of the parasite, this affects the fish processor as well as the aquaculturalist
- Cost to aquaculturalists of prophylactics

1.1.3 Quality regulations of abalone products

Quality control aspects for live, exported, animals determine that abalone should be in an impeccable condition: the shell must not be damaged or deformed, and the foot muscle (meat) must show no signs of discoloration and damage, as this part of the abalone is after all the entity of value. Damage to the foot muscle includes pustule formations or sores. Animals that are not up to standards are classified as rejects and are processed as canned products.

1.1.4 Well known parasites and diseases of abalone

A wide variety of abalone parasites and diseases have been studied in the past (see Chapter 4). Globally, the most economically important species include: *Terebrasabella heterouncinata* described as a new genus and species by Fitzhuge & Rouse, 1999 (Kuris & Culver 1999) that occurs in abalone species of California (Ruck & Cook 1998) and originated from South Africa (Fitzhuge 1996; Finlay, Mulligan & Friedman 2001) where it is an important health issue (Simon *et al*. 2004).

Boccardia knoxi Rainer, 1973, and *Polydora haplura* Claparede, 1870 (Blake & Evans 1973) are two examples of spionid mud worms that devastated abalone culture in Tasmania and inevitably led to many facilities closing or relocating in 1997 (Kuris & Culver 1999; Simons *et al*. 2004). An unidentified *Haplosporidium* sp., closely related to the genus *Urosporidium*, has been studied in *Haliotis iris* Martyn, 1784 (Diggles, Nichol, Hine, Wakefield, Cochennec-Laureau, Roberts & Friedman 2002). *Labyrinthuloides haliotidis* Bower, 1987 parasitises mainly juvenile abalone (Bower 1987a; b). Withering syndrome caused by the Rickettsiales-like prokaryotes (bacterium) *Candidatus Xenohaliotis californiensis* (Friedman 2002) has led to the devastation of wild and cultured abalone populations from California (VanBlaricom, Ruediger, Friedman, Woodard & Hedrick 1993; Moore *et al*. 2002) and of lesser economic importance is *Pseudoklossia haliotis* Friedman, Gardner, Hedrick, Stephenson, Cawthorn & Upton, 1995, that was described as a coccidian infecting the kidneys of *Haliotis* spp. (Friedman *et al*. 1995).

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Parasites have different impacts on abalone, some causing shell deformities (eg. *T. heterouncinata*) (Kuris & Culver 1999; Simon *et al*. 2004) and severe shell damage (eg. *B. knoxi* & *P. hoplura*) (Blake & Evans 1973; Kuris & Culver 1999; Lleonart, Handlinger & Powell 2003a; 2003b), others destroying the foot muscle through pustule formation eg. *Perkinsus olseni* Lester and Davis, 1981 (Lester & Davis 1981; Goggin & Lester 1987) in *Haliotis rubra* Leach, 1814 (Goggin, Sewell & Lester 1989) and *Haliotis laevigata* Donova, 1805 (Lester 1986). Protozoan parasites can damage vital organs (eg. *P. haliotis*) (Steinbeck, Groff, Friedman, McDowell & Hedrick 1992) leading to growth retardation, behavioural changes and even death (eg. Haplosporidia) (Diggles & Hine 2001).

1.1.5 Abalone and the environment

Environmental changes have also had overwhelming effects on the wild abalone population numbers. Catastrophic El Niños occurred quite frequently from the 1980s to the 1990s around California (Moore *et al*. 2002). The water temperatures elevated and nutrient levels declined (Davis *et al*. 1992) leading to the withering of kelp beds (Haaker 1997) and amongst other events, the starvation of abalone (Friedman, Thomson, Chun, Haaker & Hedrick 1997).

1.2 South African abalone industry

1.2.1 Abalone farming

Abalone farming in South Africa is quite a young industry (Cook 1998), compared with established farms across the world (see Chapter 3). Farming commenced in the late 1980s when a gravid wild *Haliotis midae* Linnaeus, 1758 that had been collected spawned spontaneously (Sales & Britz 2001a) and was spawned artificially thereafter. The industry mostly focuses on visual parasites like polychaetes (Simon *et al*. 2004) and intracellular parasites. The spionid polychaetes *Polydora hoplura*, *Dipolydora capensis* (Day) and an unidentified *Boccardia* species have been noted as being problematic in *Haliotis midae* (Lleonart *et al*. 2003a; 2003b; Simon, Ludford & Wynne 2006).

1.2.2 South African regulations on abalone quality

Methods of controlling the standard and quality of abalone production in South African farms are implemented by the Abalone Farmers Association. In 1999, a health management program was started in an attempt to control diseases and parasites (Mouton 2000). Routine health assessments took place and a range of new undescribed parasites were discovered upon sampling and examination of the abalone for possible diseases (Sales & Britz 2001a).

1.2.3 Unknown parasites of abalone in South Africa

During these routine health assessments conducted in 2001 in the Western Cape, an unknown protozoan parasite was discovered in the digestive gland of farmed *H. midae* (Mouton 2000; Sales & Britz 2001a). Initial transmission electron microscopy studies indicated that the parasite might be an apicomplexan due to the presence of organelles resembling those of the apical complex (A. Mouton, pers. comm.). Literature reviews were conducted to determine if parasites resembling this one had been described in the past. The only parasite (also unidentified) similar to the unidentified protozoan found in *H. midae* was from wild abalone in Tasmania (J. Handlinger, pers. comm.).

Diagnosis and descriptions of unknown parasites may take many months or years to complete, and afterwards the research into the biology and control of the parasite can also be extensive. Through the help and funding of Marine and Coastal Management, the current project was initiated to study this enigmatic digestive gland protozoan.

The hypotheses for this study are that the parasite is a member of the protozoan order Apicomplexa, that it has no detrimental effect on the growth and health of the host, and that *H. midae* could be its intermediate invertebrate host.

The aims of this study are thus to:
- undertake a comprehensive literature review of parasites infecting wild and farmed abalone

- undertake a comprehensive literature review of parasites infecting other important shellfish species

- describe and characterise the intracellular protozoan parasite infecting the digestive gland of *Haliotis midae* based on its ultrastructure

- evaluate the role of this parasite in disease by analysing data from histological studies

- give a preliminary indication of the life cycle of this parasite

- attempt DNA analysis of this parasite

- identify potential areas for further research into control of the parasite.

Aspects that will be covered in this dissertation include the basic biology and ecology of abalone species occurring in South Africa, how a typical abalone farm works, a literature review on abalone parasites, parasites of other economically important shellfish, techniques used in this study and the results thereof, as well as the classification of this unknown parasite.

The editorial style of this dissertation follows that of the journal *African Zoology* (see Appendix 1).
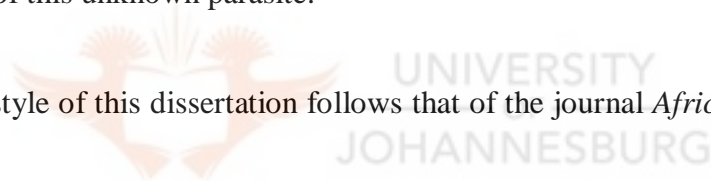
2. Wild Abalone

Nearly a hundred abalone species occur around the world (Table 2.1), but merely a few are of any commercial value (Table 2.2) (Sales & Janssens 2004). Numerous species have become vulnerable to over-exploitation and poaching (Reddy-Lopata *et al.* 2006). The French named this fascinating animal "ormeau" or "ormel", in Spain it is called "abulon", Japan "tokobushi"or "awabi" (Oakes & Ponte 1996), Australia "paua" (Grindley, Keogh & Friedman 1998) and in South Africa "perlemoen" (Steinberg 2005).

Although five different species of *Haliotis* occur along the coast of South Africa (Steinberg 2005), only one, *Haliotis midae* is economical important (Reddy-Lopata *et al.* 2006) due to its distribution and size (Tarr 1992). To appreciate the intricate nature of *Haliotis* species, it is important to understand the basic nature and life cycle of this astonishing sought-after delicacy and the research which led to its artificial cultivation.

2.1 Wild abalone species of South Africa

The five different species of *Haliotis* occurring around the coast of South Africa are *Haliotis midae* (perlemoen or Midas ear abalone), *Haliotis parva* Linnaeus, 1758 (spiral ridge siffie or canaliculate abalone), *Haliotis queketti* Smith, 1910 (Quekett's abalone), *Haliotis spadicea* Donovan, 1808 (siffie, venus ear or blood-spotted abalone) and *Haliotis speciosa* Reeve, 1846 (beautiful ear- shell abalone or splendid abalone) (Branch, Griffiths, Branch, & Beckley 2002). A sixth species, *Haliotis saldanhae* Kensley, 1972, now extinct (Sweijd, Snethlange, Harvey & Cook 1998), from the Pliocene occurred on the west coast of South Africa (Langebaan) (Geiger & Groves 1999). The species *Haliotis pustulata* Reeve (Sales & Britz 2001a) occurs from the northern South African border and throughout Mozambique. This species is thought to be a sixth *Haliotis* species to occur in South Africa, but this is not so, and it will not be discussed further in this chapter.

The habitats, food preferences and behaviour of the five *Haliotis* spp. endemic to South Africa vary due to many factors and circumstances and it is quite easy to distinguish the South African species on the morphological differences in their shells.

2.2 Identification of *Haliotis* Linnaeus, 1758 species from South Africa

Haliotis species of South Africa can be distinguished by the following morphological characteristics (Muller 1986): maximum size, shape, spire, spire ridges, spiral treads, corrugations, the number of respiratory pores (holes), raised turrets and the surface structure of the shell (Fig. 2.1).
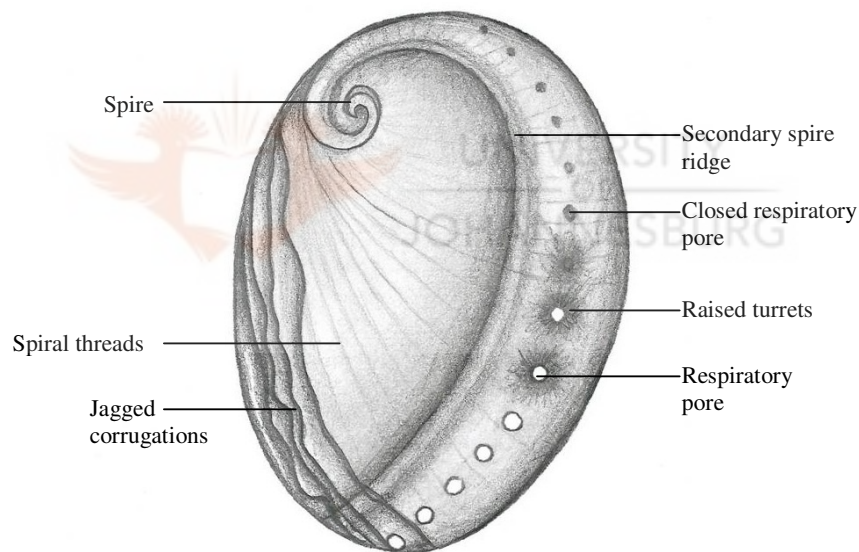
Figure 2.1, A generalized drawing of a *Haliotis* Linnaeus, 1758 shell representing the different morphological structures. The jagged corrugations are present in *Haliotis midae* Linnaeus, 1758. A secondary spire ridge and spiral treads are present on the shell of *Haliotis parva* Linnaeus, 1758. Raised turrets are most prominent on the shell of *Haliotis queketti* Smith, 1910 and less prominent on the shell of *Haliotis parva*.

2.2.1 *Haliotis midae* Linnaeus, 1758

Haliotis midae is the largest of the South African species (Muller 1986; Tarr 1989), and can grow up to 190mm (Branch *et al*. 2002) in shell length (Fig. 2.2 C) by which time, it can be 30 years or older (Barkai & Griffiths 1986; Tarr 1989). Jagged corrugations (Muller 1986) stretch across the spire (Richards 1981) (Fig. 2.2 A), the exterior shell colour can be light pink to cream-white and the inside of the shell is a mother-of pearl colour (Figs. 2.2 B & D). Juveniles lack the corrugations and the shell is smoother with a dark red colouration (Branch *et al*. 2002). The number of open respiratory pores is 8 to 11. The natural distribution range is from Saldanha Bay to Transkei (Fig. 2.5) (Kilburn & Rippey 1982).

2.2.2 *Haliotis parva* Linnaeus, 1758

Haliotis parva is the smallest South African species, with a maximum length of 45mm. The spiral ridge is prominent (Muller 1986) with a high spire (Richards 1981) (Figs. 2.3 A & B). A secondary ridge is present, as well as fine spiral threads (Kilburn & Rippey 1982). The colour of the shell may range from orange to brown, or can be mottled red and green (Richards 1981). The number of open respiratory holes is 6 to 7. The natural distribution stretch from Table Bay to East London as can be seen in (Fig. 2.5) (Kilburn & Rippey 1982).

2.2.3 *Haliotis queketti* Smith, 1910

Haliotis queketti can grow up to 46mm in shell length (Branch *et al*. 2002) and the respiratory holes are raised on turrets (Figs. 2.3 C & D). Spiral ridges are rough with a high spire (Richards 1981). The number of open respiratory pores is 4 to 6 (Kilburn & Rippey 1982). Shell colour can be dappled variations of orange, red and brown and the shell itself is rough due to the spiral ridges. The natural distribution ranges from Port Alfred to Kwa-Zulu Natal (Branch *et al*. 2002).

2.2.4 *Haliotis spadicea* Donovan, 1808

Haliotis spadicea is the most commonly found species and is ear-shaped (Figs. 2.4 A & B). (Richards 1981). This species can grow up to 80mm in length and the shell surface is smooth compared to the other species (Muller 1986). Corrugations are present on the shell surface, as well as a few spiral ridges. The number of open respiratory pores is 5 to 8 (Kilburn & Rippey 1982). Shell colour is usually reddish-brown, although splotches can also be observed. A copper-red mark is situated inside the shell, underneath the spire and the edges of the mantle are bright green (Muller 1986). The natural distribution of this species stretches from False Bay to KwaZulu Natal (Richards 1981).

2.2.5 *Haliotis speciosa* Reeve, 1846

The fifth species, *H. speciosa*, is also the rarest one, it can grow up to 86mm, which makes it the second largest species found around the South African coast. The shell is rather smooth (Figs. 2.4 C & D) and a speckled colour ranging from grey to red-brown can be observed (Branch *et al.* 2002). The number of open respiratory holes is 5 to 7. The natural distribution of this species is from Port Elizabeth to Umtata (Fig. 2.5).

2.3 Ecology of South African abalone species

2.3.1 Distributions

The *Haliotis* species of South Africa occurs naturally in the wild from Cape Columbine on the west coast to the South African boarder with Mozambique in the east (Fig. 2.5).
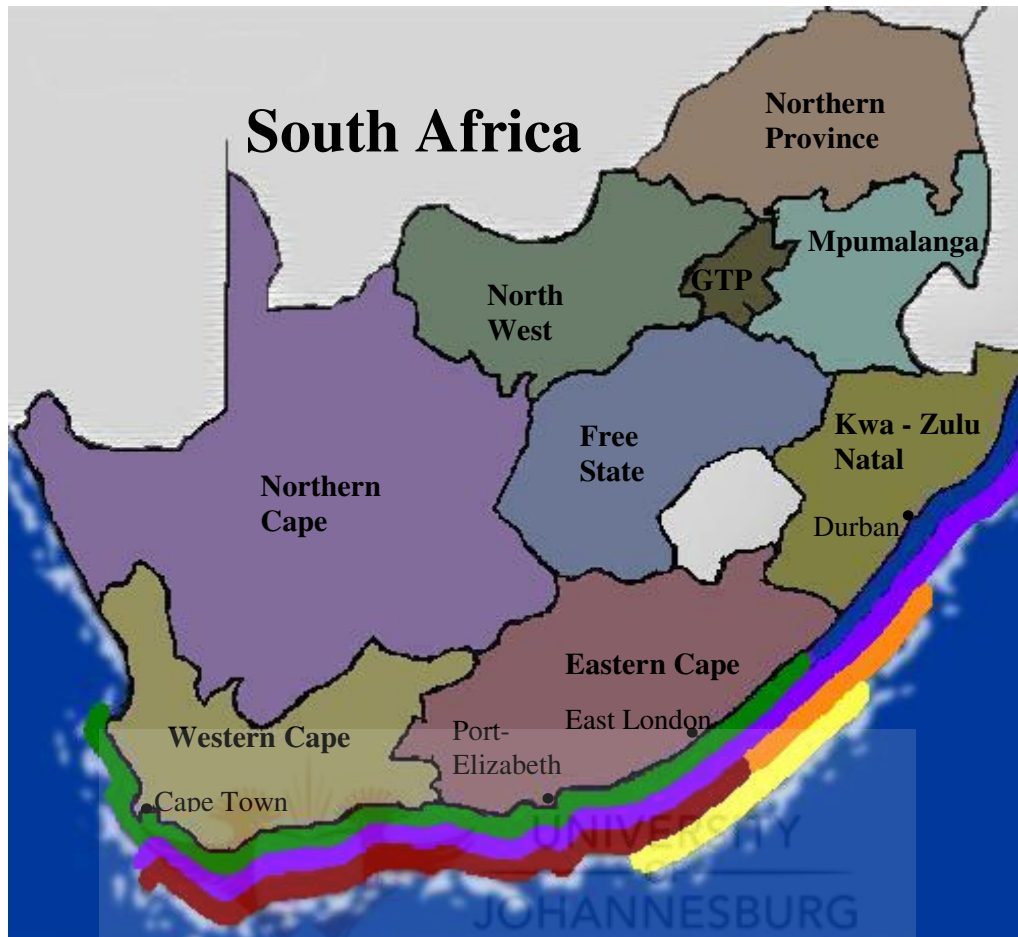
*Map compiled

Figure 2.5, The distribution of *Haliotis* Linnaeus, 1758 species around the coast of South Africa.

🟩   *Haliotis midae*: Cape Columbine – Southern Transkei

🟥   *Haliotis parva*: Cape Point – East London

🟧   *Haliotis queketti*: Port Alfred – Zululand

🟪   *Haliotis spadicea*: Cape Town – Northern boarder of KZN

🟨   *Haliotis speciosa*: Around Port Elizabeth – around Umtata

2.3.2 Habitats

The South African *Haliotis* species all occupy different habitats, according to their specific ecological needs. The species located along the west and south coast are mainly adapted to colder water temperatures brought on by the Benguela Sea current. The average water temperatures for the west coast region range from 14ºC to 17ºC (Sales & Britz 2001a). According to De Waal, Branch & Navarro (2003) temperatures in this region vary between 2 to 4ºC from the average, when comparing colder and warmer months for a ten year period.

Species occurring on the east coast are in contrast adapted to the warmer water of the Agulhas Sea current, where temperatures range from 13ºC to 21ºC (Sales & Britz 2001a). In this region, temperature variations of up to 7ºC have been recorded (De Waal *et al*. 2003). *Haliotis* spp. are found on the rocky coast in crevices, underneath stones (Muller 1986) in tidal pools, shallow reefs and in the intertidal zones where kelp species are abundant (Branch *et al*. 2002). *Haliotis midae* can be found intertidally to depths of 10 m (Tarr 1992; Dichmont, Butterworth & Cochrane 2000) whereas *Haliotis queketti* can be found up to depths of 160m (Muller 1986).

2.2.3 Food and feeding behaviour

Abalone species are herbivores (Branch *et al*. 2002) that favour night grazing and movement (Sales & Britz 2001a). Along the West-South West Coast, abalone mainly feed on the kelp species *Ecklonia maxima* (sea bamboo) (Muller 1986), as well as *Laminaria pallida* (De Waal *et al*. 2003), red algae *Plocamium* spp (Muller 1986), *Gracillaria* spp, and green algae like *Ulva* spp. (Sales & Britz 2001a). The sea bamboo does not occur naturally along the coast of the Eastern Cape (Cook 1998), but other algae are readily available (De Waal *et al*. 2003). Abalone diet thus includes a range of suitable kelp and algal species (Sales & Britz 2001a).

Abalone can entrap floating and overhanging kelp by raising the front part of their body and lengthening the foot. Seaweed washed against the foot of the abalone is instantly caught by the epipodial tentacles and then clamped down by the foot muscle

(Kilburn & Rippey 1982). A large piece of kelp trapped by one abalone can also be grazed by other individuals in the vicinity. Uneaten kelp pieces are occasionally stored underneath the foot muscle to be eaten later (Tarr 1989). Abalone use their radula to scrape kelp and other algae (Muller 1986). The radula is a tongue-like organ with rows of small teeth that can rasp, rip, pierce or cut pieces of food, which are then digested (Hichman, Roberts & Larson 2001).

2.2.4 Predators

Juvenile abalone are mostly found in crevices, under rocks and, in particular, under or close to the sea urchins *Parechinus angulosus* (Cape urchin) due to the fact that this provides protection from predation (De Waal & Cook 2001; Day & Branch 2002; Troell *et al*. 2006). Currently, there is an increase in predation on juvenile abalone in South Africa due to the influx of their main predator, *Jasus lalandii* (west coast rock lobster) (Tarr, Williams & Mackenzie 1996; Cook 1998; Anon 1, 2003). Rock lobsters are predators of note where all small invertebrates like the sea urchins and the juvenile abalone are concerned (Troel *et al*. 2006) and accordingly the shelter that the sea urchins provide to the abalone (De Waal *et al.* 2003) is no longer abundant (Day & Branch 2002). Other predators of abalone include otters, crabs, lobsters, small species of reef sharks, fish and octopuses (Day & Branch 2002).

2.3.5 The typical life cycle of abalone

Sexes are separate in all abalone species (Kilburn & Rippey 1982) and can be distinguished by the different colourations of the gonads, when they are ripe (Bevlander 1988). Testes are cream coloured and the female gonads are olive green (Bevlander 1988) (see Fig 6.1 in Chapter 6). According to Tarr (1989), perlemoen reach sexual maturity at about 8 to 10 years of age, but more recently Sales & Britz (2001a) observed that wild animals as young as seven years can be gravid and ready to spawn. Spawning can differ from species to species due to seasonality, diverse habitats and natural environmental changes (Bevlander 1988). *Haliotis midae* spawns biannually, usually during spring and autumn, and spawning activities can be reliant on temperature variations (Bevlander 1988; Tarr 1989).

As the spawning period approaches, adult behaviour changes: an example of this is aggregation, where adult abalone gather in an area and spawn together (McShane 1992). Abalone are broadcast spawners (Morse *et al*. 1979; McShane 1992) and release eggs and sperm (gametes) (Morse *et al*. 1977) into the adjacent water (Tarr 1989).

Female abalone with a shell length of 11.4cm can release about 4.3 million eggs per spawning, compared to females of about 16cm which can release as much as 15 million eggs (Tarr 1989). Tarr (1989) suggested that males spawn first and that a single spawning individual can trigger others to spawn as well, although Morse *et al*. (1979) indicated ten years previously that animals of both sexes can encourage this type of behaviour.

During spawning the gametes of both sexes are released into the water on a large scale, and external fertilisation (Bevlander 1988) of the eggs occurs (Figure 2.6). After fertilisation, eggs undergo various cleavage stages to form trochophore larvae, with prototrochs for movement (Bevlander 1988). Eggs are negatively buoyant and the trochophore larvae hatch within 24 hours (McShane 1992). Larvae then develop into free swimming veliger larvae, which move with the aid of beating cilia (McShane 1992).

The next step in the life cycle of the abalone is for the veliger larvae to settle down on a suitable substrate to start the metamorphosis process. The veliger larvae search for the most favourable surface to settle on, by constant grazing and testing of the substrate (Morse *et al*. 1979). Various abalone species juveniles will be found in coastal nursery grounds (Morse *et al*. 1979), but can also colonize other areas due to dispersal by currents (Tarr 1989).

The larvae usually settle on crustose coralline algae *Lithothamnium* sp (Morse *et al*. 1979) and feed on benthic diatoms (Sales & Britz 2001a). Concavities are present in the coralline algae and provide good shelter for settling larvae (McShane 1992). In the post-larval creeping stage, the young will graze the substrate by scraping it side to side, and then develop into juvenile abalone (Morse *et al*. 1979). The juvenile abalones feed on benthic micro-flora and various algae species (Stevence 2003) (see Fig. 2.6).

2.4 *Haliotis* species of the world

Table 2.1: The *Haliotis* Linnaeus, 1758 species with their synonyms, common names and geographical distribution across the world. This table is compiled from Muller (1986); Lindberg (1992) and Geiger & Grove (1999).

Haliotis species & synonyms	Common names	Geographical distribution
H. asinine Linnaeus, 1758 (*H. asinum* Donovan, 1808)	Ass's (donkey's) ear abalone	Indo Pacific ocean, Red Sea
H. aurantium Simone, 1998	*	*
H. australis Gmelin, 1791 (*H. aleata* Roding, 1798, *H. costata* Swanson, 1822, *H. plicata* Karsten, 1789*, H. rugosoplicata* Chemnitzi, 1788)	Australian abalone/ Silver abalone	New Zealand & Australia
H. brazieri Angas, 1869 (*H. melculus* (Iredale, 1927)	Brazier's abalone/Honey abalone	Australia
H. clathrata Reeve, 1846 (*H. venusts* Adams & Reeve, 1848)	Lovely abalone	Australia & Philippines
H. coccinea Reeve, 1846 (*H. janus Reeve*, 1846, *H. zealandica Reeve*, 1846)	Canary Island abalone	Canaries, Madeira (Europe)
H. coccoradiata Reeve, 1846	Reddish-rayed abalone/ Scarlet-rayed abalone	Australia

Table 2.1 continue

Haliotis species & synonyms	Common names	Geographical distribution
H. cracherodii cracherodii Leach, 1814 (*H. expansa* Talmadge, 1954, *H. holzneri* Hemphill, 1907, *H. imperforate* Dall, 1919, *H. lusus* Finlay, 1927, *H. rosea* Orcutt, 1900, *H. splendidula* Williamson, 1892)	Black abalone	California (America)
H. cracherodii californiensis Swainson, 1822 (*H. bonita* Orcutt, 1900)	Black abalone	California
H. crebrisculpta Sowerby, 1914	Close sculptures abalone	Indo- Pacific ocean
H. cyclobates Peron & Lesueur, 1816 (*H. excavata* Lamarck, 1822)	Whirling abalone/ Excavated abalone	Australia
H. dalli Henderson, 1915	Dall's abalone	Galapagos Islands, Western America
H. discus discus Reeve, 1846 (*H. discus hannai* Ino, 1953)	Disk abalone	China, Japan, Korea, North Pacific, Indo-Pacific
H. dissona Iredale, 1929	*	Australia, west Pacific
H. diversicolor Reeve,1846 (*H. aquatilis* Reeve, 1846, *H. tayloriana* Reeve, 1846, *H. gruneri* Philippi, 1848, *H. supertexta* Lischke, 1870)	Variously coloured abalone	Indo-Pacific ocean, West Pacific

Japan |
H. dohrniana Dunker, 1863	Dhorn's abalone	Indonesia, New Hebrides
H. elegans Philippi, 1844 (*H. clathrata* Lichtenstein, 1794)	Elegant abalone	Australia
H. exigua Dunker, 1877	*	Australia, Ryukya Islands
H. fulgens fulgens Philippi, 1845 (*H. guadalupensis* Talmadge, 1964, *H. splendens* Reeve, 1846, *H. turvei* Bartsch, 1942, *H. planilirata* Reeve, 1846)	Green abalone	California, America
H. fatui Geiger, 1999	*	*
H. gigantea Gmelin, 1791 (*H. tubifera* Lamark, 1822, *H. gigas* Roding, 1798, *H. sieboldii* Reeve, 1846)	Giant abalone	Japan, Indo- Pacific

Table 2.1 continue

Haliotis species & synonyms	Common names	Geographical distribution
H. hargravesi Cox, 1869 (*H. ethologus* Iredale, 1927)	Hargraves's abalone/ Mimic abalone	Australia
H. iris (Gmelin, 1791) (*H. iridis* Karsten, 1789)	Blackfoot abalone/ Rainbow abalone/ paua abalone	New Zealand
H. jacnensis Reeve, 1846 (*H. echinata* Sowerby, 1883, *H. hanleyi* Ancey, 1881)	Jacna abalone	Indo-Pacific Ocean
H. kamtschatkana kamtschatkana Jonas, 1845	Pinto abalone/ Japanese abalone	Alaska, California, Japan, Indo Pacific Ocean
H. kamtschatkana assimilis Dall, 1878 (*H. aulaea* Bartsch, 1940, *H. smithsoni* Bartsch, 1940)	Southern abalone/ Treaded abalone	California (America)
H. laevigata Donovan, 1808 (*H. albicans* Quoy & Gaimard, 1834, *H. excise* Gray, 1856, *H. glabra* Swainson, 1822)	Smooth Australian abalone/ Green lip abalone	Australia
H. madaka (Haba, 1977)	*	*
H. marmorata Linnaeus, 1758 (*H. decussate* Philippi, 1850, *H. guineensis* Gmelin, 1791, *H. rosacea* Reeve, 1846, *H. strigata* Weinkauff, 1883, *H. virginea* Reeve, 1846)		West Africa
H. mariae Wood, 1828 (Gray, 1831) (*H. dentate* Jonas, 1846)	Maria's abalone	Red Sea , Horn of Africa
H. midae Linnaeus, 1758 (*H. capensis* Dunker, 1844, *H. elatior* Pilsbury, 1890)	Midas ear abalone/ Perlemoen	South Africa
H. mykenosensis Owen, Hanavan and Hall, 2001	*	*
H. ovina Gmelin, 1791 (*H. caelata* Roding, 1798, *H. latilabris* Philippi, 1848, *H. patamakanthini* Dekker, Regter and Gras, 2001, *H. volcanius* Patamakanthini and Eng, 2002)	Oval abalone/ Sheep's ear abalone	Indo-Pacific Ocean
H. parva Linnaeus, 1758 (*H. kraussi* Turton, 1932, *H. parvum* Krauss, 1848)	Canaliculate abalone/Spiral ridge siffie	South Africa

Table 2.1 continue

Haliotis species & synonyms	Common names	Geographical distribution
H. planate Sowerby, 1882 (*H. grayana* Sowerby, 1882)	Planate abalone	Indo-Pacific Ocean
H. pourtalesii Dall, 1881	Pourtale's abalone	Brazil & Florida (America)
H. pulcherrima Gmelin, 1791	Most beautiful abalone	Pacific
H. pustulata Reeve, 1846 (*H. nebulatum* Reeve, 1846, *H. pertusum* Reeve, 1946, *H. pustulatum* Krauss, 1848, *H. bistriata* Barnard, 1963.	Shield abalone	Mozambique, Indian Ocean, Mediterranean
H. queketti Smith, 1910	Quekett's abalone	South Africa
H. roberti McLean,1970	*	West America (Costa Rica)
H. roei Gray, 1827 (*H. sulcosa* Philippi, 1845, *H. scabricostata* Menke, 1843)	Roe's abalone	Australia
H. rubiginosa Reeve, 1846 (*H. ethologus* Iredale, 1927*, H. howenensis* Iredale, 1929)	Lord Howe abalone	Australia
H. rubra rubra Leach, 1814 (*H. ancile* Reeve, 1846, *H. conicopora* Peron, 1816, *H. cunninghami*, Gray, 1826, *H. granti* Pritchard and Gatliff, 1903, *H. improbula* Iredale, 1924, *H. naevasa* Martyn, 1786 (Philippie 1844), *H. vixlirata* Cotton, 1943, *H. whitehousei* (Colnam, 1959))	Rubber abalone	Australia & Indian Ocean
H. rugosa Lamarck, 1822 (*H. alternata* Sowerby, 1882, *H. multiperforata* Reeve, 1846, *H. revelata* Deshayes, 1863)	Many Holed abalone	*
H. rufescens Swainson, 1822 (*H. californiana* Valenciennes, 1832, *H. hattorii* Bartsch, 1940, *H. ponderosa* Addams, 1848)	Red abalone	America
H. scalaris Leach, 1814 (*H. emmae* Reeve, 1846, *H. tricostalis* Lamark, 1822, *H. tricostata* Wood, 1828)	Staircase abalone/ Ridge ear abalone	Australia
H. semiplicata Menke, 1843 (*H. lauta* Reeve, 1946)	Serniplicate abalone	Australia
H. sorenseni Bartsch, 1940	White abalone/ Sorensen abalone	California (America)

Table 2.1 continue

Haliotis species & synonyms	Common names	Geographical distribution
H. spadicea Donovan, 1808 (*H. sanguinea* Hanley, 1840, *H. ficiformis* Menke, 1844, *H. sanguineus* Krauss, 1848, *H. pertusa* Sowerby, 1900, *H. nebulata* Turton, 1932)	Blood- spotted abalone/ Siffie/ Venus ear	South Africa
H. speciosa Richards 1981(*H. speciosum* Reeve 1846, *H. pertusa* Sowerby 1900, *H. alfredensis* Bartsch, 1915)	Splendid abalone, Beautiful ear-shell abalone	South Africa
H. squamata Reeve, 1846 (*H. elevate* Sowerby, 1883, *H. funebris* Reeve, 1846)	Scaly Australian abalone	Indo-Pacific
H. squamosa Gray, 1826 (*H. roedingi* Menke, 1844)	Squamose abalone	Australia
H. stomatiaeformis Reeve, 1844 (*H. neglecta* Philippi, 1848)	*	*
H. thailandis Dekker and Patamakanthin, 2001 (*H. tomricei* Patamakanthin, 2002)	*	*
H. tuberculata Linnaeus, 1758 (*H. incise* Reeve, 1846, *H. bisundata* Monterosato, 1942 *H. canariensis* Reeve, 1846, *H. coccinea* Reeve, 1846, *H. janus* Reeve, 1846, *H. japonica* Reeve, 1846, *H. lamellose* Lamark, 1822, *H. lucida* Requien, 1848, *H. pellucida* Von Salis, 1793, *H. reticulate* Reeve, 1846, *H. rugosa* Lamark, 1822, *H. secerneda* Monterosato, *H. straita* Linnaeus, 1758, *H. varia* Risso, *H. vulgaris* Da Costa, 1778)	European edible abalone/ Tube abalone	Canary Islands, East Atlantic, Japan, Mediterranean & Cape Verdi
H. unilateralis Lamark, 1822	*	Indian Ocean

Table 2.1 continue

Haliotis species & synonyms	Common names	Geographical distribution
H. varia varia Linnaeus, 1758 (*H. aliena* Iredal, 1925, *H. astricta* Reeve, 1846, *H. barbouri* Foster, 1946, *H. concinna* Reeve, 1846, *H. dringii* Reeve, 1846, *H. gemma* Reeve, 1846, *H. granulate* Roding, 1798, *H. papulata* Reeve, 1846 *H. pustulifera* Pilsbry, 1890, *H. semistriata* Reeve, 1846, *H. viridis* Reeve, 1846)	Variable abalone/ Barbour's abalone	Australia, Indo-Pacific, America
H. virginea virginea Gmelin, 1791 (*H. crispata* Gould, 1847, *H. gibba* Philippi, 1846, *H. huttoni* Filhol, 1880, *H. morioria* Powell, 1838, *H. subvirginea* Weinkauff, 1883)	Virgin abalone	New Zealand
H. walallensis Stearns, 1899	Northern abalone/ Flat abalone	America

* No common names or distribution found.

2.5 Economically important species of abalone

Table 2.2: The economically important abalone species. This table is compiled from Morse *et al*. (1977, 1979); Moss, Illingworth & Thong. (1995); Hauser (1997); Haaker (1997); Daniels & Floren (1998); McBride (1998); Moore *et al*. 2002); Stevence (2003); Pang, Zhang, Bao & Gao (2006), Anon 2, 2006 and Anon 3, 2007.

Haliotis species	Distribution	Wild, Farmed or recreational	State of the wild populations
Haliotis corrugata	North America, California	Wild, farmed and recreational	Critically endangered IUCN Red list. Ceased commercial diving and collection in California from 1996
Haliotis cracherodii	North America, California, Mexico	Wild, farmed and recreational	Critically endangered IUCN

Table 2.2 continue

Haliotis species	Distribution	Wild, Farmed or recreational	State of the wild populations
Haliotis discus hannai	China and Japan	Wild and farmed	*
Haliotis diversicolor	Taiwan	Wild and farmed	*
Haliotis fulgens	North America, California, Mexico	Wild and farmed and recreational	Endangered. Ceased commercial diving and collection in California from 1996
Haliotis iris	New Zealand	Wild and farmed	*
Haliotis kamtschatkana	North America	*	Endangered IUCN
Haliotis laevigata	Australia	Wild	*
Haliotis midae	South Africa	Farmed	Endangered CITES Appendices 3
Haliotis roei	Australia	Wild	*
Haliotis rubra	Australia	Wild and farmed	*
Haliotis rufescens	Chile, California (North America)	Farmed and recreational	Concern. Fisheries closed in 1997
Haliotis sorenseni	North America	Farmed and recreational	Endangered. Fisheries closed in 1996.

* No information found

2.6 South African *Haliotis* Linnaeus, 1758

2.6.1 Utilisation of abalone

Since 1953, the legal size for wild caught abalone has been set at 11.43cm in shell width (Tarr 1992). Various collection methods for wild abalone are known. The first is the collection of abalone from infratidal and subtidal zones for recreational purposes (Tietz & Robinson 1974). In South Africa, the collection of abalone by this method used to be unrestricted, but from the 1970s, seasonal quotas were implemented. Collection of abalone by recreational divers is currently not permitted, owning to the drastic decline of wild abalone populations (Troell *et al*. 2006).

In South Africa, commercial diving of abalone began in 1949 (Dichmont *et al*. 2000) in Gans Bay and then extended from Cape Columbine to Cape Agulhas (Tarr 1992).

Methods used from 1949 to collect abalone from the infratidal zone included the use of "hard hat" helmet diving equipment and the hookah system. The latter was still used recently by commercial divers. These two methods were described in detail by Tarr (1992). Abalone collected by commercial divers were processed at local processing factories (Dichmont *et al*. 2000) and since 1984, 10% of the total number collected was allowed to be sold in South Africa (Tarr 1992).

Over the years a number of fishing companies have been granted annual permits stating the number of abalone they were allowed to remove and sell. Up until the end of 2007, only about 262 permits were issued annually to individual divers (Anon 4, 2007). A total of 770t of abalone were harvested in 1953, and this rose to 2800t in 1965 (Tarr 1992). In 1971, a national quota system (Total Allowed Catch) was implemented for the commercial catches of abalone and this was set at 600mt (Cook 1998). In 1998 the commercial quota stood at 550mt/y (Sweijd *et al*. 1998) and in 2004 the quota was lowered to 230mt (Troell *et al*. 2006). During the 2006/2007 season the TAC was again lowered to 125t and in the 2007/2008 season it was lowered even more to 75t (Anon 5, December 2007). The Minister of Environmental Affairs and Tourism implemented the closure of the commercial fishery, effective from 1 February 2008, due to the drastic decline of wild abalone populations (Anon 5, 2007).

2.6.2 Poaching

The illegal collection of abalone in South Africa has been an ongoing crisis and is escalating (Anon. 1, 2003). Law enforcement authorities and the government have been pursuing this battle for many years, but despite some success, they have not been able to completely stop the illegal catching of abalone and trade thereof (Gordon & Cook 2004). In 2003 the amount of illegally caught abalone was estimated to be twice the amount of annual allowed commercial landings (Anon 1, 2003). Poaching and over exploitation of abalone could lead to the demise of the South African abalone, *Haliotis midae* in years to come (Gordon & Cook 2004).

The natural behaviour of abalone makes them an easy target for poachers (Hauck & Sweijd 1999). To illustrate this, abalone tend to aggregate in shallower waters when the spawning seasons approaches, thus exposing themselves to illegal collections (Hauck & Sweijd 1999). Poaching is a difficult matter to address by police as many factors can complicate the situation. Social issues, politics, corruption, gangs and organised crime are but a few of these factors.

A large portion of the people living in the Far East, especially China, believe that abalone possess magical attributes, and are particularly interested in them as they are a very strong aphrodisiac (Hauck & Sweijd 1999). A few highly organised syndicates in South Africa, focus their poaching effort on supplying this demand from the East (Hauck & Sweijd 1999). Abalone are sold or exchanged for other products eg. counterfeit goods, drugs, guns, diamonds and even human beings (Steinberg 2005). If a poacher is caught by law enforcement, a fine of up to R2 million for large amounts of abalone can be given, or a jail sentence of five years imposed (Steinberg 2005). Corruption of authorities includes receiving of bribes, pretence of ignorance, or the covering up of activities. Since 1997, marine poaching has received a priority crime status (Hauch & Sweijd 1999), but a long road still lies ahead for authorities to smother poaching operations in South Africa and thus protecting abalone.

3. Abalone farming

Research on the artificial spawning of the South African species *Haliotis midae* started in the late 1980s (Cook 1998) and continued into the '90s, specifically with the aim of aiding in the establishing of abalone farms (Sales & Britz 2001a). In the 1990s abalone farming operations in South Africa commenced (Sales & Britz 2001a). Currently, 22 permits for abalone farming in South Africa have been issued with numerous farms already operational and new farms being planned (Troell *et al.* 2006).

Establishment of farms and farming of *H. midae* was driven by the ongoing concern about declining wild populations, due to over-exploitation (Troell *et al.* 2006). Farming of abalone also contributes to the economy of South Africa, as well as playing a role in job creation. Presently *H. midae* is the only South African species that is being utilized for farming (Tarr 1995) and commercial purposes (Troel *et al.* 2006), mainly because it is the largest of the five known species. The other species are much smaller, difficult to harvest and have inadequate populations numbers (Cook 1998). *Haliotis midae* also occurs along the greater part of the coast and is considered, next to *Haliotis rufescens*, one of the largest economically farmed *Haliotis* species in the world (Tarr 1992).

Many problems were initially encountered when abalone farming commenced world wide. These included the difficulties associated with the spawning of wild abalone (Moss, *et al.* 1995), incapability to induce settlement of larvae on diatom films, and high mortalities in settling as well as settled larvae (Moss & Tong 1992).

The research focussing on these problems and the methods used to solve them, are discussed in this chapter. In order to understand the potential problems (diseases) that can affect abalone farming in South Africa, it is important to have a working knowledge of abalone farming in South Africa.

3.1 Commercial value of abalone

Abalone is seen as the real gold of South Africa's fisheries (Anon 6, 2000). In 1998, aquaculture output generally was about 3t and in 1999 it was estimated at 15 tons. The wild abalone catch in 1997 was about 537t with a value of R80 million (Anon 6, 2000). In 2003, more than 500mt of abalone were farmed (Troell *et al.* 2006). In China *H*. *fulgens* and *H*. *midae* receive the highest prices (Oakes & Ponte 1996; Vosloo & Vosloo 2006). A total of 890t (live mass) were exported from South Africa in 2006 at 37 US Dollars/kg with a total monetary value of R230 million (W. Barnes, pers. comm.).

3.2 Artificial spawning and settlement of larvae

3.2.1 Spawning

In nature, many factors influence the spawning process in adult abalone. Abalone usually spawn at specific times and seasons during the year, but spawning may furthermore depend on different habitats and water temperatures (Bevlander 1988; Fallu 1991). Another factor which can influence the spawning of adults, as mentioned by Morse *et al.* (1977), is broadcast sperm or egg released by a gravid male or female, which can trigger other animals in the surrounding area to spawn as well (as discussed in Chapter 2). When abalone spawn, not all the eggs may be released at once and spawning can occur at various times during the breeding period. A single female can release millions of eggs per spawning (McShane 1992; Sales & Britz 2001a).

In establishing abalone farming, artificial spawning posed a difficult problem to overcome. This was due to the fact that farmed animals were less likely to spawn and when they did, inadequate amounts of sperm and eggs were released. Morse *et al.* (1977) studied the reproduction and intricate details of abalone spawning in an effort to establish techniques by which spawning could be induced synthetically in economically farmed *Haliotis* spp. For his experiments, he used *H*. *rufescens* (Swainson, 1822) from the United States.

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The compound, prostaglandin, responsible for processes in the reproduction cycle of humans, was tested on the abalone. Prostagladin syntheses in abalone are activated by the free oxygen radicals occurring in sea water (Moss *et al.* 1995). Morse *et al.* (1977) added H_2O_2 (hydrogen peroxide) at a concentration of 5mMol to sea water with a pH of 9.1. Practically all the tested animals spawned within a few hours after being treated.

The use of ultraviolet irradiated seawater in conjunction with elevated water temperatures, and air exposure to induce spawning, are other practices. Moss *et al.* (1995) tested both the H_2O_2 and the ultraviolet methods to establish the best conditions for spawning and concluded that the first mentioned technique was most successful. During spawning, gametes move from the adult gonads to the right renal appendage and pass through the branchial chamber into the sea water via the respiratory pores (Bevlander 1988; Wood & Buxton 1996a).

3.2.3 The development of the eggs and larval stages

Fertilised eggs (Fig. 3.1 A) undergo different stages of development in order to form the first free-living larva stage (trochophore larva). Firstly, the fertilised egg cleaves into two blastomeres (Fig. 3.1 B) of the same size. During the second cleavage (Fig. 3.1 C), the egg divides into four identical blastomeres. The third cleavage takes place to give rise to eight blastomeres, consisting of four minor cells (micromeres), (Hickman, Hickman & Kats 1997), and four major cells (macromeres), (Bevlander 1988). The fourth cleavage of 16 cells leads to the formation of a morula (28 cell stages) (Fig. 3.1 D) and ultimately a blastula (Bevlander 1988; Hichman *et al.* 1997) (Fig. 3.1 E).

When the eggs hatch, a free-living trochophore larva emerges (Hichman *et al.* 2001) (Fig. 3.1 F), which mainly swims upward in the water column. The final-free living larval stage is called the veliger larva. The veliger stage larvae display creeping behaviour prior to torsion (Kang, Kim & Kim 2004). Veliger larvae mostly swim downwards in the water and this stage can be divided into two phases. Firstly, a pre-

torsion veliger larva, and then a post-torsion veliger larvae (McShane 1992) (see Fig. 2.6 Chapter 2). Larvae now have to search for a suitable substrate to settle on.

In artificial cultivation another problem arose. The veliger larvae did not settle and large mortalities occurred. Morse *et al*. (1979) performed experiments on *H. rufescens* larvae to determine a method by which planktonic abalone could be induced to settle and undergo metamorphosis. They noted that juvenile abalone are mostly found at sites where the crustose red algae *Lithothamniom* spp. and *Lithophyllum* spp. occur (Morse *et al*. 1979). Extracts and homogenate of these algae were placed into experimental containers with larvae at low concentrations, as high concentrations proved to be toxic to the larvae. Larvae stopped swimming instantaneously. Behaviour such as side-to-side scraping (grazing) on the surface of the containers and gliding locomotion were observed (Morse 1984).

Gamma aminobutyric acid, the main component found in the crustose algae was responsible for the rapid settling when 1mMol of it was added to the water (that have been treated with antibiotics) containing the larvae (Slattery 1992). In contrast, Morse *et al*. (1979) stated a few years earlier that the crustose red algae did not release large quantities of the inducing substance and thus do not "lure" the larvae to them in order to settle. Direct contact, or ingestion of the algae is needed for the larvae to settle and is aided by their behaviour. The larvae swim upwards; stop swimming movements and sink to "test" the substrate (Moss & Tong 1992). If the substrate is not desirable, they will swim up again and repeat the sequel of events in search of a suitable settling position or substrate. Oceanic drift also plays a valuable role in this settlement behaviour as well as in dispersion (Morse *et al*. 1979).

3.3 How a typical abalone farm works

The basic techniques used for abalone farming in South Africa have been adopted from other countries methods and information on new techniques developed in South Africa are not readily available due to ownership rights (Sales & Britz 2001a).

Phase 1: Spawning

Spawning occurs every fortnight in most farms that prefer to seed spawn (J. Venter, pers. comm.). The breeding pairs are usually wild abalone collected from the coastline area close to the farm. The reason for this is that wild abalone are heartier than farm animals and that pairs collected from the surrounding areas close to the farm are used to the environmental factors (Fallu 1991). Farms may also use breeding programs to select the strongest and fastest growing abalone for spawning purposes (J. Venter, pers. comm.). Spawning takes place (Fig. 3.2) in a dark room where the males and females are kept in separate tanks. To induce spawning hydrogen peroxide and a suitable buffer (eg, Tris buffer) are added to the water of the adults (Fallu 1991; J. Venter pers. comm.). The hydrogen peroxide is the stressor that leads to spawning. Sometimes small amounts of sperm cells can also be added to the tanks containing the females, to accelerate the spawning process (Fallu 1991).

Phase 2: Fertilisation and free living larvae stages

Eggs and sperm obtained from the spawning tanks are placed together. After a period of about 24 hours the fertilised eggs hatch as trochophore larvae and are then placed into a container floating in a large tank filled with water. A thin stream of water continuously flows into the floating container. Development of the trochophore larvae to veliger larvae can take five to seven days, depending on the temperature (Sales & Britz 2001a); veliger larvae are now ready to settle. The veliger larvae swim down a pipe (Fig. 3.3) attached to the floating container and into the large tank. The water with the larvae is removed from the tank by opening a tap situated at the bottom of this container.

Phase 3: Settlement of larvae

Cultured diatoms are globally used to feed metamorphoses abalone (Knauer, Britz & Hecht 1993). During the two weeks in which the larvae develop, separate tanks with acrylic plates are left to stand, in order for diatoms to grow (Daume, Brand-Gardner & Woelkerling 1999). The veliger larvae obtained from the settling tanks are then added to these separate tanks. Light, air and temperature are constantly adjusted to ensure
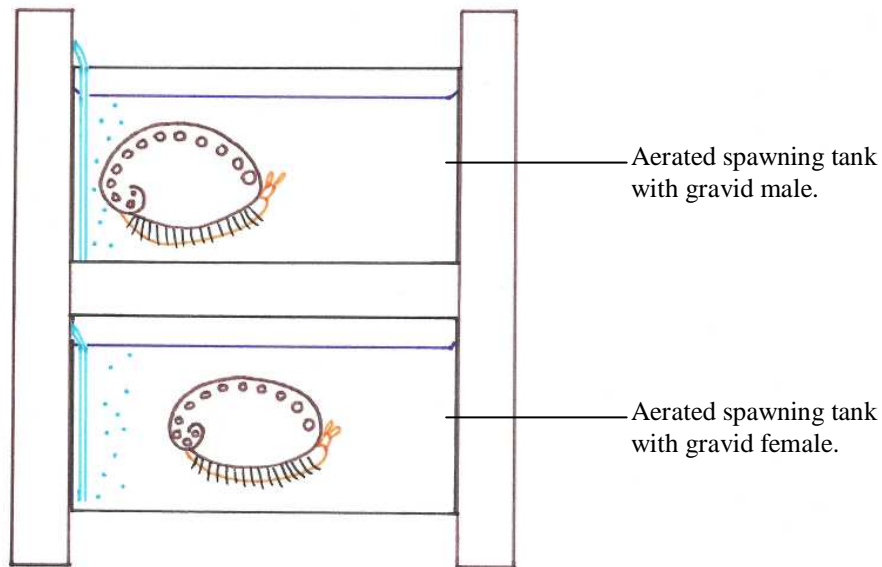
Figure 3.2, Spawning tanks containing gravid male and females.
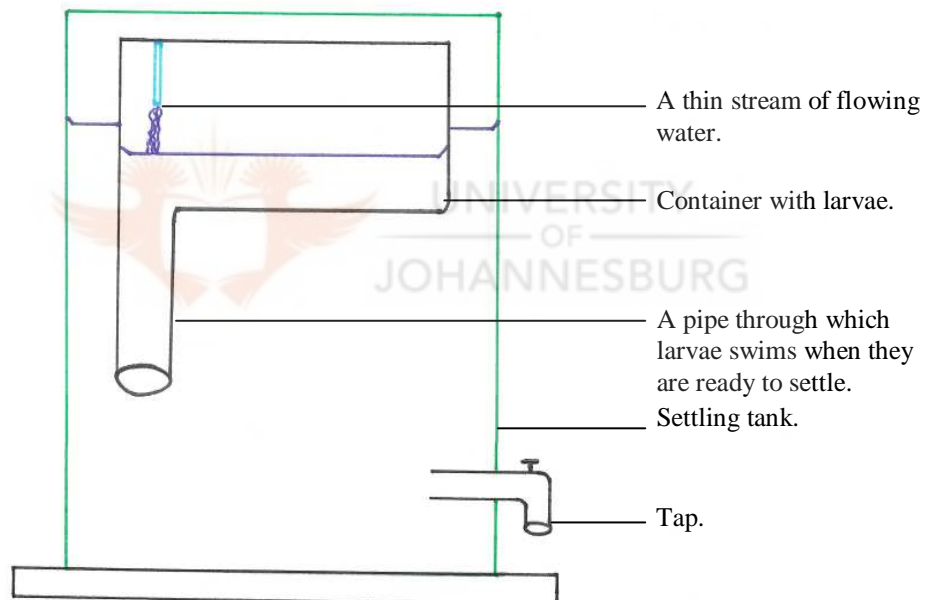
Figure 3.3, Settling tank with a floating container and a tap.

the most encouraging conditions for the larvae. Larvae settle on the plates (Cook 1998) and feed on the diatoms which grew on the plates (Kawamura, Roberts & Nicholson 1998) (Fig. 3.4). Juveniles (spat) stay in these tanks for a period of two months.

Phase 4: Juvenile weaning

The juveniles are anaesthetised with magnesium sulphate and removed from the plates

by using a small thin brush and then placed into weaning tanks. These tanks contain dark plastic cones (Fig. 3.5 A) which are submerged under the water. An air tube is connected to each cone (Fig. 3.5 B) and the larvae are placed under these cones. During the weaning process, the amount of light, air and the temperature are constantly regulated (Huchette, Koh & Day 2003). The weaning section is semi-dark to prevent large amounts of algal growth.
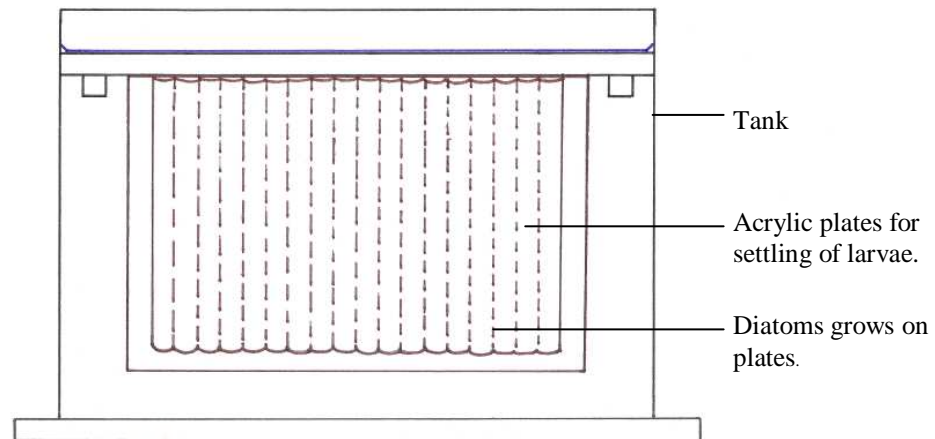
Figure 3.4, Tanks with plastic settlement plates containing diatoms.
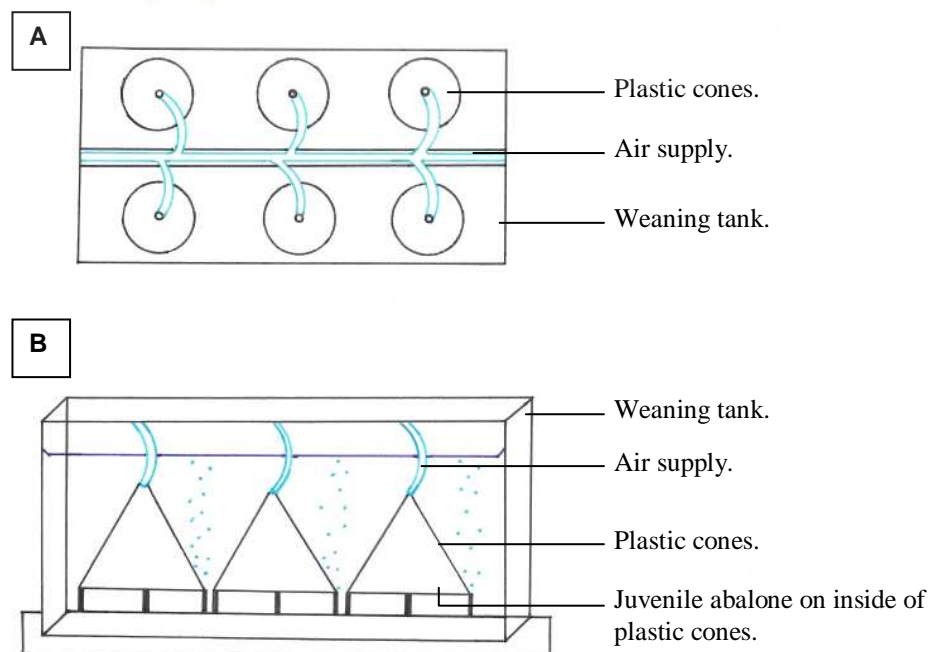
Figure 3.5, Weaning tanks with plastic cones for juveniles to hide under. A. As viewed from above B. View from the side.

∗ J. Venter Abalone Farmers Association of South Africa, Jacobsbay

The weaning process takes about two months, but during the winter months this process is prolonged. The juveniles eat the macro-algae (Knauer *et al*. 1993) in the tanks and are also fed a specific diet during this time. Abfeed®, a commercially made abalone feed (Troell *et al*. 2006), used to wean abalone from an algae diet in the majority of South African farms (Sales & Britz 2001a), is an example of an artificial diet. During the two month weaning process the juveniles' shell colour changes from red to blue. For the next four months the abalone are fed artificial feed and seagrass (*Ulva* spp. and *Porphyra* spp).

Phase 5: Spat outgrowing

After a period of about six months the juvenile abalone or spat, are transferred to raceways. A wide variety of anaesthetics have been tested in the past, but thus far only $MgSO_4$ have been found to anaesthetise abalone between the sizes of 5 to 90mm. The abalone can then be removed from the substrate without harming them when activities like maintenance of tanks, or size sorting takes place (White, Hecht & Potgieter 1996). Raceways refer to tanks through which sea water and air flows (Figs. 3.6 A), and also consists of different sections where various sizes of abalone are kept together.

In a raceway tank, a plastic container resembling a crate (Fig. 3.6 B) is placed with a plastic removable dark cover at the top (Fig. 3.7 A & B). The abalone are transferred into these crates and are fed a diet of artificial feed e.g. Abfeed® and kelp (*Ulva* spp. - sea lettuce and *Ecklonia maxima* – sea bamboo), or seaweed-based pellets called Midae Meal (Troell *et al*. 2006). Abalone are graded every four to six months and smaller abalone are placed in a tank with abalone of the same size to minimize competition. The larger the abalone becomes, the fewer individuals are placed together this also minimizes competition and ensures enough space for each individual to grow to its optimum size (Huchette *et al*. 2003).

A

Plastic lid on top.

Aerated holding tank.

B

Plastic lid on top.

Aerated holding

Crate or basket -type
container with abalone.
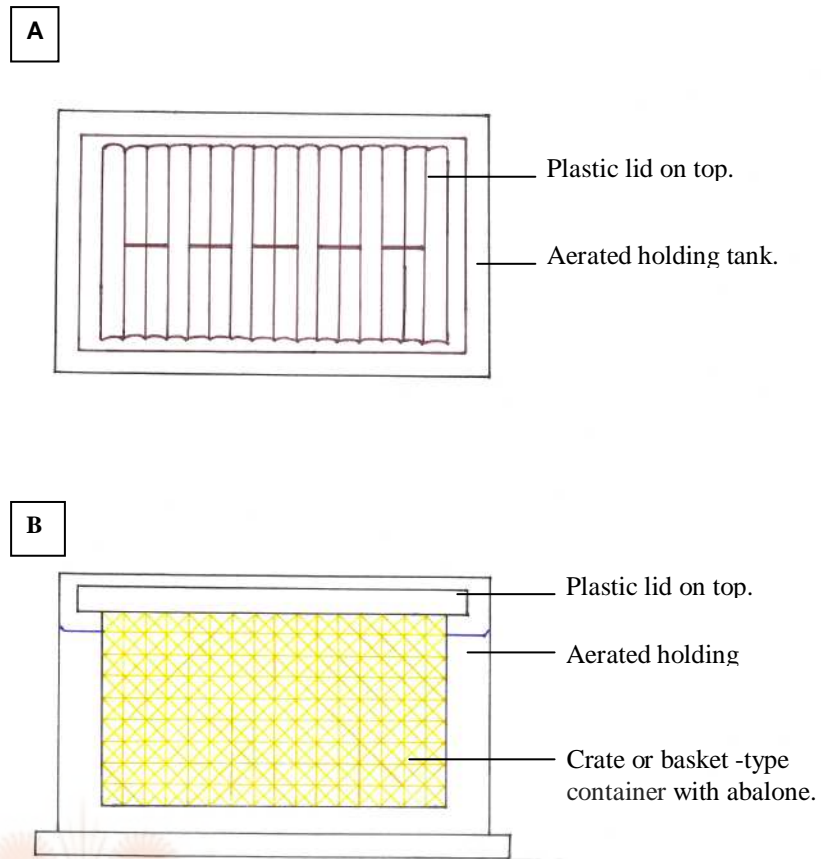
Figure 3.7, Tanks with crate-like holders for juvenile abalone to grow in to cocktail size for marketing. A.
As viewed from the top B. View from the side.

Phase 6: Sale of cocktail sized abalone

When the abalone have reached their desired size for marketing and export, they are
classed again. All animals that show signs of poor health and broken or deformed
shells are placed in reject tanks and are used to produce canned abalone products.
Animals in impeccable condition (Oakes & Ponte 1996) are weighed and placed into
transport containers according to their mass (Vosloo & Vosloo 2006). Ice sheets with
saturated jelly granules are placed into these containers along with a sponge to ensure
that the animals stay cool and moist during transport (A Mouton, pers. comm.). The
presence of divisions in the containers ensures that the abalone do not cluster together.

3.4 Abalone farms in South Africa

Abalone farms in South Africa are mostly concentrated around the Western Cape area. In Figure 3.8 the different types of abalone farms located in South Africa are indicated. Marine Growers in Port Elizabeth and Wild Coast Abalone in East London are situated in the Eastern Cape (Not indicated in Fig 3.8).
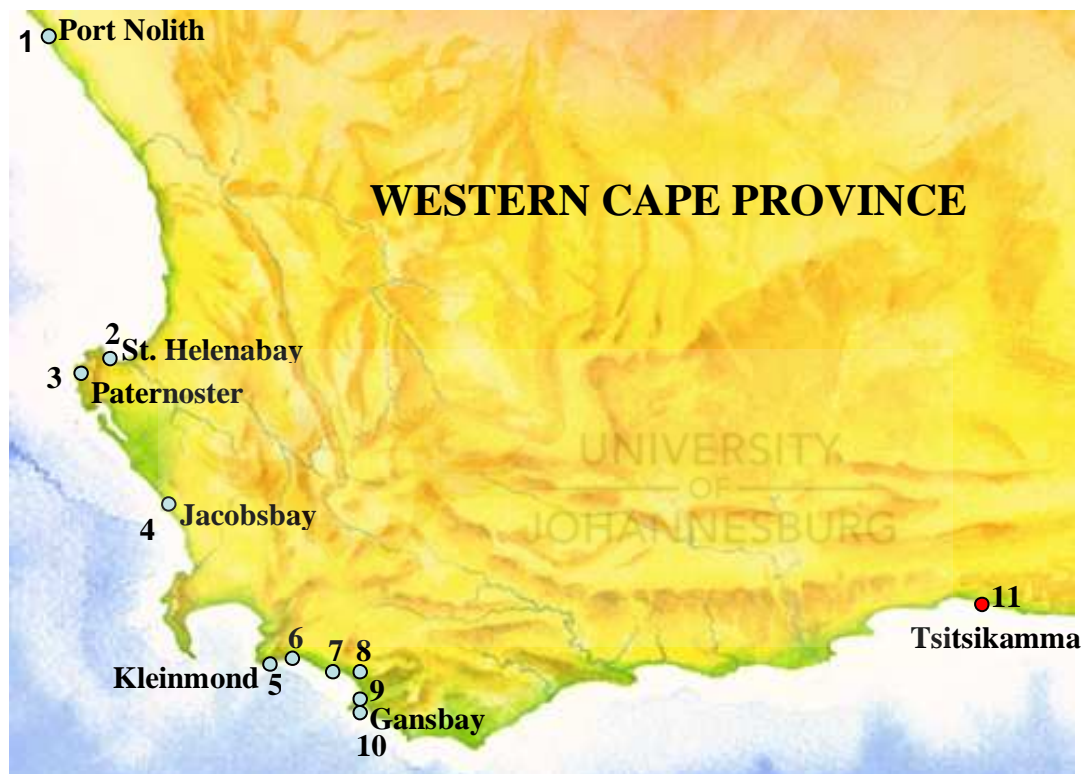
Figure 3.8, A map of abalone farms around the west coast and the Tsitsikamma National Park. **1**. Port Nolith Sea Farm **2**.Western Cape abalone in St. Helenabay **3**. Abulon Holdings & Abatech in Paternoster **4**. Jacobsbay Sea Products **5**. Global Ocean in Kleinmond **6**. Howston in Betty's Bay **7**. Abamax, Abaseed, Sea Plant Products, Abagold, Aquafarm, HIK & Hermanus Abalone in Hermanus **8**. Walker Bay **9**. Romans Bay Sea Farm **10**. Atlantic Abalone and I&J in Gansbay **11**. *Tsitsikamma National Park* (**Collection of wild abalone**).

3.4.1 Types of Abalone Farms

<u>Hatchery (Seeding) Farm:</u> At a hatchery farm, spawning is induced in mature gravid abalone. The sperm and eggs are placed together and the fertilised eggs develop to the post-larva creeping stage. This abalone stage is more commonly known as spat, and is sold to rearing farms (Troell *et al*. 2006). An example is the Abaseed Farm in Hermanus.

<u>Rearing Farm:</u> A rearing farm will buy spat (juvenile abalone) from a hatchery farm and grow them to market size (Troell *et al*. 2006). An example is the Abulon Holdings Farm in Paternoster.

<u>Ranching</u>: Ranching is when spat are bought from hatcheries. They are then placed into a confined intertidal zone and kelp beds to grow (Troell *et al*. 2006). Abalone are constantly fed with kelp and checked regularly, although they live in a natural environment (Cook 1998). When they have reached market size, they are sold. An example is the Port Nolith Sea Farm in the Western Cape (Hoffman, Swart & Brink 2000; Sales & Brits 2001a).

<u>Abalone Farms</u>: Breeding pairs taken from the wild are kept at farms. Spawning is induced, spat are weaned and the young abalone are cultivated until they have reached market (cocktail) size, and are then sold and exported. An example is Aquafarm in Hermanus.

3.4.2 Important aspects of abalone farming

It is important to note that abalone farming is intensive and in this business a lot of resources are needed to make a farm successful. Numerous aspects of business have to be dealt with on a daily basis to ensure that the farm and its animals reach their full potential and that the farm operates efficiently (Huchette *et al*. 2003).

Abalone farms only grow abalone to a size of about 100mm (cocktail size) for export purposes (Cook 1998). A period of between four and five years is required for abalone

to reach this size (Sales & Britz 2001a; Macey & Coyne 2005). A percentage of the abalone does not necessarily reach this size, owing to high mortalities in the development and settlement stages.

3.4.2.1 Water quality and the environment

Aspects like water quality, temperature, densities of the abalone, parasites and diseases have to be monitored on a regular basis to ensure the artificial environment and health of the abalone stays at optimum levels. Water quality changes include the build-up of nitrates and ammonium, and increasing or decreasing pH levels, due to the waste products of the abalone (Huchette *et al*. 2003). Industrial effluent released into the coastal environment and accidental oil spills are also factors affecting the quality of the water pumped into the farms. Toxicity testing can be conducted, using larval abalone to determine the effect of these factors (Shackleton, Schoeman, & Newman 2002).

Around the coast of South Africa an ecologically important phenomena, red tide, occurs that also has an influence on the water quality and health of the juvenile abalone in abalone farms. Red tide is caused by plantonic blooms of especially *Noctiluca* sp. and *Gonyaulax polygramma* during up-swellings (Botes, Smit & Cook 2003). Large amounts of *Noctiluca* and *Gonyaulax* blooms can consume nutrients and cause oxygen levels to decline rapidly, leading to mass mortalities in marine animals (Branch & Branch 1998). Another very important dinoflagellate species occurring on the west coast of South Africa is *Alexandrium catenella*. In this region, *A. catenella* have regularly been responsible for paralytic shellfish poisonings (Joyce, Pitcher, Du Randt & Monteiro 2005).

Adult abalone, on the other hand, are not poisoned by these algal blooms like filter feeders (Cook 1998), but toxins related to the algae blooms can be stored in the foot muscle making the abalone unsuitable for human consumption (J. Venter, pers. comm.). Red tides and algae blooms around the coast of South Africa have, however, been implicated in the death of abalone larvae in hatcheries (Cook 1998). Most abalone farms use land based pump-shore systems that can pump millions of litres of water through farm tanks per hour (Cook 1998). Water samples are collected on a

regular basis from the areas in the ocean from which farm water is pumped and are examined to determine whether red tide causing algae are present and at what levels (W. Barnes, pers. comm.). In the event that these algae are present at worrying levels in the samples, the pump systems are stopped. Water in the farm is filtered and re-circulated until the plantonic blooms have ceased or reached harmless levels in the sea water (Botes *et al*. 2003).

3.4.2.2. Nutrition

Food type and food quality also play an important role in the success of an abalone farm. Abalone diets consist of a variety of kelp and algal species and in the Western Cape especially the kelp species, *Ecklonia maxima,* which is the species with the highest nutritional value, and the abalone's food of choice (Barkai & Griffiths 1986; Wood & Buxton 1996b; De Waal & Cook 2001). Commercially developed abalone feed pellets have recently been introduced into abalone farming in order to ensure that abalone receive the best possible nutrition, thus aiding in their growth and general health (Sales & Britz 2001a & b).

3.4.3 South African abalone export

According to Troell *et al*. (2006), South Africa is currently the largest producer of abalone outside Asia and during 2003, more than 500mt were farmed. Twelve million US Dollars have been invested into South African farms (Macey & Coyne 2005). The expected production of these farms is estimated at between 500 to 800t per annum (Sales & Britz 2001a). The sale of abalone in to the public (W. Barnes, pers. comm.; A. Mouton, pers. comm.) and restaurateurs in South Africa is allowed, but very strictly regulated (J. Venter, pers. comm.). Abalone can be exported live, frozen, as a dried product, or as canned products (Oakes & Ponte 1996; Vosloo & Vosloo 2006). Live abalone exported to the Far East in 1999 had a whole mass value of R192/kg and a value of R580\kg for the shucked meat (Hauck & Sweijd 1999).

3.4.4 Current research

Various research projects are currently being conducted on different aspects of abalone biology and husbandry in an attempt to optimise the abalone industry of South Africa. These include work on the artificial spawning and seeding of abalone, seeding of juveniles in the wild (De Waal *et al*. 2003), development of methods to decrease the large mortalities involved with settlement of larvae, design of settlement plates with sufficient diatom film for larvae to settle and graze on, and the establishment of methods to remove larvae from diatom films without harming them.

The development of artificial food and its digestibility (Shipton & Britz 2001; Sales & Britz 2002a) to maximize growth and ensure that abalone receive all the nutrition they need for optimal health and growth (Britz 1996a; 1996b; Britz & Hecht 1997; Sales & Britz 2001b; 2002b; Sales & Janssens 2004), and the natural digestive gland bacteria (probiotics) aiding in digestion (Erasmus, Cook & Coyne 1997) and immunity are also important research topics (Macey & Coyne 2005). Research into water quality (Shackleton *et al*. 2002; Reddy-Lopata *et al*. 2006) and husbandry of farms are also very important (Cook 1998; Sales & Britz 2001a). As part of the research on animal husbandry, the use of chemicals for the relaxation of the abalone foot muscle in order to remove them from surfaces without harming them, and the concentrations of these anaesthetics that will have the least side effects on the animals, are being investigated (White *et al*. 1996). The sustainable utilisation of wild abalone and the conservation of natural gene pools through stock enhancement and reseeding of areas on the coastline where abalone populations have been decreasing are some of the ecological issues that are being addressed through ongoing research.

Lastly, some of the most important aspects of abalone farming being studied are the classification, prevention and control of parasites and diseases that devastated abalone farms in the past and may do so currently, and new parasites and diseases that have the potential to do so in future.

4. Abalone parasites

Over the years, abalone farming has become a very important and economically viable industry worldwide. Despite its success, abalone farmers have also encountered many problems, with the biggest of these, the loss in farm stock through diseases and parasites. Incorrect farming methods, bad water quality, insufficient nutrients due to feeding methods, and excessive handling (White *et al*. 1996) have also caused large mortalities in abalone populations. Environmental fluctuations can also result in the development of diseases and parasites in farmed abalone (Álavarez-Tinajero, Cáceres-Martínez & González-Avilés 2002). One of the biggest issues in abalone aquaculture to date is the lack of knowledge of abalone parasites and diseases. A small number of these parasites and diseases have been studied, but there are still many that are not well researched and even some that are not described or classified.

One of these undescribed parasites is an intercellular, digestive gland parasite found in South African farmed abalone. The aim of this chapter is thus to provide an overview of all the known abalone parasites worldwide, in the hope that understanding the known parasites might shed light on the enigmatic parasite that forms part of this study. The parasites will be discussed in the following order: prokaryotes, Chromista, Protozoa, digeneans, sabellid polychaetes and shell-boring polychaetes. These are also summarised in Table 4.1.

4.1 Prokaryotes

4.1.1 Withering syndrome associated with Rickettsiales-like prokaryotes

Withering syndrome is an infectious (Friedman, Roberts, Kismohandaka & Hedrick 1993), fatal, disease affecting abalone (Andree, Friedman, Moore & Hedrick 2000). It is also known as wasting foot syndrome and abalone wasting disease, and is a chronic slow-progressing disease that has a long incubation time, followed by rapid mortalities (Friedman, Biggs, Shields, & Hedrick 2002). The cell walls of the

*Note: Not all authors and common names could be obtained for genus and species used in this chapter.

that causes the disease are consistent with those of members of the order Rickettsiales. Studies done by Moore, Robbins & Friedman (2000) indicated that the modifications linked to withering syndrome in the abalone, tie in with post-oesophageal infection intensity of the rickettsia-like prokaryotes. This indicated that the rickettsia-like prokaryotes are the etiologic cause of withering syndrome (Moore *et al*. 2000). The disease was found in the Channel Islands of California and Baja California (Mexico) in the 1980s (Lafferty & Kuris 1993; Alstatt, Ambrose, Engle, Haaker & Lafferty 1996; Moore *et al*. 2002) and later in China. High mortalities occurred in black abalone populations from Acapata Island, California in 1986 (Van Blaricom *et al*. 1993) and in only five years after the first outbreak, more than 99% of the abalone population disappeared (Richards & Davis 1993).

Hosts species affected in the past include *Haliotis rufescens, H. corrugata* (pink abalone) Wood, 1828*,* and *H*. *cracherodii* (black abalone) Leach, 1814 (Moore *et al*. 2000; Friedman, Andree, Beauchamp, Moore, Robbins, Shields & Hedrick 2000a). Research by Friedman (2002) suggested dispersal of this disease might be due to human activities such as abalone "out planting" and other aquaculture activities. These activities could also cause the infection of wild abalone.

Rickettsiales-like, oval, prokaryote inclusions, 17x55μm, in diameter are colonies of intracellular Gram negative bacteria that multiply by means of binary fission (Gardner, Harshbarger, Lake, Sawyer, Price, Stephenson, Haaker & Togstad 1995; Friedman *et al*. 2000a, Moore, Cherr & Friedman 2001a). Inclusions are present in the cytoplasm of the epithelial cell linings of the digestive systems (Gardner *et al*. 1995; Antonio, Andree, Moore, Friedman & Hedrick 2000). These intracellular Rickettsiales-like prokaryotes cause the loss of nutrients like glycogen reserves in the foot muscle (Friedman 1996) as well as digestive enzymes, thus decreasing the absorption in the gut of the abalone (Gardner *et al*. 1995). The blood cell numbers decrease as well as the haemocyanin concentrations (Friedman 1996). Due to the loss of digestive enzyme secreting cells, muscle tissue catabolism cannot take place, leading to signs of withering.

Functional tissues in the digestive glands containing cells specifically intended for absorption and secretion are replaced by connective and mucosal tissues (Moore *et al*.

2000). This leads to symptoms associated with starvation, including weakness, mantle retraction, lethargy, poor gonadal development, pedal muscle atrophy (forming tumour-like masses), loss of body mass, pale colouration of the host foot and epipoduim (Lafferty & Kuris 1993) and subsequent death (Gardner *et al*. 1995; Moore *et al*. 2000). All sizes of abalone can be affected (Bower 2000). Histological studies on abalone with withering syndrome have indicated morphological changes in the digestive gland and foot muscle, as above (Moore *et al*. 2000).

Muscle degeneration due to withering syndrome increases the cellular immune system activities of the abalone (Friedman *et al*. 2002). Haemocytes of infected hosts were more active than healthy hosts, but engulfed foreign particles less sufficiently and lower respiratory bursts occurred in these hosts (Friedman, Robbins, Jacobsen & Shields 2000b). Major metabolic changes could occur before notable signs of foot atrophy were observed. Death occurred in about 42 days after clinical signs were observed in the abalone at 18-20ºC (Friedman 1996; Friedman *et al*. 2002). Wild populations can also have high mortalities rates, due to deteriorating health and strength making them more vulnerable to predators and dislodgement by strong wave actions (Van Blaricom *et al*. 1993).

This disease can be transmitted through water and no physical contact between hosts is required. Temperatures exceeding normal conditions can lead to the development of this disease (Moore *et al*. 2001b). El Niño is the most common environmental factor contributing to elevated water temperatures and another factor is the location of abalone in the vicinities of power plant discharge (Alstatt *et al*. 1996). Incidence of the Rickettsiales-like prokaryotes are extremely high at the beginning and end of these El Niño episodes (Cáceres-Martinez, Álvarez Tinajero, Guerrero Renteria & González Avilés 2000).

Andree *et al*. (2000) developed a PCR test for the rapid recognition of the Rickettsiales-like prokaryotes that cause this deadly disease. The severity of this disease in facilities can be controlled to an extent by lowering water temperatures to 15°C, if possible. Oxytetracycline administrated orally, or by means of injection, proved to be a promising treatment when the first clinical signs of withering syndrome were observed in the hosts (Friedman, Trevelyan, Robbins, Mulder & Fields 2003).

During a health program conducted in South Africa, an undescribed Rickettsiales-like prokaryote (Fig 4.1 A) was discovered in the digestive gland of *H. midae* (Mouton 2000).

4.2 Chromista

4.2.2 *Labyrinthuloides haliotidis* Bower, 1987

The parasite, *Labyrinthuloides haliotidis,* is an achlorophyllous, eukaryotic protist from the Phylum Labyrinthomorpha, Order Labyrinthulida, (see Bower 1987a). Previously, the classification of this, and related organisms, presented problems, since they were seen as lower fungi. *Labyrinthuloides* spp. are usually present in sand, sediment, dentritus and the water column, and the species *L. minuta* has been isolated from *Ulva lactuca* and other algae (Bower 1987a). Reports of labyrinthulids infecting healthy animals (abalone) first appeared in 1973. *Labyrinthuloides haliotidis* occurs along the west coast of Canada (Bower, McGladdery & Price, 1994) and in the British Columbia area (Bower 2003).

Common hosts are juvenile *Haliotis kamtschatkana* Jonas, 1845 (pinto abalone) and *H. rufescens* Swainson, 1822 (red abalone) (Bower *et al.* 1994). Various marine invertebrates can also be hosts to *L. haliotidis* (Bower 1987c). *Labyrinthuloides haliotidis* is invasive and pathogenic (Bower 1987b). Juvenile abalone younger than six months and less then five mm in shell length are mainly infected by *L. haliotidis* and high mortalities can occur (Bower 2000; 2003). In British Columbia, over 90% of 100 000 juvenile abalone died two weeks after being infected by *L. haliotidis* (Bower 1987d). Due to this infection, the abalone farm in question had to close down (Bower 2000).

In studies done by Bower (1989) on *H. kamtschtkana* and *H. rufescens* infected with *L. haliotidis*, no evidence of an inflammatory response was found in the juveniles. In experimental studies, *L. haliotidis* were inoculated into the foot and muscle of large abalone. Results showed that abalone larger than 25mm in shell length could not be

infected by these parasites (Bower 2005). It is believed that older abalone are less susceptible to this parasite, because they could have been previously exposed to the parasite, they may be survivors of past exposures (innately resistant), or they may have developed cellular and humoral defence mechanisms (Bower 1987b). Parasites are usually present in the muscles, nerve tissues and foot of the abalone (Bower 1987a) and here the vegetative stage reproduces by binary fission.

Parasites can easily be detected in juvenile abalone with the aid of light microscopy due to the transparent nature of juvenile tissue (Bower 2003). A direct antibody technique has been developed by Bower, Whitaker & Elston (1989a) to aid in the detection of all life stages of *L. haliotidis*. This technique can differentiate between this, and parasites from other similar marine organisms, and can detect low intensities of the parasite. Clinical signs of disease include enlargement and devastation of the head and foot tissue, round protists ± 10m in diameter (Bower *et al.* 1994) inside the tissue (Bower 1987b), declining state of the host and loss of righting reflexes (Bower 2003).

Labyrinthuloides haliotidis are regarded as saprobes and are common in marine habitats (Bower, Whitaker & Voltolina 1989b). Temperatures ranging from 5-24ºC promote growth of the parasite (Bower 1987c). Generally, *L. haliotidis* is not specific in its nutritional requirements and can survive for long periods of time in sterile sea water (Bower 1987c). It is thus seen as a facultative parasite. Sagenogenetosomes are surface organelles found on *L. haliotidis* (Bower 2000). The vegetative states of the parasite infect the juvenile abalone and upon contact with the host, sagenogenetosomes develop lytic activity used to disrupt and lyse the plasmalemma layers of the host's epithelial cells (Bower, McLean & Whitaker 1989c; Bower 2000). Vegetative stages are released into the surroundings as dead hosts decompose and can thus infect a new host (Bower 2000).

Various treatments, including use of detergents, have been used to interrupt the life cycle of the parasite, or to kill the parasites without harming the hosts. In a study of *L. haliotidis* and its reaction to ozone treated sea water, it was found that the parasite could survive exposure to high and low concentration of ozone for long and short periods of time. The higher concentrations of ozone (0.97mg/l for 25min) (Bower *et*

al. 1994) exterminated or damaged zoospores and a delay in their growth was noticed, but this treatment was not considered adequate enough (Bower *et al*. 1989b). Disinfectants and therapeutic agents have also been studied in an attempt to control infections with *L. haliotidis*. High resistance was shown by the parasite to formalin, iodophor, methylene blue, isopropanol, malachite green, copper sulphate and other therapeutic agents. The only effective agents tested were chlorine (Bower 1989) and cyclohexamine. Bower (2003) reported that a 20min exposure to 25ml Cl/l of sea water can destroy these parasites. Cyclohexamide treatments at 1-2mg/l for 23h/day during a five day treatment cured infected hosts (Bower 2000).

4.3 Protozoa

4.3.1 Ciliates

Representatives of the genus *Mantoscyphidia* Jankowski, 1980, are gill parasites of abalone and other marine and freshwater gastropods (Botes, Basson & Van As 2001). There are four orders of ciliates which have been noted to occur in abalone. The orders are Thigmotrichida, Peritrichida, Heterotrichida and Hypotrichida. They are present in abalone from southern Africa, British Columbia, Canada and Baja California in Mexico (Bower 2005).

Studies conducted at the De Hoop Nature reserve in South Africa indicated that *Haliotis spadicea* (blood spotted abalone) Donowan, 1808, and *H. midae* were infected with *Manthoscyphidia spadicea* and *Manthoscyphidia midae* (Fig. 4.2) (Botes *et al*. 2001). *Mantoscyphidia* spp. are usually present on the gills, mantle cavity and oesophageal pouch of the hosts. Other abalone hosts include *H. kamtschatkana* and *H. rufescens*. No control methods are available for these ciliates (Bower 2005).

4.3.2 Haplosporidians

The first haplosporidian species was discovered in the late 1800s and placed in the order Haplosporidia (Caullery and Misnil, 1899) and class Sporozoa Leuckart, 1879 (Flores, Siddall and Burreson 1996). Currently there are four haplosporidian genera:

Haplosporidium Caullery & Mesnil, 1899, *Minchina*, *Urosporidium* Caullery &Mesnil, 1905 and *Bonania* (see Hine, Wakefield, Diggles, Webb & Maas 2002; Reece, Siddall, Stokes & Burreson 2004). Severalhaplosporidian spp. are known to be pathogens of commercial molluscs and can infect wild and farmed animals (Reece & Stokes 2003). Haplosporidian parasites have uninucleated spores lacking polar capsules and polar filaments. The spore wall encloses the spores and has an anterior orifice (Flores *et al*. 1996).

Later, haplosporidians were also found as parasites of *Haliotis* spp. in New Zealand culture facilities (Hine 2001). The dominant host infected with haplosporidians was *Haliotis iris*, but others included *H. rufescens* (Hine 2001), *H. cracherodii* (Hine 2002a) and *H. tuberculata* (Azevedo, Balseiro, Casal, Gestal, Aranguren, Stokes, Carnegie, Novoa, Burreson & Figueras 2006). Heavy infections of haplosporidian parasites in *H. iris* juveniles leads to behavioural abnormalities like the loss of righting reflexes, lethargy, oedema, pale lesions in the foot and mantle, and pedal muscle degeneration (Hine 2001). Infected abalone could easily be detached from a surface (Diggles *et al*. 2002).

Histological studies done on *H. iris* showed that plasmodia were present in the gills, kidneys, foot, hemolymph, heart, mantle, epipodium and connective tissue of the abalone digestive glands (Diggles *et al*. 2002; Azevedo *et al*. 2006). Diggles & Hine (2001) pointed out that the parasite might spread through the blood stream to these organs. The plasmodium usually has two nuclei and a clear area encircles it (Bower 2003). This disease does not occur at temperatures lower than 20°C (Diggles *et al*. 2002) and is not able to transfer horizontally between hosts. It was, however suggested by Diggles & Hine (2001) that unfiltered water supplies could contribute to the transference of this parasite from wild to farmed animals. The prepatend period is about three months (Diggles *et al*. 2002) and an intermediate host may be required to transfer this pathogen from heavily infected to healthy abalone (Hine 2001).

Molecular research on this specific haplosporidian parasite was done by Reece & Strokes (2003). They found that the parasite's DNA fragments closely matched that of *Urosporidium crescens* and *Haplosporidium nelsoni* (Haskin, Stanber and Mackin, 1966). This haplosporidian was not named at that stage, but recently Azevedo *et al*.

(2006) classified it as *Haplosporidia montforti* Azevedo Balseiro, Casal, Gestal, Aranguren, Stokes, Carnegie, Novoa, Burreson & Figueras, 2006.

4.3.3 *Perkinsus* species

In the 1970s, a disease in *Haliotis rubra* (rubber abalone) Leach, 1814, from South Australia was reported (Lester & Davis 1981) and described as a *Perkinsus* species from the class Perkinsea (Levine, 1978). Later studies indicated that the species was *Perkinsus olseni* Lester & Davies, 1981 (Goggin, Sewell & Lester 1989). *Perkinsus* species are wide spread in marine molluscs (Goggin, Lester & Sewell 1990), along the coast of South Australia and the area of the Great Barrier Reef (Goggin & Lester 1987). Other hosts infected with this parasite are *H. laevigata* (green lip abalone Donovan, 1808, *H. cyclobates* (whirling abalone) Peron & Lesueur, 1816 and *H. scalaris* (ridged ear abalone) Leach, 1814 (Lester & Davis 1981; Bower 2000).

This disease affects the muscles of the adductors in abalone. Brownish masses (pustules) were present in the flesh (Lester & Davis 1981). Several parasites (Sparks 1985) grew and divided in the haemolymph (Bower *et al.* 1994). In addition, muscle abscesses occurred and high mortalities were seen (Goggin & Lester 1987). Lesions were noted on the foot and mantle of abalone studied and contained pus. Lesions closest to the shell caused brown scars on the interior surface (Lester & Davis 1981). The infected areas were surrounded by loose walls of connective tissue fibres adjacent to the infected region and inside these were numerous leukocytes, as well as clusters of round trophozoites 13-16µm in diameter and schizonts 15µm in diameter (Lester & Davis 1981).

Lester (1986) indicated that *P. olseni* Lester & Davis, 1981, can swell up to ten times its normal size within 24 hours and this coincides with the time the host starts to die. Upon decomposition of the host, the parasites are released into the surrounding sea water and can survive for up to 48 hours as free living organisms (Lester 1986). During this time, the parasite can be transmitted directly between hosts (Bower 2000). Infections with *P. olseni* in wild *H. rubra* correlate with the water temperature and size of the abalone (Bower 2003).

Widespread mortalities in *H. laevigata* infected with a similar *Perkinsus* species was also noted in South Australia (Goggin & Lester 1987). Water temperature could determine the success of this parasite's survival, as abalone in water temperatures beneath 15°C could encapsulate the parasite and destroy it, whereas abalone in water with a temperature above 20°C tended to die due to the fast proliferation and spread of these parasites in the tissue (Lester 1986). No method of control is known.

4.3.4 *Pseudoklossia haliotis* Friedman, Gardner, Hedrick, Stephenson, Cawthorn & Upton, 1995

The abalone kidney coccidian, *Pseudoklossia haliotis*, is known from California (USA) and British Columbia (Bower, Carnegie, Goh, Jones, Lowe & Mak 2004). Hosts bearing these coccidia include *Haliotis cracherodii, H. rufescens, H. corrugata, H. fulgens, H. walallensis* (flat abalone) Stearns, 1899, and *H. kamtschatkana* (Friedman *et al*. 1993). The kidney epithelial cells affected become hypertrophied and kidney damage followed (Bower *et al*. 1994). Degeneration of the foot muscle, weakness and low gonadal development have also been observed in the host (Friedman *et al*. 1995). All the life stages of this parasite (Fig. 4.3) are present in the abalone host (Bower 2000).

Healthy abalone becomes infected with this renal coccidian after being exposed to water previously occupied by infected animals (Friedman *et al*. 1995). Due to the presence of sexual and asexual stages of this coccidian in abalone, transmission can occur directly (Friedman *et al*. 1993). The parasite is not recognised by the host as being an invader and thus no humeral response takes place (Friedman *et al*. 1993; Bower 2000). Unidentified renal coccidia have been found in *Haliotis midae* farmed in South Africa (Mouton 2000). Presently there are no methods to control or prevent coccidian disease of this type (Bower 2005).

4.4 Digeneans

Digeneans from the families Allocreadidae and Opeocoilida (Botes, Basson & Van As 1999) occur in abalone from Australia and Southern Africa. Hosts reported to have

these parasites are *H. rubra*, *H. roei*, *H. tuberculata* (tube abalone) Linnaeus, 1758, and *H. spadicea* (Bower 2005). Parasites are present in the host as encystments in the foot muscle, digestive gland, gill filaments and gonads. Even though the above mentioned parasites do not appear to be very harmful to their hosts, they may cause castration (Bower 2000). The gills and gill filaments of *H. tuberculata* have also been noted to contain these digeneans in cysts (Botes *et al.* 1999).

Haliotis spadicea studied at De Hoop Nature Reserve in South Africa showed a high prevalence with trematode cercarial and metacercarial stages in the digestive gland and gill filaments (Botes 1999). Encystment of the parasites in the gills of *H. spadicea* led to destruction of epithelial cells (Botes 1999). The hosts could experience strain during the development of sporocysts and redia in the tissue (Botes *et al.* 1999). The prevention and control of these parasites are impractical, as various vertebrates such as birds and mammals are involved in the completion of the parasite life cycle (Bower 2005) and that abalone can perhaps infect themselves, as all the life stages of these trematodes have been observed in the host species (Botes *et al.* 1999).

4.5 Polychaetes

4.5.1 *Terebrasabella heterouncinata* Fitzhugh & Rouse, 1999

The sabellid polychaete *Terebrasabella heterouncinata*, Fitzhugh & Rouse, 1999 (Figs 4.1B, C; 4.4) is a member of the family Sabellidae (Fitzhugh & Rouse 1999). This endemic parasite (Simon *et al.* 2004) occurs abundantly around the coast of South Africa and has been found in wild *Haliotis midae* from the West Coast (Port Nolith) and the South Coast (Port Elizabeth), as well as in farmed animals (Ruck & Cook 1998) on both coasts. In the early 1990s, *T. heterouncinata* was accidentally transported from South Africa with abalone brood stock intended for a hybridization program to California (Fitzhugh 1996; Leighton 1998). Since then, these parasites have also been reported to occur in the USA and Baja California, Mexico (Bower 2000). Other hosts species includes *H. rufescens*, *H. fulgens* and *H. corrugata* (Day Culver, Kuris, Belcher & Morse 2000; Bower 2005).

Mature sabellid polychaetes, also known as fan worms, are about 5mm in length (Bower 2000) with a branchial crown that is used for feeding and respiration activities (Fitzhugh 1996). They are hermaphroditic (Fitzhugh 1996), filter feeding (Simon, Kaiser & Britz 2005), ectoparasites (Leighton 1998) and can reproduce to produce fully functional organisms. Self–fertilisation (Finlay, Friedman & Muligan 2000) can occur in sabellid worms and subsequently a single adult can generate a feasible population (Finlay, Mulligan & Friedman 2001).

Reproduction occurs frequently and an adult worm can brood young of different ages concurrently. Sexual maturity is reached between three and four months (Ruck & Cook 1998; Simon *et al*. 2005). The brood consists of up to seven (Simon *et al*. 2004) orange (Oakes & Fields 1996) eggs (embryos) within the adult's tube and the generation time is highly temperature dependant (Finlay *et al*. 2000). Embryos develop into mature motile larvae (Fig 4.1 B) (Finlay *et al*. 2001). Larval sabellids crawl out of their parental tube, attach to the growing edges of either the same abalone as the parent, or a different abalone shell (Kuris & Culver 1999; Day *et al*. 2000) and then cover themselves with a mucus tube (Fig 4.1 C & D), forming a thin membrane (Oakes & Field 1996; Bower 2005).

The host can deposit nacre over these newly formed membrane tubes within 12 hours (Day *et al*. 2000) and as the abalone produces new shell, the sabellid keeps the area around its anterior end open to the environment, resulting in the formation of a tube or burrow (Finlay *et al*. 2001; Simon *et al*. 2005). Oakes & Fields (1996) believe that a chemical is excreted by the worm in an attempt to soften the host shell, thus aiding in the scraping process used by the worm to keep its tube open. This results in the shell of the abalone becoming honeycombed, fragile and porous (Fig. 4.1 E arrow), and a downwards growing pattern occurs in the edges of the shell (Oakes & Fields 1996; Kuris & Culver 1999). Sabellids can infest a host population rapidly and spread from host to host through contact, attributed to the short generation time of the parasite and its extensive population growth (Shields, Buchal & Friedman 1998; Kuris & Culver 1999).

Abalone farmers have expressed their concerns regarding *T. heterouncinata* as it leads to lower productivity of abalone as well as a decline in marketability due to slow

growth rate and abnormalities in the shell morphology (Finlay *et al.* 2001; Leighton 1998). In the past, it was suggested that all the infected stock should be destroyed and facilities sterilised in an attempt to eliminate these parasites from the farms. This suggestion however can cause great financial loss to the developing abalone industry (Shields *et al.* 1998).

Detrimental effects of these sabellids to the abalone include a reduction in growth rate, deformed shells with absence of gill pores on the shells' leading edges, downwards orientation of the shell margin, and the loss in ability of the abalone to right themselves after being dislodged from their substrate (Oakes & Fields 1996; Leighton 1998). In severe cases of sabellid infections, mortalities can occur (Bower 2000). The inside of the shells can show small dark striations, and small worms are usually found upon dissecting the shells of infected hosts (Oakes & Fields 1996).

Slow growing abalone were more vulnerable to infestations (Simon *et al.* 2004) by these worms, and their diets also played a key role. Kelp-fed (*Ecklonia maxima*) abalone grew much faster than abalone on pellet diets and showed lower infestation rates. The faeces of abalone fed on a pellet diet contained high levels of proteins and it is believed that the sabellids feeds on it (Simon, Kaiser, Booth & Britz 2002). This helped the sabellids to produce more eggs and larvae than those infecting kelp-fed abalone. As a result, the pellet-fed abalone had higher numbers of sabellids infections (Simon *et al.* 2002; 2004). Although this parasite is not directly life threatening to the abalone, it can become a pest (Bower 2005).

Treatment of these parasites includes a 48h exposure to 28-29°C sea water, which is believed to kill all of the sabellid life stages (Leighton 1998), but can be life threatening to abalone which cannot tolerate such high water temperatures (Bower 2000). Leighton's (1998) studies indicated that sabellids cannot acclimatise to the temperatures variations used for thermal treatments. Tanks where infected abalone have been kept can also be disinfected with fresh water baths (Ruck & Cook 1998). McBride (1998) recommended that seed be inspected prior to selling and good husbandry be maintained. Filters must be implemented in discharges pipes to prevent wild stock from becoming infected with parasites from farmed animals. Inflow filters

can also be useful to limit sabellids (from wild stock) from entering farming environments (Ruck & Cook 1998).

Controlling *T. heterouncinata* by means of predators has been tested by Kuris & Culver (1999), but was unsuccessful. Ultrasound micro-cavitation treatments for one minute have been found to destroy the feeding crowns of adult sabellids, but had no extensive effect on the larvae (Loubser & Dormehl 2000). Usually, two treatments are necessary to kill all the sabellids, but abalone treated with this method have showed stress behaviour which questions this method's safety (Bower 2003). Other methods to treat the abalone included coating the shells with wax or exposing the worms to water soluble toxins which do not affect the abalone (Simon *et al*. 2004).

4.5.2 *Boccardia knoxi* (Rainer, 1973) *and Polydora haplura* Claparede, 1870

Shell-boring polychaetes, more commonly known as blister or mudworms, belong to the family Spionidae Grube, 1850. This family consists of three genera, namely *Polydora* Bosx, 1807 (Fig. 4.1 E, arrow 1), *Boccardia* Carazzi, 1895 (Fig 4.1 E, arrow 2) and *Pseudopolydora* Czerniavsky, 1881 (Blake & Evans 1973). They occur globally, but have been noted to pose a problem in a few locations, namely South Africa (Blake & Evans 1973), southern Japan, British Columbia and Australia (Lleonart *et al*. 2003b). The main host species affected by polychaetes are *Haliotis kamtschatkana, H. diversicolor* (variously coloured abalone) Reeve, 1846 (Bower 2005), *H. midae*, *H. rubra*, *H. laevigata*, *H. discus* and *H. iris* (Lleonart *et al*. 2003b).

In South Australia two species of mudworms, namely *Boccardia knoxi* Rainer, 1973 and *Polydora hoplura* Claparede, 1870, were studied. *Boccardia knoxi* was the dominant species found (Lleonart *et al*. 2003b). Polydorids produce eggs in capsules which are attached to the burrows in which they live (MacDiarmid, Day & Wilson 2004). Two routes of invading their hosts exist. Firstly, larvae swim into the mantle cavities and can burrow between the mantle and shell, or secondly, they settle on the outside of the shells and penetrate directly (Lleonart *et al*. 2003b).

In the past it was believed that mudworms scraped the shell of the abalone away to form burrows, but recently it was found that they use chemicals to break down the shell (MacDiarmid *et al*. 2004). These burrows are usually U, Y and pear shaped (Fig. 4.1 F), but more complex branching burrows have also been noted (MacDiarmid *et al*. 2004). Polychaete abundance is mainly influenced by the salinity, water temperature, sediment composition, the size and age of the abalone, their growth rate, seasonal changes, stocking density and diet. Due to presence of these worms, the shell becomes fragile and damaged (Lleonart *et al*. 2003a). The shells can also crack easily and deformities like nodules and dark patches can occur (Lleonart *et al*. 2003a). The muscle and flesh weight of the abalone can decrease, thus leading to lowered immunity (Bower 2005).

A few methods for the treatment of mudworms have been tested. Chemotherapeutic agents used as a bath treatment and exposure of abalone to humid air for four hours at 21°C are two of the methods (Bower 2000). No abalone died during the use of the air drying method and they responded well under controlled temperature and humidity conditions (Lleonart *et al*. 2003a). Some agents tested were potassium permanganate, methylene blue, malachite green, formalin, gentian violet and freshwater to name but a few (Lleonart *et al*. 2003a). Only the exposure to air method had the preferred outcome. The snail, *Oenopota levidensis* (Carpenter, 1864) is a natural predator of most *Polydora* species and can aid in the biological control of these parasites (Bower 2005).

Table 4.1: Summary of parasites infecting *Haliotis* spp Linnaeus, 1758, around the world and the effects they have on the host.

Parasite	Host	Effect on host, organs and structures	Location	Reference
Prokaryotes Withering syndrome associated with Rickettsiales like prokaryote, *Candidatus Xenohaliotis californiensis*	*H. cracherodii, H. corrugata, H. rufescens.*	Pedal muscle atrophy and degeneration (tumour like masses) Loss in digestive enzymes and nutrients like glycogen Decreased absorption in the gut Affects epithelial cells in the gut as well as enzyme secreting cells of the digestive diverticula Weakness and lethargy Weak gonadal development	South and Central California, Baja California, New Zealand	Lafferty & Kuris 1993 Friedman *et al*. 2000a Moore *et al*. 2000 Friedman *et al*. 2002

Table 4.1 Continue

Parasite	Host	Effect on host, organs and structures	Location	Reference
Chromista *Labyrinthuloides haliotidis*	*H. kamtschatkana, H. rufescens* (Juveniles).	High mortalities and moribund Host slightly swollen Loss of integrity around the head Vegetative stages found in muscles, gills and nerve tissue of the head and foot	British Columbia Islands, Canada	Bower 1987a Bower 1987b Bower 1987c Bower *et al*. 1994 Bower 2000
Protozoa *Perkinsus olseni*	*H. rubra, H. cyclobates, H. laevigata.*	Mortality Pale lesions in the foot and mantle Effects adductor muscle Muscle abscesses Lesions on foot and mantle Scars on internal surface of shell	Australia	Lester & Davie 1981 Goggin & Lester 1987 Goggin *et al*. 1987;1989;1990 Bower *et al*. 1994
Pseudoklossia haliotis	*H. rufescens, H. cracherodii, H. corrugata, H. fulgens, H. walallensis, H. kamtschatkana.*	Affects kidney epithelial cells (become hypertrophied) Kidney damage Degeneration of foot Weak gonadal development	California & BritishColumbia	Friedman *et al*. 1993; 1995 Bower *et al*. 1994
Haplosporidium montforti	*H. iris, H. rufescens, H. cracherodii, H. tuberculata.*	Behavioural abnormalities Loss of righting reflex Lethargy, oedema Muscle lesions in foot and mantle Pedal muscle degeneration Plasmoduim present in the gills, kidneys, foot, hemolymph, heart, mantle, epipoduim, and digestive gland	New Zealand culture facilities	Hine 2001; 2002a Diggles *et al*. 2002 Azevedo *et al*. 2006
Ciliates *Mantoscyphidia spadicea* & *Mantoscyphidia midae*	*H. spadicea,* *H. midae,*		South Africa	Botes *et al*. 2001
Mantoscyphidia spp.	*H. kamtschatkana, H. rufescens.*		British Columbia, Canada and Baja California	Bower 2005
Digeneans Matacercarial infections	*H. rubra, H. roei, H. tuberculata, H. spadicea.*	Possible castration Cysts in gills, foot muscle, digestive gland and gonads	Australia South Africa	Bower 2005 Botes *et al*. 1999
Polychaetes *Terebrasabella heterouncinata*	*H. midae, H. rufescens,*	Shell becomes honeycombed, fragile and porous	South Africa	Fitzhugh 1996

Parasite	Host	Effect on host, organs and structures	Location	Reference
	H. fulgens, *H. corrugata.*	Deformed shells Reduction in growth Loss of righting reflex Absence of gill pores Downward orientation of shell margin Mortalities in severe cases	California, USA, Baja California	Oakes & Fields 1996 Leighton 1998 Ruck & Cook 1998 Kuris & Culver 1999 Day *et al.* 2000
Boccardia knoxi & *Polydora hoplura.*	*H. rubra,* *H. midae,* *H. discus,* *H. laevigata,* *H. iris,* *H. kamtschatkana.*	Fragile shell Deformities Decreased muscle and flesh weight Stress	South Africa, Australia, Chile, Japan	Blake & Evans 1973 Lleonart *et al.* 2003a; 2003b MacDiarmid *et al.* 2004

5. Protozoan parasites of other commercially important marine invertebrates

A wide variety of protozoan parasites occur in commercially important marine invertebrates, other than abalone. The possibility exists that the intracellular digestive gland parasite, that forms the focus of this study, might not previously have been described from abalone. It is therefore necessary to discuss the protozoan parasites of other commercially important marine invertebrates as well, especially with the focus on intracellular parasites, and those occurring in the digestive system and related organs, such as the kidneys and digestive gland. The representatives of the following protozoan groups will be discussed: apicomplexans, haplosporidians, microsporidians and paramyxeans.

5.1 Apicomplexans

5.1.1 Coccidians

According to Bower *et al.* (1994), unidentified species of coccidia infect the kidneys of the European flat oyster *Ostrea edulis* Linnaeus, 1758 from Auray, France. Infected kidney epithelial cells are hypertrophied and in severe cases the kidney can become greatly damaged. However, although the damage can be extensive, it is not likely to cause mortalities in the oysters (Bower *et al.* 1994). A *Klossia*-like coccidian has also been found in the kidney lumen of the Chilean oyster, *Ostrea chilensis* in Foveaux Strait, New Zealand. This parasite was also observed in the haemocytes of the kidneys, gonads and the digestive diverticula (Hine & Jones 1994). Kidney coccidians are not restricted to oysters, but infect a variety of marine invertebrates (Friedman *et al.* 1993). Representatives of the genera *Aggregata*, *Pseudoklossia*, *Hyaloklossia* Labbé, 1896, *Myriospora* Lermantoff, 1913 and *Merocystis* Dakin, 1911, have all been described as heteroxenous parasites of most marine molluscs (Friedman *et al.* 1993), with *Pseudoklossia* having six described species (Friedman *et al.* 1995).

*Note: Not all authors and common names could be obtained for genus and species used in this chapter.

A number of kidney coccidian species are associated with clams, namely *Pseudoklossia glomerata* Leger & Duboscq, 1915, infecting *Tapes floridus* Lamarck and *T. virgineus* Linnaeus in the Mediterranean Sea (Sparks1985), *Pseudoklossia pelseneeri* (Leger, 1897) infecting *Tellina* and *Donax* spp. in France (Friedman *et al*. 1995), *Klossia tellinae* Buchanan, 1979, infecting *Tellina tenuis* da Costa, 1778, in Scotland and an unnamed coccidian spp. infecting *Protothaca staminea* (littleneck clam) Conrad, 1827, from Washington State and British Columbia. Friedman *et al*. (1993) stated that this last mentioned, unknown coccidian, is morphologically very similar to those found in abalone. Affected kidney epithelial cells show a state of hypertrophy and kidney tubules can fill up with the coccidian (Sparks 1985). Elevating levels of infection can spread to other tissues and may cause kidney damage. The clam hosts show behavioural changes and are further unable to burrow into the sand for protection (Friedman *et al*. 1993). Direct mortalities do not usually occur due to the presence of these parasites (Bower *et al*. 1994).

Most coccidians infecting mussels are also not identified to species level. These parasites have been found in *Mytilus edulis* (edible blue mussel) Linnaeus, 1758, and *Mytilus trossulus* (foolish mussel) Gould, 1850, from the East Coast of the United States and Canada, where they fill the tubules of the host's kidneys and hypertrophy of the kidney epithelial cells occurs. Kidney damage can be extensive in high infections, but death of the host is unlikely (Bower *et al*. 1994).

According to Sparks (1985) the scallop, *Pecten maximus* (great scallop) Linnaeus, 1758, from Roscoff, France, is host to the kidney coccidia *Pseudoklossia pectinis* Leger & Dubosqc, 1917. An unidentified coccidian species was also found in the North American scallop, *Argopecten irradians* (bay scallop) (Lemark, 1819), from the eastern parts of Canada and the United States. Symptoms of infection include hypertrophy of infected kidney cells, kidney tubules filling up with coccidian parasites, kidney damage and the spread of parasites to other tissues due to high infection levels. Mortalities may be due to other factors, like artificial farming conditions (Bower *et al*. 1994).

No known methods of control have yet been established to prevent or cure these molluscs of coccidian infections.

5.1.2 Gregarines

Marine bivalves are usually the intermediate hosts for gregarines from the genus *Nematopsis* Schneider, 1892 (Tuntiwaranuruk, Chalermwat, Upatham, Kruatrachue & Azevedo 2004). According to Azevedo & Cachola (1992), gregarine species are amongst the most important pathogens of all the apicomplexan parasites infecting bivalves.

Nematopsis ostrearum Prytherch, 1938, and *N. prytherchi* are the two main gregarine parasites of the oyster species *Crassostrea virginica* (eastern oyster) (Gmelin, 1791) from the Atlantic coast, the Gulf of Mexico, Louisiana and Virginia (Sparks 1985; Tuntiwaranuruk *et al*. 2004). Spores occur in phagocytes from the connective tissue and may move to the organs, and are also regularly observed in the mantle and gills of young hosts (Tuntiwaranuruk *et al*. 2004). Inflammatory responses usually occur due to infections, but pose no real threat to the health of the hosts (Bower *et al*. 1994).

Nematopsis veneris, *N. ostrearum* and *N. schneideri* infects the clam species *Cardium edule* Linnaeus, 1758, *C. lamarcki*, *Protothaca staminea*, *Saxicava rugosa*, *Tapes philippinarum* Adams & Reeve, 1850, and *Tellina* spp. The spores mainly occur in phagocytes from the connective tissue and may move to the organs, such as the gills. Infections occur in association with haemocyte infiltrations, but are not likely to have a negative effect on the health of the hosts (Bower *et al*. 1994).

Mussel species such as *Mytilus edulis*, *M. trossulus*, *M. galloprovincialis* (mediterranean mussel) Lamarck, 1819, and *Geukensia demissa* (ribbed mussel) (Dillwyn, 1817) are the hosts of *Nematopsis schneideri*, *Porospora galloprovincialis*, *P. gigantea* and other *Nematopsis* spp. All of the above parasites species also parasitise various other species of marine bivalves. The distribution of these parasites is most likely world wide (Bower *et al*. 1994). Phagocytes can contain gynospores, oocysts and sporozoites, and can move through the connective tissue to other organs, specifically the gills. The infections that occur are associated with central insignificant inflammatory responses. According to Bower *et al*. (1994), it is believed that these

infections can lead to the formation of pearls or calcareous excretions on the inner surface of *Mytilus edulis*.

Scallop species such as *Argopecten irradians*, *Chlamys varia* (Macgillivray, 1825) and *Patinopecten yessoensis* (Japanese scallop) Jay, 1857 are the hosts of *Nematopsis ostrearum*, *N. pectinis* and *N. duorari*. These parasites have been reported from Canada, the western and eastern parts of the United States and Europe. The gregarines complete their life cycles in the intestinal track of many marine arthropods and an increase in their numbers is thus restricted to the presence of suitable arthropods (Bower *et al.* 1994).

In studies done by Prasadan & Janardanan (2001), the authors described three new *Nematopsis* species infecting the intestines of crabs from India, Kerala, the Malappuram district, the Kadalundi Estuary and the Olippuram river banks. The first, *Nematopsis messor* Prasadan & Janardanan, 2001, occurs in *Metapograpsus messor*; *Nematopsis quadratum* Prasadan & Janardanan, 2001 is found in *Sesarma quadratum*; and the third species, *Nematopsis annulipes* Prasadan & Janardanan, 2001, infects *Uca annulipes* (Fig. 5.1).

There are currently no known methods for control of gregarine infections. The accessibility of crustacean hosts to complete specific life stages determines the spread of these parasites.

5.1.3 Haplosporidians

Haplosporidium costale Wood & Andrews, 1962 is a parasite of the oyster *Crassostrea virginica* and occurs in the waters of Virginia and Maryland (Sparks 1985). This disease is also known as SSO (Sea Side Organism). These parasites cause high mortality of their hosts, especially during May and June (summer) (Sparks 1985; Hine & Wesney 1992; Novoa, Balsero & Figueras 2004).

All the tissue contains sporulating stages, especially the gills and mantle (Haskin & Andrews 1988). It is suspected that salinity levels of higher than 25ppt during this time of the year may be the cause of this increase in host mortalities (Bower *et al.* 1994).

Studies conducted by Hine (1996) indicated that *Haplosporidium nelsoni* is present in the oyster *Crassostrea virginica* in Tasmania and Victoria. The parasite was first recorded in 1957 (Haskin & Andrews 1988; Hine & Wesney 1992), and the disease caused by the parasite is known as haplosporidosis or MSX (Multinucleated Sphere X) (Stokes, Siddall, & Burreson 1995; Sunila, Karolus, Lang, Mroczka & Volk 2000). Hine (1996) found that some of the oysters showed resistance to *H. nelsoni* infections. The host resistance was thought to be attributed to serum agglutinins that coat the parasite, allowing haemocytes to phagocytose them (Ford 1988). The plasmodia stages, however, have surface receptors that mimic other host cells and are thus not detected by the haemocytes in non-resistant hosts (Hine 1996).

Effects of these parasites on the hosts include the disruption of digestive gland epithelium and changes in digestive gland colour as well as mantle shrinkage, shell defects and weight loss, and ultimately, mortality (Sparks 1985; Haskin & Andrews 1988; Bower 1995). It is believed that *H. nelsoni* is intolerant to salinities lower than 10‰ (Ford & Haskin 1988; Haskin & Andrews 1988). Oysters that occur at depths of less than 45cm had lower prevalences with *H. nelsoni* than oysters growing at depths of more than 90cm. Because of this Volety, Perkins, Mann & Hershberg (2000) suggested that oysters must be farmed on artificial reefs no deeper than 45cm in order to limit infections by these parasites. Infected animals can also be transferred to water with low salinities in an attempt to lower infections. No other methods of control have been established

The clams *Tapes decussatus* Linnaeus, 1758, and *Tapes philippinarum* from the West coast of France, Portugal and Spain are hosts of *Haplosporidium tapetis* (Azevedo 2001). *Tresus capax* (fat gapper) (Gould, 1850) from Yaquina Bay in Oregon (North America) are infected by an unnamed haplosporidian. Infections levels are typically low and the pathogenicity can be minimal. Clam mortalities have not been related to haplosporidian infection (Bower *et al.* 1994).

The species of haplosporidian parasites infecting mussels include *Haplosporidium tumefacientis*, a parasite of *Mytilus californianus* (Californian mussel) Conrad, 1837, from the coast of California, and an unnamed *Haplosporidium* sp. infecting *M. edulis* from the coast of Maine. Swelling can occur in the kidney and digestive gland varying from small nodules, to swelling of the whole organ. Infected mussels digestive glands are light brown in colour compared to the green brown of uninfected animals. The infection levels are usually low, and do not have a noticeable effect on the health of the infected animals (Bower *et al.* 1994).

Haplosporidium louisiana Sprague, 1963 (Fig. 5.2) and an unnamed *Minchinia*-like species have been reported from the crabs *Callinectes sapidus* (blue crab) (M.J. Rathburn, 1896), and mud crabs *Panopeus herbstii* (Atlantic mud crab) H. Milne Edwards, 1834, *Eurypanopeus depressus* (flat back mud crab) (S.I. Smith, 1869) and *Rhithropanopeus harrisii* (estuarine mud crab) (Gould, 1841) from the East coast of the United States. Haemocytic dysfunctions can occur due to the infection by these haplosporidians. The haemolymph of infected crabs has a thick white colour with a low viscosity and is unable to clot correctly. The infected crabs can be lethargic and although the disease can be highly fatal, its occurrences are rare (Bower *et al.* 1994).

5.1.4 *Perkinsus* spp.

Perkinsus spp. has been found in approximately 34 bivalves in the Atlantic and Pacific Oceans and from 30 bivalve species on the Great Barrier Reef in Australia (Goggin & Lester 1987; Bower *et al.* 1994). Localities include the Gulf of Mexico, British Columbia, the Mediterranean Sea and along the southwest coast of Korea and Europe (Casas, Grau, Reece, Apakupakul, Azevedo & Villalba 2004).

Species infecting molluscs are *P. marinus*, *P. olseni* (syn. *P. atlanticus*) Elandalloussi, Leite, Rodrigues, Afonso, Nunes & Cancela (2005), *P. mediterraneus*, *P. karlssoni, P. qugwadi*, *P. chesapeaki* and *P. andrewsi* (Ahn & Kim 2001). Perkinosis is a disease of pandemic proportions and its pathogenicity can positively be correlated to host densities (Ngo & Choi 2004).

Perkinsus marinus, also known as "Dermo" disease (Goggin *et al.* 1990), is an apicomplexan parasite infecting mainly oysters. *Perkinsus marinus* was first described as *Dermocystidium marinus* (Andrews 1988; Perkins 1996) and also *Labyrinthomyxa marina* (Burreson & Calvo 1996), and has been found to endure a variety of environmental conditions (Goggin *et al.* 1990). These parasites were first found in 1949 in Louisiana and Virginia, but are currently also known from the East coast of the United States of America (Goggin *et al.* 1990; Ordas, Novoa & Figueras 1999) and the Gulf of Mexico (Ford 1996).

Host oyster species include *Crassostrea virginica* (Goggin *et al.* 1989) and *C. gigas* (pacific giant oyster) Thunberg, 1793, but the latter were experimentally infected (Ordás *et al.* 1999). Goggin *et al.* (1989) suggested that the parasites could possibly enter the host while it is feeding but Ngo & Choi (2004) stated that the parasites enter through the gills of the host, and will then be transported to the organs via the haemolymph. Stages of *P. marinus* have regularly been observed in the digestive epithelium (Goggin *et al.* 1989) and throughout the digestive tract (Andrews 1988). Several severe effects can be noticed in the host species, including inhibition of gonadal development, pale colouration of the digestive gland, mantle shrinkage, abscess lesions (Bower *et al.* 1994), retarded growth and mortalities in both wild and cultured animals (Goggin *et al.* 1990; Volety *et al.* 2000).

Cellular and humoral defences of the host are affected by *P. marinus* (Garreis, La Peyre & Faisal 1996). Haemocyte activity is increased due to the proliferation of the parasites (Ordas *et al.* 1999). However, encapsulation of the parasite by the haemocytes is ineffective due to the parasites' ability to lyse these cells (Anderson, Unger & Burreson 1996; Garreis *et al.* 1996). During the life cycle of *P. marinus*, immature trophozoites develop into mature trophozoites (Fig. 5.3). These will develop into tamonts, each containing 8-16 immature trophozoites (Andrews 1988; Perkins 1996). When the hosts die and decompose, the immature trohozoites, released from the tamont, mature and enlarge to form biflagelated zoospores (Burreson & Calvo 1996). The zoospores will then infect a new host (Andrews 1988; Perkins 1996).

Goggin *et al*. (1990) found oyster mortalities to be very high during warm, dry seasons and it appeared that the older oysters were more likely to die than younger ones. During these periods, water temperatures could rise above 20°C, leading to faster proliferation rates of the parasites and ultimately increasing mortalities in the host species (Goggin *et al*. 1990). Mortalities might thus be due to the disruption of epithelial and connective tissue cells, as well as a reduction in energy production by the host (Bower *et al*. 1994; Lee, Cho, Lee, Kang, Jeong, Huh & Huh 2001). Salinity levels higher than 10‰ have also been identified as a key factor in the survival and success of this parasite (Andrews & Ray 1988; Ann & Kim 2001).

Casas *et al*. (2004) described *P. mediterraneus,* from the European flat oyster, *Ostrea edulis* (edible oyster) Linnaeus, 1758, occurring in the Mediterranean Sea. DNA studies conducted by these authors focused on its classification and the different life stages that occurred in the oysters. The trophozoites were mostly found in the connective tissue of the gills, visceral mass, gonads and mantle. The hosts may become lethargic and weak when parasite numbers increase and this can lead to mass mortalities (Lee *et al*. 2001).

Perkinsus atlanticus Azevedo, 1989, occurs in the clam species *Tapes decussates* (grooved carpet shell) and *Tapes philippinarum* (little neck clam) Adams and Reeve, 1850 (Bower *et al*. 1994) from Portugal (Azevedo 1989), northwest Spain (Ordas *et al*. 1999; Leite, Afonso & Cancela 2004) and the Mediterranean Sea (Elandalloussi *et al*. 2005). Recently, *P. atlanticus* were recognised as being a synonym of *P. olseni* (Elandalloussi *et al*. 2005) that occurs in abalone species from Australia (see Chapter 4). White cysts occur on the gills (Park & Choi 2001) and in high infections also in the mantle and foot muscle (Azevedo 1989). High prevalences of *P. atlanticus* can also lead to the host being more susceptible to other pathogens (Robledo, Nunes, Cancela & Vasta 2002).

A *Perkinsus* sp. found in the gills and palps of the clam *Mya arenaria* (soft-shell clam) Linnaeus, 1758, from Chesapeak Bay (California) induced cellular host responses and encapsulation of the parasite (McLaughlin & Faisel 2000). Drought periods leading to high salinities in bays can also promote infections with *Perkinsus* species. In 1997, *Perkinsus* spp. were reported from Korea, infecting the clam

Ruditapes philippinarum (Park & Choi 2001). White nodules were observed on the gills and mantle as well as impediment of host reproduction and growth. The connective tissues of the digestive gland and gonads had the highest infection levels (Park & Choi 2001). Ngo & Choi (2004) also noted that digestive epithelium withering, haemocyte infiltration and tissue inflammation occurred.

No known methods of control have been established for *Perkinsus* spp. in general. Prevention methods that may be of some help include, not transferring, exporting, importing or transplanting infected hosts to uninfected areas, reducing the density of the hosts in farms, reducing water temperatures and lowering water salinity to beneath 10‰ (Bower *et al*. 1994). Filtration of inflow water through a 1µm cartridge followed by ultraviolet treatments (30,000μWs^{-1} cm^{-2}) has also been suggested as treatment (Ford, Xu & Debrosse 2001). Ellandallousii *et al*. (2005) reported that pyrimethamine and DFO (iron chelation) can decrease parasite loads in established infections.

5.2 *Bonamia* spp.

The disease bonamiasis (Bower 1995) is caused by various species of *Bonamia*. The European flat oyster, *Ostrea edulis*, is the most common host of *Bonamia orstrea* (Grizel, Mialhen, Chagot, Boulo & Bachere 1988; Mortensen 2000). Other hosts include the southern mud oyster, *Ostrea angasi*, from New Zealand and Australia, and the Chilean oyster *Ostrea chilensis* from the Chile and the USA (Grizel *et al*. 1988; Hine & Wesney 1992; Bower 1995).

Pathological effects include gill lesions (Grizel *et al*. 1988) and may result in high mortalities (Hine & Wesney 1992). Bonamiasis can occur throughout the year (Sparks 1985) in locations where the temperatures are as low as 4-5ºC (Grizel *et al*. 1988). According to Hine (1996), transmission of the parasite can be directly between hosts. Martyn, Bower, Clarke, Meyer, Lowe, Osborn, Chow, Hannah, Byrne, Sojonky & Robinson (2006) developed a real time PCR method, sensitive enough to detect very low infections of *Bonamia* spp. in hosts. Balseiro, Conchas, Montes, Gómez-León, Novoa & Figueras (2006) also tested PCR methods for the detection of this parasite and found that these are more sensitive than histological and cytological techniques.

Bonamia spp. poses a big threat to the wild mollusc fisheries in New Zealand. DNA analyses were conducted on a *Bonamia* sp found in *Ostrea angasi* Sowerby, 1871 from Australia (Hine & Jones 1994) and *Bonamia ostreae* from Europe. It was found that these two species were indeed different. Ultrastructural studies by Hine, Cochennec-Laureau & Berthe (2001) led to the naming of the Australian species as *Bonamia exitiosus* Hine, Cochennec-Laureau & Berthe, 2001.

Life cycle studies on *B. exitiosus* found that it is abundant in oysters during September to December. During late December the parasite is mostly present in the haemocytes of the gut (Hine & Jones 1994). When the hosts spawn the parasite enters the gonads where it reabsorbs unspawned eggs and sperm. Proliferation soon follows and the parasites are released from the excretory organs, as well as from decomposing hosts (Hine & Jones 1994). Hosts die rapidly due to infection by these parasites (Hine & Wesney 1992). *Bonamia exitiosus* was also found to infect *O. chilensis* from New Zealand and Chile (Hine *et al.* 2001). In this host, the parasites can occur in the kidneys and gonads (Hine 2002b) and inevitably lead to high host mortalities (Balseiro *et al.* 2006).

5.3 Microsporidians

Microsporidians are obliged intracellular parasites forming spores (Rohde 2005). A wide range of invertebrates can be hosts of microsporidian species and they are known to cause severe diseases (Sparks 1985).

An unidentified species of microsporidian infects the oyster *Tiostrea chilensis* from New Zealand. This parasite was observed in the connective tissue surrounding the gut, where it forms elliptical cysts that contain spores (Bower *et al.* 1994). The clams *Cardium edule* and *Macoma baltica* (Baltic macoma) Linnaeus, 1758, from France and the Tred River in the United States respectively, are hosts of another unidentified microsporidian parasite. The pathogenicity of this parasite has not yet been established (Bower *et al.* 1994).

Agmasoma spp. and *Amesoma* spp. can cause the disease more commonly known as "sick crab disease" in various crab species, as well as other marine crustaceans. In the Gulf of Mexico and the USA, *Ameson michaelis* parasitises the blue crab (Bower 1995). This microsporidian can invade or replace tissue from the gonads, hepatopancreas, gills, muscles and heart, and can cause infected tissue to appear white. Mortalities can occur and the infected hosts are unmarketable (Bower *et al.* 1994).

Cotton shrimp disease of shrimps and prawns is caused by species of the microsporidian genera *Agmasoma* and *Amesoma*. *Ameson nelsoni* occurs in shrimp species from the Gulf of Mexico (Bower 1995). *Agmasoma penaei* is a parasite of the shrimps *Penaeus setiferus* (white shrimp) (Linnaeus, 1767) and *P. duorarum* (pink shrimp) Burkenroad, 1939 (Bower 1995; Rohde 2005). Infected shrimps appear cooked. These parasites can occupy or replace the heart, gonads, muscles, hepatopancreas and gills of their hosts (Bower *et al.* 1994). Castration, deteriorating health and mortality have also been noted (Rohde 2005).

In order to control these parasites, infected hosts must be removed and destroyed. Intermediate hosts such as fin fish can also be removed or their numbers kept to a minimum. Animals from infected areas should not be transplanted to new uninfected areas (Bower *et al.* 1994).

5.4 Paramyxeans

Aber disease, or digestive gland disease, of oysters and other marine molluscs is caused by the parasite *Marteilia refringens* (Longshaw, Feist, Matthews & Figueras 2001). It is mainly found in the European flat oysters *Ostrea edulis* (Sparks 1985; Figueras & Montes 1988; Bower 1995). This disease was first discovered in 1968 (Longshaw *et al.* 2001). The condition of infected hosts can become very poor. The digestive gland can undergo discoloration, growth can be retarded, tissue damage can occur, glycogen reserves can become depleted and ultimately mortality can set in (Villalba, Mourelle, Carballal & Lopez 1993; Berthe, Le Roux, Adlard & Figueras

2004). Mortensen (2000) suggested that an unknown intermediate host forms part of this parasites life cycle.

On the East Coast of Australia *Marteilia sydneyi* Perkins & Wolf, 1976, the causative agent of QX disease (Longshaw *et al*. 2001), parasitises the Sydney rock oyster, *Crassostrea commercialis*, and the black lipped oyster *C*. *echinata* (Lester 1989; Bezemer, Butt, Nell, Adlard & Raftos 2006). Infected animals bodies become shrunken, digestive glands have a pale yellow-brown colour, gonads become reduced in size and mantle tissue appears translucent (Sparks 1985; Lester 1989). The infected digestive gland epithelial cells and tissue becomes disorganised and blocked due to heavy parasite infections (Berthe *et al*. 2004). The host animals' condition deteriorates and high mortalities are most likely to follow infections (Bower *et al*. 1994). Water temperatures above 17ºC may increase the occurrence of this parasite (Grizel *et al*. 1988).

A *Marteilia*-like protozoan infecting the giant clam *Tridacna maxima* (Röding, 1798) from Fiji induces lesions in the kidney and cysts formed by these parasites can displace host tissue (Berthe *et al*. 2004). White foci are present in the kidney tissue and the extent of the damage suggests that this disease is possibly pathogenic (Bower *et al*. 1994).

Marteilia refringens (syn: *M*. *maurini*) infects the mussels *Mytilus galloprovincialis* and *M*. *edulis* from the East Coast of Florida, and the Atlantic Ocean side of the southern United Kingdom to Portugal (Bower 1995; Longshaw *et al*. 2001; Berthe *et al*. 2004; Rayyan, Damianidis, Antoniadou & Chintiroglou 2006). Not much is known about the pathogenicity of this parasite, but it has been noted that the tubule epithelium of the digestive gland can become disrupted and gonadal development can be inhibited due to infection by this parasite (Villalba *et al*. 1993; Fuentes, Villalba, Zapata & Alvarez 1995; Bower *et al*. 1994; Rayyan *et al*. 2006).

On the East Coast of Florida a *Marteilia* sp. resembling *M*. *refringens* has been found in the Atlantic calico scallop *Argopecten gibbus* (Linnaeus,1758) (Mortensen 2000). This parasite can cause a reduction in nutritional intake of the host through filling the tubules of the digestive gland causing the starvation of the host (Berthe *et al*. 2004).

Mortality levels due to the presence of this parasite are likely to by high and death occurs rapidly. Substantial losses in the production of meat due to these parasites have been observed, and in areas where catastrophic, high levels of mortality have occurred, host populations are not likely to recover (Bower *et al*. 1994). Currently there are no methods of control and prevention for this parasite available.

6. First record of an undescribed protozoan parasite in the digestive glands of *Haliotis midae* from South Africa

During routine health assessments carried out by scientist of the Abalone Farmers Association, South Africa, low infections of an enigmatic parasite were observed in histological sections of the digestive gland of farmed *Haliotis midae* (see Chapter 1). In order to classify this parasite, an in-depth study was undertaken into its morphology, morphometrics and ultrastucture. This chapter reports on the results of this in-depth study and further discusses the possible taxonomic position of this parasite by comparing it to known parasites infecting other species of abalone (see Chapter 4) as well as to protozoans infecting other commercially important invertebrates (see Chapter 5). This chapter also aims to determine possible effects this parasite might have on the growth of infected abalone.

6.1 Collection and preparation of samples

6.1.1 Collection of farmed animals

During May 2005, farmed abalone (*Haliotis midae*) (Fig. 6.1 A) digestive gland samples were collected from the holding tanks of three abalone farms located in the Western Cape (see Fig 3.6 A & B Chapter 3 and Fig 6.2 A). Due to the sensitivity associated with abalone farming, no farm names can be used. The three farms sampled will therefore be referred to as Farm **A**, Farm **B** and Farm **C**. At each farm 30 abalone, weighing between 30 and 40g, were collected and total shell length and width were measured prior to dissection. At Farm **A,** samples were randomly collected from their holding cages, but at Farm **B** and **C** samples were only collected from animals classified as rejects. Reject animals refer to those that are, according to the abalone farmers guidelines, not in optimal condition for live export. This does not mean that these are sick animals; it only refers to slower growing animals as well as animals with broken or deformed shells. All measurements are supplied as means ± standard deviation followed by the range in brackets.

In October 2005, 31 live abalone were couriered from Farm **A** to Johannesburg. Again, random samples of about 20 to 35g wet weight were used. Since live material was available for study in the laboratory at UJ, this opportunity was used to collect more data from the animals. In addition to the shell length and width, the total weight of the animal, the weight of the meat, and the presence of visible ectoparasites were noted (Appendix II). This procedure was repeated with reject samples of about 30 to 40g from Farm **B** in January 2006 and again with reject animals from Farm **B** in February 2007.

6.1.2 Collection of wild samples

In order to determine whether this parasite could occur in wild abalone and from different species of *Haliotis* and not just the farmed *H. midae*, wild abalone were collected from the Tsitsikamma National Park. Unfortunately, due to the endangered status of abalone along the coast of South Africa, a permit for a single collection of only 30 samples per species was issued by the Scientific Services of the South African National Parks (Appendix III). Abalone were collected at low tide during April 2006. All animals were carefully removed from crevices in intertidal rock pools by means of thick blade spatulas. A total of six *H. midae* and 28 *H. spadicea* were collected. The small number of *H. midae* found during the week long fieldtrip again confirmed their endangered status, especially since this collection took place in a no-fishing National Marine Protected Area. Total shell length and width, total weight of each animal and the weight of the meat of each sample were measured. Each animal was also carefully inspected for the presence of visible ectoparasites.

6.1.3 Dissection and preservation of samples

Farmed and wild abalone were killed by cutting with a sharp blade in one swift action through their epipodium (Fig 6.1 B). Each abalone was removed from the shell by putting a blunt knife in between the shell and the abalone's body and then pushing the knife away from the shell, so as to loosen the foot muscle. The gonads of adult males and females could be clearly distinguished from one another (see 6.1 C & D), but in

samples collected throughout this project the majority of animals could not be sexed as they were still young and had not yet reached sexually maturity.

During the May 2005 collections the digestive glands were removed (Fig 6.2 B) and cut into three section. Each piece was placed into a histological cassette (Fig. 6.2 C), marked with the date, locality, and sample number and prefixed in Davidson's fixative (Austin & Austin 1989), (Fig. 6.2 D, Appendix IV) for 24 to 74 hours. All samples were post fixed in 70% ETOH for histology.

In October 2005 the digestive glands were again cut into three pieces, but this time the first piece was fixed in Karnovsky's fixative for TEM, the second piece in 4% paraformaldehyde, also for TEM, and the third in 10% neutral buffered formalin for histology (see Appendix IV for details of fixatives). The change in methodology was necessary in order to study the ultrastructure of the parasites and also since it was found that Davidson's fixative was not ideal for histology.

For the January 2006 and February 2007 samples the digestive glands were cut into four pieces. The first three pieces were fixed as above and the fourth piece was fixed in 96% ETOH for DNA analysis.

6.1.4 Histology

As mentioned above, one piece of about 5mm^3 of each digestive gland was used for histology. Tissue was washed under running tap water for two hours in the histological cassettes to remove the excess fixative. Tissue samples were dehydrated (Fig. 6.2 E) through a graded series of ethanol concentration (30% to 96%, - 45min each and twice in 100%). Once fully dehydrated, the tissue samples were transferred to a xylene clearing solution (Drury & Wallington 1980), left for 30min and subsequently placed into a 1:1 melted solution of xylene and Paramat wax for 30min (Fig. 6.2 F) (Galigher & Kozloff 1964; Leeson & Leeson 1976; Ratcliffe 1983). Samples were transferred into 100% Paramat wax melted at 56ºC (Drury & Wallington 1980; Kiernan 1981) for one hour and were left overnight in fresh 100%

Paramat wax solution. Tissue samples were finally transferred into a third fresh Paramat wax solution for one hour before being embedded into wax blocks. A metal saucepan-like tray with a hot metal plate was used for embedding, by placing two L-shaped metal plates together to form a block. (Fig. 6.3 A). The L-shaped plates and metal plate were covered with a thin film of petroleum jelly to aid in the removal of the wax blocks.

A small piece of waterproof paper (Fig 6.3 B) marked with the sample number, the type of tissue, locality and date of collection was placed together with the wax into each block. The tissue was embedded into the wax block using warm forceps. Stalled blocks were removed from the metal plates and positioned in the refrigerator over night. Stalled wax blocks were then trimmed with a blade. Metal chucks (Fig. 6.3 C) were heated over an open flame and blocks were melted onto them. Chucks were then cooled in the refrigerator for a few minutes (Leeson & Leeson 1976; Ratcliffe 1983).

Sections were cut at 5µm thickness using a Jung A.G. Heidelberg Microtome (Fig. 6.3 D). Sectioned ribbons were mounted in an albumin solution on clean glass microscope slides marked with a diamond tip pen. Two slides of each sample were prepared. The wax ribbons on the slides were stretched on a 30°C hot plate using two hand held pins. Slides were left to air dry in a slide holder and transferred into a Mini/696/Clad oven at 30°C to dry (Leeson & Leeson 1976; Ratcliffe 1983).

Slides were dewaxed in two separate xylene solutions for three minutes each and re-hydrated through descending concentrations of ETOH for staining (Fig. 6.3 E). Slides were rinsed in tap water for three minutes and stained in commercial Haematoxylin and Eosin (H&E) stain (Associated Chemical Enterprisesn (Pty) LTD) for four to six minutes depending on the tissue (tissues were viewed under a light microscope to determine whether they had stained properly). Stained slides were dipped into acid alcohol to differentiate the stain and subsequently rinsed in tap water to remove the excess acid (Galigher & Kozloff 1964; Leeson & Leeson 1976; Kiernan 1981).

Scott's solution colours the nuclei of cells in the tissue blue. Tissues were stained with Scott's solution for several minutes and rinsed in tap water. Slides were dipped in eosin (which stains the cytoplasm in the tissue pink) for 30sec to one minute, again depending on the tissue. Excess eosin was rinsed off by dipping the slides in tap water.

To dehydrate the tissue, slides were dipped into ascending concentrations of ETOH and left for two minutes in two separate 100% ETOH concentrations each. Finely, slides were cleared in two separate xylene solutions for three minutes each and cover slips were mounted using Enatellan (Merck) (Galigher & Kozloff 1964; Leeson & Leeson 1976). Slides were labelled and left to dry. All staining procedures were done under a fume hood. A Zeiss compound light microscope (Fig. 6.3 F) was used to scan the slides for the presence of parasites.

It was found that the structures in the tissue of samples collected in May 2004 that were prefixed in Davidson's solution and post fixed in 70% ETOH were damaged, probably due to the acidity of the prefix. To prevent this from happening again, 10% neutral buffered formalin was used (Appendix IV) during following collections, as this fixative is commonly used to fix a variety of different tissues with minimal damage (Leeson & Leeson, 1976).

6.1.5 Fluorescence microscopy

For fluorescence microscopy, cover slips covering digestive gland sections with a high prevalence of the protozoan parasites were removed using xylene. Samples were rehydrated and placed into acid alcohol until all the H&E stain had been removed and then subsequently dehydrated. Each slide was covered with BSA-saline (bovine serum albumin-saline) for 5min before staining with 5μl SYTO–9 (Molecular Probes, Eugene, Oregon). A cover slip was positioned onto the slide, sealed with nail varnish and left to dry in the dark, owing to the light sensitivity of the stain (Milne & Avenant-Oldewage 2006). A Zeiss Axioplan 2 epi-illuminator fluorescence microscope with Rhodamine and Fluorescein filters was used to view and photograph the slides.

6.1.6 Transmission Electron Microscopy (TEM)

As described above, the digestive glands that were used for TEM were fixed at 4°C in (a) 4% paraformaldehyde (PFA) in 0.1M Sörensen's phosphate buffer (pH 7.2-7.4) as well as (b) Karnovsky's fixative (Karnovsky 1965) to preserve their ultrastructure (Fig. 6.4 A) (see Appendix IV). *En bloc* staining was preceded by a thorough rinse (Fig. 6.4 B) of the specimens in distilled water to remove phosphate buffer, which is precipitated by uranyl salts (Hayat 1986).

Staining *en bloc* was performed by placing the tissue in 1% aqueous UrAc (uranyl acetate) (Hayat 1986) for one hour and rinsing again for 15min in buffer. Specimens were thereafter routinely processed by dehydration through graded ethanol (50%-100%), placed in propylene oxide (Luft 1961), infiltrated and embedded (Fig. 6.4 C) in a water-immiscible embedding medium (Fig. 6.4 D) (Embed-812®, Electron Microscopy Sciences U.K.).

Resin blocks were polymerized in an embedding oven at 70ºC. Ultramicrotomy of sections was performed by means of an Ultracut E (Reichert Jung, Germany) ultramicrotome (Fig. 6.4 E) using a diamond knife (Diatome Ltd., Switzerland) with a 2mm cutting edge. Semi-thin sections (1μm) (Fig. 6.4 F) were dried onto glass slides and stained with 1% toluidene blue (Appendix IV & Fig. 6.5 A & B). Slides were cleared in xylene, and cover slips mounted with Entellan® (Fig. 6.5 C). Slides were scanned to determine which blocks to use for thin sections.

Gold sections (70nm) (Fig. 6.5 D) were lifted onto copper grids 300 mesh size (#75, Electron Microscopy Sciences, U.K.) and subsequently contrasted (Fig 6.5 E) by uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963). Grids were viewed (Fig 6.5 F) by means of a Jeol JEM 1010 (Jeol Inc., Tokyo) transmission electron microscope (TEM) at 100kV. Digital micrographs were captured using a Megaview III[TM] digital camera and SiS Image Analysis Software® (Soft Imaging System Ltd.).

6.2 Results

During the 21 month period from May 2005 to February 2007, the digestive glands of 180 farmed *Haliotis midae*, 6 wild caught *H. midae* and 28 wild caught *H. spadicea* were screened for the presence of the intercellular protozoan parasite (Table 6.1). As mentioned in the materials and methods, three abalone farms were sampled as well as a single sampling at the Tsitsikamma National Park. Farm **A** was sampled twice and Farm **B** was sampled three times as they were historically the farms most likely to have infected abalone (A. Mouton pers. comm). The May 2005 samples of Farm **A** showed 10% (3/30) prevalence (Table 6.1) with the protozoan parasite. In October 2005 only a single specimen was infected (prevalence = 3.2%). None of the abalone sampled during May 2005 at Farm **B** harboured the protozoan parasite, but in January 2006 and February 2007 the prevalence was high with 10 of 29 (34.5%) and 12 of 30 (40%) abalone infected. The intracellular digestive gland protozoan parasite was not present in the digestive glands of the 30 animals sampled at Farm **C** in May 2005. From the infected samples, 17 had an intensity level of less than 10 parasites per sample (as observed from slides) and 9 had high intensity levels of more than 10 parasites per sample.

None of the six wild caught *H. midae* or the 28 *H. spadicea* from the Tsitsikamma National Park was parasitised with the unidentified digestive gland protozoan. However, a digenean similar to the one reported by Botes *et al*. (1999) from *H. spadicea* from De Hoop Nature Reserve was found in six of the 28 *H. spadicea* sampled at Tsitsikamma National Park (see Section 4.4 Chapter 4). This is the first record of such a digenean parasitising abalone from Tsitsikamma National Park.

6.2.1 Possible effects of the digestive gland parasites on growth of farmed abalone

Since the overall prevalence of infections were low (12.15%), it was impossible to obtain meaningful results using all the combined data to determine a possible effect of the digestive gland parasite on the growth rate of the abalone. It was, however, possible to use the data from January 2006 and February 2007, both sets being from

Farm B. The presence of other visible parasites, like sabellid and polychaete worms was also noted, since these annelids can play a role in the growth rate of the abalone (Table 6.1). Of the 59 abalone sampled during the two periods from Farm B, 22 were infected with the digestive gland parasite. From this data XY-scatter plots were drawn of infected (Fig. 6.6 A) and uninfected abalone (Fig. 6.6 B). No statistical difference could be found between the size-weight relationship of infected and uninfected animals. (Student's t-test, $P = 0.105$ for total weight/total length and $P = 0.09$ for meat weight/total length). However, the size of the samples (collected from the farms) were all within a very narrow range, thus making it difficult to forecast whether the parasite might have an influence over a longer growth period and a corresponding longer period of infection. The shells of 21 abalone (95.4%) infected with digestive gland parasites where also infested with sabellids and 14 (63.3%) with polychaetes, and 34 (91.9%) of the non-protozoan-infected abalone with sabellids and 24 (64.9%) with polychaetes. Because of this high prevalence of both sabellids and polychaetes in both infected and non-infected abalone it was assumed that the presence of the worms would not affect the outcome of the Student's t-test; there was no correlation between the presence of the unidentified digestive gland parasite and the presence of sabellids or polychaetes (see Fig. 4.1. C-F).
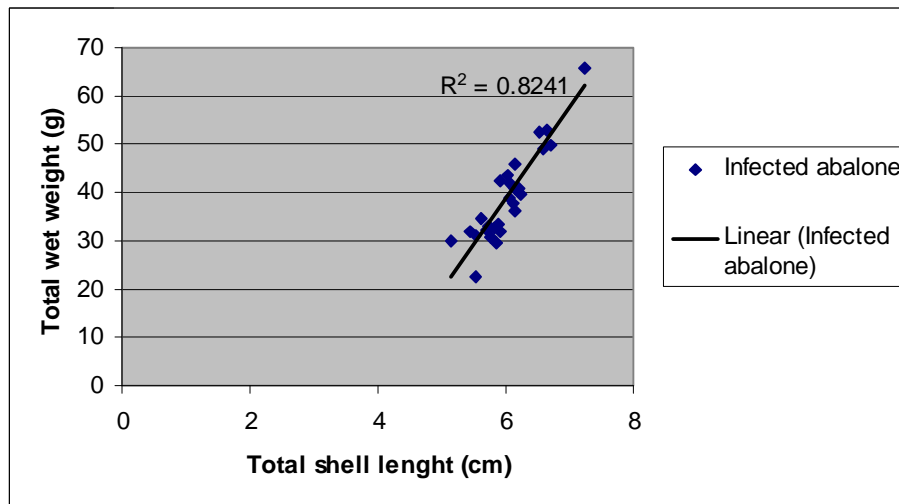
Figure 6.6 A, Scattered plot of total shell length (cm) against total wet weight (g) of abalone infected with the unidentified digestive gland parasite demonstrating the growth rate of infected abalone. R^2 value calculated at 0.8241.
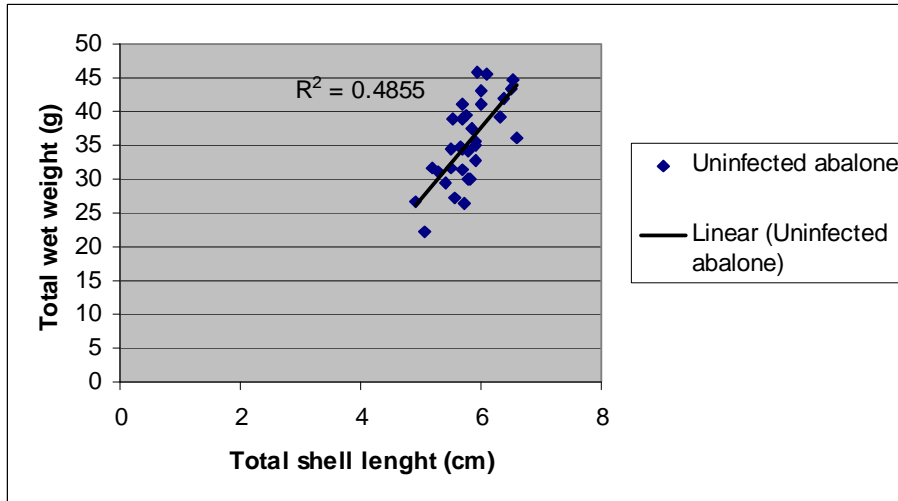
Figure 6.6 B, Scattered plot of total shell length (cm) against total wet weight (g) of abalone non-infected with the unidentified digestive gland parasite demonstrating the growth rate of uninfected abalone. R^2 value calculated at 0.4855.

6.3 Description of the intercellular digestive gland parasite

6.3.1 Morphology based on histology

Prior to examining the parasite and its structure, it was important to note where it occurred in the digestive gland, as this might indicate how the parasite entered the structure, where it was most likely to be situated in order to undergo development, and how it might then leave the digestive gland. In Figure 6.7 A, the following digestive gland structures were observed: β- cells (crypt), basal lamina, duct cells, granules and the lumen. The parasites were noted to occur either as aggregates in the digestive gland tissue (Fig 6.7 B) close to the muscle layers, lumen and basal lamina, or to be distributed singly (Fig 6.7 C), mostly close to the basal lamina and lumen. Parasites also occurred in the digestive gland tissue in between the basal lamina and the lumen (Fig 6.7 D), or in the tubules as a group (Fig 6.7 E).

Basic structures of the parasite included the presence of a parasitophorous membrane and parasitophorous vacuole (Fig 6.8 A). In Figure 6.8 B and C the parasitophorous vacuole surrounding the parasite was quite large. The parasitophorous vacuoles seen in Figure 6.8 D and E were much smaller compared to Figure 6.8 B and C. In Fig 6.8 F a few parasites are situated next to each other and the parasitophorous vacuoles can be seen.

Possible life stages of the parasite observed in the digestive gland tissue included an elongated, robust parasite with a large parasitophorous vacuole (Fig 6.9 A) and a more slender, curved form (Fig 6.9 B). In Figure 6.9 C the parasite appears to be dividing. Internal division and caps are observed in Figure 6.9 D. This type of division is also seen in Figure 6.9 E and the parasitophorous vacuole is very thin. Parasites are rod shaped in Figure 6.9 F.

 *Note: Not all authors and common names could be obtained for genus and species used in this section.

6.3.2 Morphometrics

The different life stages observed in the digestive gland were measured. The presumed first stages had an elongated slender shape with a large parasitophorous vacuole surrounding it (Fig 6.9 B & 6.10 A). Stage one measured 13.35µm ± 1.22 (11.38–15.98µm) long X 4.94µm ± 0.76 (3.4–6.75µm) wide (n = 48). The second stage (Fig 6.9 A & 6.10 B) was more rounded and robust compared to stage one and the parasitophorous vacuole surrounding the parasite was smaller. Stage two measured 18.34µm ± 1.22 (15.98–21.28µm) long X 5.24µm ± 0.98 (2.72–7.36µm) wide (n = 46). In stage three polar caps were visible at either the posterior or anterior ends, and what seemed to be internal division was also occurring (Fig 6.9 D & 6.10 C). The length of these stage were 15.11µm ± 2.45 (11.8–19.02µm) X 6.73µm ± 1.10 (4.91–8.29µm) wide (n = 16).

Presumed stage four (Fig 6.9 D & 6.10 D) compared to the presumed stage three, had polar caps at both the anterior and posterior ends, and not just at one end as seen in stage three. This stage was also possibly dividing. Presumed stage four measured 16.49µm ± 2.35 (11.38–19.85µm) long X 7.14µm ± 1.36 (4.41–9.59µm) wide (n = 21). Stage five had no polar caps and multiple division was observed inside the parasite. Parasites in stage five (Fig 6.9 E & 6.10 E) were scanty and only four were observed. These measured 19.13µm ± 0.85 (18.05–19.85µm) long X 7.81µm ± 0.82 (7.03–8.87µm) wide (n = 4). Stage six (Fig 6.9 C & 6.10 F) was a dividing form and only a single specimen was found. It measured 12.55µm long X 6.25µm wide (n = 1).

6.3.3 Fluorescence

When using an epi-illuminantion fluorescence microscope and various filters (Fig 6.11 A), the SYTO 9-treated histology slides fluoresced to reveal the parasites. According to Milne and Avenant-Oldewage (2006) the SYTO 9 green nucleic acid fluorochrome penetrates compromised plasma membranes and labels the DNA and RNA. In Figure 6.11 B use of a rhodamine-sensitive filter, allowed the parasite to be viewed by fluorescence. When using a fluorocein-sensitive filter the parasite fluoresced more clearly (Fig 6.11 C). The plasma membrane and cytoplasm were the

portions of the parasite that fluoresced most and two groves in these structures were visible (Fig 6.11 D). At the posterior part of the parasite possible caps were noted (Fig 6.11 E) and the stain gave a more three dimensional view of the parasite (Fig 6.11 F). The plasma membrane is likely used to protect the parasite from the host immune system.

6.3.4 Ultrastructure

In transmission electron micrographs the parasite was also observed close to the lumen of the digestive gland (Fig 6.12 A). In Figure 6.12 B structures resembling the formative region for haplosporosomes was observed within the parasite cytoplasm. This region was situated posterior to the nucleus. The nucleus was surrounded by the nuclear envelope and contained a likely nucleolus. The parasite also possessed an outer membrane, but this was not the same as the membrane of the parasitophorous vacuole. The anterior extremity of the parasite contained a structure representing a possible spherulosome (Fig 6.12 C). The sphlerulosome is believed to be implicated in spore encystment in haplosporidia (Perkins 2000).

6.3.5 Possible pathology

These parasites appear to exert some histological pathological effects on the host digestive gland tissue. Clusters of parasites were found in the digestive gland tubules and may block these. Blocked tubules may cause problems in the digestion as nutrients and digested food, or excreted products may not be absorbed, or excreted. Parasites also occurred between the muscle layer and basal lamina of the digestive gland and it seems that these parasites may destroy these structures.

6.3.6 Remarks

The use of histological methods and stains provided insight into the distribution of the parasite in the digestive glands and the host structures associated with it, as well as the possible pathological effect it has on the host digestive gland tissue. Firstly, it was important to examine the staining properties of the parasite and the digestive gland

tissue with the use of the H&E staining method. Haematoxylin stained the nuclei of the digestive gland tissue and the parasite purple blue, making the identification of these structures easy.

In Figure 6.7 A representing the digestive gland and its basic structures, the basal lamina stained purple-blue. Most other structures present stained pink, including the body of the parasite. In the same figure some purple staining occurred in the central part of the parasite, representing the nucleus. In most of the other figures the parasite nucleus could not be observed, possibly due to the way in which the parasite was sectioned and thus only the cytoplasmic structures could be seen.

Over the years the immunological processes of bivalves have raised controversy, but studies conducted by Travers, Da Silva, Le Goic, Marie, Donval, Huchette, Koken & Paillard (2007) on the immunity of *Haliotis tuberculata* suggested that haemocytes and granulocytes are the main phagocytic cells. It is difficult to say with certainty if phagocytic cells in the abalone host (*H. midae*) will engulf or phagocytose UDP, but no evidence of such effects were seen in the sections examined in the current study.

6.4 Discussion

In order to help with the classification of this digestive gland protozoan it is important to compare it to other known abalone protozoans. The following section indicates the differences and similarities between the digestive gland protozoan parasite of *Haliotis midae* and protozoan parasites known from other *Haliotis* species. For classification and comparison reasons the unknown digestive gland protozoan parasite of this study will further on be referred to as UDP.

6.4.1 Chromista

***Labyrinthuloides haliotidis* Bower, 1987 occurring in abalone**

Labyrinthuloides haliotidis is eukaryotic protist effecting the muscles, nerves and foot of *Haliotis kamtschatkana* Jonas, 1845 and *H. rufescens* Swainson, 1822 whereas

UDP infects only the digestive gland of *H. midae*. Hundreds of *L. haliotidis*-infected animals can die within days of being infected, especially juvenile abalone (Bower 1987a; 2003). Vegetative stages of *L. haliotidis* can penetrate epithelial cells of their hosts by means of lytic agents and reproduce by binary fission. No recognisable vegetative stages have yet been observed in the UDP infecting the digestive gland of *H. midae*. The vegetative stages of *L. haliotidis* are spheroidal in shape and measure 7.2 ± 0.8 (5.2-8.6) µm in diameter. Some individuals undergo synchronous multiple fission (sporulation) and measure 7.8 ± 0.7 (6.2-9.8) µm. Zoospores are 4.3 ± 0.5 (3.7-7.1) µm in diameter and fall within the range of UDP stage one measurements. The uni-nucleated motile biflagellate zoospores are 4.7 ± 0.5 (4.0-5.6) µm long X 3.6 ± 0.4 (2.8-4.4) µm wide (Bower 1987a). Based on the above it is clear that UDP is not related to *L. haliotidis*.

6.4.2 Apicomplexans

6.4.2.1 *Perkinsus* spp.

Perkinsus spp. generally pose a serious threat to commercially important mollusc species like abalone, clams, oyster and scallops worldwide (Robledo *et al*. 2001). *Perkinsus olseni* Lester & Davis, 1981 infect the adductor muscle of *H. laevigata* Donovan, 1808, *H. cyclobates* Peron & Leseur, 1816 and *H. scalaris* Leach, 1814 (Bower 2005), whereas UDP has not been found to infect these muscles in *H. midae*. Pustules are present in haemolymph where *P. olseni* grows and divides, and the infected areas are usually surrounded by loose connective tissue fibres; elevated numbers of leukocytes can be present. These parasites can also be grouped together in the haemolymph of the hosts.

Trophozoite diameter is 13-16µm and the spherical cells are 14-18µm in diameter (Sparks 1985). Prezoosporangia measure 56-94µm in diameter (Lester & Davis 1981). The measurements of *P. olseni* are much larger in comparison to the measurements of UDP. It is thus unlikely that the UDP is a *Perkinsus* species. Infection by *P. olseni* of the digestive gland tubules corresponds broadly to that of UDP in the digestive gland of *Haliotis midae*. This leads to inhibition of the gonadal development (Volety *et al*.

2000), pale coloration of the digestive gland (not the case for UDP), disruption of epithelial and connective tissue cells and abscess lesions in the oyster species *Crassostrea virginica* (Gmelin, 1791)*, Pinctada maxima* Röding, 1798 and *Mya arenaria* Linnaeus, 1758. (Dungan, Hamilton, Hudson, McCollough & Reece 2002).

Various life stages of *Perkinsus* spp. have been studied in the past. According to Lee *et al* (2001) trophozoites of *Perkinsus* species can occur in clusters, or singly, in the infected tissues, as is the case in UPD. The spores are biflagellated, uninucleated cells. Zoospores have an apical complex and conoid, but no preconoidal rings and polar rings occur around a conoid to which microtubules are attached. During merogony the apical complex and flagella are lost. A coccoid meront forms containing tubulovesicular mitochondria and triple bladed centrioles. Cell multiplication takes place due to cycles of karyokinesis, followed by a cycle of cytokinesis leading to the formation of morula clusters, with 4 to 64 merozoites that form within the meronts (Perkins 1991).

Perkinsus marinus Levine, 1978 parasitises almost all of the intestinal tissues such as the gonads, gill filaments, the tissues around the digestive tubules and between the digestive diverticula (Lee *et al*. 2001). Parasite DNA studies conducted by Reece, Bushek, Hundson & Graves (2001) indicated that the cells of *P. marinus* are diploid. The three life stages, trophozoites, prezoosporangia and zoospores, can induce infection (Audemard, Reece & Burreson 2004). Structures present in these parasites include an apical complex with micropores, rhoptries, micronemes and the subpellicular complex (Andrews 1988), that have not been observed in UDP. Coccoid trophozoites measure 6.4µm (3.9–11.6µm) in diameter and immature trophozoites measure 1.9-3.4µm in diameter (Perkins 1996), and are therefore not falling in the range of UDP measurements and the merozoites of *P. marinus* measured between 20-50µm (Andrews 1988).

The clam species *Mya arenari* is the host of an unknown *Perkinsus* sp. that induces a host cellular response and thus leads to the encapsulation of the parasite. Almost all of the host tissue can become infected with this parasite when high numbers are present (McLaughlin & Faisal 2000). *Perkinsus* spp. infecting the clam *Ruditapes philippinarum* (Adams & Reeve 1850) affects the connective tissue of the digestive

gland and gonads. The trophozoites measure 10µm (5-14µm) in diameter (Park & Choi 2001) and thus do not correlate with UDP measurements.

The clams *Tapes decussates* (Linnaeus, 1758), *Venerupis aurea* (Gmelin, 1791) and *Tapes philippinarum* are the hosts of *Perkinsus atlanticus* Azevedo, 1989. Cysts usually occur on the gills and mantle of the host (Bower *et al*. 1994), but no cysts were observed for UDP. The zoospores of *P. atlanticus* measure 4.5 ± 0.6µm long by 2.9 ± 0.4µm wide (Azevedo 1989) which is smaller than UDP measurements. The trophozoites of *P. medeteraneus* Casas, Grau , Reece , Apakupakul, Azevedo, Villalba 2004 occur in the connective tissues of the gills, visceral mass, gonads and mantle of the oyster *Ostrea edulis* Linnaeus, 1758 (Casas *et al*. 2004). Trophozoites measure 7.9µm ± 0.34 (4-19µm), presporangia 97.4µm ± 1.99 (62–135µm) and zoospores 4.4µm ± 1.18 (2.9–8.09µm) (Casas *et al*. 2004) and therefore do not fall in the range of UDP measurements. The intestine, stomach, digestive gland, mantle and gonad connective tissues of the scallop species *Argopecten irradians* (Lamarck 1819) have been reported to be infected by *P. irradians* (Bower *et al*. 1994).

6.4.2.2 Gregarines

Nematopsis spp. Schneider, 1892 are obligate apicomplexan parasites infecting the mussel species *Mytilus edulis* Linnaeus, 1758, *M. trossilus* Gould, 1850, *M. galloprovincialis* Lamarck, 1819 and *Geukensia demissa* (Dillwyn, 1817). Infected clam species includes *Cardium edule* Linnaeus, 1758, *C. lamarcki* (Reeve, 1844), *Saxicaca rugosa*, *Tapes philippinarum*, *Protothaca staminea* (Conrad, 1837) and *Tillina* spp. Scallops hosts are *Patinopecten yessoensis*, *Chlamys varia* (Linnaeus, 1758) and *Argopecten irradians*. Spores can be found in the phagocytes from the connective tissues of the hosts gills and mantle (Bower *et al*. 1994). An inflammatory response usually follows infections with these parasites.

The phagocytes can host the gymnospores, oocysts and sporozoites. *Nematopsis messor*, *N. annulipes* and *N. quadratum* Prasadan and Janardanan, 2001 infect the gut of the estuarine crabs *Metopograpsus messor* (Forskal, 1975), *Sesarma quadratum* (Fabricius) and *Uca annulipes* (Edwards, 1837). The measurements of *N. messor* gametocysts were 6.6–11.5µm in width; gynospores were 4.5-5.3µm in diameter and

were thus larger than the stages of UDP measured. The gametocysts of *N. quadratum* (Prasadan & Janardana, 2001) measured 20.4–40µm in width and the gymnospores measured 7.5µm in diameter correlating to the width of UDP stage five measurements. Gregarines zoites found in the oyster *O. chilensis* measured 7.9µm (6.2–9.8µm) in length by 4.9µm (4.0-5.3µm) in width (Hine 2002b) which do not correlate with UDP measurements.

6.4.2.3 *Pseudoklossia* spp. Léger & Duboscq, 1915

The coccidian parasite, *Pseudoklossia haliotis* occurs in the kidneys of *Haliotis cracherodii* Leach, 1814 *, H. rufescens* Swainson, 1822*, H. corrugata, H. fulgens* Philippi, 1845*, H. walallensis* Stearns, 1899 and *H. kamtschatkana* Jonas, 1845. The affected kidney epithelial cells become hypertrophied and kidney damage is likely to follow. UDP have thus far not been found in the kidneys of *Haliotis midae*.

Measurements of the different life stages of *P. haliotis* are noted below. The merozoites are 3 X 10µm, while spores measure between 16 and 21µm (Spark 1985). Meronts are 13.6 ± 3.1 (9.6-14.4) µm long X 11.5 ± 1.9 (9.6-16.8) µm wide. Merozoites are 7.9 ± 0.8 (6.8-9.4) µm long X 3.6 ± 0.5 (2.7-4.8) µm wide and macrogametes measure 22.8 ± 4.8 (13.5-28.0) µm long X 19.5 ± 4.0 (12.2-22.0) µm wide. Microgamonts and microgametocytes stages both have a length of 23.2 ± 3.5 (17.6–28.4) µm X 17.4 ± 2.8 (9.5-20.3) µm in width. Oocysts and sporocysts measured 27.0 ± 3.1 (21.6–29.6) µm long X 23.1 ± 2.3 (20.3–29.6) µm wide (Friedman *et al.* 1995). The range of UDP measurements is not close to the different measurements noted for *P. haliotis* life stages. The size range of *P. haliotis* spores falls within all the different sizes of the UDP stages. Although spore sizes are similar, UDP do not posses any of the following typical coccidian characteristics, granules, superpellicular microtubules, rhoptries, micronemes or myonemes (Roberts & Janovy 2000). Thus UDP can not be related to coccidians.

Pseudoklossia and *Klossia* Schneider, 1875 species infect the kidney epithelial cells of the oyster species *Ostrea edulis* and *Tiostria chilensis*, the mussel species *Mytilus edulis* and *M. trossulus* and the clams *Tapes floridus* and *T. virgineus*. The parasites infect the haemocytes of the kidney, gonads and digestive diverticula. Infected kidney

cells become hypertrophied and kidney tubules fill up with parasites. Although UDP does not occur in the kidneys of *Haliotis midae* it fills up the digestive gland tubules as *Pseudoklossia* and *Klossia* species do in the kidneys of their hosts. *Klossia* species undergo schizogony, gamont and gamete formation and sporogony. The oocysts contain the sporozoites (Sleigh 1973), but these stages have not been seen in UDP.

In *Pseudoklossia* species, development stages include schizogony, gametogony and sporogony. Divided nuclei and refractile granules are absent in the schizonts. Merozoites measure 3 X 10µm and consists of a central nucleus and cytoplasm. Mature gamonts looks similar to ova of the hosts. The oocysts harvest 20 to 24 sporocysts that will develop into schizonts. Large spores measure 16–21µm (Spark, 1985) and fall in the range of UDP stage two measurements. Sporozoites are vermiform and have one nucleus and an apical complex (Perkins 1991).

6.4.3 Microsporidians

Microsporidian parasites can infect the connective tissue neighbouring the gut of the oyster species *Tiostrea chilensis*. Clam host species include *Cardium edule* Linnaeus, 1758 and *Macoma baltica* (Linnaeus, 1758) and the various shrimp species have been noted to be infected with *Agmasomma*, *Amesoma* and *Pleistophora* spp. Schubergi. These parasites can replace the heart, muscle, gills, gonads and hepatopancreas of the shrimp hosts. Microsporidians can vary immensely in structures, but can be classified by single-celled spores, lacking an aperture in the cell wall, the presence of a polar filament and polar caps which is present in stage three and four of UDP. The occurrence of a single or double nucleus in the sporoplasm and sporulations can also be used for classification (Perkins 1991).

In general the spores range from 1.25–20µm long X 1.0-6µm in width, similar to the lengths measured for all stages of UDP, but not as wide (3–9.5µm). No mitochondria are present in microsporidians and a posterior vacuole is present in the spores. The polar sack resembles a mushroom shape and is located at the anterior part of the parasite. Spores are ingested by the hosts and released sporoplasms enter the host lumen in the intestine (Perkins 1991).

6.4.4 Paramyxeans

Marteilia **spp.**

Marteilia spp. can parasitise a range of invertebrate host including *Ostrea edulis*, *O. angasi*, *Mytilus edulis*, *Cardium edule* Linnaeus (1758), *Crassostrea gigas* (Thunberg, 1793), *C. echinata*, *Tiostrea chilensis*, *Saccostrea commercialis* Iredale & Roughley, 1933, *Tridacna maxima* and *Argopecten gibbus* (Linnaeus, 1758) to name but a few. Symptoms include the tubule epithelial of the digestive gland that can become disrupted and gonadal development which can be inhibited. The parasites can cause a reduction in nutritional intake of the hosts due to the fact that it fill the tubules of the digestive gland, which may also be the case in UDP as parasites have been noted to fill the digestive gland tubules. Lesions caused by *Marteilia* species can develop in the kidney and cysts can displace tissue (Bower *et al.* 1994).

The small plasmodia of *Marteilia refringens* are 5-8µm in size. When sporulation is initiated presporangia of about 8µm long develops being smaller than the range of UDP measurements for all stages and can become as large as 15-30µm, which is larger. Mature spores had a mean diameter of 3.9µm ± 0.08µm (Figueras & Montes 1988). Maturation of the sporangia will take place in the host digestive diverticula's and will then be excreted (Figueras & Montes 1988).

6.4.5 Haplosporidians

Haplosporidium **spp. Caullery & Mesnil, 1899.**

Haplosporidium montforti Azevedo, Balseiro, Casal, Gestal, Aranguren, Stokes, Carnegie, Novoa, Burreson and Figueras, 2006 is a parasite occurring in the connective tissue of the foot muscle, mantle and gills of *Haliotis iris* (Azevedo *et al*. 2006) and is similar to UDP as it also infects the digestive gland. *Haplosporiduim montforti* plasmodia stages are 5.5–13.5µm long X 9.0-14.6µm wide (Hine *et al*. 2002). Plasmodia are present in the gills, kidneys, foot, haemolymph, heart, mantle, epipodium and connective tissue of the digestive gland.

The plasmodia are surrounded by a clear area and usually have two nuclei. Small plasmodia stages have a mean size of 5.5 X 7µm (Diggles *et al.* 2002). The mature plasmodial stages are 17-25µm in diameter, sporonts are 30-45µm in size and the spores that occur in sporocysts are 35-50µm in diameter (Sparks 1985). No spores were observed for UPD. UDP plasmodial and *H. montforti* plasmodial measurements overlap. On comparing electron micrographs (see Perkins 1991; 2000), no haplosporosomes were seen, but a possible spherulosome and formative region for haplosporosomes were observed in UDP. Thus UDP is tentatively grouped together with the haplosporidians (see Conclusion, below).

The typical life cycle of *Haplosporidium* Caullery & Mesnil, 1899 species as described by Perkins (2000) comprise spores containing sporoplasms. Uninucleated cells form plasmodia that in turn develop, to form sporoblasts through the process of sporulation. Sporoblast combine to form zygotes will pinch off as hour-glass forms. The epispores which are formed with a spore cell barrier develop into mature spores (Perkins 2000). The orifice of each spore is covered by a hinged operculum and tails are attached to the spore wall. The presence and origin of the spore wall ornamentation and morphology are used as the main features in characterising haplosporidians (Azevedo *et al.* 2006).

Haplosporidium species infect the oyster species *Crassostrea virginica* (Gmelin, 1791), the mussel species *Mytilus californianus* and *M. edulis*, clam species includes *Tapes decussates* and *T. philippinarum.* Other hosts are the crab species *Callinectes sapidus*, *Panopeus herbstii*, *Eurypanopeus depressus* (S. I. Smith, 1869) and *Rhithropanopeus harrisii* (Gould, 1841). *Bonamia exitiosus* infects the gonads of the oyster *Ostrea angsi.* In mussel species, the kidney and digestive gland can become swollen and small white nodules are present. In the case of UDP no white nodules or swellings were observed in the digestive gland tissue. Polar capsules and filaments are absent in this phylum (Perkins1991).

Plasmodia, sporonts and sporocysts are the three stages that have been observed in *H. costalis* (Sparks 1985). Grizel *et al.* (1988) observed plasmodial stages that occur intracellularly except when the parasite is phagocytosed by the host cells. UDP are mainly intracellular, thus forming the parasitophorous vacuole. The plasmodial stages

can arise near the basal lamina and upper gut, like UDP, and are uninucleated naked cells with a width of 5µm similar to the width of stage one in UDP. The rock oysters *Saccostrea cuccullata* Born, 1778 are hosts of an unidentified *Haplosporidium* species with plasmodia measuring 6.3-15.4µm and sporonts measuring 17-22.5µm in length (Hine & Thorne 2002), correlating with UDP stage three and five measurements.

In the host *Ostrea edulis*, the spores are 4-4.5µm X 3-4µm in size (Sparks 1985), but no identifiable spores were observed for UDP. *Bonamia ostreae* has spheroidal shaped basophilic cells that measure 2-3µm in diameter and are also smaller than UDP stages. Three to five nuclei can be observed in the plasmodial stages and the plasmodia have been noted to be as large as 6µm in diameter (Grizel *et al*. 1988), which is the same as the range diameters measured for UDP stage three to six. The small plasmodia of *Bonamia perspora* Hine, Cochennec-Laureau, Berthe, 2001 parasitising the crested oyster, *Ostreola equestris* (Say, 1834) from North California are 3.7-9.7µm in width and the large plasmodia are 9.1-16.3µm in width (Carnegie, Burreson, Hine, Stokes, Audemard, Bishop & Peterson 2006). The measurements of the small plasmodia of *Bonamia perspora* overlaps with that of UDP stage three and four.

6.5 Conclusion

As can been seen from all the comparisons above between the UDP and other protozoan parasites there were no clear indications as to which of the numerous groups of protozoan parasites this species belongs to. Based on morphology and morphmetrics there might be a case for grouping the UDP together with Haplosporidians (see section 6.4.5). The use of Fluorescents techniques were valuable in the sense that the parasite could be observed three dimensionally, making its rod shape and groove more visible. TEM micrographs of UDP were compared with those from Haplosporidians and some structures looked similar (see section 6.3.4). UDP might not be part of the Apicomplexa as previously believed, however the next step will be to do molecular sequencing of the UDP and if successful it might shed some more light on the identity of this enigmatic parasite (see Chapter 7).

7. Preliminary attempts at molecular analysis of the digestive gland parasite

DNA analysis is a powerful tool in the identification and comparison of organisms based on their genetic makeup. Different molecular techniques can be used in the process of determining this type of information.

In the study of the unknown digestive gland parasite discussed in Chapter 6, histology was conducted to determine which samples were infected and what the infection levels were. Transmission electron microscopy (TEM) was performed on the samples found to be infected. The ultrastructure of this protozoan was studied from electron micrographs to aid in its identification, but no significant structures linking it to another known organism were found, except for possible haplosporidian-like features. The fluorescent studies did not show any defining structures either, and thus it was decided to attempt DNA analysis to shed some light on the identity of this protozoan.

Various problems can arise when DNA analysis is conducted with more than one organisms' DNA in the sample. In this study, the protozoan parasite was present in the digestive gland tissue of the abalone and could not be separated from the host tissue due to its small size. Another problem can be the amount of parasite DNA present in the sample, and thus available for subsequent molecular analysis. In an attempt to overcome these problems it was first necessary to determine which samples were infected with the protozoan parasite and what the infection levels were, so as to only use samples with high infection levels. This was done with the aid of histology, and only infected samples with high infection levels were used for the DNA analysis. Secondly, it was important to ensure that only the parasites's DNA will be amplified during the DNA analysis, by using specific primers designed to bind only to the DNA of the parasite and not to that of the host.

The choice of primers can have a significant effect on the success of the DNA analysis and it is thus very important to choose them wisely. Primers selected for this study was based on the results of the histology and TEM. The two primer sets used

were the protozoan primers described by Bower *et al.* (2004), as they were designed to bind to a wide range of protozoan parasites' DNA, and the primer described by Perkins & Keller (2001) which was designed for haemogregarines (group Apicomplexa) due to the possibility that the organisms might be apicomplexan, rather than haplosporidian (electron micrographs were limited in scope and therefore the haplosporidian identity was only a best match from available evidence).

As indicated above, DNA analysis is not an easy task to undertake, and this is especially true where the organism in question is undescribed. It is easier to select, or design, highly specific primers that are sure to bind to the DNA, when the organisms' taxonomical information is available. Therefore, because of its uncertain taxonomy, a total of six different techniques and two sets of primers were used in an efforts to obtain DNA sequences for the parasite from abalone.

7.1 Problems and disadvantages of DNA analysis

The polymerase chain reaction (PCR) is one of the most important steps in DNA analysis, but the capability of a PCR can be dependant on factors such as the target sequence quantity and the presence of inhibitors in the sample. Thus, a scanty amount of parasite DNA in a sample largely made up of host DNA can lead to lowered PCR sensitivity. This is due to the reduced likelihood of the primer making contact, or binding with, the template DNA (Stokes, Siddall & Burreson 1995).

DNA analysis is not a quantitative method and therefore the infection levels of a specific parasite species in a sample cannot be determined with this type of analysis. Another problem is that parasite infections can either be systemic or localised and this can further be determined by the life cycle of the parasite and the seasonality. This in turn makes it difficult to establish which life stage occurs where in the host, and ultimately from which organ to extract a sample (Stokes *et al.* 1995).

Various apicomplexans have an unusual organisation of their 18s rRNA gene. *Plasmodium* species for example have numerous replicas of nuclear rRNA genes amongst their life cycles (Perkins & Keller 2001). The choice of a restriction enzyme

in apicomplexan sequencing can be tedious and 18s genes have many insertions and deletions that can prevent accurate alignments. This is problematic as apicomplexans demonstrate high sensitivity to the alignment of the nucleotide sequence (Perkins & Keller 2001). The development of specific and sensitive analytical assays for protozoans is unlikely or usually proceeds very slowly (Bower *et al*. 2004). In instances where the host and parasite DNA is inseparable (Martyn *et al*. 2006), chosen primers may well amplify the DNA of both, and if the host DNA is in the majority it could amplify to the elimination of the parasites' DNA. Inevitably the characterisation and classification of the parasite may lag behind with the separation or amplification of the parasites' DNA from the hosts (Bower *et al*. 2004).

7.2 Successes and advantages of DNA analysis

On the other hand, the progress of PCR amplification of DNA is increasingly establishing itself as an important research and diagnostic tool (Bower *et al*. 2004) for the detection of parasites, due to the fact that it is a non-lethal method (Stokes *et al*. 1995). PCR analysis can be a compliment to histology (Bower *et al*. 2004) and can prove to be an even better analytical tool (Stokes *et al*. 1995), as organisms that could not be identified based on histology can now be identified based on comparison of results gained from other known parasites. PCR is a rapid, reproducible method that can be relatively easy (Martyn *et al.* 2006).

A large number of hosts can be screened (Wipps, Burton, Watral, St-Hilaire & Kent 2006) for specific parasites in a short period of time, without harming the hosts (Carnegie, Barber, Culloty, Figueras & Distel 2000; Wipps *et al*. 2006), especially in studies were only the infections have to be determined (Stokes *et al*. 1995). Owing to the lack of specific immune responses of marine bivalves, the above mentioned can make the efficient diagnosis of parasites more effective and will then aid in the fight against bivalve diseases (Novoa *et al*. 2004). The source of such infections, and their management, can also be endorsed (Docker, Devlin, Richard, Khattra & Kent 1997) and can assist in the control of wild and cultured host populations (Barta 2001). The diagnostic protocol of PCR has proven to be valuable in clarifying complex life

cycles of parasites (Stokes *et al*. 1995), by comparing the results with the history of other taxons (Bower *et al*. 2004).

Barta (2001) stated that DNA analysis can determine the relationship between parasites, aid in the clarification of classification schemes, and can determine the definitive host by placing a previously unidentified parasite in a class. The vast availability of sequences for different species in public databases (such as Genbank) makes rRNA gene sequencing a valuable tool for the molecular characterization of new species based on existing genera (Azevedo *et al*. 2006).

Successes in the DNA field with regards to parasites includes: the discovery that *Cryptosporidium* spp. are closer related to gregarines than coccidians (Carreno, Martin, & Barta 1999); the improved detection of withering syndrome by PCR (Andree *et al*. 2000); the successful analysis of gregarines in the tube worms (*Selenidium serpulae*) (Leander 2006); the improved detection of *Bonamia ostrea* at low prevalences in oysters (Martyn *et al*. 2006); and the classification of *Haplosporidium montforti* by Azevedo *et al*. (2006).

7.3 Lysis of tissue and extraction of DNA

Digestive gland samples intended for molecular analysis were preserved in 90% ethanol as mentioned previously (Fig 7.1 A). The first step in DNA analysis was to lyse the tissue to extract the DNA from the tissue. Lysis of tissue and extraction of DNA from samples was done with a QIAamp DNA^R Kit (Fig. 7.1B) according to the tissue protocol. Samples from Farm B (January 2006) were used for the DNA analysis. The samples lysed were numbers 2, 9, 22 & 23 as they had the highest infection levels as can be seen, for example, from Fig 6.7 (B, D & E) in Chapter 6. Tissue was cut into 0.025g pieces and one piece of tissue (per tube) was positioned in a 1.5ml micro-centrifuge tube with 180µl ATL buffer and 20µl Proteinase K. The samples were mixed by vortexing prior to incubation as well as during incubation. Incubation took place at 56ºC in a warm bath (Fig. 7.1 C) until all the tissue was completely lysed (usually overnight). After this, 200µl AL buffer was added to the

each micro-centrifuge tube. Samples were vortexed for 15s and subsequently incubated at 70ºC for 10min. A total of 200µl ethanol (96-100%) was added to each sample and followed by vortexing samples for 15s.

The mixtures were then pipetted into a QIAamp Spin Column and transferred into a 2ml collection tube. Samples were centrifuged (Eppendorf Centrifuge 5415D) (Fig. 7.1 D) for 1min at 8000rpm and both the flow-through and collection tube were discarded. The column was placed in a second 2ml collection tube with 500µl AW1 buffer and centrifuged at 8000rpm for another minute. Flow-through and collection tubes were discarded. The column was then transferred into a third collection tube and 500µl AW2 buffer were added and centrifuged for three and a half minutes at 13200rpm and again the flow-through was discarded.

The QIAamp Spin Column was placed in a new 2ml micro centrifuge tube and 150µl AE buffer were added directly onto the QIAamp Spin Column membrane. Samples were incubated at room temperature for 20min and then centrifuged for 1min at 8000rpm. Samples were stored at -20ºC for future analysis.

7.3.1 Technique 1

Samples were lysed according to the procedure described above. A total DNA electrophoresis agarose gel (Fig 7.1 E) was run by taking 5µl total DNA from each sample and mixing it with 5µl loading dye. The 10µl samples were loaded onto the gel and run at 200volts for 30min. The gel was placed under a UV lamp to determine the strength of the bands. Samples with strong bands were used for PCR. For the PCR template volumes of 0.5µl, 1µl, 1.5µl and 2µl were used for each sample of total DNA. The premix for each sample to be used in PCR was made up of 0.5µl DMSO, 22.5µl Master Mix, 0.25µl of primer 18S-EUK-581-F : 5'-GTGCCAGCAGCCGCG-3' and 0.25µl 18S-EUK-1134-R: 5'-TTTAAGTTTCAGCCTTGCG (as described by Bower *et al*. 2004), and 0.7µl BSA. The total volume of premix was 24.2µl that was added to each template and the control (only containing premix). The temperatures used for the PCR (Gene Amp. PCR System 9700 Applied Bioystems) machine (Fig. 7.1 F) consisted of a initial

denaturation step of 94ºC for 10min, followed by 40 cycles of amplification (94ºC for 1min, annealing temperature of 50ºC for 1min, an extension of 72ºC for 1min) and an final extension of 72ºC for 1min. The products were run on an agarose gel as described above and viewed with the UV light.

7.3.2 Technique 2

Procedures described above for technique 1 were followed with the exception of the temperature profile for PCR. A PCR gradient with an annealing temperature of 49-62ºC was followed to determine the optimum annealing temperature for the samples. The initial denaturation step was 94ºC for 4min followed by amplification at 94º C for 1min. The annealing temperature started at 62ºC for 1min, decreasing by 0.5ºC per cycle (16 cycles) to 49ºC for 14 cycles, extension of 72ºC for 1min) and a final extension of 72ºC for 1min.

PCR purification was conducted by adding 100µl PB buffer to 20µl PCR sample. The samples were placed into a spin column and 2ml collection tube and were centrifuged for 30s. The flow through was discarded and the column placed back into the same tube. A total of 750µl PE buffer was added to the column and centrifuged at 13200 rpm for 1min. The column was then transferred into a 1.5 ml micro-centrifuge tube and 20µl EB buffer were added and centrifuged for another minute. Dye (5µl) and elute (5µl) were mixed and loaded into the wells of an electrophoresis gel to determine the strength of the total DNA.

7.3.3 Technique 3

Procedures for technique 1 were followed as described above with the exception of the premix. A one times concentration of 0.5µl DMSO, 22.5µl Master mix, 0.3µl of 18S-EUK-581-F, 0.3µl 18S-EUK-1134-R and 0.8µl of BSA was added to each sample. The gradient PCR technique as described in technique 2 was used. Products were run on a gel and viewed with the UV light. PCR clean-up was conducted as described above in technique 2. Eight µl of the total DNA was prepared for cycle sequencing and spectrophotometer readings was taken for each sample. The template

volumes were calculated by dividing the spectrophotometer reading value by 35. The amount of distilled water to be added was calculated by subtracting the template volume and primer volume from ten. The primer mix for cycle sequencing consisted of one times concentration of 1.5µl sequence buffer, 0.3µl of primer 18S-EUK-581-F, 1µl of Pink Juice and 0.5µl of DMSO and one times concentration of 1.5µl sequence buffer, 0.3µl of primer 18S-EUK-1134-R, 1µl of Pink juice and 0.5µl of DMSO per sample. The samples were run on the sequencer.

7.3.4 Technique 4

Procedures described in technique 1 were followed, however the premix used did not contain DMSO and the temperatures for the PCR differed. A one times concentration of premix containing 12.5µl Master mix, 0.5µl of 18S-EUK-581-F, 0.5µl 18S-EUK-1134-R and 6.5µl water was made up for each sample and 5µl template DNA was used. The initial denaturation temperatures for the PCR was 94ºC for 7min, an amplification of 94º C for 1min, annealing temperature of 54ºC for 1min, extension of 72ºC for 1min and a final extension of 72ºC for 7min at 40 cycles.

7.3.5 Technique 5

The same procedures as described in technique 1 were used except for the reverse primer. The forward primer was 18S-EUK-581-F (as used in previous techniques) and the reverse primer was HEMO2: 5' CTTCTCCTTCCTTTAAGTGATAAGGTTCAC 3' (Perkins & Keller 2001). The premix did not contain DMSO and was made up of following the same procedure as in technique 4. The PCR temperature profile consisted of an initial denaturation temperature of 94ºC for 1min, an amplification of 94ºC for 1min, an annealing temperature of 58ºC for 30s, an extension temperature of 72ºC for 1min and an final extension of 72ºC for 7 min.

7.3.6 Technique 6

The Zymo Pinpoint slide DNA Isolation System^{TM} kit was used to extract DNA from histological slides. Tissue sections with a thickness of 10µm from paraffin embedded blocks were placed onto microscope slides with an albumin solution. Slides were dried for 30min at 60ºC in an oven. Slides were deparaffinised by placing them in first xylene for 30min followed by a change of xylene for another 30min. Slides were then hydrated through a graded concentration of ETOH (100%, 70% and 50%) for 2min, followed by sterile water for 2min. Slides were air-dried.

Pinpoint solution was placed on the selected area of the tissue containing parasites with a pipette at a thickness of 0.5mm. The slides were left to dry for 30-45min. A clean dissection knife was used to cut and peel of the selected tissue area. The film containing the tissue was placed in a 0.5ml tube and momentarily centrifuged to bring the film fragments to the bottom of the tube.

DNA extraction was conducted by adding 50µl Extraction buffer and 5µl Proteinase K to the samples. Samples were incubated in a water bath at 55ºC for four hours. After incubation the samples were heated in the PCR machine at 97ºC for 10min and placed on ice. Samples were then vortexed for 15s.

Purification of the DNA was conducted by adding 100µl DNA Binding buffer to each sample. These mixtures was transmitted into Zymo-Spin Columns and placed in a 2ml collection tubes. The samples were centrifuged at 13200rpm for 10s. To wash the samples, 150µl PP wash buffer was added to each column and centrifuged at 13200rpm for 10s. A second 150µl PP wash buffer was added to the samples and it was centrifuged at 13200rpm for 1 min. The columns were transferred to new 1.5ml tubes and 10µl TE buffer was added onto the membrane of the columns. Samples were left to incubate for 5min at room temperature and were spun for 10s to elute the DNA. The same primer set and PCR method described in Technique 5 was used.

7.4 Results and discussion

7.4.1 Technique 1

The primer set 18S-EUK-581-F and 18S-EUK-1134-R (general eukaryotic primers) was used for this technique. The bands for the total DNA from sample 9 & 22 (Fig. 7.2 A) were exceptionally strong. These two samples were prepared for PCR as described above and were run on a gel. These subsequent bands were faint and short (Fig 7.2 B). This might have been due to a temperature profile for the PCR that was not optimal for the primers used, and thus the primers did not bind to the parasite DNA, but to each other.

7.4.2 Technique 2

It was decided to use a gradient PCR temperature profile to insure that the primer (18S-EUK-581-F and 18S-EUK-1134-R) bound to the parasite DNA within the temperature range of 49-62ºC. The bands (following PCR) from the samples were of a better quality as those obtained in the previous technique. PCR purification protocol was followed and again the samples were run on a gel. The bands that formed were considerably weaker than the bands formed in the previous gel.

7.4.3 Technique 3

The primer set 18S-EUK-581-F and 18S-EUK-1134-R was again used in this technique. The premix for this technique was adjusted. The bands from the PCR reaction run on the gel were fairly strong. Samples were cleaned according to the PCR cleanup protocol and ran on a new gel. Samples 22 had the strongest bands (Fig 7.2 C) and were prepared for cycle sequencing as described above. No results were obtained and this might have been due to the use of DMSO, which has been found to damage animal DNA (C. Kneidinger. pers.com).

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7.4.4 Technique 4

The primer set 18S-EUK-581-F and 18S-EUK-1134-R was used in the premix of this technique. The premix for this technique did not contain DMSO, but still the bands from sample 9 were very faint and short (Fig 7.2 D). It is believed that the primers probably only bound to each other in the PCR and not to the parasite DNA, as the lengths of the bands were as short as the primers lengths when tested with a standard ladder used in one of the wells of the gel. The primer set used was probably not specific enough for the sample and was not tested again.

7.4.5 Technique 5

In this technique a different reverse primer (HEMO2 used for apicomplexans) was used with the original forward primer (18S-EUK-581-F used for eukaryotes) from previous techniques. No results were obtained due to the large difference in annealing temperatures of the two separate primers: thus the primers did not bind efficiently to the parasite DNA.

7.4.6 Technique 6

No results were obtained, indicating that the temperature profile and primer selection are of utmost importance and were unsuitable in this instance.

7.5. Conclusion

In conclusion, problems that hindered the DNA analysis of this parasite are discussed below. This unknown digestive gland organism is not uniformly distributed in the digestive gland of *H. midae*. Although only infected samples (based on histology) were used for DNA analysis, the small piece required for the analysis might not necessarily have contained sufficient of the parasite in question. No specific sensitive analysis has been developed for this parasite because these attempts were the first. The DNA of the host and the parasite could not be separated from each other, as the parasite is very small. Inevitably the host DNA dominated the sample and might have

amplified to such as extent that the parasite DNA was trivial in comparison. This might have contributed to the primers not binding successfully to the template parasite DNA and thus the PCR sensitivity was lower. The small amount of target DNA in the template could also have played a significant role in the failure of the analysis. The choice of primers was difficult (as mentioned previously) as the parasite has not been described previously and thus no specific primer could be used or designed. The general eukaryotic primers and the apicomplexan primers did not work. General haplosporidian primers (TEM had indicated that the organism might belong to this group) could unfortunately not be tested due to time and financial constraints. The PCR program could also have influenced the results, as the binding of primers to template DNA is highly temperature dependant.

8. Discussion and conclusion

Abalone are valuable shellfish, and their cultivation and processing of their products contribute to the economy of various countries across the world. As discussed in Chapter 1, outbreaks of parasites in farming facilities were common in the past and continue, and can have severe effects on the industry. Different groups of parasites have diverse effects on abalone.

Ectoparasites such as polychaete worms damage the abalone shell and thus the appearance of the abalone is unacceptable for buyers. Endoparasites injure the foot muscle and organs, and can lead to deterioration in the hosts' physical condition. It is essential for the abalone farming industry to have knowledge of abalone parasites, their effects on these molluscan hosts, and of methods suitable for control of the parasites to ensure that the farmed abalone are in optimum condition. Abalone farming operations began in the 1970s in countries across the world, but it was only in the 1980s that abalone farming commenced in South Africa.

Nearly a hundred species of abalone occur universally, but few have economical value. The only species in South Africa of commercial value is *Haliotis midae*, as mentioned in Chapters 2 and 3. When studying abalone parasites, it is important to know how abalone farming is undertaken, in order to determine what conditions promote specific parasites to accumulate and so that effective methods of control can be developed. The study of wild abalone (see Chapter 2), based on their ecology, can also provide information on the occurrence of parasites in the molluscs' natural habitat.

8.1 World abalone diseases and parasites

In Chapter 4 the most important parasites of farmed abalone were discussed. Both ecto- and endoparasites (mostly intracellular) can have tremendous effects on host condition and appearance. As was seen in Chapter 4, parasites and their associated diseases can have devastating effects on aquaculture facilities.

8.1.1 World Shellfish diseases and parasites

Abalone are the most important contributors to the world shellfish industry (as mentioned previously), but there are also other economically important shellfish species that are either cultivated, or harvested, for consumption and export purposes. Examples are scallops, clams, oysters, mussels, shrimps, prawns and crabs. The most important diseases and parasites of these latter species were discussed in Chapter 5.

It is vital to have a thorough understanding of the parasites and diseases that occur in all shellfish species, as different types of shellfish can be hosts to the same genus of parasite. The genus *Perkinsus*, for example, can infect abalone, oysters and clams. Kidney coccidians of the genus *Pseudoklossia* can contaminate abalone, oysters, clams, mussels and scallops. Haplosporidian parasites can infect abalone, oysters, clams, mussels and crabs, and abalone and oysters can be hosts of ciliate parasites. These are only a few examples illustrating how the same parasite genus can infect different shellfish hosts.

8.1.2 South African abalone diseases and parasites

The South African abalone *Haliotis midae* is the only species cultivated in aquaculture facilities around the coastline of the Republic and is also the only abalone species of any economical value as stated above and in Chapter 3. Rickettsiales-like prokaryotes, coccidia, sabellid and polychaete worms are the main parasites infecting *H. midae* in culture facilities. As far as macroscopic parasites are concerned, the endemic polychaete *Terebrasabella heterouncinata* causes the most damage to the appearance of the abalone shells. *Polydora hoplura*, *Dipolydora capensis* and an unidentified *Baccardia* species, mentioned in Chapters 1 and 4, also cause severe damage to the shell of the abalone (Simons *et al*. 2006).

During the past few years, interest has grown in the study and identification of microscopic, intracellular parasites of farmed *Haliotis midae*. To date, a Rickettsiales-like prokaryote, an unidentified kidney coccidian discussed in Chapter 4, and the

unidentified intracellular protozoan parasite (UDP) recorded in this dissertation (Chapter 8) have been discovered and studied.

In an attempt to study and identify the intracellular protozoan parasite infecting the digestive gland of *H. midae*, histological, transmission electron microscopy, fluorescence and DNA analysis were conducted (Chapters 6 and 7). The abalone collected were weighed (total mass and meat mass) and measured to determine whether infections with this parasite had an effect on the growth of the hosts. Graphs were compiled for abalone from Farm **B** on the west coast in Chapter 7. Students' t-test indicated that there was no significant difference, with infected abalone growing at a similar rate to the uninfected abalone. This suggested that this parasite might not pose a threat to the condition of the abalone host.

Results from histological studies showed no detectable infection with this parasite in abalone collected from the three farms at the end of autumn (May 2005), suggesting that lower water temperatures may limit the occurrence, or spread, of this parasite. Samples collected from Farm **A** in October 2005 (mid spring) had low infection levels. In contrast, abalone from Farm **B** had a high infection rate during mid summer for both January 2006 and February 2007 samples. From this data it can be concluded that the occurrence of the parasite is seasonal and it may proliferate mainly during the summer months, when water temperatures along the west cost are warmer.

None of the wild abalone (*H. midae* and *H. spadicea*) collected at the Tsitsikamma National Park (south coast) during April 2006 were infected with this parasite. This does not mean, however, that wild abalone do not harbour the parasite. It is important to consider that the parasite might only infect *H. midae* and not *H. spadicea*. Furthermore, the water temperatures were lower in April, and this parasite's natural geographical distribution could be limited to the west coast.

The results of methods used in attempts to identify the unidentified parasite (UDP) were discussed in Chapter 7, and the morphometrics of the parasite were compared to those of other, intracellular, parasites occurring in shellfish. Neither the histological, nor the TEM results provided a conclusive indication of the parasites position in the protozoan classification system, but some morphological and morphometric

characteristics correlated with those from the genus *Haplosporidium*. DNA analysis was therefore conducted as described in Chapter 7 in a final attempt to classify this extraordinary parasite. No results of any significance were obtained, due to difficulties in the amplification of this parasite's DNA; synthesis of suitable primers was also a problem.

8.2 Recommendations for future research

8.2.1 South African abalone

Abalone parasites have been significant contributors to financial loss for abalone farms in various countries across the world, as well as in South Africa. This emphasises the need to categorize all abalone parasites, especially those that are currently unidentified, poorly described, or not described at all. The effect that they have on the different species of abalone occurring across the globe should also be reported as quickly as possible.

Little is known about abalone parasites and diseases that are endemic to South Africa and this could be detrimental to the growth of this relatively young and small industry (compared to that of other countries), especially with the recent closure of the commercial industry along the coastlines. It is important to gain information on the life cycles of all abalone parasites from South Africa as this will aid in identifying potential vectors (see below) and as well other methods of transmission of these parasites to there abalone hosts. Information gained from the life cycle studies can aid in the control of vector populations, or even the elimination of them all together. Methods to treat infected animals should then be easier to execute

8.2.2 The parasite (UDP)

Surveys conducted at the farms considered in this study, may give a better understanding of what specific factors can influence the occurrence and proliferation of the unidentified parasite (UDP). Feeding behaviour, for example, may be important, as each farmer may feed their abalone on a different diet. Farmers may

choose one of three basic diets (kelp feed, a mixed diet of kelp and artificial feeds, or an exclusively artificial feed) and this could influence the occurrence and proliferation of this parasite in the farm.

Parasites like sabellids and polychaete that infest the abalone host should also be examined for this unidentified parasite, as they could be its potential second invertebrate host. Burreson and Ford (2004) listed the haplosporidian species that have been found in polychaetes from around the world and suggested that polychaetes might possibly serve as the much sought after, intermediate hosts.

It is also important to extend sampling of abalone for this parasite from farmed to wild abalone, especially in areas close to the existing abalone farms. If the parasite does infect wild abalone, it might be easier to conduct research on these wild specimens and establish the life cycle of this parasite by this method. This could be done by collecting and examining common abalone predators in an attempt to find the unidentified parasite, as *H. midae* might be the intermediate invertebrate host of the parasite rather than its final, or only host.

Ideally, in future, sampling of wild and farmed abalone should be done around the coast of South Africa to estimate the geographical distribution of the unidentified parasite. Furthermore, husbandry methods used by aquaculturists can play a vital role in controlling all abalone parasites, as well as the parasite studied in this dissertation. Simple precaution methods like installing good filtering systems with very small mesh sizes on inlet pipes, exposing inlet water to UV light, high-quality cleaning and maintenance of holding tanks can improve general health and control parasite loads.

8.3 Hypotheses and aims revisited

The original hypotheses of outlined in this study were that the UDP is a member of the protozoan grouping, the Apicomplexa, that it has no detrimental effect on the growth and health of the host, and that *H. midae* could be its intermediate invertebrate host. Through an extensive study that included histology, fluorescence microscopy, TEM and molecular analysis the first hypothesis is rejected. It seems that this parasite

is more likely to belong to the haplosporidian group of parasites than to the apicomplexans (see Chapter 6). Based on the limited data available, the second hypothesis that this parasite has no detrimental effect on the growth and health of the host can be accepted (see Chapter 6). Since no hosts for the UDP other than *H. midae* were identified within the scope of this dissertation (although suggested hosts are noted above), it is neither possible to accept, nor reject, the final hypothesis on the life cycle of this parasite. However, all the original aims and objectives identified for this project were achieved (see Chapter 1).

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Appendix I

African Zoology

Instructions to Authors

Publication policy

African Zoology continues *Zoologica Africana* and *South African Journal of Zoology*. Full-length papers and short communications on original research on any aspect of zoology in Africa (or that is relevant to Africa) and its surrounding oceans, seas and islands will be considered for publication, especially studies in: ecology, ethology, physiology, functional morphology, genetics, taxonomy, systematics and phylogeny, biodiversity and conservation. Review articles are also accepted. Contributions will be sent to two referees for review, and papers will normally be published in order of acceptance. Authors may provide a list of three potential referees for consideration by the editors. Submission of a manuscript will be taken to imply that the material is original and will not be submitted in similar form for publication elsewhere. Copyright of papers that are published in the Journal is vested in the Zoological Society of Southern Africa. Contributions should be written in clear and concise English.

Presentation of manuscripts

Manuscripts may be submitted electronically to either of the editors, or in hard copy (in which case please include an identical copy on disc). Text should be double spaced (this includes the **Abstract**, **References**, **Tables**, **Figure captions** and **Book Reviews**). Use either Word-Perfect or MSWord for Windows on the PC platform, and include tables and/or figures in the main text document. Your letter accompanying the submitted manuscript should acknowledge your understanding of page charges levied in *African Zoology*, as outlined below.

Consult a recent issue of the Journal for typographic conventions. Use the active voice whenever feasible, and write in the first person, using British conventions of spelling and grammar. Manuscripts should be structured as **Short Communications** when the scope of a study is limited, sample sizes are small, or for preliminary reports of important scientific developments. These should ideally not exceed 2500 words, start with a short (<200 words) summary of the research findings, and must employ minimal heading structure (not more than two levels).

The **title** must be short but sufficiently informative for use in title lists or coding for information storage and retrieval.

The **abstract** may be up to 200 words long and should give the content of the paper factually and concisely in English. It should be suitable for separate publication and adequate for indexing and include up to five **key words**.

The **first page** should contain the title of the paper, the author(s) name(s) and address(es) (including e-mail address if available), an indication to which author corre-

spondence should be addressed and the abstract. Start the paper itself on a new page. Number pages consecutively and indent all paragraphs.

Cite **references** by name and year of publication, e.g., Smith (1999). When the reference is in brackets there is no comma separating the name and date, e.g. (Smith 1999). When several references are in brackets, the references are separated by a semi-colon and in chronological order, e.g. (Smith 1999; Jones & Jack 2000). If there are more than two authors, *et al.* should be used. If the reference cited, however, is also the taxonomic authority, a comma should separate the author's name and date (see also **Scientific names** below). Personal communications and reference to unpublished data should be cited in the text giving the initials and name (e.g. J.D. Smith, pers. comm. or A.B. Jones, unpubl. data) and the affiliation of the persons should be given in the acknowledgements section. Work submitted or in preparation should not be cited in the text nor presented in the reference list.

Use the SI metric system for units of measurement. Spell out numbers from one to nine; use numerals for larger numbers, groups of numbers, fractions or units, e.g., three; 8 to 16; 4 kg/ha; 27 impala. Statistical symbols, if not available on the word-processing program, e.g. χ, should be handwritten and identified in the margin (Greek l.c. chi), and **variables in formulae** should be italicized.

Scientific names should be given in full in the text when a genus or species is first mentioned. Authors should consult taxonomic authorities such as the Bulletin of Zoological Nomenclature, the International Code of Zoological Nomenclature (both published by the International Trust for Zoological Nomenclature, c/o British Museum (Natural History), Cromwell Road, London SW7 5BD) and the Nomenclator Zoologicus. The taxonomic authority, when cited, must follow the name of the taxon without intervening punctuation and not be abbreviated. If the year is added, a comma must separate author's name and year.

Ethical Standards. If the research has involved vertebrate experimentation the authors should indicate that such experiments were approved by an ethical standards committee, giving an approval reference number (if available).

Voucher Specimens. Authors of taxonomic works are encouraged to deposit voucher specimens in an established permanent collection in the country of origin of the material. This collection should be cited in the publication. All nucleic acid sequences should be placed in Genbank and accession numbers included in the manuscript

References should be listed alphabetically by authors'

surnames. Authors' names appear in capital letters, the rest of the reference in lower case letters. Journal names must be in italics and given in full. Book titles should be in italics; do not italicize thesis titles. Examples follow:

BRONNER, G.N., MALONEY, S.K. & BUFFENSTEIN, R. 1999. Survival tactics within thermally-challenging roosts: heat tolerance and cold sensitivity in the Angolan free-tailed bat, *Mops condylurus*. *South African Journal of Zoology* **34**: 1–10.

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With the exception of electronic journals, reference to Internet web sites should be avoided.

Tables should be carefully constructed so that the data presented may be easily understood. Take care not to overload tables with information or to have an unnecessary proliferation of tables. Wherever possible, tables should be arranged to fit the page vertically within the dimensions of the printed page (205 × 142 mm). Tables may include up to four horizontal lines but no vertical lines. Consult recent issues of the Journal for examples of the layout of tables and, in particular, the layout of column headings. Tables should be numbered consecutively as they are mentioned in the text, using Arabic numerals, e.g. Table 1, Table 2. All tables must be typed or printed (in double spacing) on separate pages and grouped together at the end of the manuscript.

Illustrations must be prepared on separate A4 sheets and numbered chronologically as they are referred to in the text using arabic numerals, e.g. Fig. 1, Figs 2, 3, or where drawings or photographs are grouped as Fig. 1a,b etc. The Journal does not differentiate between figures and plates and all illustrations should be referred to as figures. Black-and-white illustrations, e.g. drawings, diagrams, maps, graphs, must be originals executed in black on a clean white background, and one set of original illustrations and two sets of copies must be submitted, the **originals being essential for good quality reproduction** in the Journal. Photographs should be of excellent quality on glossy paper with clear details and adequate contrast, and three sets of prints should be submitted. Illustrations can be submitted in the following electronic formats: TIFF, CDR, EPS, WMF OR EMF, preferably in one of the vector (not bitmap) formats, **once the manuscript has been accepted for publication**.

It is important that lines are thick enough and symbols large enough to withstand reduction. Where very fine lines or small dots are used for shading these may disappear completely with reduction. Proper drawing equipment giving uniform lettering and lines must be used. **Freehand and typewritten lettering and lines are not acceptable**. Authors are requested to pay particular attention to the proportions of illustrations so that they can be accommodated in single (68 mm) or double (142 mm) columns after reduction. **Capital letter height should not exceed 5 mm or be smaller than 3 mm.**

All figures should bear on the reverse side and written in soft pencil, the name of the author, the figure number and, where confusion might result, an arrow indicating the top of the figure. Captions for figures must be collected together and typed on a separate sheet headed 'Legends'.

Acknowledgements should be brief and direct, and should be made only for assistance outside the normal duties of the parties concerned.

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Appendix II

South African
NATIONAL PARKS

Dr. N.J. Smit
University of Johannesburg

Dear Dr. Smit:

PROJECT TITLE :
THE CHARACTERISATION OF AN INTRACELLULAR PROTOZOAN PARASITE
INFECTING THE DIGESTIVE GLAND OF ABALONE, HALIOTIS MIDAE

Herewith I would like to confirm that your application for a once of collection of *Haliotis* sps. in Tsitsikamma National Park was successful. Only **30 animals per species** may be collected

Please submit a progress reports on your project covering the
period from April 2006 - December 2006. This report will be due before 28 February 2007.

Please follow the prescribed reporting format. Please remember to include copies of any papers, presentations and publications that have emanated from this project.

Not only are progress reports and related publications read with interest and disseminated to the relevant parks, but they are also a useful gauge for assessing future applications to SANParks.

If your project has been completed, please state that this is you final report, and please indicate if there are any scientific publications in preparation.

Thank you.

Regards

Tania Walton
Admin
Conservation Services : Rondevlei
South African National Parks
Sedgefield

addo elephant

agulhas

augrabies falls

bontebok

cape peninsula

golden gate highlands

karoo

kgalagadi transfrontier

knysna

kruger

marakele

mountain zebra

namaqua

tankwa karoo

tsitsikamma

richtersveld

vaalbos

vhembe dongola

west coast

wilderness

Conservation
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Appendix III

Table A, Measurements taken from farmed *Haliotis midae* from Farm **A** October 2005 and notes on the presents of Sabellid and Polychaete worms.

Farm A	Total mass (g)	Meat mass (g)	Shell length (mm)	Shell width (mm)	Sabellids	Polychaetes
1	36.89	24.03	55	38	Yes	No
2	36.58	24.53	54	38	Yes	Yes
3	24.2	15.41	50	36	Yes	Yes
4	36.18	25.97	55.9	39	Yes	Yes
5	26.42	18.15	52.7	37.2	Yes	No
6	32.82	22.71	57.9	37.1	Yes	No
7	31.8	22.48	54.4	38.8	Yes	No
8	36.04	27.1	55.6	39.4	Yes	No
9	34.07	22.56	57.1	41	Yes	Yes
10	33.3	23.33	58.2	40.9	Yes	No
11	36.51	26.03	52.4	37.2	Yes	No
12	25.92	17.53	52.6	37.1	Yes	No
13	22.05	15.22	49.1	32	Yes	No
14	31.45	22.07	54.9	39.4	Yes	No
15	35.66	26.01	53.9	35.5	Yes	No
16	29.9	20.9	53	40.9	Yes	No
17	34.32	23.87	54	40.2	Yes	No
18	28.62	19.8	56.7	35	Yes	No
19	38.78	29.14	57.5	41.2	Yes	Yes
20	29.45	20.43	52	36.4	Yes	Yes
21	27.42	19.04	55	38	Yes	No
22	30.94	21.4	55	37	Yes	No
23	21.48	15.87	51.5	31.9	Yes	No
24	24.53	16.83	49.9	32.2	Yes	No
25	24.82	18.03	51.2	35.9	Yes	No
26	27.82	18.83	52	36.8	Yes	No
27	35.29	26.6	55	37.8	Yes	No
28	21.17	15.33	47	32.2	Yes	No
29	34.58	24.01	52.8	36.3	Yes	No
30	26.47	17.99	51.1	37	Yes	No
31	22.3	15.5	51	34.1	Yes	No
AVER	30.25	21.18	53.50	37.08		
STDEV	5.30	4.00	2.70	2.65		
MIN	21.17	15.22	47	31.9		
MAX	38.78	29.14	58.2	41.2		

Table B, Measurements taken from farmed *Haliotis midae* from Farm **B** January 2006 and notes on the presents of Sabellid and Polychaete worms.

Farm B	Total mass (g)	Meat mass (g)	Shell length (mm)	Shell width (mm)	Sabellids	Plychaetes
1	52.67	37.08	6.54	4.59	Yes	Yes
2	45.92	29.86	6.14	4.33	Yes	Yes
3	22.18	14.92	5.06	3.6	Yes	No
4	31.54	23.28	5.2	3.7	Yes	No
5	45.91	32.71	5.93	4.19	Yes	No
6	32.88	19.96	5.9	4.47	Yes	Yes
7	39.64	25.67	6.24	4.77	Yes	Yes
8	41.12	27.36	5.7	4.5	Yes	Yes
9	41.86	29.22	6.07	4.32	Yes	Yes
10	32.54	18.13	5.82	4.26	Yes	Yes
11	43.34	28.03	6.5	4.69	Yes	Yes
12	30.1	22.28	5.16	3.68	Yes	Yes
13	37.38	20.97	5.83	4.4	Yes	Yes
14	43.67	29.3	6.04	4.55	Yes	Yes
15	44.76	27.05	6.54	4.8	Yes	Yes
16	27.16	14.12	5.56	4.2	Yes	Yes
17	39.48	26.26	5.76	4.16	Yes	Yes
18	38.83	27	5.7	3.3	Yes	Yes
19	26.71	17.1	4.9	3.8	Yes	Yes
20	34.47	24.19	5.69	4	Yes	Yes
21	35.49	25.13	5.9	4.09	Yes	Yes
22	30.63	17.97	5.77	4.2	Yes	Yes
23	22.37	13.24	5.54	4.03	Yes	Yes
24	35.06	18.82	5.9	4.42	Yes	Yes
25	42.94	26.9	6	4.4	Yes	Yes
26	40.98	27.97	6	4.2	Yes	Yes
27	38.84	27.33	5.54	4.53	Yes	Yes
28	42.41	28.39	5.9	4.4	Yes	Yes
29	45.46	31	6.1	4.54	Yes	Yes
AVER	37.46	24.53	5.83	4.25		
STDEV	7.46	5.85	0.40	0.36		
MIN	22.18	13.24	4.9	3.3		
MAX	52.67	37.08	6.54	4.8		

Table C, Measurements taken from farmed *Haliotis midae* from The Tsitsikamma National Park April 2006 and notes on the presents of Sabellid and Polychaete worms.

Tsitsikamma	Total mass (g)	Meat mass (g)	Shell length (mm)	Shell width (mm)	Sabellids	Polychaetes
1	38	23	61.27	42.55	No	No
2	19	13	47.53	33.57	Yes	No
3	22	14	48.13	31.36	Yes	No
4	38	22	57.28	40.81	Yes	No
5	9	6	38.18	25.92	Yes	No
6	364	174	130.27	99.7	No	Yes
AVER	81.67	42	63.78	45.65		
STDEV	138.78	64.97	33.57	27.18		
MIN	9	6	38.18	25.92		
MAX	364	174	130.27	99.7		

Table D, Measurements taken from farmed *Haliotis spadicea* from The Tsitsikamma National Park April 2006 and notes on the presents of Sabellid and Polychaete worms

Tsitsikamma	Total mass (g)	Meat mass (g)	Shell length (mm)	Shell width (mm)	Sabellids	Polychaete
1	10	7	37.53	24.4	Yes	No
2	5	3	30.11	19.42	No	No
3	31	22	49.42	31.8	No	No
4	39	24	56.93	33.33	No	No
5	33	20	51.32	30.57	No	No
6	52	38	60,81	37.19	No	No
7	34	24	51.81	33.36	No	No
8	53	33	69.97	37.75	No	No
9	49	27	59.89	35.15	No	Yes
10	34	24	59.35	34.3	No	No
11	18	11	43.97	26.94	No	No
12	37	27	40.7	26.78	No	No
13	14	9	56.7	34.33	No	No
14	53	35	59.59	36.49	No	No
15	41	28	56.5	26.18	No	Yes
16	35	23	50.77	32.28	No	No
17	52	42	57.66	37.08	No	No
18	48	34	56.26	35.11	No	No
19	31	21	50.31	34.33	No	Yes
20	41	26	52.81	32.96	No	Yes
21	42	28	57.82	36.1	No	No
22	29	16	51.02	32.93	No	Yes
23	48	32	59.14	36.03	No	No
24	39	26	57.5	32.71	No	No
25	49	36	57.46	36.34	No	No
26	39	29	52.26	32.7	No	Yes
27	50	34	64.6	38.27	No	No
28	30	21	58.59	33.46	Yes	Yes
AVER	37	25	53.70	32.80		
STEV	13.05	9.45	8.27	4.42		
MIN	5	3	30.11	19.42		
MAX	53	42	69.97	38.27		

Table E, Measurements taken from farmed *Haliotis midae* from Farm **B** February 2007 and notes on the presents of Sabellid and Polychaete worms.

Farm B	Total mass (g)	Meat mass (g)	Shell length (mm)	Shell width (mm)	Sabellids	Plychaetes
1	37.72	26.58	6.10	4.61	Yes	No
2	32.18	23.43	5.69	3.65	Yes	No
3	36.08	24.57	6.57	4.49	Yes	No
4	31.14	22.33	5.29	3.69	Yes	No
5	40.83	27.93	6.14	4.46	Yes	No
6	29.63	20.1	5.87	4.27	Yes	No
7	36.22	26.19	6.15	4.41	Yes	No
8	33.19	23.53	5.73	3.99	Yes	No
9	65.73	42.63	7.25	5.69	Yes	Yes
10	32	23.39	5.45	3.46	Yes	No
11	26.26	18.57	5.72	3.96	No	No
12	39.13	26.08	6.32	4.43	No	Yes
13	31.53	22.64	5.51	3.89	Yes	Yes
14	31.41	22.35	5.67	4.17	Yes	Yes
15	34.73	22.91	5.67	4.09	Yes	Yes
16	34.51	26.2	5.60	4.27	Yes	Yes
17	30.05	22.26	5.81	3.97	Yes	No
18	29.58	20.88	5.40	3.69	Yes	No
19	29.9	22.57	5.79	3.80	Yes	Yes
20	34.13	24.5	5.79	4.09	Yes	No
21	41.93	29.12	6.38	4.56	Yes	Yes
22	49.65	35.23	6.69	4.52	No	Yes
23	31.05	21.77	5.54	4.04	Yes	No
24	31.88	22.72	5.90	4.10	Yes	Yes
25	40.69	30.33	6.21	4.42	Yes	No
26	34.47	24.76	5.49	3.89	Yes	Yes
27	38.8	29.1	6.05	4.14	Yes	Yes
28	33.43	23.24	5.87	4.16	Yes	Yes
29	48.81	35.04	6.59	4.75	Yes	Yes
30	52.88	37.35	6.64	4.92	Yes	Yes
AVERAGE	36.65	25.94	5.96	4.22		
STDEV	8.34	5.46	0.46	0.44		
MIN	26.26	18.57	5.29	3.46		
MAX	65.73	42.63	7.25	5.69		

Appendix IV

A. *Davidson's solution for marine use according to Austin & Austin 1989*

    a. Formalin………………………...20 parts
    b. Glycerol…………………………10 parts
    c. Glacial acetic acid………………10 parts
    d. Absolute alcohol……………….. 30 parts
    e. Seawater……………………….. 30 parts

B. *10% Neutral Buffered Formalin according to Humanson 1979*

    a. $NaH_2PO_4\ 2H_2O$………………..112g

    b. $Na_2HPO_4\ 12H_2O$ …………….....196g

    c. Two liters of 40% Formalin

Add all to a 20 liter container and fill with dH_2O to make up 20 liters.

C. *Solutions:*

    (a)   *0.1M Phosphate buffer (Sörensen) according to Hayat (2000)-*

    Solution A: Sodium phosphate, dibasic (Na_2HPO_4. $2H_2O$) ….11.876g

         Distilled water to make 1000 ml

    Solution B: Potassium phosphate monobasic (KH_2PO_4) …... 9.08g

         Distilled water to make 1000 ml

The desired pH of 7.4 can be obtained by adding 81.8ml of solution A to 19.2ml of solution B, to make a total volume of 100ml.

    (b)   *4% Phosphate buffered Paraformaldehyde (PFA) Fixative-*

Heat 4 g of paraformaldehyde powder in 100 ml 0.1 M Sörensen's buffer until dissolved.

    (c)   *Karnovsky fixative (Karnovsky, 1965)*

    1. 10%   PFA   in H_2O …………………………………………….20ml

    2. Prepare 0,2 M cacodylate buffer at pH 7, 4 as follows:

Na-cacodylate ……………………………..2,14 g

Add H_2O ……………………………..25 ml

Add 0,2M HCl to obtain pH of 7, 4 (± 4 drops)

Add H_2O to obtain final volume of 50ml

To prepare fixative combine:

0,2M cacodylate buffer ……………………….50 ml

10% PFA in H_2O ……………………….20 ml

Gluteraldehyde (25%) …………………….10 ml

Distilled H_2O to obtain final volume of 100 ml.

(d)   Toluidene blue stain

Staining Solution:

Toluidene blue ………………………………..   1 g

Borax ………………………………………..   1 g

Distilled water …………………………………   100 ml

BORAX= Sodium tetraborate ($Na_2B_4O_7.10H_2O$)   +   Boric acid (H_3BO_3)

Dissolve the borax in water and add dye with constant agitation. When dissolved, filter solution and store in an amber container. Cover dried sections on a slide with a large droplet of stain, and heat slide at60ºC until golden-coloured rim appears around the stain edges. Wash off excess stain and mount the sections with Entellan® and a coverslip for light microscopy.